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Proceedings of the 12th International TNF Conference, 2009

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Preface by David Wallach

The Biennial International TNF Conferences and Their Proceedings

This volume presents a collection of essays by the speakers at the 12th Biennial International TNF conference, which took place at San Lorenzo del Escorial, Spain, in April 2009.

The TNF conferences have seen an immense advance in our knowledge of the TNF family. At the time of the first meeting (at Heidelberg, Germany, in September 1987), the only known ligands of the TNF family were tumor necrosis factor itself (TNF) and lymphotoxin (LT). There was as yet no indication that there were any other related ligands, and certainly no hint of the existence of a family of receptors to which these ligands bind. In fact, scientists were still arguing over the question of whether TNF was a cytokine, i.e., a cellular regulator that acts by triggering a specific cell-surface receptor; indeed, practically nothing was known of the mechanisms by which TNF acts. Moreover, most of the numerous activities of TNF (and of the other members of the TNF family) were not yet known. TNF and LT were still mainly perceived as agents destined to combat tumors, either by killing their cells or by initiating their hemorrhagic necrosis.[1] This primordial state of knowledge was

[1] In that connection I wish to pay tribute to the late George Gifford, a wonderful person who, together with Holger Kirchner, raised the idea of holding conferences on TNF and was largely instrumental in organizing the first conference. The scientific organizing committee for that meeting comprised scientists who had begun to study TNF at a time that we subsequently liked to refer to as 'B.C.' (before cloning of TNF and LT). At the time of the conference all of them were still studying either the anti-tumor function or the cytotoxic activity of TNF (or in my case, the ability of TNF to induce resistance to its cytotoxic effect, a function that we now mainly ascribe to its activation of NF-κB). A few years before that first conference, George had written a comprehensive review of the state of knowledge of TNF at that time. Two hypotheses from that review illustrate the conceptual gap between the 'B.C.' and 'A.C.' periods: "...Could the protein component of TNF serve as a specific carrier for phospholipid toxin...alternatively, could TNF exist as a zymogen in the more traditional sense...perhaps proenzyme?" (1). (Also mentioned in his review was the possibility that TNF is a cytokine that acts by binding to specific receptors.) And yet, although he was probably the oldest of the conference organizers and had thus had the longest acquaintance with such 'B.C concepts,' George was the one most alert to the new advances. I recall him saying,

reflected in the title of that meeting, 'Conference on TNF and Related Cytotoxins' [1], which differed from that of all subsequent meetings in the series. Since then, studies by the numerous groups who were increasingly attracted to the TNF research field have gradually revealed the existence of many TNF-related ligands and as many interrelated receptors through which the ligands induce their effects. It was further disclosed that triggering of these receptors leads to a plethora of functional consequences affecting virtually every cell in the body and practically all known immune functions as well as multiple embryonic developmental and homeostatic processes. Vast knowledge has also been acquired of the molecular mechanisms by which these effects are induced and of their potential relevance for disease and therapy.

The scientific community studying the functions of the TNF family has grown immensely and the research subjects upon which these functions have a bearing are remarkably heterogeneous. Nevertheless, there is still much of common interest that induces these scientists to meet with their peers. Such meetings enable them to share information and ideas on common proximal signaling molecules activated by different ligands and receptors of these families, mechanisms by which these molecules act, modes of regulation of receptor and ligand activities, cooperation among the various molecules, techniques for therapeutic modulation of ligand functions, and many other common pursuits. Despite the fact that these scientists possess neither an official organization nor funds to support their cooperation, they have continued to convene approximately every second year, each time in a different part of the world. For close to a quarter of a century these international conferences on TNF have successfully taken place, thanks to untiring efforts on the part of ad hoc chosen chairpersons and organizing committees.

Inevitably, the scientific progress made over this period required some changes in organization of the meetings. In particular, to accommodate the vast expansion of knowledge about the TNF family within acceptable time limits it became necessary to prune discussions of subjects that were related only tangentially to the central issues. At the initial meetings the sessions had encompassed the relationship of TNF functions to those of bacterial endotoxin and other pathogen-associated molecules and insults, the functions of other inflammatory cytokines such as IL1 and IL6, as well as infectious diseases, tumor immunology, and many other subjects. Discussions of these fringe issues and their implications for the field of TNF research had promoted a more profound understanding of TNF function. At the more recent meetings, however, their inclusion was a luxury that the organizers could not afford. Stringent restrictions were now placed also on presentations of progress in the TNF field itself. Most chairpersons chose to do this by focusing on those research aspects showing the most dramatic advances while devoting very

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"There is a guy called Tony Cerami who, together with his PhD student Bruce Beutler, reached findings that provide a new point of view of the function of TNF. They actually call it 'cachectin'. We must also discuss the potential pathogenic role of TNF in our conference. Why don't we invite Tony to join us in organizing this meeting?" (which we did).

little time to the numerous other aspects in which progress has been more difficult to achieve and might therefore appear less impressive.

Despite the continuing proliferation of laboratories devoted to studying the functions of the TNF family, the numbers of conference participants have remained constant for several years, and in recent times have even decreased. In the course of publicizing the current international conference and while searching the Internet for laboratories supported by the National Institutes of Health or the European Union for research on ligands of the TNF family, we were astonished not only to see how numerous these laboratories have become but also to discover how many of them have never been represented at a TNF conference. In pondering this paradox we concluded that planners of these meetings, by opting to focus on the more spectacular advances in the field, have inadvertently restricted the circle of scientists who attend the meetings.

Therefore, in order to reach out to the many research laboratories which, despite their intimate involvement in subjects central to the TNF field, do not participate in these meetings, we decided that at the 12th TNF conference we would not restrict the scope of subjects, but would instead strictly limit the presentation length. To compensate for this restriction it was decided that each session would be followed by a round-table discussion in which the principal open questions pertaining to that session would be thoroughly discussed. To our satisfaction, this approach substantially boosted participation in the meeting and greatly extended the scope of the subjects discussed. A short review of these subjects is presented in [2].

Each of the first four TNF conferences (held between 1987 and 1992) was followed by the publication of *Proceedings* in which talks from all of the sessions were summarized [3–6]. This practice was then discontinued, perhaps because as time went on the focus of the conferences became more restricted, as explained above. Since the range of subjects addressed at the 12th conference represented a wide overview of the current state of TNF research, and because a compendium of articles in this field had not been published for quite some time, we opted to publish the *Proceedings* of this meeting. In addition to the articles written by the speakers, this volume contains some short overviews contributed by the scientists who chaired the discussions held after each session.

Although almost two decades have passed since the first four conferences were held, their *Proceedings* are still of interest, both as source of reference for data or hypotheses and as snapshots of the state of knowledge at the time of their publication. We hope that the *Proceedings* of this most recent meeting will be at least as valuable, and that it will serve as an effective complement to the conference itself in facilitating interactions among the numerous scientists who contribute to the different facets of research on the TNF family.

Rehovot, Israel David Wallach

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Preface by Marc Feldmann

Introduction

The TNF/TNF-R Family: A Gold Mine for Clinical Therapeutic Targets?

The potential importance of TNF in therapy was first described in animal models of sepsis [1] and the attempts to replicate it in humans started the evaluation of the clinical relevance of TNF in disease. The first success was with anti-TNF antibody in rheumatoid arthritis, soon followed by anti-TNF antibody in Crohn's disease, and this documented the importance of the TNF family in major diseases.

From that time, therapeutic and clinical studies have grown and TNF blockade, by either monoclonal antibodies or TNF receptor fusion proteins, has become a huge therapeutic market, about 2 million treated patients and drug sales of $17 billion in 2008 [2].

But TNF is not the only therapeutic target in the TNF/TNF-R family. The monoclonal antibody to RANK ligand is very effective in many clinical trials at preventing bone loss for osteoporosis or bone cancer and is likely to be another 'blockbuster.' There are other promising leads, but some do not progress. Thus anti-LTβ was not successful in arthritis, but has a potential against cancer.

The ligands also are potential therapeutics. TNFα was not useful systemical for cancer due to life-threatening effects, but local infusion has been successful in limbs, provided sensitizing IFNγ pre-treatment is administered and TNF is not allowed to disseminate systemically. TRAIL, inducing apoptosis in cancer cells, may be more useful, and preliminary studies were encouraging.

But what is the future for the TNF/TNF-R in the therapeutic domain? It looks bright. Inhibition of TNF-R may be a better alternative to TNF blockade, as a number of mouse knockout experiments suggest that, but proof with selective inhibitors is lacking. Inhibiting other TNF-R family members for a variety of diseases is being explored. As the TNF family receptors are susceptible to activation on being cross-linked, monomeric blockers are essential, but the reciprocal opens up

another opportunity, of activation by cross-linking receptors, for inducing apoptosis in cancer cells, for example.

Thus there will be interesting reports in the future in the field of therapy for the TNF and TNF receptor family.

London, UK Marc Feldmann

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12th International TNF Conference. San Lorenzo de El Escorial, Spain. Marc Feldmann and David Wallach, co-chairs. April 26–29, 2009. (Group picture)

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Part I
Roles of the TNF Family in Tissue Homeostasis and Normal Development

Chapter 1
Workshop Summary: *Roles of the TNF Family in Normal Development and Cancer*

Nancy H. Ruddle

Lymphotoxin and TNF, the first TNF family members to be described, were originally assigned roles in inflammation or tumor rejection. The discovery that LT-deficient mice lacked lymph nodes and the discovery of multiple additional members of the family with distinctly non-inflammatory roles brought about the realization that many members of the family were crucial for normal development. Furthermore, TNF's role in tumor defense has come under scrutiny in recent years with the realization that in some cases it can act as a tumor promoter.

What is known about the TNF superfamily members' roles in normal development? The RANK–RANKL system has been known for some time to play an important role in bone development. Josef Penninger expanded on this subject and emphasized the essential roles of these molecules in the immune system, particularly lymph node development and regulation of AIRE in medullary thymic epithelial cells. Interestingly, RANKL is also crucial for lactation. Furthermore, RANK is expressed in the brain where it may act as a physiological thermal receptor. C.J. Mueller continued the discussion of RANK in his presentation of data regarding its role in hair growth. Mice deficient in RANKL have reduced epidermal thickness. Studies with mice transgenic for RANK in hair follicles show increased hair renewal and epidermal hyperplasia. Bcl-3 appears to be involved in this process. M. Mikkola presented data on the role of the Edar, Xedar, and Troy subfamily of TNF receptors in hair development. EDA is crucial for initiation of hair follicle development, through EDAR and induction of chemokines. Troy, which is also important in hair follicle development, has been considered an orphan receptor, although a recent report suggests that LTα is a physiological ligand.

What is known about TNF's effects on the vascular system? C. Hughes discussed the seemingly contradictory roles of TNFα in angiogenesis whereas it appears to be proangiogenic in vivo, but anti-angiogenic in vitro. Endothelial sprouting in vitro was evaluated in the continual presence or a 2- to 3-day pulse of TNF. The duration

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of exposure to TNF was critical. TNF initially inhibited angiogenesis, but when added as a pulse-induced sprouting through induction of "tip cells." These data emphasize the importance of local dosage effects of cytokines. N. Ruddle described experiments that evaluated the role of lymphotoxin (LT) in lymphatic endothelial cells and high endothelial venules (HEVs) in lymph nodes. A new mouse model with green fluorescent HEVs was described.

What is known about the role of TNF family members in development of organisms other than man and mouse? What is known about the phylogeny of TNF family members? M. Mikkola continued the discussion of the Edar, Xedar, and Troy family with a discussion of the role of Eda/Edar in feather, fish scale, and fin development. M. Miura discussed Eiger, a TNF superfamily member in *Drosophila.* Eiger is expressed in the eye where it signals through Wengen and then JNK to induce cell death. It is expressed in the fat body where it plays a role in energy homeostasis.

TNF has been implicated in both protection and progression on tumors. What is known about other TNF superfamily members in this context? A. Garcia-Castro reported that many human breast cancer tumors and cell lines have high levels of APRIL. Cell lines proliferated in the presence of exogenous APRIL, suggesting that APRIL may contribute to autocrine growth. B. Dougall demonstrated a role for RANKL in spontaneous and induced mammary tumors. Treatment with an inhibitor of RANKL delayed the onset of chemically viral-induced tumors. Q. Liu et al. reported that FasL signals in vivo contribute to lung tumor growth.

What are the mechanisms by which TNF superfamily members promote tumors? Z. Qin reported that mice deficient in TNFRI and II spontaneously rejected plasmacytomas. Apparently TNF is required for the induction of myeloid derived suppressor cells (MDSC) that inhibit tumor growth. Liu and colleagues suggested a similar mechanism for FasL in enhancing lung cancer in its ability to induce production of PGE2 that recruit MDSC.

Inflammation can be both good and bad for cancer. How do we determine when TNF is a tumor promoter and when it contributes to tumor defense?

How do we determine when in tumor progression and for which types of cancer TNF agonism or antagonism is an optimal therapeutic approach? Are there diagnostic markers that might help with this? TNF contributes to squamous cell carcinomas (Ulvmar), lung cancer (Maru), and ovarian cancer (Balkwill). In the case of squamous cell carcinomas, TNF appears to promote tumors through its induction of an inflammatory response that inhibits cell mediated immunity. In the case of lung cancer, TNF enhances metastases by inducing S100A8/A9, which recruits tumor cells. Inhibition of TNF resulted in decreased ovarian tumor growth in mice. TNF produced by the tumors appeared to be acting through the TNFR1 in a paracrine factor to enhance tumor growth. Small trials of TNF inhibitors in humans with ovarian cancer have resulted in disease stabilization. Thus far, diagnostic markers are not available that will allow the distinction between TNF's tumor inhibiting and enhancing activities.

Chapter 2
TNF Conference 2009: Beyond Bones – RANKL/RANK in the Immune System

Andreas Leibbrandt and Josef M. Penninger

The Role of the RANKL–RANK–OPG Axis in Bone Remodeling

Given the huge impact that bone loss and fractures associated with bone loss have on our healthcare system, it is not surprising that many laboratories concentrated their efforts to understand the underlying molecular mechanisms of bone metabolism in order to devise new strategies to attenuate bone loss. One major research focus has been (and still is) the identification of genes controlling osteoclast function and osteoclastogenesis, respectively. The landmark discovery of three essential genes at the end of last century has moved bone research into a new era. These are the genes for (i) receptor activator of NF-κB (RANK) [1]; (ii) its ligand RANKL [1–3]; and (iii) osteoprotegerin (OPG), the natural decoy receptor for RANKL [4].

In particular, the generation of RANKL knockout mice for the first time revealed that this molecule is essential for osteoclastogenesis in vivo: $RANKL^{-/-}$ mice develop severe osteopetrosis and have a defect in tooth eruption resulting from a complete lack of osteoclasts [5]. These findings were later corroborated by the phenotype of mice in which the signaling receptor for RANKL, RANK, was deleted from the mouse germline: $RANK^{-/-}$ mice phenocopied $RANKL^{-/-}$ mice, i.e., they are osteopetrotic, have a defect in tooth eruption, and lack all osteoclasts [6, 7]. These findings unambiguously established the pivotal role of RANKL–RANK interactions for the development of bone-resorbing osteoclasts. This process is naturally counteracted and balanced by OPG, which – as a decoy receptor – sequesters RANKL and thereby prevents binding of RANKL to its cognate signaling receptor RANK. Importantly, these knockout experiments also showed that in contrast to calciotropic factors or cytokines, such as vitamin D3, PTHrP, IL-1β, or TNF-α, which all affect osteoclastogenesis at distinct stages of development [8, 9], only RANKL and RANK are essential for osteoclast development [5– 7].

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RANKL–RANK Signaling in the Immune System

Lymph Node Development

The detailed anatomical analysis of both RANKL$^{-/-}$ and RANK$^{-/-}$ knockout mice revealed an entirely unexpected phenotype – the complete absence of all lymph nodes [5–7]. Other TNF family molecules had been previously shown to play important roles in the development and organization of secondary lymphoid tissues, such as lymphotoxin-α (LTα) [10], LTβ [11, 12], TNFR1 [13], or LTβ receptor (LTβR) [14]. For example, LTα$^{-/-}$ mice lack all lymph nodes, Peyer's patches, and follicular dendritic cells, and present with an altered splenic architecture [12]. LTβ$^{-/-}$ mice also lack Peyer's patches (PP), peripheral lymph nodes, splenic germinal centers, and follicular dendritic cells. However, most LTβ$^{-/-}$ mice still retain mesenteric (mLNs) and cervical lymph nodes (cLNs), pointing towards an LTβ-independent function of LTα in the development of cLNs and mLNs [11, 12]. The knockout of the receptor for LT$\alpha_1\beta_2$ heterotrimers, LTβR [15], results in mice lacking all lymph nodes, and these mice also lack Peyer's patches (PPs) and colon-associated lymphoid tissues [14]. Thus, the lack of lymph nodes in LTα$^{-/-}$ mice might be best explained by defective LTβR signaling since LTβR$^{-/-}$ mice also lack all lymph nodes while LTβ$^{-/-}$ mice still retain the ability to form cLNs and mLNs. The observation of some LNs in LTβ-deficient mice would, however, argue that another cytokine cooperates with LTα in the development of mLNs and cLNs.

Since mice with disrupted LT signaling all seem to lack PPs, it was initially assumed that PP and LN development would always be genetically linked. The analysis of RANKL$^{-/-}$ and RANK$^{-/-}$ mice, however, proved that these processes are not necessarily genetically coupled: both RANKL and RANK mutant mice display intact splenic architecture, lack all lymph nodes but develop PPs normally, though these PPs are smaller than in wild-type mice [5–7]. In addition to mutant mice, patients with an osteoclast-poor form of autosomal-recessive osteopetrosis (ARO) due to RANKL mutations [16] also lack lymph nodes, showing that RANKL–RANK signaling also controls lymph node formation in humans [16].

Several cell types, such as fibroblasts, macrophages, reticular cells, and endothelial cells, are required to form lymph nodes [17]. After formation of the LN anlage, the primordial tissue is seeded by T and B cells and CD4^{+}3^{-}LTb^{+} cells that differentiate into NK cells, APCs, and follicular cells to form mature compact nodes [18]. Cells expressing RANKL and RANK are present in "mature" LNs, located mainly in the cortical areas adjacent to subcapsular sinuses [3]. Since both RANKL and RANK are also expressed in spleen and PPs, restricted RANKL–RANK expression cannot account for the selective lack of LNs in RANKL and RANK knockout animals. Transfer experiments of RANKL$^{-/-}$ fetal liver cells into immunodeficient Rag1$^{-/-}$ mice indicate that the lack of LNs in RANKL$^{-/-}$ cannot be attributed to a cellular homing defect of RANKL-deficient lymphocytes. Transfer of wild-type bone marrow cells into newborn RANKL-mutant mice also does not rescue LN formation [5].

Another study correlated defective LN development in RANKL$^{-/-}$ mice with a significant reduction of $\alpha_4\beta_7{}^+CD45^+4^+3^-$ inducer cells [19]. In LT$\alpha^{-/-}$ mice, these hematolymphoid precursor cells also showed impaired colonization and cluster formation in the LN anlage. Transgenic overexpression of RANKL did not restore LN development in LT$\alpha^{-/-}$ mice, suggesting that LT$\alpha_1\beta_2$ expression is required on $\alpha_4\beta_7{}^+CD45^+4^+3^-$ inducer cells [19]. Thus, it appears that both RANKL and LTs regulate LN genesis by controlling the colonization and cluster formation of $\alpha_4\beta_7{}^+CD45^+4^+3^-$ cells during development of the early LN anlage. Since the defects in the RANKL/RANK or LT/LTβR system did apparently not affect the initiation of colonization by $\alpha_4\beta_7{}^+CD45^+4^+3^-$ cells, neither RANKL nor LT seems to be intrinsically required for the formation of the LN anlage, but rather required for further development of the LN anlage [19]. Although some light has been shed on LN development over the last years, the exact cellular and molecular roles of RANKL–RANK interactions on LN organogenesis as well as the link between LN and PP formation still await experimental resolution.

RANKL–RANK Signaling in T-Cell–Dendritic Cell Interactions

Multiple subtypes of dendritic cells (DCs) exist, including DCs that are specialized cells for capturing and processing foreign antigens. In tissues, DCs present in an immature state are unable to stimulate T cells, but if DCs encounter antigens, they mature in response to inflammatory stimuli or danger signals [20]. Mature DCs that have captured antigens present them to antigen-specific T cells in the T-cell zone of secondary lymphoid organs to which they have migrated through afferent lymphatics. These T-cell zones represent a special environment for DC, T-, and B-cell interactions required to coordinate and initiate adaptive immune responses [20]. Antigen-loaded DCs directly interact with naive antigen-specific T cells within the T-cell areas of lymph nodes, and shortly after such T–DC interactions, these DCs are eliminated [21]. DC elimination through apoptosis requires production of TNF family molecules TRAIL, FasL, and TNF-α by activated T cells. Induction of DC death shortly after antigen presentation is essential as evidenced in patients with autoimmune lymphoproliferative syndrome (ALPS). ALPS is an inherited disease with defects in lymphocyte homeostasis and apoptosis. Patients manifest with lymphocytosis and autoimmune disorders. In type II ALPS, accumulation and prolonged survival of DCs was mapped to mutations in caspase 10 rendering DCs resistant to TRAIL-mediated apoptosis [22]. Thus, mature antigen-presenting DCs have to be effectively eliminated for excessive immune responses to be avoided, and the life span of DCs might thus be an important checkpoint to control immunotolerance, priming of the immune response, and inflammation.

While RANKL is not expressed on naive $CD4^+$ or $CD8^+$ T cells, surface RANKL is detected on $CD4^+$ cells as early as 4 h after anti-CD3/CD28 stimulation, with a peak at 48 h and sustained at high levels until at least 96 h. RANKL expression on $CD8^+$ T cells follows similar kinetics upon stimulation [23]. RANK surface

expression is found on DCs from various sources – on mature bone marrow-derived DCs and on freshly isolated lymph node or splenic DCs – and RANK expression on DCs can be increased by overnight culture. However, RANK is absent from freshly isolated lymph node B cells, lymph node-derived T cells, and most thymocyte subsets [24]. As for T–DC interactions, RANKL on the surface of activated T cells interacts with RANK on DCs and by doing so mediates at least in part DC survival via Bcl-x_L induction and upregulation of the costimulatory factor CD40 on DCs [1, 24, 25].

Since RANKL–RANK signaling leads to the activation of the anti-apoptotic Ser/Thr kinase Akt/PKB, NF-kB, and ERK through signaling complexes involving TRAF6, c-Cbl, Gab2, and c-Src on mature DCs and osteoclasts [26], induction of anti-apoptotic signaling cascades seems to be at least partially responsible for RANKL-mediated DC survival [8]. Moreover, treatment of antigen-pulsed mature DCs with soluble RANKL in vitro was shown to enhance the number and persistence of antigen-presenting DCs in draining LNs in vivo [25]. RANKL treatment also increased antigen-specific primary T-cell responses. Significant memory responses, however, were observed only in mice injected with DCs previously treated with RANKL [25]. Since RANKL stimulates production of cytokines in DCs, such as IL-1, IL-6, IL-12, and IL-15 [23, 27], it might well be that increased primary and memory T-cell responses following vaccination with RANKL-treated DCs are due to enhanced/altered cytokine production. Moreover, the observed enhanced T-cell responses could be explained by increased numbers of antigen-pulsed DCs in draining LNs [25].

Initially it has been proposed that RANKL and CD40L, a TNF family member and close relative of RANKL, share functional similarities. Both CD40L and RANKL are expressed on activated T cells and enhance the activation and survival of DCs [1, 24]. However, in contrast to the CD40L-CD40 system, RANKL–RANK signaling from T cells to DCs does not alter the expression of cell surface molecules, such as MHC class II, CD80, CD86, and CD54. Whereas CD40L is primarily expressed on activated $CD4^+$ T cells, RANKL is expressed on both activated $CD4^+$ and $CD8^+$ T cells [23, 28]. In addition, the kinetics of RANKL and CD40L expression on activated T cells are different: RANKL expression following initial T-cell activation peaked around 48 h and remained high at least until 96 h, while CD40L reached maximal levels between 6 and 8 h and thereafter was downregulated [23, 29].

CD40L–CD40 interactions might control the initial priming stage whereas RANKL–RANK could act at later time points during the immune response. For instance, although CD40L–CD40 interactions are crucial for the generation of antigen-specific T-cell responses in vivo [30], both CD40L- and CD40-deficient mice can still mount protective $CD4^+$ T-cell responses upon viral challenge in vivo [31]. Thus, some pathogens must be able to activate $CD4^+$ T-cell responses independent of CD40L–CD40 expression. One system to potentially mediate CD40L–CD40-independent $CD4^+$ T-cell priming was suggested to be RANKL and RANK. This was based on the following experimental results: blocking RANKL in vivo by soluble RANK-Fc had no effect on the priming of lymphocytic

choriomeningitis virus-specific T cells but impaired proliferation of $CD4^+$ T cells to the viral antigen at later time points after the initial infection, something that was particularly apparent when CD40 was not present [27]. In addition, at later stages of the immune response, it has been reported that RANKL leads to CD40L-independent activation of $CD4^+$ T helper cells [27]. Although CD40L and RANKL might cooperate, it has been suggested that these two ligand-receptor systems also exhibit fundamentally different functions in the control of immune responses: CD40L might regulate priming of T- and B-cell responses while RANKL could have a role in memory T-cell responses.

The decoy receptor for RANKL, OPG, is also expressed in DCs [32]. OPG was shown to bind the TNF family molecule TRAIL, which is commonly produced by activated T cells to induce apoptosis of mature antigen-presenting DCs [32, 33]. This would in fact suggest that OPG can fine-tune DC survival through sequestration of RANKL and/or TRAIL. Since the binding affinity of OPG to TRAIL is ~10,000 times less than its affinity to RANKL [3], it is unclear whether OPG–TRAIL interactions have any functional relevance in vivo. Nonetheless, various groups are trying to control DC fate via RANKL–RANK–OPG to modulate DC survival in vivo and to enhance the efficacy of DC-based vaccinations for anti-tumor therapy or the treatment of autoimmune diseases. In the final analyses of all the published genetic and functional studies on RANKL, RANK, and OPG, it appears that although these molecules can influence some aspects of lymphocyte and DC functions, none of these molecules plays a yet clearly established essential role in T-, B-, or dendritic cell development and functions that cannot be compensated for by other molecules such as CD40L/CD40. Since we and others have shown that expression of RANKL and OPG is strongly influenced by sex hormones [34], it is tempting to speculate that the RANKL–RANK–OPG system may contribute to gender-specific differences in immunity and might help explain the higher incidence of autoimmune diseases like arthritis in women.

RANKL–RANK Control the Development of $AIRE^+$ Medullary Epithelial Cells

In our initial chapter describing the RANKL knockout phenotype, we also described altered T-cell development and expression of RANKL on primarily $CD4^-8^-$ thymocyte progenitors [5]. Since RANK knockout mice exhibited apparently normal thymocyte development [6, 7], it was unclear whether RANKL–RANK might indeed have some primary functions in the thymus. Recent studies surprisingly provided evidence for such a primary function of RANKL–RANK in the thymus: RANKL–RANK controls the development of AIRE-positive thymic medullary epithelial cells.

Cortical epithelial cells (cTECs) positively select thymocytes, which subsequently migrate to the medulla where thymocytes interact with medullary thymic epithelial cells (mTECs) expressing costimulatory molecules and self-tissue-restricted antigens (TRAs) [35]. Expression of self-antigens on medullary thymic

epithelial cells is regulated in part by the transcription factor AIRE (autoimmune regulator). Mutations in AIRE result in multiorgan autoimmune disease in humans and autoimmunity in AIRE$^{-/-}$ mice [36]. Despite the identification of a common progenitor of cTECs and mTECs [37], the developmental pathway leading to AIRE^{+} mTECs was not entirely clear. Intriguingly, an intrathymic CD4^{+}3^{-} lymphoid tissue inducer (LTi) cell population expresses RANKL and it appears that RANKL from thymic LTi cells is required to trigger the development of CD80^{-}AIRE^{-} mTECs into CD80^{+}AIRE^{+} mTECs [38]. In line with these data, AIRE is absent in thymi from RANK$^{-/-}$ mice and transplantation of RANK$^{-/-}$ thymic tissue under the kidney capsule of nude mice resulted in inflammatory infiltrates in liver and autoantibodies to several tissues, which parallels the phenotype observed after transplantation of AIRE$^{-/-}$ thymic stroma [36, 38].

These findings suggest a role of RANKL–RANK in the regulation of central tolerance, for which LTα–LTβR signals have also been shown to be important [39, 40]. However, AIRE^{+} mTECs can develop in the absence of LTβR and LTα [38, 40], suggesting that some aspects of mTEC development and organization may involve both RANK and LTβR signals. Moreover, in the absence of TRAF6, the key downstream signaling adaptor of RANK, AIRE^{+} mTECs also do not develop leading to the onset of organ-specific autoimmunity [41]. Thus, RANKL expressed by LTi cells and possibly other thymic cell types can activate RANK on thymic epithelial progenitor cells to develop into AIRE^{+} mTECs. These results have been further substantiated by a series of recent papers. In one study, the authors could demonstrate that mTEC development required both RANK and CD40 signaling [42]. RANK signaling was shown to be essential for mTEC development during embryogenesis since mTECs failed to develop properly in embryonic thymus while mTECs – albeit at reduced numbers – were still detectable in postnatal mice even in the absence of RANKL. The molecule capable of compensating the loss of RANKL expression in the postnatal thymus appears to be CD40L, since development of mTECs was almost completely abolished in double mutant CD40$^{-/-}$ RANKL$^{-/-}$ mice. Moreover, CD40L- or RANKL-mediated mTEC development depended on TRAF6, NIK, and IKKβ, respectively. Thus, depending on the stage of development, cooperation between RANK and CD40 regulates mTEC development [42].

In another study, the authors showed that positively selected thymocytes express RANKL and that such RANKL-expressing T cells expand mTEC cellularity required to form the mature adult thymic medulla [43]. This effect depended on interactions with RANK expressed on mTECs, since neutralization of RANKL by RANK-Fc expression perturbed mTEC numbers and RANKL expression in mice deficient for positive selection resulted in normalization of the thymic medulla. Thus, in addition to inducer cells, RANKL produced by positively selected thymocytes can also trigger thymic medulla formation [43]. To address the potential contribution of single-positive CD4^{+} and CD8^{+} thymocytes in the process of postnatal mTEC development, a third study could show that although either CD4^{+} or CD8^{+} thymocytes were sufficient to sustain formation of a well-defined medulla, expansion of the mature mTEC population required autoantigen-specific interactions

between positively selected $CD4^+$ thymocytes bearing autoreactive T-cell receptor (TCR) and mTECs displaying cognate self-peptide-MHC class II complexes. These interactions involved the engagement of CD40 on mTECs by CD40L, which in turn was induced on the positively selected $CD4^+$ thymocytes. This antigen-specific TCR-MHC class II-mediated crosstalk between $CD4^+$ thymocytes and mTECs defines a checkpoint in thymic stromal development that appears to be essential for generating mature mTEC cells involved in the establishment of thymic tolerance [44].

RANKL–RANK Signaling in UV-Induced Immunosuppression

Surprisingly, a novel function of RANKL–RANK has been described in the skin, i.e., RANKL expression in the skin influences the number of regulatory T cells (Tregs) upon exposure to UV light [45]. Tregs are a T-cell subpopulation that maintain immunological self-tolerance and suppress excessive immune responses such as allergies or autoimmune diseases [46]. In addition to the role of DCs to induce and orchestrate immunity, it has been shown that DCs can also induce expansion of $CD4^+25^+$ Tregs [47]. In view of the fact that the activation of epidermal Langerhans cells, the skin resident (dendritic cells population, by CD40L results in severe systemic autoimmunity [48] and given the importance of RANKL–RANK (and CD40L-CD40) signaling in T-cell–DC interactions, the role of RANKL and RANK in skin was also assessed. Moreover, sun light, i.e., exposure to UV radiation, induces vitamin D3 production in the skin; vitamin D3 is one of the key triggers of osteoblastic RANKL expression during osteoclastogenesis [49].

In keratinocytes, RANKL expression is strongly upregulated following UV irradiation [45, 50]. Importantly, in contrast to transgenic overexpression of CD40L, RANKL overexpression in keratinocytes abrogated cutaneous contact hypersensitivity responses and resulted in a marked increase in Treg numbers [45]. RANK is expressed on Langerhans cells (LCs) of the skin and interactions between RANKL-overexpressing keratinocytes and RANK-expressing LCs increased their survival and rendered LCs more effective in enhancing Treg proliferation [45]. Moreover, RANKL overexpression in keratinocytes rescued the autoimmune phenotype caused by CD40L overexpression in K14-RANKL/CD40L double transgenic mice [45]. These findings provided a rationale for the long known immunosuppressive effect of exposure to ultraviolet light: UV irradiation upregulates RANKL in keratinocytes which in turn activates RANK-expressing LCs through RANKL–RANK interactions. RANKL-activated LCs preferentially trigger expansion of Tregs and thereby suppress immune reactions in the skin and other tissues. UV-mediated immunosuppression, that is, alleviation of DTH reactions in the ear after UV exposure, is impaired in mice transplanted with RANKL knockout skin. Thus, RANKL–RANK might be one link to solve a long known conundrum – why sun exposure (sun burns) are immunosuppressive. Local induction of the RANKL–RANK system in the skin could be used as a new approach for the treatment of allergies or

systemic autoimmunity through increasing Treg numbers while avoiding systemic side effects [45].

The importance of Tregs and of RANKL–RANK signals on Treg number and regulatory capacity has previously been suggested in an inflammation-induced model of type 1 diabetes. In this mouse model, islet-specific expression of TNFα can be switched off upon doxycycline administration (Tet-TNFα); in Tet-TNFα/CD80 double transgenic mice, which constitutively co-express TNFα and the co-stimulatory molecule CD80 on β cells in the islets of Langerhans, progression to diabetes depends on the duration of TNFα expression [51, 52]. In this model, inflammation activates self-reactive $CD8^+$ T cells to induce autoimmunity and diabetes, respectively, but $CD4^+25^+$ Tregs can successfully prevent β cell destruction. These Tregs have been shown to accumulate preferentially in the pancreatic lymph nodes (PLN) and islets. Their capacity to prevent diabetes appears to be dependent on RANKL signals [52]. Blockade of RANKL–RANK interactions through RANK-Fc resulted in a decrease of $CD4^+25^+$ Tregs in the PLN, consequently resulting in intra-islet differentiation of $CD8^+$ T cells into cytotoxic T cells and rapid progression to diabetes [52]. Inflammation may thus result in the RANKL–RANK-dependent generation and activation of $CD4^+25^+$ Tregs which then migrate to the inflamed tissue and draining LNs for the prevention of tissue destruction and autoimmunity by autoaggressive T cells. Moreover, RANK expression on epithelial cells might be a signal to locally tune the immune system at epithelial barriers such as the skin and the gut.

Recent data suggest that the RANKL–RANK system might also be involved in controlling intestinal Treg function [53]. RANKL is predominantly expressed on $CD4^+25^+$ Tregs and colitogenic $CD4^+$ T cells, while RANK expression is found on DCs [53]. Although neutralizing anti-RANKL monoclonal antibodies did not affect the regulatory capacity of $CD4^+25^+$ Tregs in vitro, in vivo RANKL blockage by anti-RANKL blocking mAbs abrogated $CD4^+25^+$ Treg-mediated suppression of colitis induced by the adoptive transfer of $CD4^+45RB^{high}$ T cells into SCID mice [53]. Adoptive transfer experiment using $Ly5.1^+CD4^+45RB^{high}$ cells and $Ly5.2^+CD4^+25^+$ Tregs revealed that the ratio of $CD4^+25^+$ Tregs in total $CD4^+$ T cells in inflamed mucosa was significantly decreased by anti-RANKL mAb treatment [53]. The expression of RANK on lamina propria $CD11c^+$ cells from colitic mice was significantly increased as compared to normal mice and in vitro treatment with anti-RANKL mAb suppressed the expansion of $CD4^+Foxp3^+$ Tregs in culture with colitic lamina propria $CD11c^+$ cells, suggesting that the RANK–RANKL signaling pathway might be involved in regulating the function of $CD4^+25^+$ Tregs in colitis [53]. These data now await genetic confirmation.

Functions of RANKL–RANK in Extramedullary Hematopoiesis and B Cells

In addition to T cells, $RANKL^{-/-}$ and $RANK^{-/-}$ mice have reduced numbers of mature $B220^+IgD^+$ and $B220^+IgM^+$ B cells in the spleen and lymph nodes and

disorganized B-cell areas in the spleen [5, 6, 54]. Since RANKL$^{-/-}$ and RANK$^{-/-}$ mice have no bone marrow cavities, the reduced cellularity of B cells could be due to an altered microenvironment or due to changes in the composition of stromal cells outside the bone marrow cavity that affect B-cell differentiation. For example, RANKL$^{-/-}$ mice can form extramedullary hematopoietic tissues localized at the outer surfaces of vertebral bodies [5]. This tissue exhibits morphological and phenotypic features characteristic of hematopoiesis and proliferating precursor cells. Whether these hematopoietic islands in RANKL$^{-/-}$ mice represent a defect in the homing of precursors during the switch from hepatic to bone marrow hematopoiesis, or an event secondary to defective seeding of bone marrow cavities needs to be determined in tissue specific knockout experiments.

In fetal liver cell chimeras, RANKL was found to regulate early B-cell differentiation from the B220^{+}CD43^{+}25^{-} pro-B cell to the B220^{+}CD43^{-}25^{+} pre-B-cell stage of development suggesting that the TNF-family cytokine RANKL can influence B-cell development [5]. Results in an OPG-mutant mouse strain confirmed the notion that the interplay between RANKL–RANK and its molecular decoy receptor OPG may regulate the development of B lymphocytes: ex vivo, OPG$^{-/-}$ pro-B cells exhibit enhanced proliferation to IL-7 and type 1 transitional B cells accumulate in the spleens of OPG$^{-/-}$ mice. Thus, loss of OPG may control B-cell maturation [55]. OPG is also a CD40-regulated gene in B cells and dendritic cells. Moreover, prostaglandin E2 treatment can increase the amount of RANKL messenger RNA in B220^{+} B cells in an estrogen-dependent manner [55, 56].

RANKL–RANK Signaling, T Cells and Bone Loss – The Foundation of Osteoimmunology

RANKL, RANK, and OPG are essential for controlling bone remodeling – RANKL expressed on osteoblasts activates RANK-expressing osteoclastic progenitors to differentiate into mature osteoclasts, which in turn degrade bone matrix. The decoy receptor OPG secreted from osteoblasts and other cell types binds to RANKL and thus inhibits osteoclast activation and bone resorption. However, a key finding was that RANKL is not exclusively expressed on osteoblasts, but RANKL is also strongly induced in T cells following antigen receptor engagement.

We therefore originally posed the question if T-cell-derived RANKL could regulate the development and activation of osteoclasts, i.e., would activated T cells modulate bone turnover via RANKL? In an in vitro cell culture system of hematopoietic bone marrow precursors, we were indeed able to show that activated CD4^{+} T cells can induce osteoclastogenesis. On the other hand, osteoclastogenesis was efficiently blocked by addition of the physiological decoy receptor OPG and was not dependent on T-cell-derived cytokines, such as IL-1 or TNF-α, which also upregulate RANKL expression in stromal cells [28]. Activated T cells also affect bone physiology in vivo, as judged by the severe osteoporotic phenotype of Ctla4$^{-/-}$ mice, in which T cells are spontaneously activated. The adoptive transfer of Ctla4$^{-/-}$ T cells decreased bone mineral density in lymphocyte-deficient

Rag1$^{-/-}$ mice and blockage of RANKL–RANK signaling by continuous OPG administration diminished the osteoporotic phenotype of Ctla4$^{-/-}$ mice [28, 57]. These results established the pivotal role of systemically activated T cells in resorbing bone through upregulation of RANKL, thereby stressing the importance of T cells as crucial mediators of bone loss in vivo. Moreover, these results also provided a novel paradigm for immune cells as key regulators of bone physiology and gave birth to the field of osteoimmunology to account for the interplay between the immune system and bone metabolism [58]. It also gave a new perspective to certain inflammatory or autoimmune diseases, such as rheumatoid arthritis, which besides their own characteristic etiopathologies show bone loss as an accessory symptom in patients.

RANKL–RANK Are the Molecular Triggers of Bone Loss in Arthritis

Rheumatoid arthritis (RA) is a very frequent autoimmune disease that affects around 1% of people. RA is characterized by chronic inflammation of synovial joints, progressive destruction of cartilage and bone, severe joint pain, and ultimately life-long crippling [59]. Since osteoclasts are found at areas of bone erosion in RA patients [60], it was tempting to speculate that RANKL might be a key mediator of bone erosion in RA patients. In addition, T cells present in the inflamed joints and draining lymph nodes produce many pro-inflammatory cytokines known to induce RANKL expression [61]. Consequently, we therefore analyzed the contribution of RANKL to bone loss in the adjuvant-induced arthritis model (AdA) in Lewis rats as this experimental model mimics many of the clinical and pathological features of human RA, i.e., severe inflammation in bone marrow and soft tissues surrounding joints, accompanied by extensive local bone and cartilage destruction, loss of bone mineral density and crippling [62].

Inhibition of RANKL through OPG did not influence the severity of inflammation as determined by joint swelling. However, OPG treatment abolished the loss of mineral bone in inflamed joints of arthritic rats in a dose-dependent manner. Bone destruction in untreated arthritic animals correlated with a dramatic increase in osteoclast numbers, which was not observed in AdA rats receiving OPG treatment [28]. As a consequence, OPG-treated arthritic rats exhibited minimal loss of cortical and trabecular bone, whereas untreated AdA animals developed severe bone lesions characterized by partial to complete destruction of cortical and trabecular bone, and positively affected erosion of the articular cartilages. In further pre-clinical studies in AdA rats, a single OPG injection was efficient to inhibit joint erosions for several days and produced sustained anti-erosive activity; this treatment was most effective when initiated early in the disease [63]. These results unequivocally demonstrated the importance of RANKL in mediating joint destruction and bone loss in AdA arthritis.

An important step in the etiology of arthritis is the alteration of cartilage structures leading to cartilage collapse in the joints. It is not quite clear whether cartilage destruction occurs independently of bone loss, or whether damage to the subchondral bone indirectly causes cartilage deterioration [64]. In AdA rats, partial or complete erosion of cartilage in the central and peripheral regions of joint surfaces is observed, which can be preserved by OPG administration. Neither cartilage erosion nor matrix degeneration in the centers of joint surfaces occurred in OPG-treated AdA rats [28]. OPG could protect the cartilage by maintaining the underlying subchondral bone and insulating the overlying cartilage from the inflammatory cell infiltrates in the bone marrow. Since both RANKL and RANK are expressed on chondrocytes [3, 54], and RANKL$^{-/-}$ and RANK$^{-/-}$ mice exhibit significant changes in the columnar alignment of chondrocytes at the growth plate [5, 7], it is possible that the RANKL–RANK pathway plays a direct role in cartilage growth and cartilage homeostasis. Arthritis can also develop in the absence of activated T cells, as shown in the K/B×N serum transfer model of spontaneous autoimmunity [65]. Although RANKL-deficient mice still develop inflammation in the K/B×N serum transfer arthritic model, RANKL$^{-/-}$ mice showed a dramatic reduction in bone erosion [66]. However, in contrast to the AdA model cartilage damage was still observed in both arthritic RANKL$^{-/-}$ and arthritic control mice, but a trend toward milder cartilage damage in the RANKL$^{-/-}$ mice was noted. Thus, RANKLis not essential for cartilage destruction, but most likely has an indirect modulatory role [66].

In all animal model of arthritis examined, such as in TNF-α- or collagen-induced arthritis [67], inhibition of RANKL prevented bone erosion [68]. The correlation between RANKL expression in inflamed joints and arthritis appears to be absolute. RANKL is also expressed in inflammatory cells isolated from the synovial fluid of patients with adult or juvenile RA and patients with osteoarthritis [28]. Consistent with results obtained in rats, both synovial T and non-T-cell populations from RA patients expressed RANKL. In line with this, the capacity of human T cells expressing RANKL to directly induce osteoclastogenesis from human monocytes has been confirmed [69]. Moreover, RANKL expression is also upregulated in rheumatoid synovial fibroblasts which in turn can efficiently induce osteoclastogenesis in vitro [70]. All these results confirmed the original findings in rodent adjuvant arthritis and showed that RANKL/RANK signaling from inflammatory cells such as T cells and synoviocytes is the essential pathway of bone destruction in arthritis. Taken together, RANKL is the trigger of bone loss and crippling in all animal models of arthritis studied so far, making RANKL a prime drug candidate for therapeutic intervention in different forms of arthritis. Importantly, recent phase II clinical trials showed that inhibition of RANKL in human RA patients has no apparent effects on the inflammation but prevents bone loss at the site of inflammation [71].

These findings also provided a molecular explanation for the observed bone loss in many other human diseases with chronic activation of the immune system, such as adult and childhood leukemia [72], chronic infections such as HIV [73], autoimmune disorders such as diabetes mellitus, Chron's disease or lupus erythematosus [74], and allergic diseases such as asthma [75]. These bone disorders can all cause

irreversible crippling and thereby pose a tremendous burden on the quality of life of a huge number of patients. For example, some patients with lupus require hip replacement surgery and essentially all children that survive leukemia experience severe bone loss and growth retardation. In addition, T-cell-derived RANKL also contributes to alveolar bone resorption and tooth loss in an animal model that mimics periodontal disease in humans [76]. This was initially shown by transplanting human peripheral blood lymphocytes from periodontitis patients into immune-compromised NOD/SCID mice and by challenging these mice with a bacterium (*Actinobacillus actinomycetemcomitans*) that can cause periodontitis in humans. In response to stimulation by that microorganism, $CD4^+$ T cells upregulated RANKL and induced osteoclastogenesis and bone destruction at the site of infection. Most importantly, inhibition of RANKL significantly reduced alveolar bone resorption around the teeth [76].

Further experiments showed that blocking RANKL might also help to prevent periodontitis in diabetic patients, which are at high risk of developing periodontitis [77]. Nonobese diabetic (NOD) mice – an experimental model for human type 1 diabetes – were orally infected with *A. actinomycetemcomitans* and it turned out that diabetic NOD mice manifested significantly higher alveolar bone loss than non-diabetic control mice. The observed bone loss was correlated with pathogen-specific proliferation and RANKL expression in local $CD4^+$ T cells and could be reduced by inhibition of RANKL [77]. These data demonstrated that specific interference with RANK activation might be of therapeutic value for treating inflammatory bone disorders, such as human periodontitis, or even bone loss in diabetic patients at high risk.

Since disease pathogenesis correlates with the activation of T cells in many osteopenic disorders, the obvious question arises why then T cells in our body – of which a certain proportion is activated at any time due to fighting off the universe of foreign antigens to which we are permanently exposed – do not cause extensive bone loss? Likewise, in some chronic T-cell and TNF-α-mediated diseases such as ankylosis spondylitis [78], T-cell activation does not result in bone loss. One mechanism that counteracts RANKL-mediated bone resorption of activated T cells is the upregulation of interferon-γ (IFN-γ). IFN-γ blocks RANKL-induced osteoclastogenesis in vitro and IFN-$\gamma R^{-/-}$ mice are more prone to osteoclast formation in a model of endotoxin-induced bone resorption as compared to their wild-type littermates [79]. In line with this study, IFN-γ receptor knockout mice exhibited also enhanced severity in the collagen-induced model of T-cell-mediated autoimmune arthritis [80]. Mechanistically, IFN-γ triggers TRAF6 degradation and therefore blocks RANK signaling, thereby preventing uncontrolled bone loss during inflammatory T-cell responses. Moreover, T-cell-derived IL12 alone and IL12 in synergy with IL18 can inhibit osteoclast formation in vitro [81], and IL-4 can abrogate osteoclastogenesis through STAT6-dependent inhibition of NF-κB signaling [82]. Thus, multiple T-cell-derived cytokines might be able to interfere with RANK(L) signaling and therefore block osteoclastogenesis and osteoclast functions.

A defined subset of $CD4^+$ T helper cells, namely Th17, appears to function as osteoclastogenic helper T cells [83]. Derived from naive T cells by a distinct

mechanism than Th1 or Th2 cells [84], Th17 cells are defined by IL-17 production and are causative triggers for a variety of autoimmune diseases [85]. Since IL-17 has been described as a potent inducer of RANKL expression and can be found in the synovial fluid from RA patients [86], Th17 cells were a prime candidate population for the osteoclastogenic Th cell subset. Indeed, Th17 cells, but not Th1, Th2, or Treg cells, can stimulate osteoclastogenesis in vitro [83], indicating that Th17 cells may act as mediators of bone destruction in RA patients. The positive effect of Th17 cells on osteoclastogenesis is believed to be balanced by Th1 and Th2 cells mainly through their production of the cytokines IFN-γ and IL-4, respectively [83]. Thus, targeting Th17 might also be a potent approach to prevent bone destruction associated with T-cell activation in RA and other inflammatory bone diseases.

RANKL Inhibition as a New Therapy to Control Bone Loss in Human Patients

Several years ago, a fully human monoclonal IgG2 antibody to block human RANKL, Denosumab, has been developed and is currently in late-stage clinical trials for postmenopausal osteoporosis, cancer-metastases-induced bone loss, and RA [71, 87, 88]. Importantly, binding of Denosumab to RANKL is selective to RANKL [89]. Subcutaneous application of Denosumab at 3- or 6-month intervals over a period of 12 months to 412 postmenopausal women with low bone mineral density (BMD) in a randomized, placebo-controlled, dose-ranging phase 2 study resulted in a sustained decrease in bone turnover and a rapid increase in BMD [90]. In another 2-year randomized, double-blind, placebo-controlled study with 332 osteoporotic, postmenopausal women, twice-yearly subcutaneous application of Denosumab significantly increased BMD and decreased bone turnover markers in postmenopausal women [87]. In a similar study in patients with breast cancer and patients with multiple myeloma, a single dose of Denosumab resulted in the rapid and sustained decrease of bone turnover [91]. Lastly, in a multicenter, randomized, double-blind, placebo-controlled, phase II study with 218 patients with RA receiving methotrexate treatment, RANKL inhibition by Denosumab also increased BMD and protected from bone loss at the site of the inflamed joint without affecting the severity of inflammation [71]. In all cases, Denosumab administration was well tolerated and as good or superior to current standard medication.

Conclusions

The identification of RANKL, its receptor RANK, and the decoy receptor OPG as the key regulators for osteoclast development and the activation of mature osteoclasts has provided the rational molecular framework to understand bone physiology and opened the doors for the development of highly effective drugs to treat bone loss in literally millions of patients. The finding that RANKL is produced by activated

T cells and that activated T cells in turn can directly induce osteoclastogenesis, also provided a novel molecular paradigm for bone loss associated with diseases having immune system involvement, such as T-cell leukemias, autoimmunity, various viral infections, RA, or periodontitis. In addition, RANKL–RANK control development of mammary glands in pregnancy and the formation of lymph nodes and AIRE+ thymic medullary epithelial cells. Moreover, RANKL might be one molecular link between sun exposure and Treg mediated immunosuppression. Importantly based on all available data, inhibition of RANKL function might be the most rational therapy to ameliorate many osteopenic conditions and prevent bone destruction and cartilage damage, e.g., in osteoporosis and arthritis, thereby dramatically enhancing the lives of millions of patients.

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Chapter 3
The Edar Subfamily in Hair and Exocrine Gland Development

Marja L. Mikkola

Introduction

The expansion of the tumor necrosis factor receptor (TNFR) family in vertebrate lineage appears to be correlated with acquisition of vertebrate evolutionary novelties, such as the adaptive immune system, bone, and cutaneous appendages [29]. Skin appendages are class-defining features of vertebrates and many TNFRs have recently been implicated in morphogenesis of feathers, hair follicles, teeth, and mammary glands [33]. Edar, Xedar, and Troy form a subfamily within the TNFRs based on the sequence similarity of their ligand-binding domains [29]. Like other TNFRs, these receptors utilize Trafs to activate downstream pathways such as NF-κB ([33]; for details on NF-κB signaling, see [24]). The ligands for mammalian Edar and Xedar, Eda-A1, and Eda-A2 are generated through alternative splicing of ectodysplasin (Eda) and show absolute receptor specificity[3, 54]. The physiological ligand for Troy has remained elusive (see below). As several reviews on the role the Edar subfamily/NF-κB in morphogenesis of skin appendages have been published [7, 9, 33, 34, 36], I will here briefly summarize only the most recent progress in understanding the developmental function of the Edar subfamily in vertebrates, with focus in hair follicles and glands.

Conservation of the Edar Subfamily

Sequences for the Edar subfamily members can be found in all osteichthyan clades (mammals, birds, amphibians, and teleost fishes) with the exception that Xedar appears either to be highly divergent or lost in teleost species [41]. Also Eda-A1 and Eda-A2 splice variants have been experimentally identified in all

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osteichthyans tested, but the receptor specificity of the non-mammalian ligands has been questioned [41]. Only one Edar-like gene has been detected in invertebrate chordates (e.g., *Ciona* and lancelet) but it is yet to be examined whether it is involved in epithelial morphogenesis. Edar and Troy are co-expressed in many developing organs, including teeth, hair follicles, mammary buds, and feather follicles [14, 33]. Due to the apparently low expression levels of *Xedar*, no clear picture on its expression pattern in mouse embryos has emerged [42, 54] but in feather buds its expression succeeds that of *Edar* and *Troy* [14].

Current evidence suggests that Eda-A1/Edar has an ancestral role in regulating the development of skin appendages in all vertebrates while considerably less is known about the function of Troy and Xedar. Inactivating mutations in Eda, Edar, or the Edar-specific signal transducer Edaradd result in a highly similar condition in all vertebrates (exemplified by human, mouse, dog, and bovine) known as hypohidrotic (or anhidrotic) ectodermal dysplasia (HED) [4, 15, 36]. Mutation of *Eda* is the most common cause of HED, which typically manifests in boys because of the X-chromosomal location of the *Eda* gene. HED is characterized by sparse and thin body and scalp hair, missing and malformed teeth, dry skin, reduced salivation, lacrimation, and mucus production in respiratory and gastrointestinal tracts, and diminished or absent eccrine sweat pores [6]. HED can also be caused by mutation in genes essential for canonical NF-κB activity [8], or in mice by expression of a non-degradable I-κBα under the ubiquitous β-catenin promoter [47] (referred to here as I-κBα ΔN mice) suggesting that at least in mammals, the downstream effects of Edar are largely mediated by NF-κB [34]. In chicken, knockdown experiments have not been reported, but expression of dominant negative or constitutively active forms of Edar, Xedar, or Troy in ovo all disturb the normal patterning of feather follicles in similar albeit not an identical manner [14]. Analysis of *Eda* and *Edar* mutant zebrafish uncovered a critical role of this pathway in scale and (pharyngeal) tooth morphogenesis, and in other dermal elements [22]. Fish scales are bony elements that develop in the dermis underlying the epidermis. Perhaps surprisingly, deficiency in the Eda pathway affected the development of all fins, including the paired ones [22] while there has not been any previous indication of a role of the Edar subfamily in limb development. Apical ectodermal ridge is an important signaling center regulating patterning of tetrapod limbs as well as of paired fins. *Edar* and *Troy* are both expressed in the apical ectodermal ridge of the mouse limb bud, but this is not associated with active NF-κB signaling[36] (Mikkola, unpublished data).

Edar Subfamily in Hair Follicle Development

The development of hair follicles, as other skin appendages, is initiated by the formation of dense epithelial patches known as placodes that appear concomitant with condensation of the underlying dermal cells. In the next stage, proliferation of placode cells generates a hair follicle bud that grows rapidly downwards. The tip of the hair germ encases the dermal condensate which from now on is called

dermal papilla. The lower portion of the hair follicle renews throughout lifetime via repeated cycles of regression (catagen), rest (telogen), and regrowth (anagen) (for reviews see [17, 49].

The role of Eda-A1/Edar/NF-κB in hair follicle development has been extensively studied using both loss- and gain-of-function mouse models, and it is evident that the Edar pathway is important in several stages of hair follicle development from specification of placode cells to hair shaft differentiation [9, 34, 49]. The mouse coat consists of four different hair types (guard, awl, auchene, and zigzag) that are produced in three successive waves of hair follicle induction. A hallmark of the Eda pathway deficiency is the lack of the first hair follicles (and thereby guard hairs) due to defective primary hair placode formation. Recent studies using transgenic mice with inducible Eda-A1 expression [12], or injection of recombinant Fc-Eda-A1 into pregnant dams [19], have revealed that guard hairs depend on Eda only shortly during placode formation and its initial downgrowth, but they seem to acquire Eda autonomy concomitant with the establishment of the dermal papilla [25]. In the absence of Eda, the primary hair placodes are induced though, and follicular keratinocytes show some sign of clustering, but get arrested and no proper placodes form [48, 56]. The aberrant pre-placodes can be visualized by the expression of certain placode marker genes such as *Dickkopf-4* (*Dkk4*) and *Lrp4* [16]. Accordingly, some aggregation of basal epithelial cells could be detected in *Eda* and *Edar* mutant zebrafish, but placodes were disorganized and failed to develop further indicating an evolutionary conserved role for Edar in placode formation [22]. On the other hand, excess of Edar pathway activity either in vitro or in vivo generates enlarged hair placodes [37, 39]. Collectively, these data indicate that Edar activity, via a mechanism that is still incompletely understood, instructs keratinocytes to adopt a hair follicle fate instead of epidermal fate and leads to stabilization of nascent hair placodes.

Intricate Interplay Between Edar and Wnt Pathways Regulates Hair Development

Recent studies have revealed a complex interplay and interdependence between the Wnt and Eda-A1/Edar pathways. In embryonic hair (and tooth and mammary) epithelium, high Wnt signaling activity correlates with Edar/NF-κB activity [42, 56]. The canonical Wnt pathway, mediated by β-catenin and Tcf/Lef family of transcription factors, is absolutely necessary for the initiation of all hair follicle types [49] and its forced activation in embryonic ectoderm leads to precocious and superfluous hair placode induction [40, 55]. It seems that Wnts, along with a positive autoregulatory loop of Edar [37] and activinβA expressed in the dermal condensate [28], are responsible for the focal upregulation of *Edar* expression in hair placodes, while Edar on the other hand induces the expression of *Wnt10b* in the placode [56]. Thus, Edar activity is required to refine the pattern of Wnt/β-catenin activity and to maintain this activity after placode induction. Surprisingly, Edar also strongly

upregulates the expression of the Wnt inhibitor *Dkk4* [16], which when ectopically expressed, inhibits hair placode formation [50]. The correct spacing of hair follicles is thought to arise as a result of competition between both placode promoting (such as Wnt) and inhibiting signals (such as Dkk), and Eda and Wnt pathways have emerged as the key players involved in this process. While the Wnt pathway is evidently upstream of the Edar/NF-κB pathway in placode induction [56], the two pathways may act in parallel in stabilizing emerging placodes possibly through regulation of the same or related target genes, as forced stabilization of epithelial β-catenin activity can compensate for absence of Eda during primary hair placode formation [40].

Interaction between Wnt and Edar is implicated also in determining hair fiber characteristics. Mouse undercoat consists of zigzag hairs typified by multiple bends. Straightening of zigzag hair fibers is observed in both *Eda* null and *Eda* overexpressing mice and it is apparent that while initiation and morphogenesis of zigzag hair follicles is independent of Eda-A1/Edar, generation of zigzag hair shafts requires continuous, but strictly spatio-temporally controlled Edar activity [12, 34, 38]. The *Eda* null zigzag hair phenotype is shared by a mouse model with suppressed, but not completely abolished Wnt activity. This characteristic appears to be mediated at least in part by Sonic hedgehog (Shh), a likely target gene of both Eda and Wnt pathways [21, 44].

Transcriptional Targets of Edar

Several laboratories have been in the hunt for the target genes of Edar [11, 16, 37, 44, 56] and these efforts have revealed some genes that are likely direct transcriptional targets such as *Shh* (essential for hair placode downgrowth), *Dkk4* (knockout phenotype not known), *lymphotoxin-β* (required for proper hair fiber production), as well as *Ctgf/Ccn2*, antagonist of the Tgf-β/Bmp pathway suspected to play a role in hair placode formation [34]. We performed microarray profiling of genes differentially expressed in *Eda-/-* embryonic skin explants at the time of primary hair placode formation and found about 20 and 140 genes to be upregulated upon 90 min and 4 h exposure to recombinant Eda protein, respectively. These genes included several genes previously proposed to be downstream of Eda, known feedback inhibitors of the NF-κB pathway such as *I-κBα* and *A20*, and genes associated with the key signaling pathways (Wnt, Bmp, Fgf, Shh) regulating hair follicle development, including *Dkk4* discussed above [16, 44] (and Fliniaux et al., unpublished data). Interestingly, several chemokines were strongly induced by Eda and two of them, *Cxcl10* and *Cxcl11*, showed focal expression in primary hair placodes, as did their common receptor *CxcR3* (Lefebvre, Mikkola et al. unpublished). We are currently testing the functional relevance of these chemokines in hair follicle morphogenesis by skin explant cultures and by analysis of *Cxcr3* deficient mice. Thus far, there are no reports on the involvement of any chemokine in hair

follicle development with the exception of *Cxcl12* which regulates the migration of melanocyte progenitors in mouse hair follicles [1].

Functional Redundancy Between Troy and Edar

As discussed above, it has long been recognized that Edar is necessary for formation of primary hair placodes but curiously not for the other types of hair follicles. This puzzle was solved, at least partially, when the phenotype of *Eda* and *Troy* double-mutant mice was analyzed. While *Troy* single mutant mice have no apparent skin phenotype [23, 42], compound *Eda* and *Troy* mutants lacked both primary and secondary hair follicles, and all hair follicles in the top of the vertex [42]. However, no other additional skin appendage defects were found. It has been hypothesized that Xedar and Troy have redundant functions [23], but the ability of recombinant Eda-A1, but not Eda-A2, to restore secondary hair placode formation in skin explants of *Eda*/*Troy* compound mutants confirmed the specific involvement of Eda-A1/Edar in secondary hair follicles [42]. Up to date, neither loss- nor gain-of-function studies have revealed any role for Eda-A2/Xedar in ectodermal organogenesis [33].

Troy has long remained as an orphan receptor and it did not show specific interaction with any of the TNF family members [3]. A recent report suggested, however, that Troy is activated by lymphotoxin-α [23]. This discrepancy is likely to arise from the different experimental approaches utilized: Bossen et al. [3] tested the ability of Fc-conjugated TNFs to bind GPI-anchored Troy, whereas Hashimoto et al. [23] used co-immunoprecipitation of tagged extracellular domains of LT-α and Troy secreted into the culture medium. *LT-α* was shown to be expressed in the dermal papilla next to Troy-positive follicular epithelium [23], but it is not known whether *LT-α* is expressed at the correct time and location to initiate secondary hair placode formation. Generation of mice deficient in both *Eda*(*r*) and *LT-α* and their comparison with *Eda(r)*/*Troy* compound mutants should clarify whether LT-α is the physiological ligand of Troy in developing hair follicles.

Interestingly, the secondary hair follicles as well as the mid-cranial hair follicles are present in I-κBα ΔN mice [47] indicating that either Edar or Troy, or both, activate some other, still unknown, NF-κB independent pathway(s) essential for secondary hair placode formation. On the other hand, the number of tertiary hair follicles is strongly reduced in I-κBα ΔN mutants [48], which may suggest the involvement of a third TNFR (or some other activator of NF-κB) in hair placode initiation. However, the possibility that the phenotypic differences between *Eda*/*Troy* and I-κBα ΔN mutant mice reflect to some extent diverse backgrounds of each mouse model cannot currently be fully ruled out. A recent report described another mouse model with suppressed NF-κB activity (achieved via transgenic expression of non-degradable I-κBα under a skin specific promoter) which did display focal absence of hair in the mid-cranium similar to *Eda*/*Troy* mutants, but it remained unclear whether this was due to defective hair fiber growth or true absence of

hair follicles [52]. A more detailed analysis of these mice, as well as conditional Ikkγ mutants, should be helpful in delineating the exact role of canonical NF-κB signaling in hair development.

Edar Subfamily in Glandular Appendages

Over 20 different exocrine glands have been reported to be affected in HED (MIM 305100 and 224900; [6, 20]) but the pathology behind these defects has not been analyzed in detail in any organ system, their research being hampered by the fact that the molecular details on the development of many of these glands are poorly understood. In general, the glands are either completely missing or hypoplastic. On the other hand, mouse models with increased Edar activity are characterized by hyperplasia or increased secretory activity of many glands including the sebaceous, Meibomian (special kind of sebaceous glands that produce lipid-rich secretions at the rim of eyelid to prevent evaporation of the eye's tear film), sweat, salivary, and mammary glands [5, 10, 38]. Interestingly, a gain-of-function *Edar* allele causing a single amino acid change in the death domain of Edar (EdarV370A) is found at high frequency in human populations in East Asia and among Native Americans and is thought to reflect positive selection on this allele >10 000 years ago in Northeast Asia [5, 18] (and references therein). While this allele clearly associates with increased hair thickness [18], it was recently proposed that the selective forces could have acted on altered glandular activity (such as increased lubrication and humidification) that might have been beneficial in the cold and dry environment present at the time when the derived *Edar* allele was estimated to attain high frequency [5]. Any association of the derived *Edar* allele with changes in glandular function in human populations is yet to be demonstrated.

As the name of the syndrome indicates, reduced or even absent sweating is a distinctive mark of HED, and may cause life-threatening fever in affected children [6]. The development of sweat glands, located in the footpads in mice, is initiated about two days before birth in wild type but not in *Eda* null mice, causing their complete absence in the adult [20, 27]. However, sweat gland formation can be rescued in *Eda* null pups by postnatal injection of recombinant Eda protein, and this can be blocked by co-injection with an excess of the decoy receptor Fc-Edar [19, 51]. Remarkably, when administration of Fc-Edar is postponed only by 3.5 h, notable sweating activity could be restored, as was also formation of tail hairs which are normally absent in *Eda* null mice [51]. These finding highlight that the essential time-window required for Eda signaling activity to initiate sweat gland development is very narrow.

HED patients, as well as *Eda* deficient dogs, are prone to respiratory tract infections apparently due to lack of nasal, tracheal, and bronchial glands [4, 6, 30]. Treatment of *Eda* mutant dogs with recombinant Eda protein during their first 2 weeks of life led not only to significant correction of the missing glands but also prevented respiratory disease [4, 30]. Analysis of mice defective in Edar signaling

has revealed lack of the initial outgrowth of tracheal submucosal glands [45], indicating that Eda has an equivalent early role in submucosal glands as in sweat glands and hair placodes. The molecular targets of Edar in these glands are not known, but intriguingly, local administration of epidermal growth factor into footpads of newborn *Eda-/-* pups was shown to restore sweating ability [2].

In contrast to sweat and submucosal glands, Edar activity is dispensable for the initiation of most glands affected in HED, but is required either for epithelial morphogenesis (e.g., branching) and/or in cytodifferentiation [20]. HED patients have decreased saliva secretion, and it is evident that Eda regulates morphogenesis of the salivary gland [26, 33]. Eda null submandibular salivary glands (SMG) are hypoplastic, whereas excessive Eda-A1 promotes SMG branching both in vitro and in vivo. Using an NF-κB reporter mouse line, we have noticed high Eda-dependent NF-κB activity in embryonic SMG epithelium ([42] (and Häärä, Mikkola unpublished data). Surprisingly, based on a quantitative profiling of suspected NF-κB responsive genes in *Eda* null and control SMGs, a recent study concluded that NF-κB is an unlikely mediator of Edar activity in developing SMGs [31]. We are currently analyzing in detail the SMG phenotype of *Eda* null, I-κBα ΔN, and Eda-A1 overexpressing mice to clarify the involvement of NF-κB downstream of Eda-A1/Edar in salivary gland morphogenesis.

Mammary Gland Development

The number of mammary glands varies greatly in mammals, but in all species they develop bilaterally in the region between the axilla and the groin. In the embryo, this area is called the mammary line which in many species, including human, appears as an anatomically distinct epithelial ridge that coalesces into individual organ primordia that form in highly stereotypic positions, five pairs in the mouse [53]. In the next step, mammary placode invaginates into the dense mesenchyme to form a bud which later sprouts down and once it has reached the secondary mammary mesenchyme, the developing fat pad, branching morphogenesis begins. A rudimentary ductal tree with 10–15 branches is formed by birth, but most of the epithelial expansion takes place postnatally during puberty and pregnancy under the influence of systemic hormonal cues [46, 53].

Eda mutant mice have no obvious deviations in the number or position of the mammary glands (Voutilainen, Mikkola unpublished observations) and can foster their pups without any obvious difficulty (as do *Eda*/*Troy* compound mutants). Therefore, it came as a surprise that transgenic overexpression of Eda-A1 in the embryonic ectoderm (K14-*Eda-A1* mice) resulted in the formation of enlarged and supernumerary mammary placodes that developed into ectopic glands in the adult [38, 39]. In an as yet unpublished study we found that branching morphogenesis is highly accelerated in K14-*Eda-A1* embryos. In 3-week-old Eda transgenic mice, the number of ductal tips is about three times higher than in the controls, while reduced branching is observed in *Eda* null mice (Voutilainen, Mikkola unpublished data). Similar findings were recently reported in virgin 6-week-old mice with increased

Edar activity [5]. In the micro array screen described above, we identified parathyroid hormone related peptide (PTHrP) and two Egf-like ligands as putative target genes of Edar. Both PTHrP and Egf pathways are known to regulate mammary branching morphogenesis [35, 46]. We are currently analyzing these genes more in detail in the context of Eda signaling.

Embryonic mammary gland epithelium exhibits high Eda-dependent NF-κB activity ([42] our unpublished data) but previous studies have suggested that suppression of NF-κB activity in the mammary epithelium does not affect either early development or morphogenesis of the mammary gland, except for a transient delay in epithelial branching during early pregnancy [13, 43]. Therefore it remains to be clarified whether NF-κB plays any role in mammary development downstream of Edar.

Perhaps due to scarcity of female HED patients, there are no comprehensive reports on the breast abnormalities associated with HED. A recent case report suggested that inactivating mutations in Edar may lead to absence of breasts concomitant with a rudimentary extranumerary areola and nipple [32]. Also some 30% of male HED patients are reported to have nipple defects (absent, simple, or accessory nipples) [6]. Collectively, these findings together with data from mouse models suggest that Eda-A1/Edar pathway is an important early regulator of mammary gland development, but its loss may be compensated for by some other pathway.

Concluding Remarks

During recent years it has become apparent that mammalian hair and tooth, chick feather, and fish scale and fin development depend on the same TNF pathway. Whether this conservation extends to the level of downstream target genes, or even to NF-κB activation, is currently not known. The impact of complete abrogation of the Edar pathway, at least on mammalian hair and tooth development, is rather well described, and also the shortage of knowledge on cutaneous glands is being remediated. This, together with the analysis of gain-of-function models paves the way to understanding the consequences of more subtle changes in the pathway activity. Indeed, association studies indicate a role for Edar signaling in causing phenotypic variation in human populations. Owing to the conserved nature of this system, it is rather safe to predict that genetic fine-tuning of the Edar pathway is likely to explain some of the amazing diversity of integumentary appendages seen in vertebrates [25].

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Chapter 4
High Endothelial Venule Reporter Mice to Probe Regulation of Lymph Node Vasculature

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Abbreviations

LN	lymph node
PLN	peripheral lymph node
LTβR	lymphotoxin-β receptor
LV	lymphatic vessel
HEV	high endothelial venule
PNAd	peripheral node addressin
HEC-6ST	high endothelial cell sulfotransferase, HEC-GlcNA6ST, GlcNAc6ST-2, N-acetyl glucosamine 6-O-sulfotransferase-2; carbohydrate (chondroitin 6/keratan) sulfotransferase 4
MAdCAM-1	mucosal addressin cell adhesion molecule 1
GlyCAM-1	glycosylation-dependent cell adhesion molecule-1
OX	oxazolone
LYVE-1	lymphatic vessel endothelial hyaluronan receptor 1
GFP	green fluorescent protein
L-sel	L-selectin
PROX1	prospero-related homeobox 1

Introduction

Lymph nodes (LNs) are dynamic structures that respond to environmental insults by facilitating interactions of naïve lymphocytes with antigen. Crucial to this interaction is the entrance of naïve cells into the LN via specialized blood vessels called high endothelial venules (HEVs) and the entrance of antigen and antigen presenting

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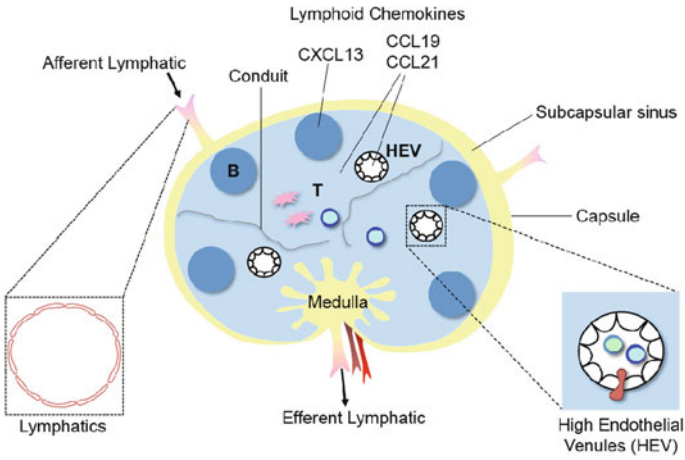

Fig. 4.1 Diagram of a lymph node emphasizing HEVs and lymphatic vessels

cells through afferent lymphatic vessels (LVs) (Fig. 4.1). Understanding the regulation of these vessels will provide insight into the dynamics of antigen–lymphocyte interaction. Lymphotoxin (LT) is well understood with regard to its role in inflammation and lymphoid organ development. $LT\alpha^{-/-}$ mice lack all lymph nodes and Peyer's patches and have severely disorganized spleens and nasal-associated lymphoid tissue. $LT\beta^{-/-}$ mice have similar defects but retain cervical, mesenteric, and sacral LNs [1].

Several adhesion molecules and chemokines are expressed on HEVs that allow the entrance of naïve lymphocytes into the parenchyma of the LN. HEVs in peripheral LNs (PLNs) undergo changes in their adhesion molecules in the first days after birth in the mouse. Predominant mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expression detected by monoclonal antibody MECA 367 is replaced by peripheral node addressin (PNAd) expression detected by the monoclonal antibody MECA 79 [2]. PNAd expression results from several modifications of a variety of glycoproteins, such as CD34 and GlyCAM-1. One modification, crucial for the recognition of PNAd by the monoclonal antibody MECA 79, is sulfation, mediated by an HEV restricted sulfotransferase GlcNAc6ST-2 (also called HEC-6ST, LSST, *Chst4*) [3, 4]. This sulfation is crucial for the interaction of naïve L-selectin$^+$ cells with PNAd. One difference between PLNs and mucosal LNs (MLNs) is the retention on HEVs of MAdCAM-1 that is replaced in the early days of life by PNAd in HEVs of PLNs.

We are particularly interested in the molecular regulation of the LN vasculature. We have demonstrated that $LT\alpha_3$ can induce MAdCAM-1 and abluminal PNAd through TNFR1 (Fig. 4.2a) [4–9], but that $LT\alpha_1\beta_2$ is necessary for induction of pericellular PNAd that allows interaction of L-sel$^+$ cells with the luminal surface of HEVs through LTβR (Fig. 4.2b) [7]. Luminal expression of PNAd is dependent on multiple modifications of glycoproteins that include GlyCAM-1. One modification, as noted above, is sulfation. $LT\alpha_1\beta_2$ induces GlyCAM-1 and HEC-6ST via the

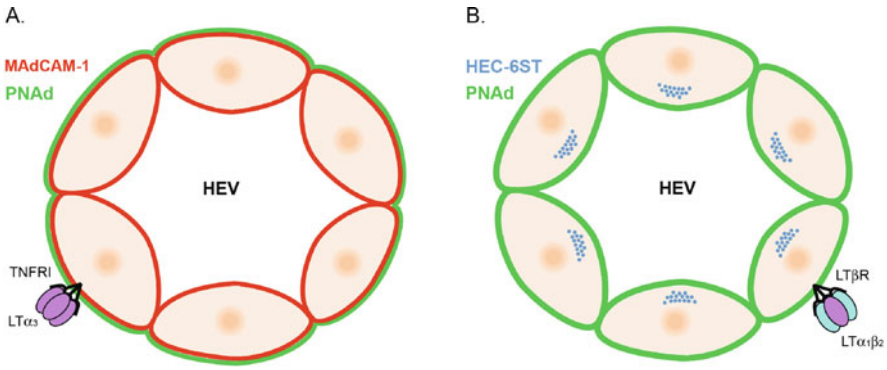

Fig. 4.2 The roles of $LT\alpha_3$ and $LT\alpha_1\beta_2$ in inducing HEV adhesion molecules. (**a**) LTα induces MAdCAM-1 (*red*) and abluminal PNAd (*green*) through TNFR1. (**b**) $LT\alpha_1\beta_2$ induces pericellular PNAd (*green*) in part by its induction of HEC-6ST through LTβR

alternative NFκb pathway [10] resulting in the mature form of PNAd in peripheral LNs. Though the process of lymphangiogenesis in ontogeny has been well described, its regulation in inflammation is less well understood. We are currently evaluating the role of LT in several models of steady state and inflammation-induced lymphangiogenesis [11].

After immunization, dramatic changes occur in lymphoid organs that include extensive remodeling of the vasculature. HEVs, which in the mature resting node are MAdCAM-1^{lo} $PNAd^{hi}$ HEC-$6ST^{hi}$, revert to an immature phenotype (MAdCAM-1^{lo}, $LT\beta R^{lo}$ HEC-$6ST^{lo}$) and following a recovery period that includes expansion of the number of HEVs, resume their mature phenotype which includes regained PNAd and HEC-6ST expression [12]. Our previously reported data reveal that this process is influenced by an initial afferent lymphatic vessel insufficiency. At later times after immunization, extensive lymphangiogenesis occurs at the site of immunization and in the draining lymph node [12].

Our eventual goal is to evaluate the dramatic changes in the vasculature during immunization in living mice. Here we summarize our studies on the cytokine regulation of the LN vasculature. We also provide data regarding the development of mice with HEVs that express a green fluorescent protein (gfp) under the control of the above-mentioned HEV restricted *Chst4* gene (here called HEC-6ST).

Materials and Methods

Mice

C57BL/6 mice were purchased from Jackson Laboratories. $LT\alpha^{-/-}$ and $LT\beta^{-/-}$ mice were previously described and are maintained at Yale University [13, 14]. The Yale University Institutional Animal Care and Use Committee approved all animal use and husbandry protocols.

Immunization with Oxazolone

OX immunization was performed by abdominal skin painting with 50 μl of 4% oxazolone (Sigma) in acetone. Mice were anesthetized by i.p. injection of ketamine/xylazine. OX was applied on shaved skin on the abdomen at the inguinal and brachial LN areas. LNs were harvested at different time points after OX ($n \geq 5$ per time point).

Development of HEC6ST-GFP Reporter Mice

The HEC6ST-eGFP transgene was constructed using the parental pClasper clone, pCLA-HEC6ST, described in Liao et al (2007) and modified therein with a β-galactosidase reporter gene to produce pCLA-HEC6ST-LacZ. pCLA-HEC6ST contains a 60 kb genomic insert spanning the entire HEC-6ST gene (gene symbol *Chst4*, carbohydrate (chondroitin 6/keratan) sulfotransferase 4) with upstream and downstream non-coding sequences. We have demonstrated faithful recapitulation of endogenous HEC-6ST expression with this genomic clone [15]. To make pCLA-HEC6ST-eGFP, we prepared a cassette containing the promoterless enhanced green fluorescent protein gene (eGFP; Clontech) mated to the SV40 polyadenylation signal. The orotidine-5′-phosphate decarboxylase (URA3) gene from *Saccharomyces cerevisiae* was ligated in reverse orientation at the 3′-end of the polyadenylation signal to provide selectability in yeast. The eGFP-URA3 cassette is 2250 bp in size – 726 bp eGFP, 392 bp SV40 polyadenylation signal, and 1132 bp URA3. The cassette was amplified by PCR for insertion in-frame in the coding sequence of exon II of HEC6ST by homologous recombination in yeast [16, 17]. Each primer used for cassette amplification consisted of two parts – one part was homologous to the site of insertion in the HEC6ST gene for recombination and the other part for PCR amplification of the cassette. The forward primer is CCCTCTGCTTGCTCTTTCAAGGTCTTCTCCTTCTTCCGCAGGATGATGCTG TTGAAGAAATGGTGAGCAAGGGCGAGG (78 bp). Nucleotides 1–60 correspond to the HEC6ST sequence up to amino acid 6, while nucleotides 60–78 will anneal to eGFP starting at the translation initiation codon. The reverse primer is CT GTGGACGGACATATGGATGAAGAGAGCTACAACGATGACCTGGGAACCC AGGAACATCAGCAGCCTCCCTACCACAGCTTTTCAATTCAATTC (95 bp). Nucleotides 1–71 correspond to the HEC-6ST sequence from amino acid 7 to 30, while nucleotides 71–95 anneal to the URA3 promoter. pCLA-HEC6ST-eGFP was verified by restriction enzyme analysis and sequencing of the cassette insertion junctions (data not shown).

HEC6ST-eGFP transgenic mice were generated by pronuclear microinjection of C57Bl/6 J × SJL/J F2 fertilized ova (Transgenic Mouse Service, Yale University). DNA prepared from tails of potential transgenic mice was screened by PCR and Southern blot. Screening primers were GFPF (ATGGTGAGCAAGGGCGAGG) and GFPR (TTATTATTACTTGTACAGCTCGTCC), amplifying the eGFP gene portion (726 bp) of the HEC6ST-eGFP transgene. The same primers were used to

amplify eGFP from a plasmid template to probe Southern blots of transgenic tail DNA (10 μg) digested with *Bam*HI. A unique 4.4 kb *Bam*HI fragment identified positive transgenic mice.

Immunofluorescence Analysis

LNs were fixed in 1% PLP (paraformaldehyde-lysine-periodate in phosphate buffer), subjected to a sucrose gradient (10–30%), frozen in O.C.T (Sakura) and 7 μm cryocut sections were prepared. Blocking buffer was 3% BSA (Sigma) and 5% mouse serum (Zymed) in PBS, or 5% BSA and 4% goat serum (Sigma) in PBS. Antibodies were rat anti-mouse MAdCAM-1, rat anti-mouse PNAd MECA-79 (BD Pharmingen); rabbit anti-human LYVE-1 (cross-reactive with mouse LYVE-1) (Upstate); and rat anti-mouse LYVE-1 (R&D systems). Rabbit anti-mouse HEC-6ST antibody was developed in our laboratory [7, 18]. Biotin-conjugated secondary antibodies were goat anti-rat IgG (Caltag Laboratories), goat anti-hamster IgG, and goat anti-rabbit IgG (Jackson ImmunoResearch). The tertiary antibodies for immunofluorescence were Cy3-goat anti-rat IgM, Cy3-goat anti-rat IgG, and Cy2-streptavidin (Jackson ImmunoResearch). After staining, sections were cover slipped with Fluorosave (Calbiochem). Images were captured with a Zeiss fluorescence Axioskope or a Zeiss LSM 510 Meta Confocal Microscope.

Results and Discussion

Vessels Positive for Markers of both HEVs and LVs Are Apparent After Immunization

We have previously reported on the apparent synchrony of HEVs and lymphatic vessels after immunization with OX [12]. Curiously, at the early times after immunization we noted the appearance of vessels that stained with antibodies that detect both PNAd and LYVE-1 (Fig. 4.3). The occurrence of such "double positive" vessels appears to be dependent on signaling through the LTβR as there were many fewer such vessels in PLNs of mice treated with LTβR-Ig before immunization [12]. What is the origin and function of such double positive vessels? Trivial explanations such as physical overlap may be distinguishable by confocal microscopy. Another possibility is that the process of lymphangiogenesis in inflammation is recapitulating that of ontogeny where lymphatic vessels are derived from veins [19]. Understanding whether there is a physical connection between LVs and HEVs may provide insight into the function of these vessels. It may be that low molecular weight materials, in addition to their passage from lymph to HEVs via conduits, may also pass via these double positive vessels during lymphangiogenesis. These various possibilities are outlined in Fig. 4.4. One way to distinguish these possibilities is to evaluate the process in living mice during an immune response. To that end, we are developing mice

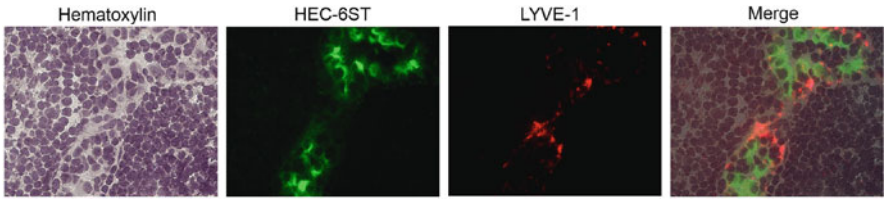

Fig. 4.3 At 4 days after immunization with OX, vessels with characteristics of HEVs and LVs are apparent. Vessel with the morphological appearance of an HEV by hematoxylin stains positive for HEV marker (HEC-6ST-*green*) and for an LV marker (LYVE-1-*red*)

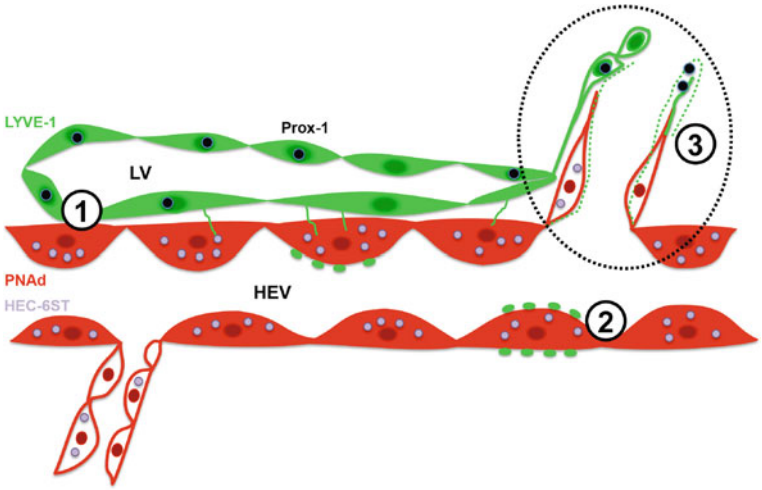

Fig. 4.4 Explanations for the observation of "double-positive" vessels after immunization. (1) HEVs and LVs are touching, but not actually the same vessel. (2) HEVs revert to a primitive phenotype and express both blood endothelial (PNAd) and lymphatic endothelial markers (LYVE-1, PROX-1). (3) The process in ontogeny by which LVs sprout from veins is recapitulated in acute inflammation

with green fluorescent HEVs and red fluorescent LVs to evaluate their interaction in vivo by multi-photon microscopy.

HEC6ST-eGFP Mice Have Been Developed

To develop HEV fluorescent reporter mice, we took advantage of our previously published construct in which we used the entire HEC6ST gene to drive expression of β-galactosidase [15]. These mice showed faithful expression of the transgene in HEVs and unexpected staining in the villi of the small intestine. In the current project, we substituted a gene for green fluorescent protein for the LacZ gene (Fig. 4.5). The eGFP reporter gene was inserted after the codon for amino acid 6 in the HEC-6ST gene, the same position as previously was done for LacZ. Thus, we

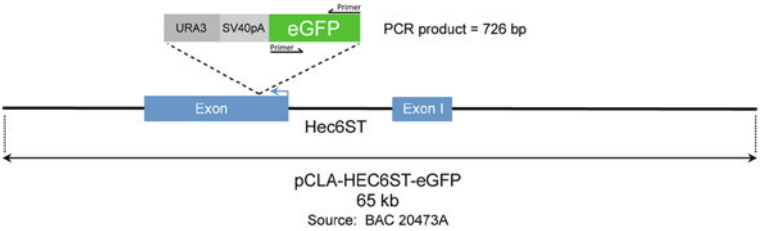

Fig. 4.5 Construct that includes eGFP driven by the *Chst4* (HEC-6ST) gene

expected similar faithful expression of green fluorescent protein as was observed with LacZ. Transgenic mouse tail DNA was evaluated by Southern blot analysis (data not shown) and PCR for the presence of the transgene and five of 50 (10%) mice were positive. Data from three of the positive founders are shown in Fig. 4.6. These HEC6ST-eGFP founders varied by copy number as assessed by Southern blot comparison and were designated as low (approximately 2 copies), medium (approximately 15 copies), or high (more than 20 copies). Three of the five mice bred well and germ line transmission was achieved with each. No obvious gross phenotypic differences were observed in HEC6ST-eGFP mice as compared to transgene-negative littermates. No phenotypic differences were observed among progeny of the founders, and fairly equal reporter expression was observed among the progeny of these transgenic mice despite differences in copy number. One founder line (#23, medium copy number) has been crossed to C57BL/6 mice and continued through eight generations. The progeny of this mouse was used for all analyses described here.

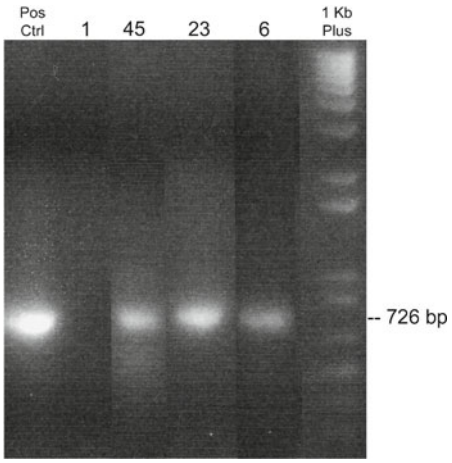

Fig. 4.6 PCR genotyping reveals the presence of the transgene. In this composite gel DNAs of three founders were analyzed by PCR with primers that detect eGFP. Mice #s 45, 23, 6 are positive for the transgene. Mouse #1 is negative. These results were confirmed by Southern blotting

HEC6ST-eGFP Mice Recapitulate Endogenous HEC6ST Expression

Immunofluorescent analysis has been carried out on peripheral LNs of progeny of all three HEC6ST-eGFP founders to evaluate the presence of the transgene and to determine if expression is concurrent with the endogenous HEC-6ST gene. First, LNs were removed from mice and immediately examined by fluorescence microscopy. Green HEVs were apparent without any further manipulation (Fig. 4.7a) indicating that the intensity of the GFP transgene is adequate for multi-photon in vivo imaging. Next, we asked whether the transgene was expressed in the same cells as PNAd. Expression of the transgene and PNAd (detected with MECA 79 antibody) were apparent in the same vessel (Fig. 4.7b). We stained for the endogenous gene with an antibody to HEC-6ST and visualized the transgene by its fluorescence; both the endogenous gene and the transgene were expressed in the same vessels and showed overlapping patterns (Fig. 4.7c). These data indicate that the transgene is appropriately expressed. We have already carried out a few experiments in vivo, and it is clear that the LNs of HEC6ST-eGFP mice are appropriate for analysis. We are currently in the process of using the pClasper technique to develop mice with red fluorescent LVs. We will isolate the BAC clone for PROX-1, one of the first genes expressed in LVs in ontogeny. Our plan is to modify this clone by inserting a red fluorescent protein. If successful, mice will be available to image the interaction of LVs and HEVs in the course of immunization.

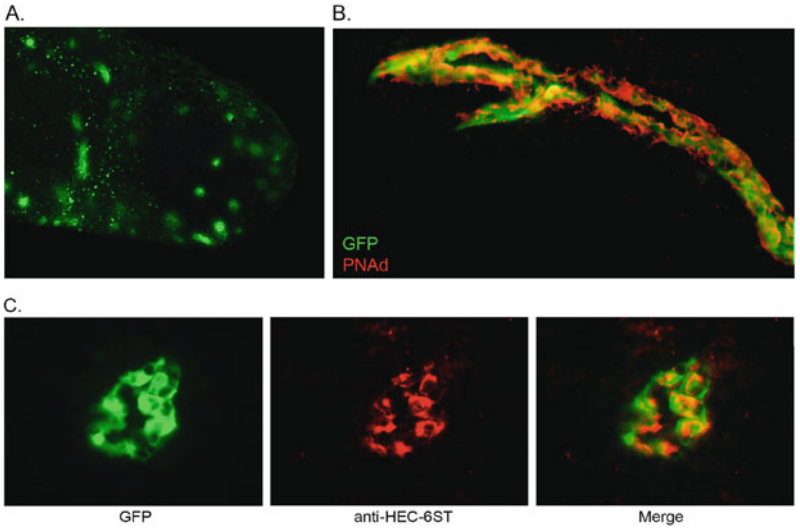

Fig. 4.7 LN HEVs of mice fluoresce and stain for endogenous HEC-6ST and PNAd: (**a**) whole mount of lymph node under uv – original magnification 10×, (**b**) confocal image of LN section of HEV-coincident fluorescence due to GFP (*green*) and staining for PNAd by MECA 79 (*red*), and (**c**) confocal image of LN section of HEV with coincident fluorescence of GFP (*green*) and endogenous HEC-6ST (*red*). Original magnification of images in (**b**) and (**c**), 63×

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Chapter 5
Eiger and Wengen: The *Drosophila* Orthologs of TNF/TNFR

Tatsushi Igaki, Hiroshi Kanda, Hideyuki Okano, Tian Xu, and Masayuki Miura

Identification and Signaling Mechanisms of Eiger

Drosophila provides a powerful genetic model for studying the in vivo regulation of biological processes, such as cell proliferation, cell differentiation, and cell death. In a large-scale ectopic-expression screen in *Drosophila* for identifying genes regulating cell death, the fly homolog of tumor necrosis factor (TNF) encoded by *eiger* (*eda-like cell death trigger*) was identified [7]. *eiger* was also isolated independently through a molecular approach [15]. *eiger* encodes a protein of 409 amino acids with a C-terminal TNF homology domain and a hydrophobic transmembrane domain. The sequence of the Eiger's TNF domain shows highest homology with human EDA-A2 (28%) and also shows significant homology with all known mammalian TNF superfamily members. Eiger is predominantly expressed in the nervous systems, as well as in the posterior region of the eye imaginal disc where photoreceptor cells are differentiated into neurons. Ectopic expression of Eiger in imaginal discs causes cell death through activation of the c-Jun N-terminal kinase (JNK) pathway. Genetic studies identified components of the JNK pathway [Misshapen (Msn, JNKKKK), dTAK1 (JNKKK), Hemipterous (Hep, JNKK), and Basket (Bsk, JNK)] that mediate Eiger-induced cell death. Further genetic efforts for identifying dominant modifiers of Eiger-induced cell death resulted in isolation of the fly TNF receptor (TNFR) homolog *wengen* (named after a village at the foot of Mt. Eiger) [10]. Eiger and Wengen are found to be the sole counterparts of the TNF/TNFR superfamily members in *Drosophila*. Another genetic screen for dominant modifiers of Eiger identified the *Drosophila* homolog of TAB2/3 (dTAB2) as a downstream signaling component [6]. Furthermore, the *Drosophila* ortholog of the TNF receptor-associated factor (TRAF) DTRAF2 and the deubiquitinating enzyme dCYLD, a tumor suppressor mutated in familial cylindromatosis, were identified

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as components of the Eiger–JNK pathway [18]. dCYLD facilitates Eiger–JNK signaling by deubiquitination of DTRAF2, which leads to stabilization of the DTRAF2 protein. Thus, genetic studies have dissected the signaling pathway of Eiger-induced cell death (Fig. 5.1). However, despite its potent cell death-inducing ability, an in vivo role for Eiger-induced cell-death signaling has been missing until recently.

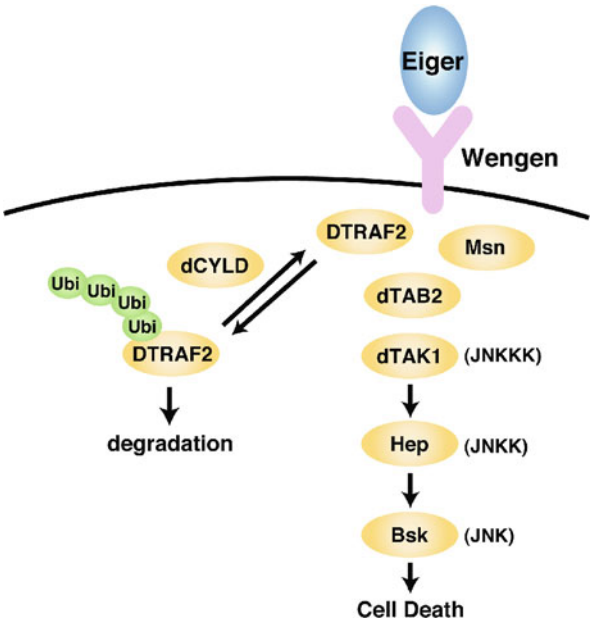

Fig. 5.1 Eiger-induced cell death signaling. Upon ligation of Eiger to its receptor Wengen, the JNK pathway (composed of Msn, dTAK1, Hep, and Bsk) is activated through DRAF2 and dTAB2, resulting in cell death. dCYLD stabilizes DTRAF2 by deubiquitination to facilitate the pathway

Role of Eiger in Epithelial Tumor Suppression

Most cancers originate from epithelial tissues that normally exhibit pronounced apico-basal polarity. In these tissues, loss of apico-basal polarity is often associated with tumor development and malignancy. Against tumor development, epithelium seems to exhibit 'intrinsic tumor suppression' mechanisms that eliminate such oncogenic polarity-deficient cells from the tissue [13]. A genetic study in *Drosophila* discovered that Eiger–JNK cell-death signaling plays a central role in the intrinsic tumor suppression (see below).

In *Drosophila* imaginal epithelia, clones of cells mutant for evolutionarily conserved tumor suppressors, such as *scribble* (*scrib*) or *discs large* (*dlg*), lose their apico-basal polarity and are eliminated from the tissue. This elimination of mutant cells is caused by JNK-dependent cell death. Interestingly, this elimination of

polarity-deficient cells occurs only when these mutant cells are surrounded by normal tissue; removal of surrounding wild-type tissue by inducing cell death allows polarity-deficient clones to grow. As a result, the tissue mutated entirely for *scrib* not only escapes elimination but grows into tumors [5]. These observations suggest that normal epithelium has an intrinsic tumor suppression mechanism that eliminates oncogenic polarity-deficient cells from the tissue. Interestingly, this intrinsic tumor suppression is abolished in *eiger* mutant flies. *scrib* or *dlg* clones generated in *eiger* deficient imaginal discs are not eliminated from the tissue but instead grow aggressively and develop into tumors. In this background, polarity-deficient clones no longer activate JNK signaling, showing that Eiger is the upstream trigger of the JNK pathway in these cells [8].

What is the mechanism of Eiger-signaling activation in these cells? It was found that Eiger changes its subcellular localization from the plasma membrane to endosomes in *scrib* clones. In addition, JNK activation is frequently observed in endosomes in these clones. Furthermore, endocytic activity is increased in *scrib* clones. These observations led to the hypothesis that Eiger–JNK signaling is activated by the endocytic pathway in these cells. Indeed, this hypothesis has been supported experimentally in three ways. First, *scrib* clones are hypersensitive to Eiger signaling, as elimination of these cells is strongly enhanced by moderate expression of exogenous Eiger that does not cause cell death on its own. Second, Eiger-induced cell death is significantly enhanced by increasing endocytic activity by overexpression of Rab5. Third, elimination of *scrib* clones is suppressed by blocking endocytic activity by overexpression of a dominant-negative form of Rab5 [8] (Fig. 5.2). Endocytic activation of cell-surface receptor signaling has been observed for EGF and β_2-adrenergic receptor signaling in mammals [14]. The endocytic pathway translocates these ligand/receptor complexes from the plasma membrane to endosomes, where they meet adaptor or scaffold proteins that recruit

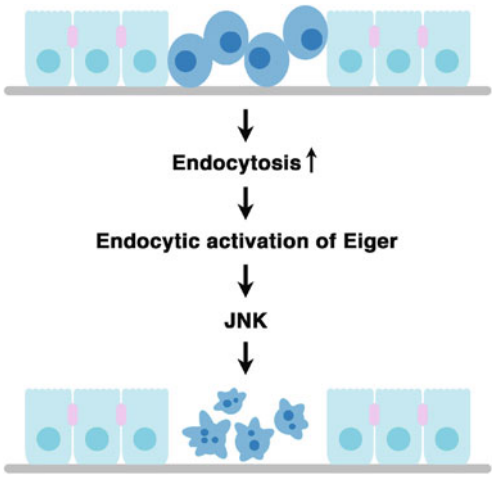

Fig. 5.2 Epithelial tumor suppression by Eiger. Oncogenic polarity-deficient cells generated in epithelium are eliminated by endocytic activation of Eiger–JNK cell-death signaling

downstream signaling components. Thus, Eiger–JNK signaling might also be activated in a similar manner. Indeed, similar endocytic activation of caspase by TNFR signaling has been reported [17].

Given that *eiger* deficient flies show no morphological or cell death defects [7], Eiger–JNK cell-death signaling might be latent under normal developmental conditions and is activated only when the organism needs to eliminate aberrant cells from the tissue. This argues that Eiger functions as an intrinsic tumor suppressor in a fashion similar to mammalian p53 or ATM, which causes no phenotype when mutated, but protect animals as tumor suppressors when their somatic cells are damaged.

Other Physiological Roles of Eiger

As mammalian TNF family proteins regulate many of biological activity, recent studies have indicated the various physiological functions of Eiger in *Drosophila*.

Roles in Host Defense

Drosophila has largely contributed to the study of immune system. Flies have several immune mechanisms such as the humoral immune response, melanization, and the cellular immune response. Among these, one of the best-characterized processes is the humoral immune response, which is composed of two major pathways, the immune deficiency (IMD) and the Toll pathways. These pathways independently regulate distinct classes of NF-κB proteins. The IMD pathway is activated by Gram-negative bacteria and induces Relish. The Toll pathway is triggered by fungi or Gram-positive bacteria and leads to Dorsal and Dif activation [9, 12]. Eiger is involved in the fly immune machinery. Microarray analysis showed that *eiger* is up-regulated after exposure of mbn-2 cells to LPS [9]. Eiger protects cells against extracellular pathogens independently of Toll and IMD signals. Eiger mutant flies show decreased phagocytosis, which results in exclusion of extracellular pathogens, indicating that *eiger* has a role in limiting the growth of pathogens via regulation of phagocytosis [16]. However, in the case of *Vibrio cholerae* infection, mutation of *eiger* increased susceptibility of flies to *V. cholerae* infection [2].

Eiger does not affect sensitivity to the intracellular pathogens (*Salmonella typhimurium*). However, mutation of *eiger* delays the lethality of host animals, suggesting that, like septic shock, excess expression of *eiger* could be harmful for flies [4]. Thus, the immune responses of the fly show significant similarities to those of mammals in terms of cytokine secretion mechanisms and autoinflammatory diseases.

The prophenoloxidase-activating cascade is also a key component of insect immunity. *Drosophila* prophenoloxidase is stored in crystal cells and is released through cell rupture. The JNK activation pathway is required for this process and *eiger* plays critical roles for this [3].

Role in Cell Proliferation

Glial cells respond to neuronal cell death. During *Drosophila* development, neuronal cell deaths occur right after eclosion especially in the area around the root of the antennal nerve. Surrounding glial cells undergo cell division in response to the neuronal cell death, as inhibition of neuronal cell death by caspase inhibitory molecule p35 blocks surrounding glial cell proliferation. In *eiger* mutant background, neuronal cell death is not altered; however, glial proliferation is severely impaired. It has been shown that brain injury causes axonal degeneration and glial cell proliferation. *eiger* mutation also suppresses the glial cell proliferation induced by neuronal injury. These results suggest that Eiger is one of the mediators that regulate glial cell proliferation after neuronal cell death (which is induced by both developmental PCD and injury) [11].

Role in Pain Sensitization

Nociceptors are the specialized sensory neurons that are activated in response to harmful stimuli. In *Drosophila* larvae, UV-induced tissue damage develops both thermal hyperplasia and thermal allodynia. Allodynia requires apical caspase *dronc* within epidermal cells, and *eiger* seems to be expressed in damaged cells. Knockdown of *eiger* receptor *wengen* in nociceptive sensory neurons abolishes development of thermal allodynia after UV treatment. Furthermore, ectopic expression of *eiger* in nociceptive sensory neurons developed allodynia even in the absence of UV irradiation. These results indicate that *eiger* mediates a subset of nociceptive sensitization responses (UV-induced nociceptive sensitization) [1].

Further Genetic Analysis of Eiger Signaling

To address the molecular mechanism of Eiger-induced cell death, we have conducted a genome-wide deficiency screen for dominant modifiers of Eiger-induced small-eye phenotype. The dominant modifier chromosome regions of each deficiency isolated were further narrowed down by screening small deficiencies and available mutants. Intriguingly, several genes relating mitochondria-mediated energy production were identified as dominant modifiers. The result of the screen and our subsequent analyses suggest that mitochondria involve a novel role in the Eiger-induced cell death pathway.

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Part II
Novel Aspects of Signaling Activation by the TNF Family: *Novel Aspects of the Functions of the TRAFs and cIAPs*

Chapter 6
Workshop Summary: *Novel Aspects of the Functions of the TRAFs and cIAPs*

John Silke and Domagoj Vucic

> Two paths diverged in a wood, and I—
> I took the one less traveled by,
> Oh, I kept the first for another day!
> Yet knowing how way leads on to way,
> I doubted if I should ever come back.
> *With apologies to Robert Frost*

Session 8 of the TNF 2009 conference focused on novel aspects of the functions of TRAFs and cIAPs. Consistent with the overarching theme of the TNF conference, nearly all the data presented in this session were previously unpublished which really added to the interest that this particular session generated. One of the main reasons that this particular field has exploded has been the development of IAP antagonist compounds. The scientific foundation for this novel class of reagents with great therapeutic potential was generated by earlier studies in insect systems as well by seminal work from the Vaux and Wang labs that identified the mammalian IAP antagonist Smac/DIABLO [7, 35]. Subsequently, work from the Shi and other labs defined the minimal amino acids required for binding of Smac/DIABLO to the BIR3 of XIAP and provided the structural basis for future drug development efforts [42].

Small molecule IAP antagonists were originally conceived largely as reagents that would sensitize cells to apoptotic stimuli by antagonizing XIAP, the best characterized IAP protein and the only one that is able to inhibit activated caspases. By sensitizing cancer cells to apoptosis induced by chemotherapeutic drugs it was hoped that these new reagents could be used to increase therapeutic response to conventional compounds. It came as an enormous surprise therefore when these

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antagonist compounds were shown to be able to kill cells as single agents and did so by promoting the degradation of cIAPs (Petersen et al. 2007) [2, 9, 33, 38]. These compounds have now emerged as very specific reagents to target cIAP1/2 and proved a great boon to researchers investigating the function of IAPs in cell signaling pathways.

The ability of these compounds to inhibit XIAP remains an attractive feature of these reagents and could contribute to their efficacy and utility in the clinic. Along these lines *Domagoj Vucic* showed using IAP antagonists with high selectivity for cIAPs over XIAP that these selective compounds were less efficient in inducing cell death than the pan IAP antagonists. These new selective compounds, as might be expected, are still efficient inducers of cIAP auto-ubiquitylation and degradation. They are also potent activators of NF-κB, which is consistent with the emerging picture that antagonism of cIAPs is critical for stimulation of NF-κB activation by IAP antagonism [21, 32, 33, 38]. The level at which XIAP plays a role is probably by blocking caspase-3 because it has recently been shown that, in the absence of BID, Fas ligand killing can be blocked by XIAP [12]. This paper and results presented from the Vucic group provide evidence that XIAP is a critical determinant for FasL and DR5 agonistic antibody stimulated apoptosis and that neutralization of XIAP through knockout/knockdown or by IAP antagonists eliminates the need for mitochondrial amplification of the death receptor pro-apoptotic signal.

The cIAPs were originally identified bound to TRAF2 and for a long time TRAF2 was believed to be the dominant partner in this relationship. For example, it was thought that TRAF2 provides the E3 ligase activity needed to promote RIPK1 K63 ubiquitylation in the TNF-R1 signaling complex [13, 22, 40]. However, none of these studies demonstrated directly that TRAF2 E3 ligase activity is responsible for RIPK1 ubiquitylation. Furthermore, recent data show that cIAP1 and 2 are critical ligases for RIPK1 ubiquitylation [2, 24, 34] and that loss of cIAPs prevents TNF-induced RIPK1 ubiquitylation and activation of NF-κB [2, 18, 34]. While Park et al. showed that cIAPs could promote K48 linked ubiquitylation of RIPK1, the groups of Barker and Vucic demonstrated that cIAPs could also promote K63 linked ubiquitylation of RIPK1 [2, 34] which would indicate that cIAPs are the key E3 ligases in TNF-induced activation of NF-κB. One way to reconcile the older data suggesting a role for TRAF2 with the newer data showing a key role for cIAPs would be to propose that the role of TRAF2 in RIPK1 ubiquitylation is to recruit cIAPs to RIPK1, in an analogous manner, perhaps, to the way in which they recruit cIAPs to NIK [32, 33, 46]. One of the confounding issues in working out the role of TRAF2 has been the fact that TRAF2 and TRAF5 are redundant in TNF-induced activation of NF-κB [30] yet TRAF5 does not appear to bind cIAPs directly. Furthermore the fact that RING domains are known to have the ability to homo- and heterodimerize means that TRAF2 RING could heterodimerize with the RINGs of cIAPs and thereby form a functional E3 ligase for RIPK1.

Evidence for the idea that the main role of TRAF2 in TNF-induced NF-κB is to recruit cIAPs was provided by *John Silke* whose lab identified a new region in TRAF2 that binds to the BIR1 of cIAPs that is required for binding cIAPs [37]. Using TRAF2 mutants that were unable to bind TRAF2 and also ΔRING

TRAF2 to reconstitute TRAF2/TRAF5 double knock-out MEFs that are defective in TNF-induced activation of NF-κB, he showed that TRAF2 mutants unable to bind cIAP1 had completely lost the ability to ubiquitylate RIPK1 while ΔRING TRAF2 mutants were as competent as wild type TRAF2 at reconstituting TNF-induced RIPK1 ubiquitylation. This finding supports the previous studies using IAP antagonist compounds, which indicated that cIAPs were critically important for RIPK1 ubiquitylation. These data also indicate that the RING of TRAF2 does not form a functional E3 for RIPK1 by heterodimerizing with the cIAP RING. The proposal that the RING of TRAF2 is dispensable for TNF-induced NF-κB is now supported by a recent publication showing that TRAF2 has significant differences in its structure to the TRAF6 RING that prevent it from interacting with Ubc13 and other related E2s [44]. A further interesting finding that arose from the Silke study was that although TRAF2/TRAF5 knock-outs reconstituted with ΔRING TRAF2 activated NF-κB normally in response to TNF, as assayed by p65 phosphorylation, IκBα degradation, and p65 translocation, these cells were not protected from TNF-induced apoptosis [37]. If activation of all NF-κB target genes was normal in these cells, this indicates for the first time that activation of NF-κB by TNF is not sufficient to protect cells from TNF cytotoxicity although it is required for protection.

Tencho Tenev further explored the intimate interaction between TRAF2 and cIAPs. He was looking for chemotherapeutics that synergized with cIAP deletion. He found that depletion of TRAF2, cIAP1, or cIAP2 alone did not substantially sensitize cancer cell lines to etoposide. However, knocking down all three simultaneously did result in a strong sensitization. These results show that depletion of both cIAPs and TRAF2 sensitize cells to apoptotic insults, and it will be interesting to determine whether this sensitization is the result of a more effective knock-down in the same signaling pathway or whether increased sensitivity is due to the fact that TRAF2 and cIAPs are functioning in independent pathways. Etoposide boosted activation of the NIK-dependent non-canonical NF-kB pathway, and stimulated TNF-dependent cell death upon depletion of TRAF2/cIAPs in certain cell lines. Furthermore, neutralization of TNF with TNF-R2-Fc suppressed etoposide-induced killing upon depletion of TRAF2/cIAP in these cells. These results suggest that combination of anti-cancer drugs that promote NF-κB activation and the production of pro-inflammatory cytokines together with IAP antagonists may increase the effectiveness of both these reagents when used in the treatment of cancer.

There are also physiological scenarios where loss of TRAF2 and cIAPs occurs. For example, TNF-like weak inducer of apoptosis (TWEAK) has been shown to promote loss of both cIAPs and TRAF2 [36, 41]. *Andreas Wicovsky* further explored this particular aspect of TRAF2/cIAP biology. He showed that soluble TWEAK trimers induce translocation of TRAF2 to a Triton X-100-insoluble compartment without a change in the total cellular TRAF2 content [41]. Vince et al. showed that Fc-tagged TWEAK, which mimics the membrane-bound form of the ligand, promoted both TRAF2 translocation and degradation and this finding was confirmed by Andreas [40].

This finding of different effects caused by different forms of the same TNFSF ligand was also made by other presenters in the meeting, e.g., *Emmanuel Dejardin*,

and was also raised during the round table discussion. The main point gleaned from the round table discussion is that much more work needs to be done to understand which is the relevant ligand in physiological situations. O'Reilly et al. recently published a potentially fruitful (albeit arduous) direction for this type of study. These authors generated knock in mice with either an uncleavable FasL or a constitutively soluble FasL and showed that mice expressing a constitutively membrane bound form of the ligand were essentially the same as wild type animals while those with the constitutively soluble form developed rare histiocytic sarcomas [23].

Although Fc-TWEAK and soluble TWEAK have different effects at the level of TRAF2, both forms of ligand cause a strong increase in p100 processing to p52. These results indicate that TRAF2 redistribution is sufficient for activation of the alternative NF-κB pathway and are in accordance with results obtained using Fc-TWEAK [36]. As described earlier, loss of cIAPs results in reduced activation of canonical NF-κB by TNF and the same effect was seen by Dr. Wicovsky when he looked at TNF induction following TWEAK priming. Similarly, and consistent with this mechanistic effect, TWEAK-primed cells were sensitized for TNF-R1-induced cell death [36, 41].

One possible explanation for the lack of TNF-induced activation in cIAP/TRAF2-depleted cells is a large reduction in TNF-induced RIPK1 modification in such cells. This reduction in RIPK1 modification in cIAP-depleted cells has been independently shown by several groups [2, 18, 34] and was also shown for TWEAK primed cells by Dr. Wicovsky. However a startling finding presented by the Silke lab in this session hints that the explanation for loss of TNF-induced NF-κB is likely to be more complex. The Silke lab showed, despite the belief that TNF-induced activation of NF-κB absolutely requires RIPK1, that RIPK1$^{-/-}$ MEFs activated NF-κB relatively normally. Whether they looked at IκBα degradation by Western blot, p65 translocation by immunohistochemistry or induction of NF-κB responsive TNF target genes such as A20, in all cases NF-κB was activated and to similar levels as in wild type MEFs. With reference to the earlier discussion about the effects of soluble versus membrane bound forms of the ligand it was noteworthy that this was true whether Fc-TNF or soluble TNF was used. These results suggest that RIPK1 is not required in all cell types for TNF-induced activation of NF-κB and that the essential nature of the role of RIPK1 has been over-interpreted.

A unifying concept might be drawn from this presentation and presentations from three independent labs that analyzed the TRADD knockout mice. All three presenters, Nikoletta Papadopoulou, Christine Chio, and You-Sun Kim, showed that TNF-induced activation of NF-κB was lost in many TRADD$^{-/-}$ cell types, including MEFs, as expected. Unexpectedly, however, TNF-induced activation of NF-κB occurred normally in macrophages [4, 8, 25]. These results indicate that the composition of the TNF-R1 signaling complex is more elastic than previously anticipated and that it will be important to look at many different cell types before the requirement for a particular protein this pathway is assured.

Although it is widely believed that RIPK1 is required for TNF-induced activation of NF-κB, the new data presented actually make sense of the fact that RIPK1$^{-/-}$ mice are born at Mendelian ratios and look normal at birth, only failing to thrive

subsequently. Knockouts of proteins that are essential for NF-κB activation in mice, including RelA/p65 itself, are early embryonic lethal [6]. In the case of RelA, this lethality is due to massive hepatocyte death and liver destruction at E14 and this can be rescued by loss of TNF [6]. This aspect of the biology of RIPK1$^{-/-}$ mice had previously been overlooked and suggests that, at the very least, hepatocytes are likely to be able to activate NF-κB normally even in the absence of RIPK1$^{-/-}$.

Despite the caveat imposed by these results, it is clear that RIPK1 can play a number of different roles in TNF signaling. This particular point was made by Xiadong Wang who, along with others, has shown that RIPK1 is required for death induced by IAP antagonists and TNF [2, 9, 39]. In his presentation he extended this finding to show that another member of the RIP Kinase family, RIPK3, was required for this programmed necrotic cell death. This programmed necrotic death not only occurs readily when cells are treated with TNF, IAP antagonists, and caspase inhibitors but also occurs in some cells treated with TNF and zVAD alone. The data he presented also tied in nicely with data presented in another session by Francis Chan. Both of these groups showed that RIPK1 and RIPK3 form a complex in cells treated with TNF and caspase inhibitors [5, 11]. Using siRNA Dr. Wang showed that both RIPK1 and RIPK3 were essential for the alternative necrotic response. He further showed, as is the case for RIPK1, that the kinase activity of RIPK3 was essential for the execution of this necrotic pathway. Dr. Wang's data suggest that RIPK3 is downstream of RIPK1 in this particular pathway, because he showed that RIPK3 was a target for the kinase activity of RIPK1. Dr. Chan showed that RIPK1 was a substrate for RIPK3, but because neither researcher examined phosphorylation of the other component it is quite possible that in fact both events occur.

The bifurcation of different signaling arms of TNF signaling complexes exemplified by the differing roles RIPK1 plays in regulating both the survival response to TNF and also the apoptotic/necrotic response was also a common theme of this session (Fig. 6.1), and prompted the reference to the Robert Frost poem. Seminal work from the Tschopp lab showed that the survival signaling complex (complex 1) is formed at the plasma membrane while the apoptotic-inducing complex is formed

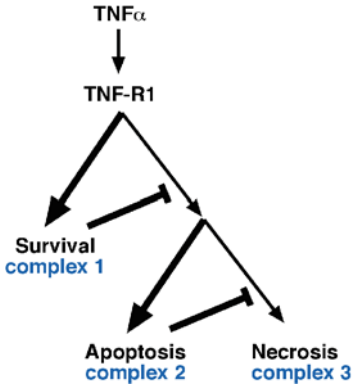

Fig. 6.1 The diverging pathways of TNF-R1 signaling

within the cytoplasm (complex 2) [20]. This finding suggested that the cellular location of the signaling component affects the ultimate outcome of a signaling pathway and, by extension, that localization of signaling complexes might be regulated. The idea that the compartmentalization of the signaling components is important to TNFSF signaling has recently been reviewed by Schütze et al. [27], and further support for the concept was provided by *Michael Karin* who showed that signaling by CD40 involves cytoplasmic translocation of a receptor-assembled signaling complex [19]. CD40 forms a complex that contains TRAF2, TRAF3, Ubc13, NEMO, MEKK1, and cIAP1/2. Subsequent activation of MAPK cascade requires translocation of this multi-protein complex from the CD40 receptor into the cytosol. This translocation and kinase activation involve cIAP1/2-mediated ubiquitylation and consequent degradation of TRAF3. In a similar fashion, Dr. Karin reported that during MyD88-dependent signaling from plasma membrane localized Toll-like receptors (TLRs) TRAF3 is subjected to degradative K48-linked polyubiquitylation that depends on TRAF6 and cIAP1 and cIAP2. As with CD40 signaling, degradation of TRAF3 is essential for cytoplasmic translocation of plasma membrane receptor-associated signaling complexes that are needed for MAPK activation via TAK1 and MEKK1.

Dr. Karin's talk demonstrated that the influence of TRAFs and cIAP proteins and their ability to regulate ubiquitylation of various signaling components extends much further than previously appreciated and a more detailed structural picture of these proteins and complexes is clearly needed. Hao Wu presented data addressing this need with crystal structures of the N-terminal region of TRAF6, encompassing the RING and the zinc finger domains, and its complex with Ubc13 [45]. Interestingly, the interaction of TRAF6 with Ubc13 requires not only the RING, but also the first zinc finger and residues preceding the RING. This RING–RING interaction seems a common theme of these E3 ligases and several RING domains have been shown to form dimers [15, 17, 29]. As previously observed for cIAP1 and cIAP2, TRAF6 RING dimerization was critical for its E3 activity [3, 17, 45]. However, TRAF6 auto-ubiquitylation also required the coiled coil domain suggesting the role of higher order aggregation for the formation of multimeric scaffold for the assembly of multi-component complexes. Dr. Wu also reported that only TRAF6 is capable of direct interaction with Ubc13 while other TRAF family members are not [45]. This finding agrees with earlier work demonstrating that Ubc13 was not required for TNF signaling at least in MEFs [43] and with a study from the Vucic lab, which demonstrated that cIAPs collaborate with another E2 enzyme, UbcH5a in mediating K63-linked polyubiquitylation of RIPK1 [34].

The picture that emerged from this session was that the E3 ligase activity of cIAPs is critical for normal TNF-induced NF-κB activation. In the round table discussion, the role of other E3 ligases in TNF signaling was also discussed from the controversial role of CARP E3 ligases [1, 14] to the requirement for the RING finger protein RNF11 in A20 mediated downregulation of NF-κB [28]. One particular new class of E3 ligase described by *Henning Walczak* in the Novel Aspects of Signaling session also provoked much discussion in the cIAP/TRAF2 session

because he showed that cIAP E3 ligase activity was required for recruitment of the RING In Between RING finger ligases HOIL-1/HOIP. These E3 ligases form a linear ubiquitylating complex and have been shown to linearly ubiquitylate NEMO. This ubiquitylation contributes to full activation of the NF-κB by TNF-R1 [29] but how HOIL-1 and HOIP were able to facilitate this following TNF stimulation was unknown. Dr. Walczak's data have provided this link by showing that the HOIL-1/HOIP complex was recruited to the native TNF-R1 signaling complex following TNF stimulation. He also presented data showing that HOIL-1/HOIP were able to bind K63-linked ubiquitin chains and that cIAPs and their E3 ligase activity were required for HOIL-1/HOIP recruitment to the TNF-R1 signaling complex [9], neatly explaining at least some of the requirement for cIAPs in TNF-R1-mediated activation of NF-κB. Together with recent data showing that NEMO preferentially binds to linear ubiquitin chains [15, 24] this finding has certainly opened up new paths to follow on the way to find out how TNF signaling works. Judging from this session, one of the stops on the way will definitely be TNF2011 in Japan.

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Chapter 7
TRAF2 and Cellular IAPs: A Critical Link in TNFR Family Signaling

Domagoj Vucic

Discovery of Cellular IAP Proteins and TRAF2

Cellular inhibitors of apoptosis (c-IAP1 and c-IAP2) proteins and tumor necrosis factor receptor associated factor 2 (TRAF2) were first identified through their recruitment to TNFR2 [58, 59]. This recruitment is direct in the case of TRAF2 and indirect in the case of c-IAPs. Parallel efforts using genomic searches for the human homologues of baculovirus encoded Op-IAP and Cp-IAP, founding members of the IAP family [12, 15], lead to the identification of cellular IAPs by several independent research groups [20, 46, 67]. Some of these reports suggested that overexpression of cellular IAPs blocks caspase expression-stimulated apoptosis. However, extensive research on the functional role of c-IAP proteins revealed that they are not direct caspase inhibitors, and that they are most predominantly involved in regulating TNFR-mediated signaling [72]. TNFR1 and TNFR2 are the founders of the tumor necrosis factor receptor super family whose members have important roles in immune development and homeostasis [84]. Most of these receptors are type 1 transmembrane proteins and they typically contain multiple repeats of cysteine-rich domains (CRDs) in their extracellular domain that are responsible for ligand binding [17]. Some members of the TNFR superfamily contain a death domain (DD) in their cytoplasmic region, which enables these death receptors to promote apoptosis or programmed cell death [2, 83].

Structural Properties of Cellular IAPs and TRAF2

Both cellular IAPs and TRAF2 are multi-domain proteins that engage a multitude of binding partners. c-IAP1 and c-IAP2 have three IAP signature baculovirus

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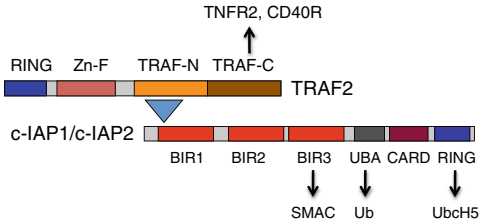

Fig. 7.1 Schematic representation of TRAF2 and c-IAP1/2 protein domains. TRAF stands for tumor necrosis factor receptor-associated factor, c-IAP for cellular inhibitor of apoptosis, RING for really interesting new gene, Zn-F for zinc fingers, BIR for baculovirus IAP repeat, UBA for ubiquitinin-associated, and CARD for caspase recruitment domain. The *arrows* point to the preferred binding partner for particular domains

IAP repeat (BIR) domains (Fig. 7.1). BIR domains of IAP proteins are approximately 80-amino acid modules with a conserved alpha/beta fold [51]. In addition, c-IAP proteins contain a centrally located ubiquitin-associated (UBA) domain and a caspase-recruitment domain (CARD) of unknown function. At the carboxy-terminal end of the protein, c-IAPs possess an ubiquitin ligase RING domain [74]. Cellular IAPs engage most of their binding partners through the BIR domains. The TRAF2-binding region in c-IAPs has been mapped to the BIR1 domain [61, 73]. Mutational analysis of the residues conserved in c-IAP1 and 2 identified the first two alpha helices in the BIR1 domain as a region crucial for TRAF1 and TRAF2 binding, and for engaging other signaling molecules [70, 73]. Even though the binding partners for the BIR2 domain of c-IAPs are not as well defined, several interactors have been identified through proteomic screens [76]. The BIR2 domain of c-IAP proteins binds second mitochondrial activator of caspases/direct IAP-binding protein with low PI (SMAC/DIABLO) and SMAC amino-terminal peptides with very low affinity [70]. Yet, the overall uncertainty regarding the true functional relevance of this domain suggests that more needs to be learnt about this region. As for the BIR3 domain, it is clear that it binds SMAC/DIABLO and HtrA2/Omi as well as SMAC-mimicking IAP antagonistic compounds [19, 75]. Structural studies on the BIR domains of c-IAP proteins reported to date have focused on the BIR3 domain [32, 43]. These studies have shown that the BIR3 domain of c-IAP1 and 2 is defined by two alpha helices and three beta strands at the amino-terminal portion of the domain, and by three alpha helices at the carboxyl end. As with the other BIR domains, this conserved alpha/beta fold supports Cys-Cys-His-Cys zinc-coordinating motif with zinc ion bridging two parts of the domain.

The carboxyl end of the c-IAP proteins contains UBA, CARD, and RING domains (Fig. 7.1). The UBA domain of c-IAPs binds monoubiquitin, as well as Lys48- or Lys63-linked polyubiquitin chains, with Lys63 chains showing a slight binding preference [7, 26]. Several functions have been attributed to the c-IAP1/2 UBA domain, including the regulation of proteasomal degradation of cellular IAPs and the modulation of c-IAP1/2-mediated signaling [7, 26]. The UBA domain of c-IAP1 contains a highly conserved Met-Gly-Phe/Tyr loop that is instrumental for

ubiquitin binding. Structural studies of the c-IAP1 UBA domain revealed that this UBA domain has a compact three alpha-helix fold that binds the classical hydrophobic patch surrounding the Ile44 of ubiquitin [7]. The CARD domain of c-IAP proteins remains one of the more enigmatic domains in cellular signaling: it was identified over a decade ago and still no binding partners or functional properties have been attributed to this domain [33]. The last domain of c-IAP1/2 is the RING domain. The RING domain of cellular IAPs acts as a classical single-unit ubiquitin ligase (E3) that is capable of mediating autoubiquitination and ubiquitination of several other proteins. Functional/biochemical studies have determined that this RING domain can mediate polyubiquitination with a variety of linkages, and that it preferentially interacts with ubiquitin conjugating enzymes (E2s) of the UbcH5 family [7, 48, 94]. Interestingly, c-IAP1/2 RINGs form dimers using the amino acid patches immediately preceding or following the RING domain [48]. RING homodimerization is essential for c-IAP1/2 proteins to function as E3 ligases. In addition, it was reported that the same region mediates IAP heterodimerization and regulates the stability of IAP proteins [57, 65].

Like c-IAPs, TRAF2 possesses very different domains at its amino (RING and zinc finger domains) and carboxyl (TRAF domains) ends (Fig. 7.1). The amino-terminally located RING domain of TRAF2 was proposed to act as an E3 ligase in TNF receptors signaling complexes. However, direct E3 ligase activity of TRAF2 RING has never been demonstrated. A likely explanation for the observed E3-like activity of TRAF2 is the constitutive association with c-IAP1/2, whose RING domains are bona fide ubiquitin ligases [64, 74]. A recent report describing the crystal structure of the TRAF2 RING and the adjacent zinc finger domain provided conclusive evidence that TRAF2 is not an E3 ligase [96]. In that report Wu and colleagues showed that structural features of TRAF2 RING cause steric clashes and unfavorable interfaces that preclude association or promotion of ubiquitination with any of the classical E2 enzymes, including Ubc13 [96].

Following the RING and four zinc fingers domains, TRAF2 possesses TRAF-N and TRAF-C domains [59]. The TRAF-N domain is comprised of coiled-coil that binds several of its signaling partners including c-IAP1/2 [58]. A mutational analysis of the evolutionarily conserved residues in this coiled-coil region identified the amino acid patch that is important for association of TRAF2 with c-IAP1, and for TRAF2 mediated NF-κB signaling events [78]. However, as in the case of the TRAF2-binding region in the BIR1 domain of c-IAP1/2, the crystal structure of c-IAP1/2 BIR1 domain, in complex with the coiled-coil region of TRAF2, should verify the identity of interacting residues and provide the mechanistic explanation for the nature of this interaction. Much more is known about the TRAF-C domain, which mediates binding of TRAF2 to TNF receptors such as TNFR2. The crystal structure of this domain revealed a trimeric self-association of the TRAF domains, and the formation of an eight-stranded antiparallel beta-sandwich structure by TRAF-C domain [55]. The same study identified a conserved shallow surface depression on the TRAF-C domain as a binding site for TNFR2 peptide [55]. Thus, TRAF2 engages TNF family receptors through its TRAF-C domain and brings cellular IAPs via its TRAF-N domain to create an E3 complex in the

receptor-associated complex that promotes various signaling events. The role of TRAF2 RING domain is not clear at this moment but it is possible that TRAF2 RING might recruit additional binding partners to receptor-associated complexes with ability to fine-tune signaling events.

TRAF2 and c-IAP Proteins in Signaling Pathways

Even though cellular IAP proteins and TRAF2 are vital components in multiple signaling processes, their role and importance are best established in NF-κB pathways [72]. NF-κB signaling plays a critical role in differentiation, proliferation, and survival of immune system cells, as well as in the development of inflammation-associated tumors. The NF-κB transcription factor family consists of five members: RelA/p65, RelB, cRel, p50, and p52 [29]. These NF-κB proteins form homodimeric or heterodimeric complexes, and in unstimulated cells they are retained in the cytoplasm by IκB (Inhibitors of kB) proteins [4]. The activation of NF-κB is mediated via canonical (classical) or noncanonical (alternative) signaling pathways that can be initiated by a variety of stimuli including the TNF receptor family [40] (Fig. 7.2). Ubiquitin-dependent proteosomal degradation of IκB proteins is a critical component of NF-κB activation. In the canonical pathway, IKKβ, which is part of the IκB kinase (IKK) complex, phosphorylates IκBα, thus generating a signal that is recognized by the ubiquitin ligase machinery prompting IκBα degradation [62]. In the case of alternative NF-κB signaling, IKKα phosphorylates the p100 protein, a precursor subunit of the p52/RelB heterodimer, leading to p100 ubiquitination,

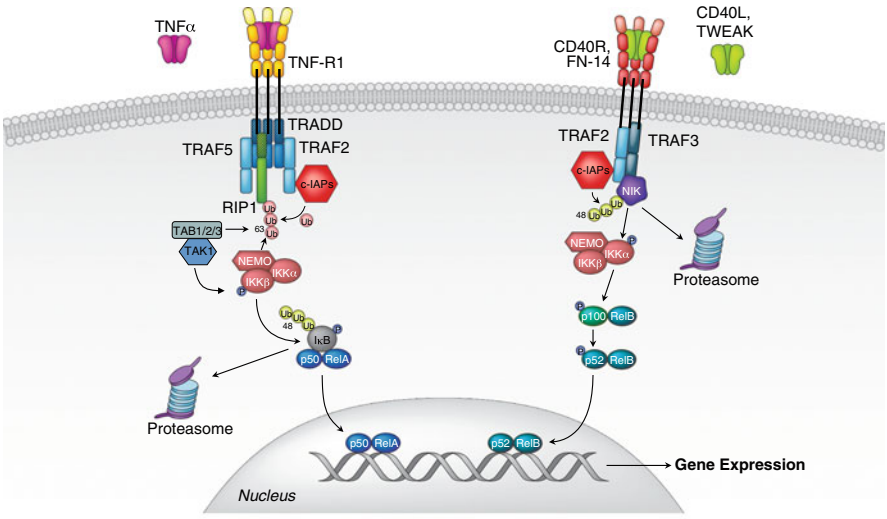

Fig. 7.2 Canonical and noncanonical NF-κB pathways

partial proteosomal processing, and the generation of an active p52 protein [18]. In both cases liberated NF-κB dimers translocate to the nucleus and induce gene expression.

Canonical NF-κB activation by TNFα is mediated by the TNFR-associated signaling complex [72]. Binding of TNFα to TNFR1 induces recruitment of the adaptor protein TNF Receptor Associated Death Domain (TRADD) to the death domain of TNFR1, followed by the recruitment of two other adaptor molecules, RIP1 and TRAF2. c-IAP1 and c-IAP2 are recruited to TNFR1 complex through their constitutive association with TRAF2 [58, 64]. TRAF2 was initially postulated to promote the ubiquitination of itself and RIP1 in the TNFR1 signaling complex [9]. However, several independent studies clearly established c-IAP1 and c-IAP2 as E3 enzymes for RIP1 and as predominant ubiquitin ligases in TNF signaling. These studies demonstrated that elimination of c-IAP1 and 2 by gene targeting, siRNA knockdown, or IAP antagonist-induced protein degradation abolished TNFα-induced RIP1 ubiquitination and NF-κB signaling [6, 49, 71, 72]. In addition, they also showed that c-IAP proteins, in combination with the ubiquitin-conjugating enzyme UbcH5, promote in vitro polyubiquitination of RIP1 with both Lys48 and Lys63 ubiquitin linkages [6, 71, 72]. These findings support earlier reports that indicated UbcH5 as a key activator of IKK complex and showed the lack of effect on TNFα-stimulated NF-κB activation from the genetic ablation of UbcH13 [10, 93]. A recent study using engineered ubiquitin modifications confirmed these findings and showed that the c-IAP/UbcH5 ubiquitination complex is essential and specific for TNFα mediated NF-κB activation [92].

Lys63-linked polyubiquitinated RIP1 serves as a docking platform for the assembly of two protein kinase complexes: TGFβ-activating kinase 1/TAK1 Binding Protein 1, 2 and 3 (TAK1/TAB1/2/3) and IKK/NF-κB Essential Modifier (NEMO). TAK1 phosphorylates and activates IKKβ, which in turn phosphorylates IκBα (Fig. 7.2). A proposed critical role of RIP1 in TNFα-mediated NF-κB activation has been challenged lately. Genetic ablation of RIP1 was reportedly detrimental for the activation of canonical NF-κB pathway by TNFα. However, a recent report carefully examined the necessity of RIP1 for TNFR1 mediated NF-κB activation and demonstrated that RIP1 might not be absolutely essential [87]. Rather, RIP1 requirement seems to be tissue-specific, as some cell types or tissues like lungs rely on RIP1 for NF-κB activation while others such as thymus or mouse embryonic fibroblasts (MEFs) do not [87]. Tissue-specific or tissue-preferred signaling events are not an anomaly in TNF-initiated pathways, as TRADD deficiency also seems to exert different effects in MEFs and bone marrow-derived macrophages (BMDMs) [22, 56]. Therefore, the TNFR1-recruited signaling complex appears to possess a certain level of flexibility. Such plasticity could allow interchangeable use of various signaling molecules for similar tasks, making the signaling process more robust and resistant to genetic deficiencies in particular components. One example of such signaling by-pass is HOIP–HOIL1 ubiquitin ligase pair that gets recruited to TNFR1 complex through c-IAP1/2 ubiquitin ligase activity [27]. By anchoring themselves in this signaling complex, HOIP and HOIL1 gain access to their substrate, Nemo, and modify it by promoting its linear polyubiquitination thus contributing to NF-κB

activation [66]. The role of cellular IAPs in the activation of canonical NF-κB signaling by other TNF receptor family members is not as well understood. Nevertheless, at least in the case of TWEAK signaling c-IAP1/2 seem to be recruited to FN14 with a functional consequences for the NF-κB activation [77]. It is well established that TRAF2, on the other hand, binds and transduces signaling for a majority of TNF receptor family members [3]. It is worth noting, however, that TRAF2 function in these receptor-signaling complexes was predicted to rely on its RING domain activity. Now, after learning about the inaptness of TRAF2 as an ubiquitin ligase, these functional models will have to be re-examined with the most likely role for TRAF2 being a signaling adapter.

In noncanonical NF-κB signaling, IKKα is activated through phosphorylation by the NF-κB inducing kinase (NIK) [50, 63, 89]. NIK also serves as a docking molecule needed for the recruitment of IKKα to p100. The exact mechanism of NIK activation is not fully known, although it is believed to involve an autophosphorylation step. In unstimulated cells, NIK protein levels are low due to constitutive proteosomal degradation. Two RING-domain containing molecules, TRAF2 and TRAF3, are critical regulators of NIK stability [3, 30]. Targeted deletion or downregulation of TRAF2 and TRAF3 resulted in NIK accumulation and constitutive processing of p100 to p52 [25, 28, 31, 45, 90]. However, these two TRAF molecules do not function as E3s for NIK, but as adaptor proteins that bring ubiquitin ligases c-IAP1 or c-IAP2 to their substrate NIK [70]. The NIK/TRAF3/TRAF2/c-IAP1/2 complex is present in unstimulated cells, where it causes proteasomal degradation of NIK [68, 97]. The maintenance of depressed NIK protein levels precludes IKKα phosphorylation and prevents uncontrollable activation of noncanonical NF-κB signaling, a setting that may lead to unregulated lymphoproliferative growth [68, 97]. Activation of noncanonical NF-κB TNF family ligands CD40 or TWEAK leads to NIK stabilization and eventual processing of p100 to p52. The mechanistic aspects of these signaling events are not entirely clear at the moment but they definitely involve autoubiquitination and subsequent proteasomal degradation of cellular IAPs, thus liberating NIK and allowing noncanonical NF-κB activation. In support of this model, treatment of cells with SMAC-mimicking IAP antagonists that trigger autoubiquitination and subsequent proteasomal degradation of c-IAP1/2 leads to NIK stabilization and to the activation of signaling [70, 79]. In addition, a significant proportion of multiple myeloma patients with inactivating biallelic mutations in both c-IAP1/2 have a constitutive activation of the noncanonical NF-κB, further establishing cellular IAPs as seminal regulators of this signaling pathway [1, 42].

Therefore, cellular IAPs are positive regulators of TNFα-induced canonical NF-κB activation, where they promote polyubiquitination of RIP1 and possibly other signaling molecules. On the other hand, in noncanonical NF-κB pathway, c-IAP proteins are negative regulators that mediate NIK ubiquitination and proteasomal degradation. Only after cellular IAPs undergo autoubiquitination and proteasomal degradation can NIK levels stabilize, which leads to the activation of noncanonical NF-κB pathway.

In addition to NF-κB, TRAF2 and cellular IAPs are important for other signaling pathways such as mitogen-activated protein kinase (MAPK) activation. Genetic deficiency of TRAF2 clearly established its critical role for JNK/SAPK activation following TNFR1 stimulation [54, 95]. On the other hand, the significance of c-IAP proteins in this process is not entirely clear. The knockouts of c-IAP1 or c-IAP2 did not reveal impairment of JNK activation, although that might be explained by their functional redundancy [13, 14]. This assumed redundancy of c-IAP1 and 2 was not apparent in the nucleotide-binding and oligomerization domain (NOD) mediated innate immunity signaling [5]. Genetic ablation of c-IAP1 or c-IAP2 blunted response to NOD agonists such as muramyl dipeptide (MDP). This finding suggests that c-IAP1 and c-IAP2 might work together in regulating NOD pathways, maybe as a part of an IAP-some complex [57]. This has not been observed in other signaling pathways (i.e., NF-κB) where c-IAP1 and 2 can functionally substitute for each other [49, 71]. Therefore, it is possible that the combined and/or individual role of cellular IAP proteins could be specific for a particular signaling process. Clearly, more studies and the combined ablation of c-IAP1 and c-IAP2 are needed to definitively establish their role in various signaling pathways.

Regulation of Apoptotic Pathways by c-IAPs and TRAF2

Programmed cell death or apoptosis is mediated by two well-characterized pathways: the intrinsic and extrinsic apoptotic pathways (Fig. 7.3). Both of these pathways eventually converge, resulting in activation of caspases, and cysteine-dependent aspartyl-specific proteases that represent the effector arm of apoptotic signaling [60]. The intrinsic or mitochondrial pathway is initiated by developmental cues or cellular stress signals that activate Bcl-2-homology 3 (BH3) proteins [41]. BH3 proteins neutralize the anti-apoptotic proteins Bcl-2, Bcl-x_L or Mcl-1 leading to the activation of pro-apoptotic proteins, Bax and Bak, and ensuing disruption of mitochondrial membrane potential. Subsequent release of cytochrome c from the mitochondria into the cytoplasm leads to Apaf-1-mediated caspase-9 activation, consequent activation of effector caspases 3 and 7, and culminates in cell death. The extrinsic apoptotic pathway is triggered when death receptors such as Fas, DR5, or TNF receptor 1 are engaged by their respective ligands, resulting in recruitment of the adaptor protein Fas-associated death domain (FADD) and the apical caspases 8 or 10 [2]. Incorporation of these caspases into the death receptor-associated death-inducing signaling complex (DISC) causes their self-activation and leads to ensuing activation of caspases 3 and 7.

IAP proteins, especially X chromosome-linked IAP (XIAP), represent the ultimate line of defense against cellular suicide by regulating caspase activity and preventing caspase activation [60]. XIAP is the only true endogenous inhibitor of caspases, as other IAP proteins exhibit weak binding to and inhibition of caspases [21]. XIAP inhibits caspases 3 and 7 using the linker region between BIR1 and BIR2 as well as the BIR2 domain, while inhibition of caspase-9 relies on the

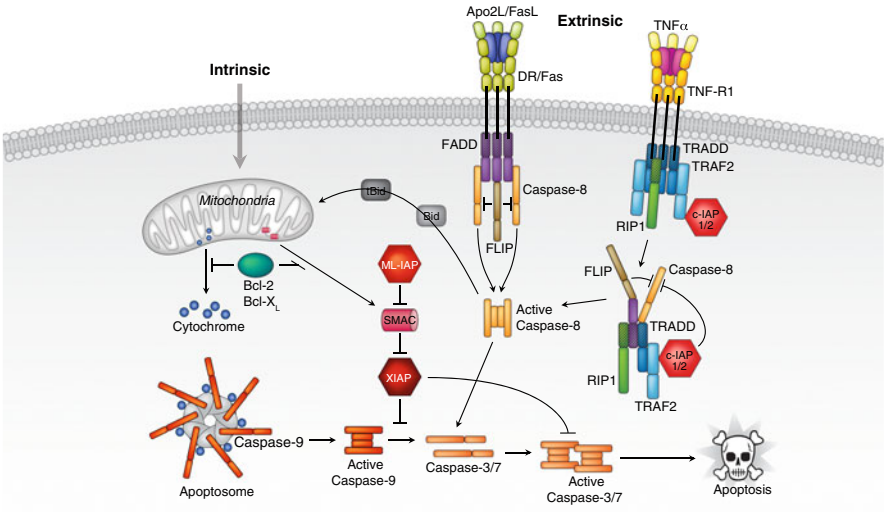

Fig. 7.3 Intrinsic and extrinsic cell death pathways

binding of the BIR3 domain to an amino-terminal IAP-binding motif (IBM) of partially processed caspase-9 [21]. Caspase-inhibitory activity of XIAP is blocked by SMAC [19, 75]. During induction of apoptosis proteolytically processed SMAC is released from mitochondria into the cytoplasm, where it can bind BIR2 and BIR3 of XIAP and antagonize XIAP via an exposed IBM [19, 75]. Some IAP proteins, like ML-IAP as well as c-IAP1 and 2, have a high affinity for SMAC but do not interact strongly with caspases [70, 72, 82]. Instead, these IAP proteins can compete with XIAP for SMAC binding, and thereby alleviate the antagonism of XIAP [16, 82].

Through their interaction with TRAF2, cellular IAP1 and 2 are recruited to TNFR complexes, where they modulate apoptotic signaling and caspase-8 activation [8]. As stated above, c-IAP1 and c-IAP2 are E3 ubiquitin ligases that mediate RIP1 ubiquitination, a critical step in the activation of canonical NF-κB signaling and in the inhibition of TNFR1-mediated cell death. The absence of c-IAP1 and 2 eliminates RIP1 ubiquitination and allows association of RIP1 with FADD and caspase-8, leading to induction of apoptosis [6, 49, 71, 85]. Since cellular IAPs are not direct inhibitors of caspases, their anti-apoptotic activity is predominantly exerted through the ubiquitin ligase activity. The survival role of TRAF2, a protein devoid of caspase inhibitory or ubiquitin ligase activity, is probably best described through its adaptor functions. By bringing c-IAP proteins to receptor signaling complexes, TRAF2 facilitates their access to the substrates whose ubiquitination potentially regulates protein stability, cellular localization, and/or association with binding partners [40]. Assembly of TNF receptor complexes may lead to c-IAP-mediated ubiquitination of TRAF2, ASK1, and RIP1, potentially resulting in dampened NF-κB activation,

deregulated MAPK signaling, and dampened caspase-8 activation [86]. Besides regulating apoptotic signaling downstream of TNFR1, cellular IAPs and TRAF2 are key components of TWEAK-stimulated FN14 receptor complex [77]. TWEAK induces c-IAP1-dependent degradation of c-IAP1 and TRAF2, which in turn sensitizes cells to TNFα-stimulated cell death [77]. On the other hand, cellular IAPs do not appear to play a crucial role in Fas or Apo2L/TRAIL-induced apoptosis [39, 69]. This might not be surprising given that Fas and Apo2L/TRAIL induce more direct and pronounced caspase-8 activation compared to TNF and considering the fact that NF-κB pathways only tangentially affect the pro-apoptotic activity of these death ligands.

In addition to regulating the protein stability of numerous TNFR signaling components, c-IAP1 can also mediate ubiquitination and proteasomal degradation of c-IAP2 and XIAP [11, 14, 65]. This fratricide might ensure the appropriate levels of IAP proteins in cells and prevent uncontrolled signaling. The c-IAP proteins also regulate the ubiquitination and stability of proteins involved in the mitochondrial apoptotic pathway and Myc-mediated signaling. Endogenous antagonist of IAP proteins, SMAC, is subject to ubiquitination and proteasomal degradation by c-IAP1 and 2 [34]. At the same time, SMAC may induce c-IAP1 and c-IAP2 ubiquitination leading to a reduction in levels of these proteins [94]. By acting as an ubiquitin ligase for a cellular antagonist of Myc, Max dimerization protein-1 or Mad1, c-IAP1 cooperates with Myc to promote cellular proliferation [91].

Targeting c-IAPs and TRAF2 for Therapeutic Intervention

IAP proteins are expressed at elevated levels in the majority of human malignancies, which makes them attractive targets for developing a novel class of cancer therapeutics [35]. In addition, overexpression of IAPs in human cancers has been shown to suppress apoptosis induced by a variety of stimuli [35, 80]. For example, genetic loci for c-IAP1 and c-IAP2 are the target of genetic amplification, and in the case of c-IAP2 genetic translocation [36, 37, 38]. These aberrations are associated with enhanced tumorigenicity and with the development of inflammation-associated tumors [35, 38]. Thus, the contribution of cellular IAP proteins to the development of human malignancies stems from their elevated expression levels in combination with cell death inhibition and the promotion of survival signaling pathways (e.g., NF-κB).

The interaction between SMAC and IAP proteins is limited to the AVPI tetrapeptide at the amino-terminus of mature, processed SMAC [47, 88]. Efforts to identify small-molecule antagonists of IAPs have targeted the BIR3 domains of XIAP, c-IAP1, and c-IAP2, or the single BIR domain of ML-IAP with a peptidomimetic approach designed to mimic the AVPI motif [44, 52, 81]. Two general classes of these mimics have been reported, monovalent and bivalent [44, 52, 81]. The monovalent IAP antagonist compounds are small molecules designed to mimic one AVPI tetrapeptide, while the bivalent compounds comprise two of the AVPI mimetics

linked together with a chemical spacer. Monovalent antagonists bind with high affinity to the BIR3 domain of c-IAP proteins and with very low affinities to their BIR2 domain [70]. The monovalent and bivalent antagonists can disrupt the association between multiple IAP proteins and SMAC, as well as XIAP and caspases. Binding of IAP antagonists results in a dramatic induction of c-IAP auto-ubiquitination activity and rapid proteasomal degradation of the c-IAP proteins [6, 70, 79]. Besides neutralizing these anti-apoptotic proteins, the IAP antagonists activate NF-κB pathways and induce cell death that is dependent on TNF signaling and de novo protein biosynthesis [6, 24, 70, 79]. IAP antagonists induce cell death in a variety of human cancer cell lines and inhibit tumor growth in a number of xenograft cancer models [44, 52, 81].

A novel class of IAP antagonists with high selectivity for cellular IAP proteins was recently reported. The c-IAP selective antagonist CS3 (Fig. 7.4) was developed using structure-based design by taking advantage of differences in the SMAC binding groove on the surface of the BIR3 domain of XIAP and c-IAP1/2 [53]. The c-IAP-selective antagonist can promote c-IAP1 and c-IAP2 degradation and activation of canonical and noncanonical NF-κB signaling pathways. These findings demonstrated that IAP antagonist-stimulated activation of NF-κB signaling relies on the antagonism of c-IAP1 and 2 but not XIAP. The CS3 compound also stimulated apoptosis in tumor cells as a single agent. However, when compared with a pan-selective antagonist it was shown that the c-IAP-selective compound was significantly less potent in inhibiting short-term as well as long-term survival, which suggest that antagonism of both XIAP and c-IAP proteins is required for efficient induction of cancer cell death by IAP antagonists [53]. The ultimate test for IAP antagonists and the validation of targeting this cell death pathway will be human clinical trials. These trials will explore the applicability of monovalent and bivalent IAP antagonists for treatment of human malignancies and pave the way for future clinical investigations of IAP-regulated apoptotic pathways [23, 52].

TRAF2 plays a critical physiological role in the regulation of cell signaling and survival in response to a number of TNF family ligands [3]. Its importance for TNF and CD40 signaling, and for overall survival, was demonstrated over a decade ago in gene ablation studies [54, 95]. These TRAF2-dependent signaling events are

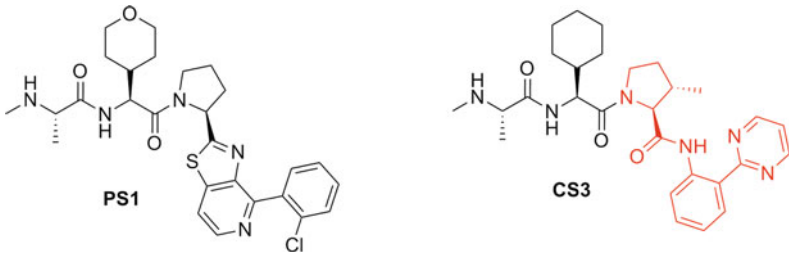

Fig. 7.4 Structural representation of c-IAP selective (CS3) and pan-IAP antagonist (PS1) compounds. The portion of CS3 compound shown in *red* is responsible for conferring c-IAP selectivity

critical in inflammation, innate immunity, and cell survival, and their deregulation can lead to the development of autoimmune diseases and cancer. However, TRAF2 does not possess any obvious small-molecule binding grooves that would make it amenable for pharmacological targeting. Thus, TRAF2 is likely to be an exciting but extremely challenging target. One possible avenue for therapeutic intervention in these pathways might be the disruption of c-IAP1/2:TRAF2 association. Recently identified binding regions in TRAF2 and c-IAP1/2 might make this strategy feasible [73, 78], with the caveat that structural analyses are needed to reveal the exact nature of this association. Future studies on the physiological and biochemical properties of cellular IAPs and TRAF2 should increase our understanding of the role and structure of these important signaling molecules, potentially revealing novel targeting opportunities for the development of therapeutics.

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Chapter 8
New Perspectives in TNF-R1-Induced NF-κB Signaling

Ian E. Gentle and John Silke

Signaling from TNF-R1 receptors has been intensively studied in the past 15 years and the model that has emerged from these studies is that ligation of TNF to TNF-R1 leads to sequential recruitment of molecules to the cytoplasmic portion of the receptor that promote the activation of a number of transcriptional responses including NF-κB, JNK, and MAPK signaling. These signaling responses mediate diverse outcomes including inflammation, immune signaling, cell proliferation, and cell death. The NF-κB pathway has been most well characterized and represents a major component of the transcriptional response. This chapter focuses on the activation of NF-κB in response to TNF and new discoveries on how NF-κB activation is regulated at the receptor.

TNF-R1-Induced Activation of Canonical NF-κB

The simple model for activation of NF-κB by TNF is that binding of TNF to its receptor, TNF-R1, drives the recruitment of the adaptor protein TRADD, the ubiquitin E3 ligases TRAF2, TRAF5, cIAP1, cIAP2, and the kinase RIPK1. TRADD is believed to act as a seed molecule binding to TNF-R1 via a Death Domain (DD)–DD interaction. TRADD then recruits the DD-containing kinase RIPK1 via a DD–DD interaction [4]. The E3 ubiquitin ligases TRAF2 and TRAF5 are also recruited to TRADD via their C-terminal TRAF domains and the N-terminal region of TRADD [14, 20]. Recruitment of the inhibitor of apoptosis proteins cIAP1 and cIAP2 is mediated via interaction with TRAFs [15, 21, 24]. Extensive ubiquitin modification within the complex occurs and, in particular, ubiquitylation of RIPK1 by K63 linked ubiquitin chains has been thought to be essential for TNF-induced activation of NF-κB [6, 8, 26, 28, 30]. K63 ubiquitin chains attached to RIPK1 can recruit the

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TAB1/2/TAK1 complex, via TAB2 and IκB kinase complex (IKK1/IKK2/NEMO) via interaction of the IKKγ/NEMO subunit. TAK1 phosphorylates and activates IKK2. The active IKK complex phosphorylates the inhibitor of NF-κB, IκBα, marking it for proteasomal degradation. The liberated NF-κB RelA/p65 dimers translocate to the nucleus and promote transcription of target genes (Fig. 8.1). In this simple model, ubiquitylation of RIPK1 is a critical event in the activation of NF-κB from TNF-R1 (Fig. 8.1).

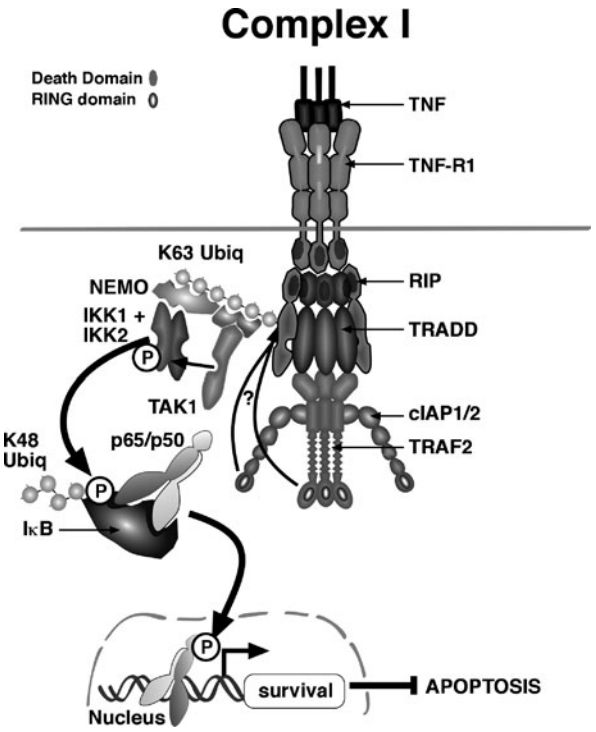

Fig. 8.1 Canonical NF-κB signaling. Recruitment of TRADD to TNFR1 is followed by recruitment of RIPK1, TRAF2, and cIAP1/2. cIAP1/2 and TRAFs K63 ubiquitylate RIPK1. K63 ubiquitin chains recruit TAB2/TAB3/TAK1 and IKK1/IKK2/NEMO complexes. TAK1 phosphorylates IKK and activating it. Active IKK complexes phosphorylate IκB leading to its K48 ubiquitylation and degradation. Liberated p65/RelA dimers translocate to the nucleus and transcribe target genes

Ubiquitylation of RIPK1 was shown to be mediated by TRAF2, cIAP1, and cIAP2 [1, 8, 10, 22, 28]. However the precise function of each individual E3 ligase in this ubiquitylation is unclear. It is a difficult task to find out relative contributions because TRAF2 binds cIAPs and without TRAF2 cIAPs are no longer recruited to TNF-R1. TRAF2$^{-/-}$ MEFs show slightly reduced and delayed activation of NF-κB in response to TNF. Part of the explanation for the residual activation of NF-κB in TRAF2$^{-/-}$ MEFs appears to be due to the function of the closely related TRAF5. TRAF5$^{-/-}$ MEFs also activate NF-κB normally in response to TNF [11, 33], but

TRAF2/5 DKO MEFs showed significant reduction in activation of NF-κB after TNF stimulation, indicating redundancy in TRAF2/TRAF5 functions at this level [18].

More recently a role for the IAPs has been shown in ubiquitylation of RIPK1 [1, 10, 22]. cIAP1 and cIAP2 both contain C-terminal RING domains that function as E3 ubiquitin ligases, analogously to the RING domains of TRAFs. Several recent studies have now shown that cIAPs are required for RIPK1 ubiquitylation in response to TNF because their loss, whether induced by synthetic IAP antagonist compounds or a combination of genetic knock-out and/or siRNA approaches, reduces TNF-R1 activated RIPK1 ubiquitylation. Furthermore loss of cIAPs blocks TNF-induced activation of canonical NF-κB and renders cells sensitive to TNF-induced apoptosis [10, 12, 22, 23, 25, 27]. Several models could account for these different observations. For example, TRAF2 could simply recruit cIAPs and be required only to do this. Alternatively, TRAF2's RING might heterodimerize with the RING of cIAP1 or cIAP2 and this might be the functional E3 ligase for RIPK1. It is also possible that TRAF2 is required to ubiquitylate cIAPs and this ubiquitylation is required to activate the cIAPs, or vice versa; cIAPs are required to ubiquitylate and activate TRAF2.

cIAP1/2 Are Required for RIPK1 Ubiquitylation and NF-κB Activation

It has been shown that the BIR1 domain of cIAP1 or 2 is sufficient for interaction of cIAPs with TRAF2; however, the BIR1 interacting region on TRAF2 has not been identified. We identified a cIAP interacting motif (CIM) in the N-terminal region of TRAF2 using a yeast two-hybrid and sequence alignment approach because we hypothesized that the cIAP1 interacting region would be highly conserved (Fig. 8.2). Mutation of this motif abolished TRAF2 and cIAP1 interaction confirming its function in cIAP recruitment [24]. Reconstitution of $TRAF2^{-/-}$ MEFs with a CIM mutant TRAF2 failed to restore NF-κB activation and RIPK1 ubiquitylation [24]. Furthermore the level of RIPK1 recruited to TNF-R1 was significantly reduced if cIAPs were recruited by TRAF2 (Fig. 8.2b). This suggests that cIAP1/2 are responsible for RIPK1 ubiquitylation, but does not rule out the possibility of cIAP1/2 acting on TRAF2 to activate its E3 activity. $TRAF2^{-/-}$ MEFs reconstituted with a RING deletion mutant of TRAF2, which retains the ability to recruit IAPs but lacks E3 ligase activity, were more informative on this point. The ΔRING TRAF2 construct restored the TNF mediated RIPK1 ubiquitylation and NF-κB activation [24] and also reduced total RIPK1 recruitment to TNF-R1 (Fig. 8.2b). These results strongly suggest that the main function of TRAF2 in TNF-induced NF-κB is to recruit cIAPs which serve as the E3 ligase for RIPK1.

These results however beg the question of the role of TRAF5 and its apparent redundancy with TRAF2 in TNF-R1 signaling because TRAF5 does not contain a cIAP interaction motif (Fig. 8.2a) and does not bind cIAP1 (Fig. 8.2c). If TRAF5

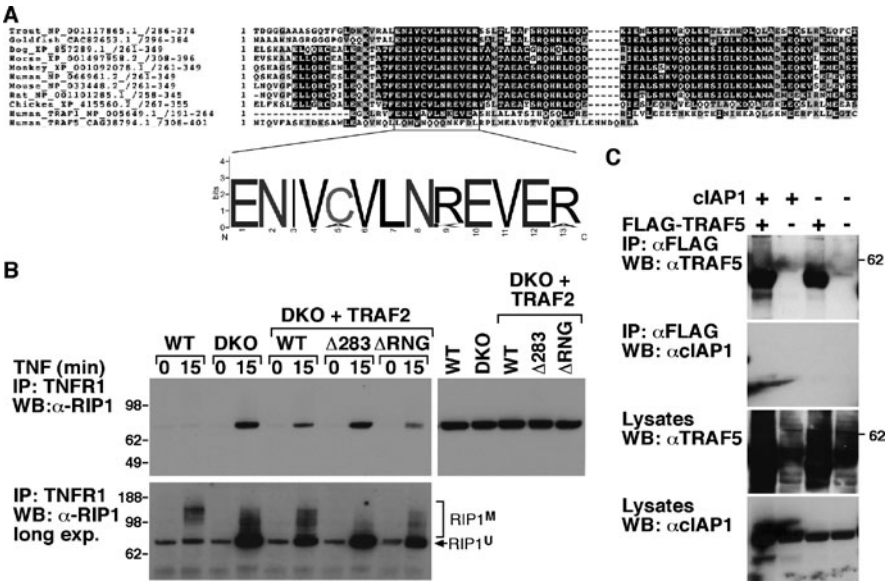

Fig. 8.2 TRAF2 CIM domain. (**a**) The TRAF-N domains of TRAF2 from multiple species were aligned including the corresponding region of human TRAF1 and TRAF5. A sequence logo of the highly conserved cIAP Interaction Motif (CIM) is shown. (**b**) cIAP recruitment is required for efficient RIPK1 ubiquitylation. TNFR1 was immunoprecipitated from wild-type and TRAF2/5 DKO MEFs alone or reconstituted with wild-type and mutant TRAF2 constructs. Immunoprecipitates were analyzed for RIPK1. (**c**) TRAF5 does not bind to cIAP1. 293T cells were transfected with FLAG-TRAF5, cIAP1, and TRAF5 immunoprecipitated with anti-flag. Immunoprecipitates were separated by SDS/PAGE, transferred to membrane, and analyzed by Western blotting with anti-Flag and anti-cIAP1

does not recruit cIAPs, it is not clear why its loss combined with TRAF2 loss is so disruptive of TNF-R1 signaling.

These results also raise an interesting point about experiments performed with Dominant Negative (DN) TRAF2 constructs. The widely used DN TRAF2 construct which was also used to generate the transgenic mouse [7] is exactly the same as the ΔRING TRAF2 construct used in this study. Because this construct is able to rescue TNF-induced activation of NF-κB when expressed to similar levels as the endogenous protein [24] it suggests that if it interferes with TNF-induced activation of NF-κB it might be because it is over-expressed to such high levels that it squelches the available cIAP1 and thereby prevents recruitment of cIAP1 to TNF-R1. If this is the case it suggests that even high over-expression of TRAF2 could block TNF-R1-induced activation of NF-κB. Even with these caveats in mind it is noteworthy that thymocytes from the TRAF2 DN transgenic mouse activated NF-κB normally in response to TNF which is also consistent with the data presented here (Fig. 8.2b; [7, 24]) and the idea that TRAF2's RING is not required for TNF-R1-induced NF-κB.

A function for the TRAF2 RING domain was however revealed when cells were analyzed for cell death induced by TNF. TRAF2$^{-/-}$ MEFs are highly sensitive to

death induced by TNF and cycloheximide, but show less sensitivity to TNF alone [18]. E15 TRAF2$^{-/-}$ MEFs immortalized with SV40 large T antigen are however very sensitive to TNF alone [24], implying that TRAF5 plays a less important role in TNF signaling in these cells than in other MEFs. Despite the fact that activation of NF-κB and RIPK1 ubiquitylation was restored when these immortalized TRAF2$^{-/-}$ were reconstituted with an inducible ΔRING TRAF2 construct, these reconstituted cells were not protected from death induced by TNF [24]. TRAF2's RING domain therefore has a function in mediating an anti-apoptotic response, independent of RIPK1 ubiquitylation and NF-κB activation. Together these results show that recruitment of cIAPs as well as TRAF2 RING function are necessary for resistance to TNF-induced cell death.

RIPK1 Is Not Required for NF-κB Activation

K63 ubiquitylation of RIPK1 has been assumed to be absolutely required for TNF-induced NF-κB because it directs recruitment of the TAB/TAK and IKK complexes. The data obtained with the TRAF2 mutants described above also support the association of RIPK1 ubiquitylation and NF-κB activation because loss of RIPK1 ubiquitylation correlated with loss of NF-κB activation.

However mice that fail to mount a normal protective NF-κB response to TNF, because they are deficient in proteins such as p65, TAB2 or NEMO die at or before E15 because of massive, TNF-induced, hepatocyte death [2, 16, 17]. RIPK1$^{-/-}$ mice on the other hand are born at Mendelian ratios and look normal although they only survive for up to three days post-natally (Fig. 8.3a; [5]).

This observation suggests that the requirement for RIPK1 in TNF-R1 signaling may not be as absolute as previously thought and using RIPK1$^{-/-}$ MEFs we were able to test this idea. RIPK1$^{-/-}$ MEFs have been shown to be highly sensitive

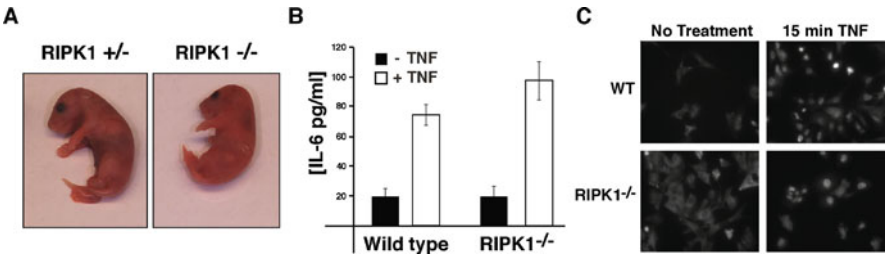

Fig. 8.3 RIPK1 is not required for NF-κB activation. (**a**) Heterozygote and RIPK1$^{-/-}$ pups immediately after birth (data adapted from Wong et al.) (**b**) TNF induced IL-6 production. 50,000 Wild-type and RIPK1$^{-/-}$ MEFs were seeded in 12 well plates and incubated with 10 ng/ml Fc-hsTNF for 24 h. Supernatants were harvested and IL-6 levels were analyzed using ELISA (data adapted from Wong et al.). (**c**) p65 nuclear translocation. Wild-type and RIPK1$^{-/-}$ MEFs were grown on cover slips and left untreated or treated with 100 ng/ml Fc-hsTNF for 20 min. Cells were fixed and stained with anti-p65 and FITC conjugated secondary for visualization

to TNF and low doses (250 ng/ml) of Cycloheximide (CHX), a dose at which wild-type cells were resistant to TNF [5]. Using RIPK1$^{-/-}$ E15 MEFs immortalized with SV40 large T antigen we confirmed this result. Loss of TRAF2 or cIAPs sensitizes SV40 immortalized MEFs to TNF even in the absence of cycloheximide. We reasoned therefore that if RIPK1 is essential for TNF-induced NF-κB even low doses of cycloheximide should be unnecessary to sensitize these cells to TNF. Surprisingly, however, RIPK1 $^{-/-}$ MEFs treated with TNF alone behaved like wild-type cells and were not killed by TNF [29]. The survival of RIPK1$^{-/-}$ MEFs following TNF treatment suggested that NF-κB activation was occurring normally in these cells. To test this we treated RIPK1$^{-/-}$ MEFs with Fc-TNF and assayed for IκBα degradation by Western blotting. Surprisingly to us, RIPK1$^{-/-}$ MEFs showed close to wild-type levels of IκBα processing [29]. To confirm that loss of IκBα led to p65 nuclear translocation in these cells we also examined p65 translocation by immunohistochemistry (Fig. 8.3b; [29]) and this occurred within the same timeframe as in wild-type cells (Fig. 8.3b). The survival of RIPK1$^{-/-}$ MEFs and the apparently normal activation of NF-κB in response to TNF shows that while RIPK1 ubiquitylation is a useful marker for TNF-R1-induced activation of NF-κB signaling and indicates recruitment of functional cIAPs, it is not an essential step in NF-κB activation [29]. This was further confirmed by examining production of a known NF-κB-dependant TNF-induced target gene IL-6 [13]. IL-6 production in response to TNF was measured in RIPK1$^{-/-}$ MEFs by ELISA. RIPK1$^{-/-}$ MEFS showed similar to wild-type levels of IL-6 production when measured by ELISA (Fig. 8.3c).

These findings suggest that the reason that RIPK1$^{-/-}$ survive a few days after birth is that hepatocytes do not require RIPK1 for TNF-induced NF-κB. To examine TNF-induced NF-κB activation in other cell types in addition to MEFs, embryos from timed matings of RIP$^{+/-}$ mice were taken. Consistent with published data RIPK1$^{-/-}$ pups appeared grossly normal immediately after birth when compared with wild-type littermates and showed no macroscopic abnormalities (Fig. 8.3**a**; [5]). Furthermore thymus and liver harvested from RIPK1$^{-/-}$ mice and treated with exogenous TNF degraded IκBα with the same kinetics as in wild-type tissue [29]. Surprisingly, wild-type E18 embryonic hepatocytes had no or undetectable RIPK1 expression, thus even wild-type E18 hepatocytes are functionally RIPK1$^{-/-}$. This is likely to be a developmental stage specific lack of expression, because adult hepatocytes express easily detectable levels of RIPK1 [29]. RIPK1$^{-/-}$ lung cells showed little degradation of IκBα in response to TNF suggesting a requirement for RIPK1 in these cells.

Alternative Mechanisms for NF-κB Activation

While a non-essential role for RIPK1, and by extension RIPK1 K63 ubiquitylation, in TNF-induced NF-κB activation was unexpected, several recent studies support this idea. Ubiquitylation is probably an essential component of the signaling pathway as demonstrated by the requirement for cIAPs; however, the type of

ubiquitylation and the E2/E3 pairs and their targets have recently been shown to be more complex than previously thought. One finding that indicated early on that TRAF2 may not be involved in K63 ubiquitylating RIPK1 was that knock-out of UBC13 which is the E2 required for TRAF6-mediated K63 ubiquitylation, and the presumed E2 for TRAF2 did not affect TNF-induced NF-κB [32]. In addition the structure of the RING of TRAF2 has recently been solved and it has several features that make it unlikely that it binds to UBC13 or any other related UBC such as Ubc5 [34]. However phosphorylation of TRAF2 by protein kinase C stimulates its K63 polyubiquitylation, enhancing recruitment of IKK1 and IKK2 and activation of NF-κB in response to TNF. Taken together with the fact that RIPK1$^{-/-}$ MEFs can activate NF-κB in response to TNF this suggests that K63 ubiquitin chains on TRAF2 might be sufficient to allow recruitment of a functional NF-κB activating complex [9]. It is possible that the identity of the K63 ubiquitylated target is relatively unimportant in many cells because it was also recently shown that free K63-linked ubiquitin chains are able to activate TAK1 [31].

In addition to K63 linked chains, linear ubiquitin chains catalyzed by the LUBAC complex containing HOIL-1 and HOIP are important for TNF-R1 signaling. HOIL-1 and HOIP form the LUBAC complex, which is recruited to TNF-R1 [3] and conjugates linear ubiquitin chains onto NEMO [19]. Linear ubiquitylation by LUBAC is also independent of UBC13 and mice deficient in HOIL-1 showed significantly impaired NF-κB responses to TNF [19].

In collaboration with the Walczak group we have now shown that both TRAF2 and cIAPs are required for recruitment of HOIL-1/HOIP to the TNF-R1 signaling complex [3]. Furthermore, the requirements for RIPK1 ubiquitylation are the same as for HOIL-1/HOIP recruitment, namely a TRAF2 cIAP interaction is required and cIAP's RING function is required but TRAF2 RING function is not. Haas et al. also showed that HOIL-1/HOIP bound to K63 linked ubiquitin chains suggesting that HOIL-1/HOIP can be recruited to RIPK1. However, consistent with the fact that RIPK1 is dispensable for TNF-R1-induced NF-κB in MEFs, HOIL-1/HOIP were still recruited in the absence of RIPK1. Indeed the identification of HOIL-1/HOIP as E3 ligases that can form linear ubiquitin chains that can recruit the IKK complex to TNF-R1 and which can be recruited in a RIPK1-independent manner provides a potential explanation for the redundancy of RIPK1 for TNF signaling in some cell types. These new data now provide the basis for a new model for TNF signaling (Fig. 8.4).

NF-κB and TNF-Mediated Cell Death

In order to sensitize normal wild-type cells to TNF-mediated cell death, the standard approach is to use inhibitors of transcription such as actinomycin D or translation such as cycloheximide. This sensitization was thought to be a result of the loss of NF-κB-mediated up-regulation of pro-survival genes such as cFLIP, A20, and cIAP2. However the results discussed in this chapter suggest that additional

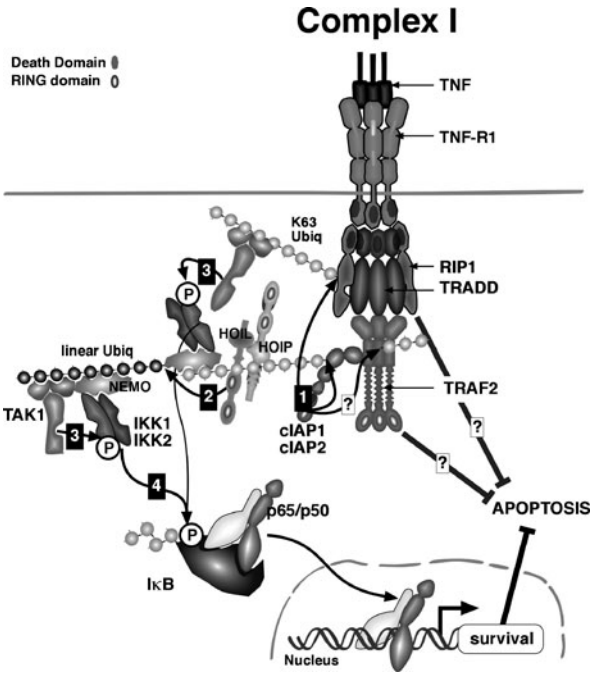

Fig. 8.4 Alternative model for RIPK1 independent NF-κB activation. TNF binds to TNF-R1 recruiting TRADD, RIPK1, TRAF2, and cIAP1/2. (1) The RING domains of cIAP1/2 act to K63 ubiquitylate RIPK1 and also themselves. K63 ubiquitin chains recruit HOIL-1, HOIP, TAB/TAK1, and IKK complexes. (2) HOIL-1 and HOIP linearly ubiquitylate NEMO, promoting additional NEMO recruitment to linear ubiquitin chains thus amplifying the signal. (3) TAK1 phosphorylates IKK activating it. (4) Active IKK complexes K48 ubiquitylate IκBα, degrading it and freeing NF-κB to translocate to the nucleus. Both TRAF2 and RIPK1 are shown playing an unknown direct role in inhibiting apoptosis

effects may be at play. The observed lack of protection of TRAF2$^{-/-}$ cells reconstituted with a ΔRING TRAF2, despite apparently normal NF-κB activation and the increase in sensitivity to CHX and TNF in RIPK1$^{-/-}$ MEFs compared to wild-type MEFs, despite approximately normal NF-κB activation in the RIPK1$^{-/-}$ cells, suggest that the prosurvival signaling from TNF-R1 is not just about NF-κB. These findings also highlight how complex receptor signal transduction is and how much remains to be learnt.

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Chapter 9
Structural Studies of NEMO and TRAF6: Implications in NF-κB Activation

Hao Wu, Yu-Chih Lo, and Qian Yin

NF-κB proteins are evolutionarily conserved master regulators of immune and inflammatory responses [2]. They play critical roles in a wide array of biological processes including innate and adaptive immunity, oncogenesis, and development. They are activated in response to ligation of many receptors including T-cell receptors, B-cell receptors, members of the tumor necrosis factor (TNF) receptor superfamily, and the Toll-like receptor/interleukin-1 receptor (TLR/IL-1R) superfamily.

The Ser/Thr-specific IκB kinase (IKK) signalosome is at the bottleneck for NF-κB activation because activated IKK phosphorylates IκB, leading to Lys48 (K48)-linked polyubiquitination and subsequent degradation of IκB by the proteasome [4]. The freed NF-κB dimers translocate to the nucleus to mediate specific target gene transcription. The IKK signalosome contains the kinase, IKKα, and/or IKKβ, and the highly conserved regulatory protein NEMO (also known as IKKγ or FIP-3) [4]. IKK activity relies on the interaction between the kinase and NEMO. In cells lacking NEMO, IKKα, and IKKβ cannot be activated by any of the classical NF-κB inducers. Sequence analysis of NEMO indicates a high helical content with an N-terminal kinase-binding domain (KBD), three coiled coil regions (CC1, CC2 and LZ), and a zinc finger (ZF) domain [4].

Recent studies have shown that the ability of the CC2-LZ region of NEMO to interact with both linear and Lys63-linked polyubiquitin chains is crucial for IKK recruitment to receptor signaling complexes and for IKK activation [1, 5, 6]. In an effort to understand how NEMO interacts with both linear and Lys63-linked polyubiquitin chains, we performed and reported both biochemical and structural studies on the CC2-LZ region of NEMO [3]. We showed that CC2 and LZ together form a continuous coiled-coil structure with a prominent bent at the junction between CC2 and LZ. Affinity measurement by isothermal titration calorimetry showed that CC2-LZ interacts with linear diubiquitin and Lys63-linked diubiquitin at

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dissociation constants of 1.4 and 131 μM, respectively. No interaction could be detected between NEMO and monoubiquitin.

We used structure-based mutagenesis and nuclear magnetic resonance (NMR) to map the interaction between CC2-LZ and linear or Lys63-linked diubiquitin. These experiments revealed that the binding sites for diubiquitins at LZ are composites of both chains and that the proximal and distal ubiquitins interact with CC2-LZ differently. For tandem diubiquitin, the distal ubiquitin uses the conserved hydrophobic patch and the C-terminal tail while the proximal ubiquitin uses an adjacent surface patch. For Lys63-linked diubiquitin, the proximal ubiquitin uses its conserved hydrophobic patch while the distal ubiquitin mostly employs the C-terminal arm including the K63-linkage residue. These studies uncover the energetics for mutual recognition of NEMO and diubiquitins.

In a separate study, we elucidate how TRAF6 acts as an E3 in mediating Lys63-linked polyubiquitination.

Tumor necrosis factor (TNF) receptor associated factors (TRAFs) act upstream of IKK in the same receptor-mediated NF-κB signaling pathways. TRAF6, in particular, has been shown to mediate Lys63 (K63)-linked polyubiquitination via its N-terminal RING and zinc finger domains. To elucidate the ubiquitin ligase (E3) function of TRAF6, we reported the crystal structures of TRAF6, alone and in complex with the ubiquitin conjugating enzyme (E2) Ubc13 [7]. The RING and zinc fingers of TRAF6 assume a rigid, strikingly elongated structure. Interaction of TRAF6 with Ubc13 involves direct contacts of the RING and the preceding residues while the first zinc finger plays a structural role. Surprisingly, this region of TRAF6 is dimeric both in the crystal and in solution, different from the trimeric C-terminal TRAF domain. Structure-based mutagenesis reveals that TRAF6 dimerization is critical for polyubiquitin synthesis and auto-ubiquitination. Fluorescence energy transfer analysis shows that TRAF6 dimerization induces higher order oligomerization of full-length TRAF6. The mismatch of dimeric and trimeric symmetry may provide a mode of infinite oligomerization that facilitates ligand-dependent signal transduction of these receptors.

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Part III
Novel Aspects of Signaling Activation by the TNF: *Ubiquitin Modification of Signaling Proteins*

Chapter 10
Modification by Single Ubiquitin Moieties Rather Than Polyubiquitination Is Sufficient for Proteasomal Processing of the p105 NF-κB Precursor

Yelena Kravtsova-Ivantsiv, Shai Cohen, and Aaron Ciechanover

Introduction

NF-κB proteins are transcription factors playing an important role in regulation of the inflammatory response and apoptosis. Some authors link them to the pathogenesis of cancer (Naugler and Karin, 2008). The NF-κB transcriptional complexes are dimeric combinations of subunits p50, p52, and/or members of the Rel family of proteins. p50 and p52 are produced from two precursors, NF-κB1 (p105) and NF-κB2 (p100), by limited proteolytic processing at the proteasome, and represent the N-terminal domains of their precursors, whereas the C-terminal segments are degraded.

Both signal-induced and constitutive types of processing are observed. Following stimulation, p105 undergoes phosphorylation on specific serine residues by IκB kinase (IKK) with subsequent recruitment of the $SCF^{\beta\text{-}TrCP}$ ubiquitin ligase. This results in both complete degradation and enhanced processing of the protein (Cohen et al., 2004; MacKichan et al., 1996; Orian et al., 2000). The mechanisms and enzymes involved in a constitutive processing are not studied well. A glycine-alanine rich repeat (GRR) is essential for processing (Lin and Ghosh, 1996) and probably serves as a "stop signal" for the 26S proteasome (Orian et al., 1999). Lin and colleagues (Lin et al., 1998, 2000) suggested that p50 is not a product of p105 processing, but rather is generated co-translationally by the proteasome via a mechanism involving ribosome halting. Moorthy and colleagues (Moorthy et al., 2006) claim that the processing is mediated by the 20S rather than the 26S proteasome, and the endoproteolytic cleavage is not dependent on prior ubiquitination of the

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precursor. Other studies have shown that ubiquitination of p105 is required for its proteasomal processing (Coux and Goldberg, 1998; Orian et al., 1995; Palombella et al., 1994; Sears et al., 1998).

Ubiquitin-mediated proteolysis is a major pathway involved in regulating numerous biological processes (Mayer, 2005, 2006a, b, 2008). The proteins destined for degradation are signaled for recognition by the 26S proteasome by generation of a polyubiquitin chain. This chain is anchored in most cases to an internal lysine residue in the substrate via an isopeptide bond between the C-terminal glycine residue of the first ubiquitin moiety and an ε-NH_2 group of an internal lysine in the target substrate. Within the chain, each ubiquitin moiety is bound to the previously conjugated molecule via a similar bond with the ε-NH_2 group of internal lysine 48 (Chau et al., 1989). Recent studies of protein degradation revealed the chains based on lysine 63 (Hofmann and Pickart, 2001; Kim et al., 2007), chains lacking lysine 48-based linkages, and heterogeneous ones based on lysines 11, 48, and 63 (Kirkpatrick et al., 2006). It is also accepted that the shortest signal that is recognized by the proteasome is a tetraubiquitin chain (Thrower et al., 2000). Monoubiquitination has been shown to control non-proteolytic processes such as epigenetic control mediated via histone modification (Robzyk et al., 2000), receptor routing (Terrell et al., 1998), and viral budding (Patnaik et al., 2000). As for degradation, the methylated ubiquitin (MeUb), in which all the amino groups in the internal lysines were modified – resulting in its inability to polymerize – could stimulate in vitro degradation of lysozyme, although at a lower efficiency compared with WT ubiquitin (Hershko and Heller, 1985). There exists a single and specific monoubiquitination of Pax3 – a key regulator of myogenesis – which targets it for proteasomal degradation (Boutet et al., 2007). Interestingly, the degradation of certain proteins by the proteasome appears to be ubiquitin independent (reviewed recently in Jariel-Encontre et al. (2008)), though the only firmly established case is ornithine decarboxylase (ODC) (Murakami et al., 1992). Experimental evidence strongly suggests that the 19S regulatory particle of the proteasome recognizes the ubiquitinated substrates. Subunit Rpn10/S5a/Mcb1 was shown to bind ubiquitin chains through ubiquitin-interacting motifs (UIMs) with a preference to polymeric ubiquitin (Deveraux et al., 1994). Since deletion of this subunit had only a mild effect on protein degradation in yeast (van Nocker et al., 1996), it is clear that additional ubiquitin-binding subunit(s) exist. Indeed, it was shown that the 19S ATPase subunit S6'/Rpt5 binds polyubiquitin chains in an interaction that is modulated by ATP hydrolysis (Lam et al., 2002); however the biological significance of this association has not been further unraveled. It was reported recently that Rpn13/ARM1 functions as a novel ubiquitin receptor (Husnjak et al., 2008). Rpn13 carries a conserved amino-terminal domain termed the pleckstrin-like receptor for ubiquitin (Pru). This motif binds monoubiquitin and K48-linked diubiquitin with high affinity compared to other ubiquitin receptors (Husnjak et al., 2008; Schreiner et al., 2008). Present chapter demonstrates that proteasomal processing of p105 can proceed following multiple monoubiquitinations, suggesting that a cluster of single moieties and not necessarily a polyubiquitin chain can serve as a proteasomal recognition proteolytic signal.

Results

Involvement of the Ubiquitin System in Processing of p105

As described in the Introduction, certain basic aspects related to the mechanism of p105 processing are still controversial. These controversies prompted us to re-examine the role of the ubiquitin–proteasome system (UPS) in the process. Processing of ^{35}S-labeled p105 in crude reticulocyte fraction II (that lacks ubiquitin) is strongly dependent on the addition of ubiquitin (Fig. 10.1a). It requires the presence of E1 and E2 (Fig. 10.1b, compare lane 8 to lane 4) and probably also of an E3 present in fraction II (Orian et al., 1995). The reaction is ATP dependent (Fan and Maniatis, 1991; Orian et al., 1995), suggesting again that the 26S proteasome catalyzes p105 processing. The involvement of the 20S proteasome in the process was confirmed by the finding that MG132, *clasto*-lactacystin β-lactone, and epoxomicin inhibited processing significantly (Fig. 10.1b, lane 5 and Fig. 10.1c).

The experiment shown in Fig. 10.1b was carried out using WT p105. The same results (Fig. 10.1d) were obtained in an identical experiment in which we used p105S927A, a mutant that cannot be specifically phosphorylated at this site by IKK, and therefore cannot recruit the βTrCP ubiquitin ligase and undergo signal-induced processing and/or degradation (Heissmeyer et al., 2001; Lang et al., 2003; Salmeron et al., 2001). It should be noted that p105S927A can be phosphorylated on other Thr/Ser/Tyr residues, yet these modifications are probably irrelevant to βTrCP recruitment (Heissmeyer et al., 2001; Lang et al., 2003).

These experiments clearly demonstrate that processing of p105 in a cell-free system is dependent on ubiquitination and cleavage by the 26S proteasome.

Processing of p105 Does Not Require Polyubiquitination

Once it was established that processing requires ubiquitination, it was important to determine whether it also requires generation of a polyubiquitin chain(s). As can be seen in Fig. 10.2a, processing proceeds even in the presence of ubiquitin species that cannot polymerize – UbK48R (lane 4), MeUb (lane 5), and UbK0 (lane 6). We hypothesized that p105 is subjected to processing mediated via two independent mechanisms: (i) signal-mediated (which can occur also in the crude extract) that leads to phosphorylation and cleavage of p105 that requires polyubiquitination and is mediated by the βTrCP ubiquitin ligase and (ii) basal/signal-independent processing that requires monoubiquitination and is mediated by an as yet to be identified ligase. WT ubiquitin can promote both processes, and therefore the cleavage in its presence is more efficient than in the presence of the non-polymerizable ubiquitin species. Supporting this hypothesis is our finding that p105 is partially phosphorylated in crude extract (Orian et al., 2000), and that this modification can result in increased processing (Cohen et al., 2004). To demonstrate that this is indeed the case and p105 can undergo two types of processing that are apparently dependent on

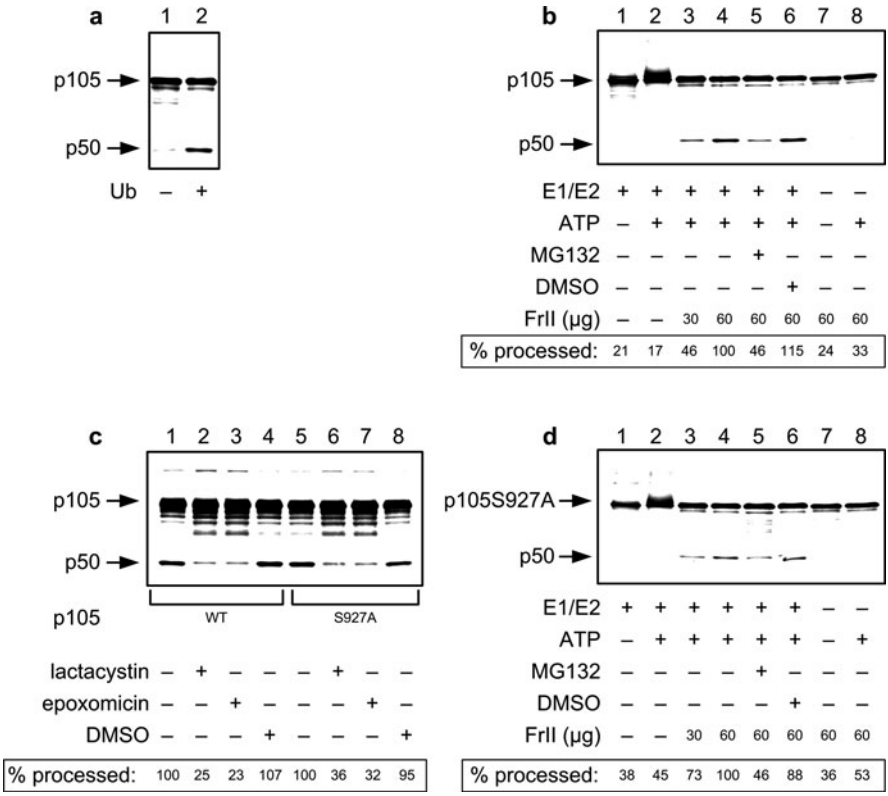

Fig. 10.1 Ubiquitin system-mediated processing of WT and S927A p105s to p50 in a reconstituted cell-free system. (**a**) Processing of p105 requires ubiquitin. In vitro-translated and ^{35}S-labeled p105 was processed in the presence or absence of ubiquitin as indicated. Processing of WT (**b**, **c**) or S927A (**c**, **d**) p105s is ATP-, proteasome-, E1-, E2-, and fraction II-dependent. ^{35}S-labeled p105s were processed in the presence or absence of ATP and ATP-regenerating system, MG132, *clasto*-lactacystin β-lactone, epoxomicin, DMSO, and E1 and E2, as indicated. Ub denotes ubiquitin and FrII denotes fraction II; % of processing is the ratio of radioactivity of p50/radioactivity of p50 + p105 multiplied by 100. All values relate to 100% which is the set point for processing of p105 in a complete system

two different modes of ubiquitination, we utilized p105S927A (Heissmeyer et al., 2001; Lang et al., 2003; Salmeron et al., 2001). Indeed, efficiency of processing of this p105 mutant is similar using either WT or non-polymerizable ubiquitin species (Fig. 10.2b, lanes 5 and 6, and Fig. 10.2c).

To further demonstrate that p105 can be processed following monoubiquitination, we monitored the generation of p50 from p105S927A in the presence of WT ubiquitin and increasing concentrations of MeUb. This ubiquitin species can be activated by E1 and conjugated to the substrate, and therefore can be used to inhibit degradation of substrates that require polyubiquitination for their recognition by the proteasome (Ben-Saadon et al., 2004; Breitschopf et al., 1998; Herman-Bachinsky

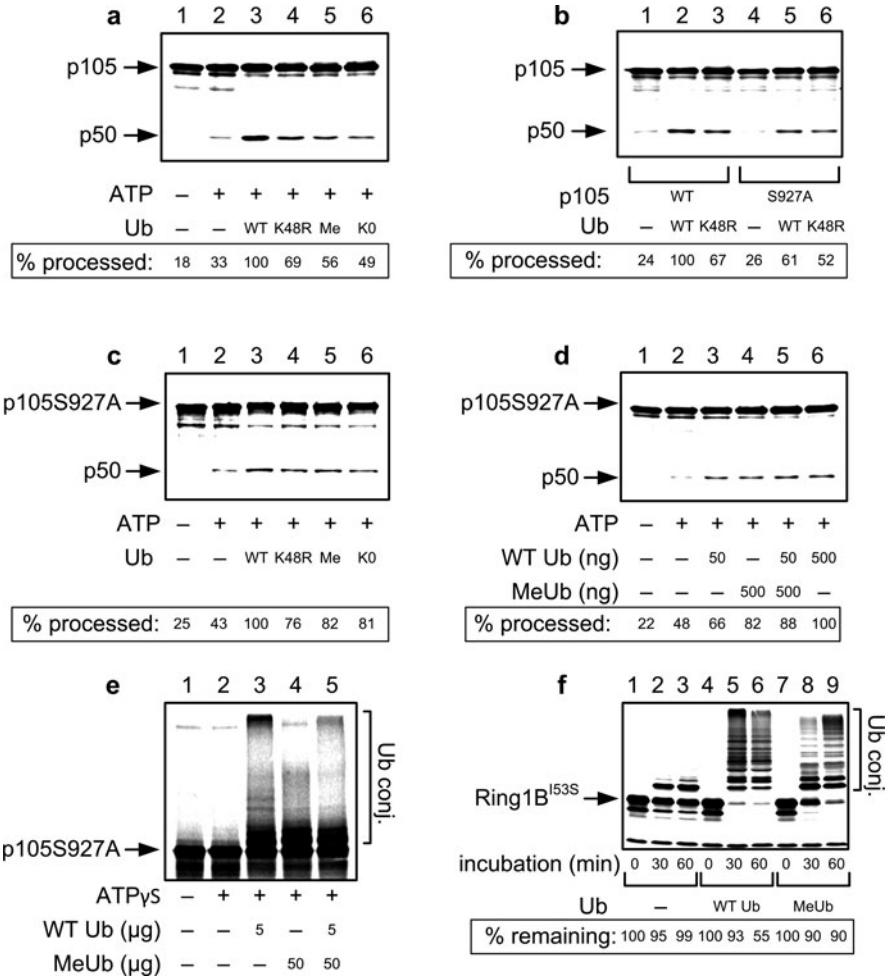

Fig. 10.2 Processing of p105 in a cell-free system does not require generation of polyubiquitin chains. (**a**–**c**) Processing of ^{35}S-labeled WT (**a**, **b**) and p105S927A (**b**, **c**) was monitored in a cell-free system in the presence of WT, K48R, methylated, and K0 ubiquitins as indicated. (**d**) Processing of p105S927A mediated by WT ubiquitin is not affected by competition with MeUb. ^{35}S-labeled p105S927A was processed in the presence of the indicated ubiquitin species. (**e**) Generation of high MW ubiquitin adducts of p105S927A is inhibited by MeUb and the inhibition is alleviated by WT ubiquitin. ^{35}S-labeled p105S927A was ubiquitinated in a cell-free system using WT and methylated ubiquitins as indicated. (**f**) Degradation of Ring1B^{I53S}in a cell-free system requires WT Ub. In vitro-translated and ^{35}S-labeled Ring1B^{I53S}was degraded in a cell-free system in the presence of WT or methylated ubiquitins as indicated. Degradation was calculated based on the radioactivity remained in the lane along time relative to time 0. MeUb denotes methylated ubiquitin and Ub conj. denotes ubiquitin conjugates. Processing (panels **a**–**d**) was calculated as described under Fig. 10.1, except that the 100% set point reflects processing in a system that contains WT ubiquitin

et al., 2007; Hershko and Heller, 1985). As can be seen in Fig. 10.2d, MeUb did not have any inhibitory effect on processing of p105. To demonstrate that the modified ubiquitin was indeed active, we showed that it inhibits generation of high molecular mass ubiquitin adducts of p105 (Fig. 10.2e, compare lane 4 to lane 3), and this inhibition can be alleviated by competition with WT ubiquitin (Fig. 10.2e, compare lane 5 to lane 4).

As a control, we tested the requirement for WT ubiquitin for in vitro degradation of Ring1B (Ben-Saadon et al., 2006). Since Ring1B is an ubiquitin ligase that catalyzes also self-ubiquitination, we used a RING finger mutant of this enzyme ($Ring1B^{I53S}$) in which this activity is abrogated. As is shown in Fig. 10.2f, WT ubiquitin catalyzes initially formation of high MW conjugates (lane 5) that disappear along time (lane 6). In striking contrast, the methylated species of ubiquitin leads to accumulation of non-degradable, lower MW adducts (lanes 8 and 9), representing multiply monoubiquitinated species of Ring1B that cannot be digested by the proteasome.

Monoubiquitination(s) Is Sufficient for Processing of p105 In Vivo

To test the role of a certain species of ubiquitin in any process, a most useful tool appears to be a yeast strain in which all the endogenous ubiquitin genes were deleted (Spence et al., 1995), and only the studied ubiquitin species is expressed. The yeast cells are kept alive by expression of WT ubiquitin under a galactose-regulated promoter. This promoter can be turned off by the addition of glucose with subsequent rapid depletion of cellular ubiquitin (Hanna et al., 2003; Hanna et al., 2007). Following depletion of endogenous cellular ubiquitin, any species of ubiquitin (such as WT, K48R, or K0 ubiquitins) can be expressed under the *CUP1* promoter, for example, along with a proteolytic target substrate. Since it was shown that p105 is faithfully processed to p50 in the yeast *Saccharomyces cerevisiae* (Sears et al., 1998), we chose the yeast cell as our initial experimental platform.

As can be seen in Fig. 10.3a, processing of p105S927A proceeded unaffected regardless of whether WT or K48R ubiquitins were expressed. Yet, K48R ubiquitin can still be involved in generation of other than K48-linked chains recognized by the proteasome. To ascertain that this does not occur, we expressed in yeast UbK0. As can be seen in Fig. 10.3b, processing of p105S927A proceeds in a similar rate to that observed when WT Ub is expressed, further strengthening the notion that it occurs following monoubiquitination(s). In contrast, degradation of short-lived proteins that apparently require generation of lysine 48-based polyubiquitin chains, such as Cln2 (Fig. 10.3c, d), Gcn4 (Fig. 10.3e), and MyoD (Fig. 10.3f; Sadeh et al., 2008), was significantly inhibited following expression of UbK48R or UbK0.

Similar results were obtained in mammalian cells, where processing of p50 proceeded unaffected in the presence of K0 ubiquitin (Fig. 10.4ai). Two lines of experimental evidence demonstrate that the expressed UbK0 affected conjugate formation and degradation in cells: (i) the polyubiquitin chains conjugated to the

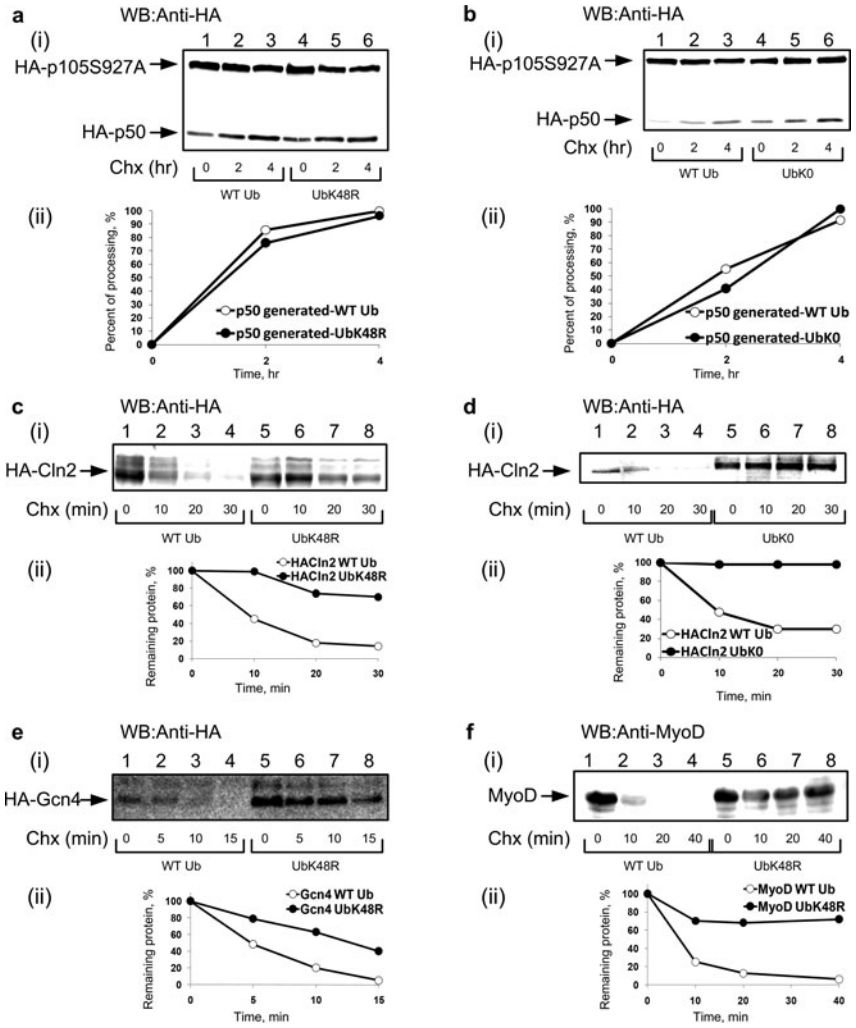

Fig. 10.3 Monoubiquitination is sufficient for processing of p105 in yeast cells. (**a**) and (**b**) (i) Generation of p50 in *Saccharomyces cerevisiae* does not require synthesis of a polyubiquitin chain(s). Processing of HA-p105S927A was monitored in a yeast cell that expresses either UbK48R or UbK0 as indicated. (ii) Quantitative analysis of p105 processing. Processing was calculated as described under Fig. 10.1. For each time point, the respective value of processing at time 0 was subtracted, and the 100% set point represents processing in a system that contains WT ubiquitin that was incubated for 4 h following addition of CHX. Hollow and solid circles represent processing in the presence of WT ubiquitin and UbK48R (or UbK0), respectively. (**c**) and (**d**) Stabilization of HA-Cln2 in a yeast cell that expresses K48R (**c**) or K0 (**d**) ubiquitins. Degradation of HA-Cln2 was monitored following addition of cycloheximide (i panels). (ii) Quantitative analysis of the degradation experiments. Hollow and solid circles represent degradation in the presence of WT ubiquitin and UbK48R (or UbK0), respectively. (**e**) and (**f**) Stabilization of HA-Gcn4 (**e**) and MyoD (**f**) in a yeast cell that expresses UbK48R. Degradation of the indicated proteins was monitored following addition of cycloheximide (i panels). (ii) Quantitative analysis of the degradation experiments. Chx denotes cycloheximide and WB denotes Western Blot

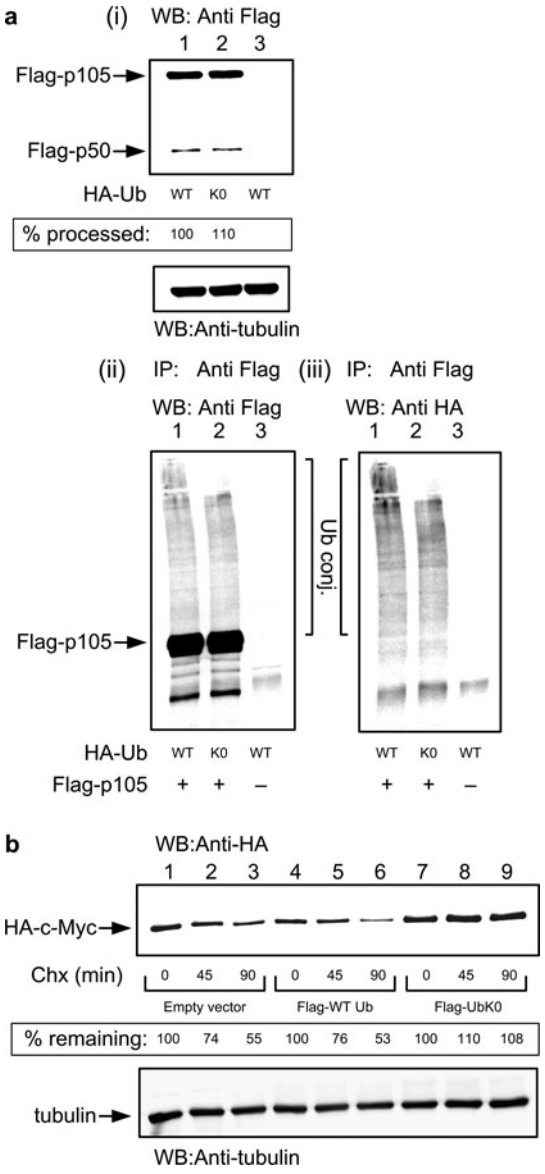

Fig. 10.4 Processing of p105S927A in mammalian cells does not require generation of a polyubiquitin chain(s). (**a**) HEK 293 cells were transfected with cDNAs coding for Flag-p105 (lanes 1–2), and HA-WT Ub (lanes 1 and 3) or HA-UbK0 (lane 2). (i) Analysis of expression and processing of p105. 10% of the cell lysates were resolved via SDS-PAGE, blotted onto nitrocellulose membrane, and p105 and p50 were detected using anti-Flag. Processing was calculated as described under Fig. 10.2. p105 and its conjugates were immunoprecipitated from the remaining portion of the lysate (90%) using anti-Flag, and detected by either anti-Flag (ii) or anti-HA (iii). (**b**) Stabilization of HA-c-Myc in cells that express UbK0. HEK 293 cells were transfected with cDNAs coding for HA-c-Myc (lanes 1–9), along with an empty vector (lanes 1–3), cDNAs coding for Flag-WT Ub (lanes 4–6), or Flag-UbK0 (lanes 7–9). Degradation of HA-c-Myc was monitored following the addition of cycloheximide. Tubulin was used to assess equal protein loading. IP denotes immunoprecipitation

expressed p105 are of lower MW compared to those generated in the presence of WT Ub (Fig. 10.4aii, aiii) and (ii) degradation of c-Myc, a bona fide short-lived substrate of the ubiquitin system (Gross-Mesilaty et al., 1998), was significantly inhibited following expression of UbK0 (Fig. 10.4b).

Taken together, these findings strongly suggest that processing of p105 in cells can be promoted by monoubiquitination(s).

Processing of p105 Requires Multiple Monoubiquitinations

In order to distinguish whether proteasomal processing of p105 requires multiple monoubiquitinations or whether a modification by a single moiety is sufficient, we tested the cell-free processing of a series of p105 mutants in which we eliminated progressively the number of lysine residues in the degradable C-terminal domain (Fig. 10.5a). The lysines in this domain were shown to be the targets of ubiquitination that leads to processing (Cohen et al., 2004; Cohen et al., 2006). The in vitro reaction was carried out using UbK48R. As can be seen in the experiment presented in Fig. 10.5b, processing efficiency was in direct correlation with the number of lysines in p105. A similar result was obtained using the yeast strain that lacks endogenous ubiquitin (Fig. 10.5c; see above).

The 26S Proteasome Binds Multiply Monoubiquitinated p105

One can assume that if multiply monoubiquitinated p105 is recognized and processed by the 26S proteasome, the enzyme complex will also bind the ubiquitin-tagged substrate. To test this assumption experimentally, we carried out ubiquitination of immobilized p105 in a cell-free reconstituted system and looked for association of the ubiquitin-tagged protein with the proteasome. As can be seen in Fig. 10.6a, p105 that was ubiquitinated with either WT or MeUb (lanes 2 and 3) could bind proteasomes as is evident by the detection of the α2 and α6 subunits of the 20S complex in the precipitated complex (Fig. 10.6b, lanes 2 and 3). When the mixture did not contain ubiquitin (Fig. 10.6a, lane 1), binding did not occur either (Fig. 10.6b, lane 1).

Discussion

Our findings further corroborate the notion that processing of p105 is mediated by the ubiquitin–proteasome system (Fig. 10.1). Surprisingly, ubiquitin species that cannot generate polyubiquitin chains could also support processing (Fig. 10.2). To demonstrate in cells the validity of the results obtained in the cell-free system, we monitored processing of p105 in a yeast strain in which all the endogenous ubiquitin genes were substituted with exogenous genes coding for either WT, K48R, or K0 ubiquitins. Efficiency of processing of p105 in the presence of UbK48R and UbK0 was not different from that monitored in the presence of WT ubiquitin (Fig. 10.3a, b). In contrast, the degradation of polyubiquitin chain-dependent substrates, such as Cln2, Gcn4, and MyoD, was severely affected (Fig. 10.3c–f). Similar findings were observed in a mammalian cell (Fig. 10.4). Using a series of p105 mutants in which we progressively substituted/eliminated all lysine residues in the C-terminal degradable domain, we found a direct relationship between the efficiency of generation of p50 and the number of ubiquitin anchors in the tail (Fig. 10.5), suggesting that a cluster rather than a single ubiquitin moiety is recognized by the proteasome.

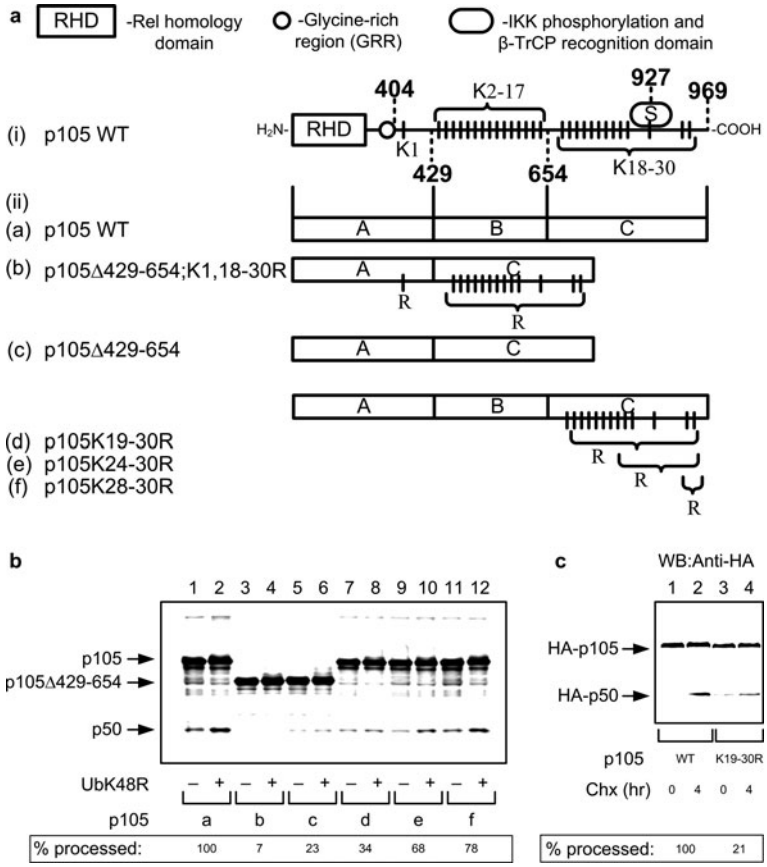

Fig. 10.5 Processing of p105 requires multiple monoubiquitinations. (**a**) (i) Schematic representation of WT p105. Numbers that follow "K" denote sequential lysine residues downstream to the GRR. K425 is denoted by K1. Numbers in bold marked by dashed lines denote the respective residue along the protein sequence. (ii) (**a**–**f**) p105 constructs in which the indicated lysines were mutated/deleted alone or in clusters. (**b**) Efficiency of processing of p105 in vitro is dependent on the number of lysine residues in the C-terminal domain. ^{35}S-labeled p105s (as described under **a**) were processed in a cell-free reconstituted system in the presence or absence of UbK48R as indicated. Quantitative analysis was carried out in two steps. First, for each reaction mixture to which UbK48R was not added, the amount of p50 generated was calculated as the ratio between the p50 signal to that of the summed signal of p50 and p105. Then, the same calculation was carried out for the corresponding reaction to which UbK48R was added. For each mutant, the net processing was calculated by subtracting the amount (%) of p50 generated in the presence from that generated in the absence of Ub. Arbitrarily, the net processing of WT p105 was denoted as 100%. In the second step we calculated the net processing for each mutant relative to the processing of WT p105. The numbers shown represent these values. (**c**) Efficiency of processing of p105 in vivo is dependent on the number of lysine residues in the C-terminal domain. Processing of HA-p105 and HA-p105K19-30R was monitored in a *Saccharomyces cerevisiae* strain that lacks endogenous *UBI* genes. Calculation of processing was carried out in a similar manner to that described under **b**, except that comparison was made to processing in time 0 rather than to processing in a reaction to which Ub was not added

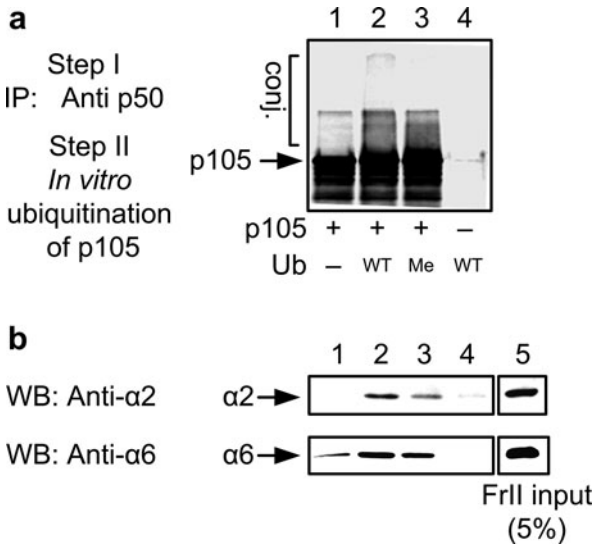

Fig. 10.6 Multiple monoubiquitinations are sufficient to mediate interaction of p105 with the proteasome. ^{35}S-labeled p105 was immunoprecipitated (panel **a**, lanes 1–3) and ubiquitinated (lanes 2–3) in the presence of WT or methylated ubiquitin as indicated. Lane 4 represents a reaction mixture that contained all components except for p105 and was processed identically to the other mixtures. FrII served as the source for the 26S proteasome. Proteins were resolved via SDS-PAGE and blotted onto nitrocellulose membrane. Labeled p105 and its conjugates were detected using PhosphorImager. The membrane was incubated with antibodies to the proteasome subunits α2 and α6 that were visualized (**b**) using the appropriate secondary antibody. Lane 5 in (**b**) represents separation of 5% of the input of FrII used for the ubiquitination reaction

Last, we have shown that the multiply monoubiquitinated p105 can bind to the proteasome (Fig. 10.6).

At this stage it appeared that it will not be useful to identify precisely the lysine residues targeted. That is because processing efficiency increased with the number of ubiquitin anchors regardless of their site, suggesting that recognition is not dependent on a particular sequence of amino acids and specific lysines. Thus, deletion/mutation of lysine residues in two clusters, B and C (Fig. 10.5aii, mutants c and d), resulted in similar processing efficiency (Fig. 10.5b lanes 5, 6 and 7, 8). In addition, lack of knowledge of the structure of the multiply monoubiquitinated region and of the identity of the proteasome subunit(s) that recognizes the modified p105 would have hampered further elucidation of the mechanism involved.

Taken together, these findings strongly suggest that the proteasome can recognize multiple single ubiquitin moieties, and not only a polyubiquitin chain.

These findings raise several interesting questions. One is whether monoubiquitination that leads to proteasomal targeting occurs in cells, and if so, what restricts elongation of the chain by the ligase. The same question can be asked for other processes where monoubiquitination has been suggested to occur, such as endocytosis of membrane proteins that serves to target them to the lysosome/vacuole. In these

cases – where some experimental manipulations were also used to restrict polyubiquitination – it was hypothesized that ubiquitin-binding domains of the downstream proteins that associate with the conjugated ubiquitin mask Ile44 on the surface of the ubiquitin molecule that is required for chain elongation, or Lys48 which serves as the primary ubiquitin anchor, and thus restrict further ubiquitination. Alternatively, the E2-E3 that catalyze ubiquitination may limit the extent of modification, or the polyubiquitin chain that is nevertheless formed, is trimmed by a DUB (Hicke et al., 2005). Isolation and characterization of the enzymes involved in p105 targeting will be necessary in order to shed light on this unsolved question. Another important problem will be to identify the proteasome subunit to which the multiply monoubiquitinated p105 binds, whether it is one of the already described ubiquitin-binding subunits or rather a novel one. A likely possibility is that the multiple ubiquitins are binding to multiple different ubiquitin recognition sites that may even reside on different subunits, which collectively results in a high degree of avidity. Following identification of the binding subunit(s), it will be important to identify its "ligand" – the cluster of ubiquitin moieties that bind to it. Here one would like to know how "fixed" or "flexible" is the cluster and what are the principles that govern p105 binding to the proteasome – density of the modifying ubiquitins, their distance from one another, whether they generate a specific 3D structure, etc. Last, it will be interesting to study whether monoubiquitination is confined to proteins that undergo limited processing, or it targets also substrates that are completely destroyed. In this context, studying the mechanism(s) of degradation of another protein that undergoes limited processing – the yeast Spt23 transcription factor involved in regulating the level of unsaturated fatty acids (Hoppe et al., 2000) – will be useful.

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For the complete list of referenced studies, please see Kravtsova-Ivantsiv, Y., Cohen, S., Ciechanover, A. (2009) Modification by Single Ubiquitin Moieties Rather Than Polyubiquitination is Sufficient for Proteasomal Processing of the p105 NF-κB Precursor. Mol Cell 33, 496–504.

Chapter 11
Selective Binding of Linear Ubiquitin Chains to NEMO in NF-kappaB Activation

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Introduction

NF-kappaB signaling pathway plays a critical role in various biological situations, such as cancer, inflammation, and development [11, 17, 21]. NF-kappaB is activated by cytokines including TNF family members, lipopolysaccharide, interleukins, and other agonists. This signaling pathway has been extensively studied in different aspects. For example, in gene targeting mouse models of TRAFs, IKKs, and IkappaB-alpha, and NF-kappaB components, p50/p52 and p65, the NF-kappaB pathway is shown to be inactive by certain stimuli [2, 21]. From molecular and biochemical studies, phosphorylation and ubiquitination of adaptor molecules play a critical role in mediating NF-kappaB activation [3, 4]. After TNF-alpha binds to its receptor, adaptor molecules, such as TRADD, TRAFs, RIP, cIAP, TAB1/2, TAK1, and IKK complex, are recruited to the receptor complex [4, 11]. This complex formation is induced not only by direct interactions of NF-kappaB regulating molecules but also by the distinct ubiquitin chains that are formed on these proteins [15]. Ubiquitin is an 8.5 kDa small modifier protein, which is expressed ubiquitously in cells [12]. Ubiquitin is known to be conjugated to lysine (Lys) residues of target proteins through a three-step enzymatic action containing activating (E1), conjugating (E2), and ligating (E3) enzymes. Similar to phosphorylation, ubiquitin modification (ubiquitylation/ubiquitination) can be reversible by an action of deubiquitin enzymes, DUBs [19, 23]. The outcome of homeostatic activities between ligases and DUBs leads to dynamic modification of substrate proteins in cells via either addition of a single ubiquitin (monoubiquitination), multiple ubiquitins to multiple lysines (multiple monoubiquitination), or formation of ubiquitin chains (polyubiquitination, [10]). Ubiquitin molecule itself contains seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48, and Lys63) that can be used for the formation of different linkage type of ubiquitin chains [15]. This leads

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to the formation of multiple ubiquitin signals in the cells with distinct structural folds. These signals are in turn recognized by the linkage-specific ubiquitin binding domains, present in effector proteins known as ubiquitin receptors, which transmit the signals to the downstream of the ubiquitin modification [5]. Among the seven different linkage types of ubiquitin chains, Lys48-linked ubiquitin chain is the most characterized [12]. Lys48-linked ubiquitin chains are recognized by proteasome receptors, Rpn10 and Rpn13, so that ubiquitinated proteins via this type of chain are degradated by proteasomal system [9, 14]. Other linkage-types of ubiquitin chains are also biologically relevant. For example, Lys63-linked ubiquitin chains are shown to be involved in various signal regulations, such as DNA repair and NF-kappaB [3, 4, 10]. In the case of activation of NF-kappaB signaling, ubiquitination takes place in three steps. In the first step, Lys63-linked ubiquitination occurs; adapter molecules such as TRAFs and RIP are modified by Lys63-linked ubiquitin chains. This is critical in the NF-kappaB signaling to lead to a signal complex formation. In the second step, NEMO (IKK-gamma), a subunit of IKK kinase complex, is ubiquitinated and also binds to ubiquitin chains subsequently activating associated IKK kinases [16, 22]. The last step where ubiquitin plays a role in the pathway is ubiquitination of inhibitory factor kappaB-alpha (IkappaB-alpha) by Lys48-linked ubiquitin chains that leads to a degradation of IkappaB-alpha and release of p50/p65 transcription factors [3, 4, 11] . It has been shown that NEMO is not only ubiquitinated [1, 13, 25, 28] but also recruited to the upstream signal complex by binding to ubiquitin chains [7, 27]. We have identified a novel ubiquitin binding domain present in NEMO and ABIN proteins (UBAN) [26]. The UBAN domain showed a great specificity in binding to linear ubiquitin chains but not to monoubiquitin or other linkage-type of ubiquitin chains [20, 22]. The UBAN motif is an evolutionarily conserved structural fold found in several proteins (NEMO, ABINs, and optineurin), which are all involved in the regulation of the NF-kappaB activation [26].

Main Text

The NEMO–UBAN Domain Selectively Binds to Linear Ubiquitin Chains

To further characterize the functions of the NEMO–UBAN domain in the regulation of NF-kappaB signaling, we studied the NEMO–UBAN domain by using a combination of biochemical and structural analysis [22]. We tested interactions between the NEMO–UBAN domain and different linkage types of diubiquitins. Purified GST–NEMO–UBAN bound specifically to linear diubiquitin but not to Lys63-linked or Lys48-linked diubiquitin chains. An affinity between the NEMO–UBAN domain and linear-diubiquitin chains was confirmed by surface plasmon resonance (SPR) with a dissociation constant of 1.6 μM, while binding to Lys63- or Lys48-linked diubiquitin molecules was below the detection level. These results revealed a specificity of the NEMO–UBAN domain in linear diubiquitin binding.

The NEMO–UBAN Domain Forms a-Helical Dimer

Structural analysis revealed that the NEMO–UBAN, the C-terminal part of the NEMO-CoZi, aa250-339 (Fig. 11.1a), forms a parallel coiled-coil homo-dimer with a near-perfect twofold symmetry (Fig. 11.1b). To characterize the linear-ubiquitin recognition by the NEMO–UBAN domain, we co-crystallized the NEMO-CoZi and liner-diubiquitin chains. The NEMO dimer accommodates two diubiquitin molecules, one on either side of the coiled-coil (Fig. 11.1b). Interestingly, diubiquitin binding induces a slight unwinding of the coiled-coil of the NEMO-CoZi domain. This might contribute to the activation of IKK kinases that binds the N-terminus of NEMO as discussed [22].

Determination of Linear-Ubiquitin Binding Surfaces in the NEMO–UBAN Domain

We found that the distal and proximal ubiquitin molecules bind to the NEMO–UBAN in a remarkably divergent fashion. The distal ubiquitin interacts with the NEMO–UBAN using its C-terminal tail together with the canonical hydrophobic Ile44 surface. On the other hand, the proximal ubiquitin employs a novel surface adjacent but not overlapping with the Ile44 surface. Leu73 and Arg74 residues of the distal ubiquitin are the only residues that bind both molecules of the NEMO–UBAN dimer simultaneously. In contrast, the last two C-terminal residues of distal ubiquitin, Gly75 and Gly76, are not involved in NEMO binding. To confirm the NEMO–UBAN binding surface in linear diubiquitin, we introduced mutations in Arg72, Leu73, and Arg74 of the distal ubiquitin as well as Gln2 in proximal ubiquitin. These mutations impaired the binding to the NEMO–UBAN [22]. These observations indicate that both ubiquitin moieties in a linear diubiquitin are involved in binding to the NEMO–UBAN. In the case of the binding surface of the distal ubiquitin in the NEMO–UBAN domain, residues such as Val293, Tyr301, Lys302, and Phe305 are included. On the other hand, the proximal ubiquitin recognition surface in the NEMO–UBAN contains Glu313, Glu317, and Glu320 residues. There is also a surface in the NEMO–UBAN domain that contacts both the distal and the proximal ubiquitins and includes Glu308, Arg309, and Arg312 residues [22]. Introduction of mutations in the NEMO–UBAN motif indicated above abolished linear ubiquitin binding in vitro.

Role of Linear Ubiquitin Binding to NEMO in Regulation of Signaling

To understand the role of liner-ubiquitin chain recognition by the NEMO–UBAN in the regulation of NF-kappaB signaling, we introduced mutations on the linear ubiquitin binding surface in the NEMO–UBAN motif. We used NEMO knockout

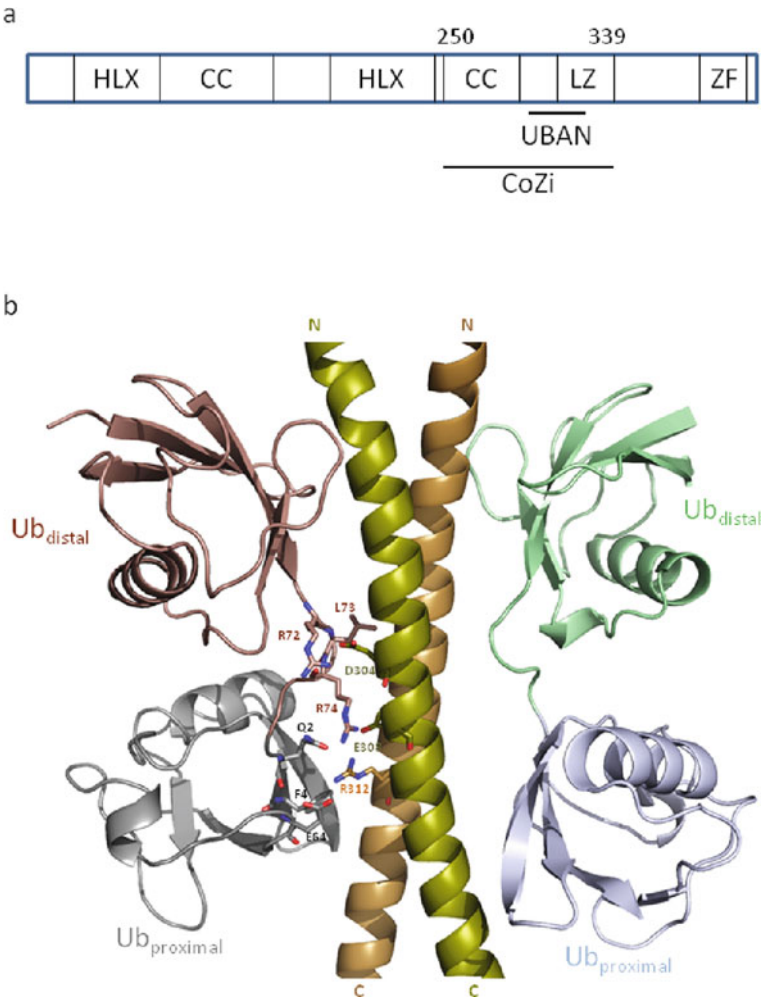

Fig. 11.1 Structure of NEMO-CoZi and linear diubiquitin. (**a**) NEMO consists of two helices (HLX), two coiled-coils (CC), a leucine zipper (LZ), and a zinc finger (ZF). The UBAN motif is within CoZi domain. (**b**) Structure of NEMO-CoZi in complex with linear-diubiquitins is shown. Mutations in NEMO UBAN motif found in ectodermal dysplasia with imunodeficiency (D304, E308 and R312) and their interacting partners from linear-diubiquitin (R72, L73, and R74 in distal Ub and Q2, F4, and E64 in proximal Ub) are indicated as sticks. The two chains of the NEMO dimer are colored in *green* and *brown*. The distal ubiquitins are shown in *purple* and *light green* and proximal ubiquitins in *gray* and *light blue*

MEF cells and NEMO deficient pre-B cells and reconstituted with either wild-type NEMO or different UBAN mutants. Among these cells, only the NEMO wild-type reconstituted cells were able to respond to NF-kappaB activation by TNF-alpha, IL1-beta, and LPS stimulations. On the other hand, all the cells reconstituted with the NEMO–UBAN mutants in the linear ubiquitin recognition surface abolished its

Table 11.1 NEMO–UBAN mutants used in the study. Mutations in mouse NEMO–UBAN used in the study and corresponding human residues are shown. Bindings of these mutants to linear tetra ubiquitin chain (Lin-4Ub) and Lys63-linked tetra ubiquitin chain (K63-4Ub) are indicated followed by an involvement of the mutants in NF-kappaB and MAP-kinase (MAPK) activations

Mutation in UBAN		Ub bindings		NF-kappaB activation	MAPK activation
Mouse	Human	Lin-4Ub	K63-4Ub		
V293Y301K302	V300Y308K309	−	+	−	+
F305	F312	−	−	−	+
R309R312R313	R316R319R320	−	++	−	+
Wt	Wt	+++++	++	+	+

ability to mediate NF-kappaB activation (Table 11.1). However, TNF-alpha- and IL1-beta induced MAPK signaling including JNK and P38 activations were intact in the MEF cells reconstituted with the NEMO–UBAN mutants. These data strongly suggest that linear-ubiquitin binding to NEMO plays a critical role in NF-kappaB signaling in multiple cells and in response to various stimuli.

Discussion and Future Perspectives

We have characterized the NEMO–UBAN motif using structural and biochemical assays and found that NEMO selectively binds to linear ubiquitin chains [22]. We demonstrated that the two moieties of ubiquitin as a minimum length for the NEMO–UBAN to recognize linear ubiquitin chains. Similarly, we have observed that the Pru domain from Rpn13 protein binds with higher affinity to diubiqitin units in longer Lys48-linked ubiquitin chains [14, 24]. It is likely that the diubiqitin unit represents the linkage specific fold that is distinguished by the selective UBDs [5]. Recent work by Iwai's laboratory identified a novel linear ubiquitin specific E3 ligase complex, LUBAC [18]. They showed that NEMO is one of the targets of LUBAC in vivo and that linear-ubiquitination of NEMO at the residues Lys285 and Lys309 (Lys278 and Lys302 in mouse) is important for cytokine-induced NF-kappaB activation in vivo (Fig. 11.2). They have shown that LUBAC induces canonical NF-kappaB signaling independently of Ubc13, an E2 enzyme specific for the formation of Lys63-linked ubiquitin chains [25]. These observations together with our findings that NEMO specifically binds to linear ubiquitin chains led us to propose a model whereby LUBAC-induced linear ubiquitination of NEMO will lead to *trans* and/or *cis* binding between neighboring NEMO dimers, which in turn can induce a conformational change and subsequent activation of associated IKK kinases (Fig. 11.2). Moreover, the NEMO–UBAN domain interface in binding to linear ubiquitin chains is also medically relevant. Several studies have identified mutations in the UBAN motif in patients suffering from ectodermal dysplasia

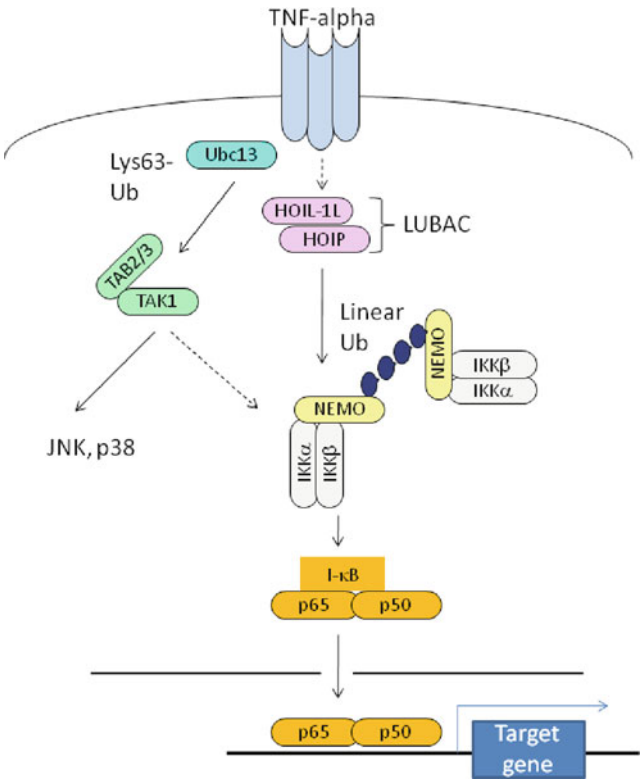

Fig. 11.2 A schematic model of linear-ubiquitin chain involvement in NF-kappaB signaling pathway. Upon TNF-alpha stimulation, the linear-ubiquitin specific E3 ligase (LUBAC) ubiquitinates NEMO. Linear-ubiquitin chain binding of NEMO plays a critical role in the regulation of NF-kappaB activation. Regulations of how LUBAC complex (HOIL-1L and HOIP) as well as IKK are activated needs to be further studied

and immunodeficiency (Fig. 11.1b, [6, 8]). These observations will further prove a critical role of the NEMO–UBAN in a biological setting.

To further understand the role of linear ubiquitin recognition in NF-kappaB signaling, detailed analysis of other UBAN containing proteins, such as ABINs and Optineurin needs to be performed. Up to date, there has been only one E3 ligase complex identified as linear-ubiquitin chain specific ligase [18, 25]. Identifications of other linear ubiquitin specific E3 ligases as well as their targets are expected. At the same time, one awaits for the broader understandings of protein networks formed via linear ubiquitin chains and their roles in various biological circumstances.

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Chapter 12
The Linear Ubiquitin Chain Assembly Complex (LUBAC) Forms Part of the TNF-R1 Signalling Complex and Is Required for Effective TNF-Induced Gene Induction and Prevents TNF-Induced Apoptosis

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Introduction

In 1974, a 76 residue polypeptide was isolated from bovine thymus during the isolation of thymopoietin and termed ubiquitin [7, 24]. This small protein was later to become an important player not only for cellular protein degradation processes but also in signal transduction induced by TNF-receptor 1 (TNF-R1) as well as many other ligand–receptor systems. Ubiquitin is a highly conserved protein that can be covalently attached to substrate proteins to regulate a wide variety of biological processes such as endocytic trafficking, NF-κB signalling, gene expression, DNA repair, and apoptosis [5]. Generally, ubiquitin is attached via its C-terminal glycine residue to the ε-amino group of a substrate lysine (K) residue. The transfer of ubiquitin to a substrate or a pre-existing ubiquitin chain is mediated by the sequential action of three enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). Ubiquitin itself has seven lysine residues and one terminal amino group, all of which can be used to attach additional ubiquitin molecules, producing eight types of poly-ubiquitin chains [12, 14]. Based on recent studies it now becomes clear how these different types of ubiquitin chains are capable of mediating different intracellular signals [6, 15, 25, 29]. Depending on the lysine residue, which serves as acceptor for further attachment of ubiquitin molecules, differently linked polyubiquitin chains adopt distinct structures that enable interactions with linkage-specific ubiquitin receptors. The resulting collection of heterogeneous recognition patterns can be bound by a variety of ubiquitin-binding domains (UBDs) present in the receptor proteins. Structurally, K63-linked ubiquitin chains are much more elongated

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than K48-linked ubiquitin chains leading to distinguishable intersubunit interfaces of K63-linked polyubiquitin chains compared to K48-linked chains [25, 29]. As ubiquitinated proteins are linked to different cellular signalling processes via their respective ubiquitin receptors, structural differences translate into distinct signalling functions: K48-linked polyubiquitin chains, which are most abundant in vivo, serve as a signal for proteasomal degradation. In contrast, K63-linked polyubiquitin chains rather seem to function as scaffolds for the recruitment of other signalling proteins upon cytokine stimulation in the context of signal transduction [5, 11].

Due to a number of recent studies, our understanding of the remarkable specificity within the ubiquitin system has been boosted, with enormous impact on the field of TNF-R1 signalling. These data particularly question the established model on how ubiquitin-dependent processes trigger TNF-signalling within and from the TNF-RSC. Current models postulate that binding of trimeric TNF to TNF-R1 results in the recruitment and assembly of a membrane-associated complex (complex I), consisting of the adaptor protein TRADD, the E3 ligases TRAF2, TRAF5, cIAP1, cIAP2, and the protein kinase RIP1. Subsequently, TRAF2 and cIAP1/2 are believed to attach K63-linked ubiquitin chains to RIP1, serving as a recruitment platform for the IKK and TAK/TAB signalling complexes [2, 11, 30, 34]. Therefore, RIP1 is commonly thought to be required for the activation of the canonical NF-κB pathway and for inhibition of TNF-R1-induced apoptosis. However, the role of K63-polyubiquitinated RIP1 in TNF signalling has now been challenged in several recent studies. As shown by Wong et al. [35], RIP1 is not essential for NF-κB activation after TNF stimulation in mouse embryonic fibroblasts (MEFs). In addition, using a ubiquitin-replacement strategy, Xu et al. found that, in contrast to IL-1β signalling, K63-linked ubiquitin chains are not required for IKK activation upon TNF stimulation [36]. Although the E3 activities of cIAP1/2 are essential for TNF-mediated ubiquitination of RIP1 and NF-κB activation [2, 20, 30], it is currently unclear which type of ubiquitin chain is synthesised by cIAP1/2. Despite the intensive studies outlined above and the accumulated knowledge with respect to ubiquitin-dependent mechanisms of signal transduction and the composition of the TNF-RSC, a number of open questions concerning the activation of different signalling pathways by TNF remain. Due to these gaps in understanding, it seemed that we may not know all components of the complex molecular machine that the TNF-RSC represents. We reckoned that only if all components of the TNF-RSC are known, a thorough understanding of downstream events will be within reach. Recently a new type of ubiquitin ligase generating linear ubiquitin chains, in which the C-terminus of ubiquitin is not attached to a lysine within the preceding molecule but rather to its N-terminus, has been discovered [14]. Although originally thought to act as a degradation signal like K48-linked ubiquitin chains [14] these “head-to-tail”-linked chains were now shown to be a third type of polyubiquitin linkage involved in TNF-induced signal transduction [9, 26]. Here we will describe how we, together with our collaboration partners, discovered this role [9].

Identification of HOIL-1 and HOIP as Novel Components of the Native TNF-RSC

To be able to comprehend the complex network of signalling pathways emanating from the TNF-RSC, the complex and its molecular composition need to be understood. Therefore we devised a novel modified tandem affinity purification (moTAP) procedure which allowed us to physically determine the molecular composition of the TNF-RSC in an unbiased manner. moTAP is based on a modified TAP-tag directly attached to TNF and facilitates the purification of native RSCs without the need to tag or over-express any of its intracellular constituents. The moTAP technique is a sensitive and selective method to purify multi-protein complexes formed under physiological conditions. It combines a first high-affinity purification via a FLAG-tag with mild elution using a site-specific protease which works at low temperature and a second affinity purification via a biotinylated Avi-tag to obtain protein complexes with high efficiency and specificity. These parameters are highly discriminating against unspecific binding of proteins throughout the biochemical purification procedure yet retain all essential components of the complex.

We purified the TNF-RSC using moTAP-TNF and subsequently identified the components of the isolated protein complex by nanoscale liquid-chromatography tandem mass spectrometry (nanoLC-MS/MS). Besides many of the known components of the TNF-RSC, including TNF-R1, TRADD, TRAF2, RIP1, ubiquitin, cIAP2, ABIN1, and the IKK and TAK/TAB complexes, this analysis also revealed the presence of two ubiquitin RING E3 ligases: haeme-oxidised IRP2 ubiquitin ligase 1 (HOIL-1/RBCK1/XAP3) and HOIL-1-interacting protein (HOIP/RNF31/Zibra). HOIL-1 and HOIP belong to the In-Between-RING (IBR) subfamily of RING-domain proteins and were shown to form a protein complex that has a peculiar E3 activity, as it catalyses the formation of linear head-to-tail ubiquitin chains. Due to this activity the protein complex formed by HOIL-1 and HOIP was named “linear ubiquitin chain assembly complex” (LUBAC) [14]. Using newly generated HOIL-1- and HOIP-specific antibodies we were able to show that HOIL-1 and HOIP are recruited to the native TNF-RSC in a stimulation-dependent manner.

LUBAC Recruitment to the TNF-RSC Is Dependent on TRADD, TRAF2/5, and cIAP1/2

To understand the mechanism of how HOIL-1 and HOIP are recruited to the TNF-RSC and to identify potential interaction partners we immunoprecipitated the TNF-RSC from several mouse embryonic fibroblast (MEF) cell lines, deficient for one of the main components of the receptor complex, respectively. In cells lacking the main adaptor protein TRADD, HOIL-1 was not associated with the TNF-R1,

indicating that LUBAC does not directly interact with the TNF-R1 upon TNF stimulation. The TNF-RSC was next immunoprecipitated from wild-type or RIP1-deficient fibroblasts. Interestingly, we could not detect any significant differences in the recruitment of HOIL-1 in the absence of RIP1. Furthermore, also deficiency in NEMO did not prevent recruitment of HOIL-1 to the TNF-RSC. Thus, although a recent report suggested an interaction between LUBAC and NEMO [26], this result shows that NEMO cannot be the sole interaction partner of HOIL-1 and HOIP within the TNF-RSC.

We next found the recruitment of HOIL-1 to be significantly reduced in TRAF2 deficient and strongly diminished in cIAP1/2-deficient MEFs compared to wild-type cells. These results suggested that ubiquitination events mediated by the E3 ligases TRAF2 and especially cIAP1/2 are of great importance for the recruitment of the LUBAC. Thus, to test whether and which of the ubiquitin–ligase activities of these proteins are required for LUBAC recruitment we stably re-expressed wt or catalytically inactive mutants of cIAP1 (F610A) or TRAF2 (ΔRING) in MEFs deficient for cIAP1/2 or TRAF2/5, respectively [19]. In case of cIAP1, re-expression of wt cIAP1 was sufficient to restore both the ubiquitination of RIP1 and the stimulation-dependent recruitment of HOIL-1 to the TNF-RSC whereas reconstitution with catalytically inactive cIAP1 (F610A) failed to rescue either of these two events. In contrast re-expression of both wt or the ΔRING mutant of TRAF2 was sufficient for RIP1 ubiquitination and HOIL-1 recruitment to occur. This clearly demonstrates that the E3 ligase function of cIAPs is required for recruitment of HOIL-1 to the TNF-RSC whereas the catalytic activity of TRAF2 is not essential. These data also show that the reduction in HOIL-1 recruitment and RIP1 ubiquitination observed in TRAF2-deficient cells is due to TRAF2's role as a recruitment platform for cIAPs via its cIAP interaction motif (CIM) and that its E3 activity is not required for this [31].

HOIL-1 and HOIP Directly Interact with Specific Polyubiquitin Chains

The finding that the absence of the E3 activity of cIAP1 and cIAP2 reduces the amount of HOIL-1 recruited to the TNF-RSC led us to hypothesise that the interaction between LUBAC and TNF-R1 may be mediated by ubiquitin chains generated by cIAPs. Indeed, HOIL-1 and HOIP could be precipitated in a ubiquitin-pulldown assay and further analysis showed that recombinantly expressed HOIL-1 and the zinc finger (ZnF) domain of HOIP bound to ubiquitin chains of different linkage types including K63-, K48-, and linearly linked ubiquitin chains. Together, these data indicate that LUBAC is recruited to a ubiquitin chain platform generated by cIAP1/2 in the TNF-RSC and that the generation of this platform requires recruitment of TRADD, TRAF2, and catalytically active cIAPs to TNF-R1.

HOIL-1 and HOIP Mediate TNF-Induced Signalling Events, Gene Induction, and Protect from TNF-Induced Apoptosis

When examining the functional role of LUBAC in the TNF-RSC we observed that the knockdown of HOIL-1 and/or HOIP reduced TNF-induced NF-κB activation. We also found that stable knockdown of HOIL-1 resulted in a severe reduction in TNF-induced JNK activity and cJun phosphorylation. This indicates that LUBAC is not only required for effective TNF-induced activation of NF-κB but also of JNK. Concomitant knockdown of HOIL-1 and HOIP had a marked suppressive effect on TNF-induced expression of various TNF target genes and on TNF-induced secretion of IL-8. Consistent with a reduction in NF-κB activation, HOIL-1 knockdown cells were sensitised to TNF-induced cell death. These results clearly demonstrate that loss of HOIL-1 and HOIP results in inefficient activation of NF-κB and JNK, leading to a reduced TNF-induced gene expression and sensitisation of cells to TNF-induced apoptosis.

HOIL-1 and HOIP Are Required for Stable TNF-RSC Formation

The loss of TNF-induced JNK and NF-κB signalling in the absence of LUBAC, together with the discovery that LUBAC forms an integral component of the TNF-RSC, suggests that it functions within the membrane proximal receptor complex to activate these signalling pathways. We therefore investigated the composition of the TNF-RSC in cells stably overexpressing HOIL-1 and HOIP and also in cells lacking HOIL-1 and HOIP. Overexpression of wt LUBAC increased and prolonged overall ubiquitination of TNF-RSC components and RIP1, TRAF2, and TAK1 were retained within the complex substantially longer than in control cells, whereas TRADD retention was not greatly affected. Conversely, transient knockdown of HOIL-1 and HOIP severely reduced recruitment and/or retention of RIP1, TRAF2, and TAK1 in the complex. TRADD recruitment was, however, not altered in the absence of LUBAC suggesting that LUBAC acts downstream of TRADD to specifically stabilise the complex and to allow for full activation of downstream signalling pathways. Because LUBAC generates linear ubiquitin chains and NEMO preferentially binds to this type of polyubiquitin chains [26], we tested whether the reduced NF-κB activation caused by depletion of LUBAC might be due to impaired recruitment of the IKK complex to the TNF-RSC. In stable HOIL-1/HOIP double knockdown cells, much less IKK1 was detected in the TNF-RSC when the complex was immunoprecipitated. In addition, immunoprecipitation of NEMO revealed that in TNF-stimulated HOIL-1/HOIP double knockdown cells substantially less RIP1, TRAF2, TRADD, and TNF-R1 were associated with NEMO than in control cells.

Finally we asked whether TNF-RSC stabilisation results from the mere presence of LUBAC in the complex or from LUBAC's function as an E3 ligase. To answer this we expressed wt HOIL-1 together with wt or mutR HOIP, a mutant,

which renders LUBAC catalytically inactive. In contrast to untransfected cells or cells overexpressing wt LUBAC, expression of HOIL-1/HOIP mutR resulted in normal initial recruitment and modification of RIP1 but rapid loss of RIP1 from the complex, demonstrating that it is the E3 activity of LUBAC, hence linear ubiquitin chains, that is required for TNF-RSC stabilisation.

In summary, our experiments show that recruitment of LUBAC to the TNF-RSC via TRADD, TRAF2, cIAPs, and cIAP-generated ubiquitin chains stabilises the complex, that this stabilisation requires LUBAC's linear ubiquitin chain forming activity and that LUBAC recruitment to the TNF-RSC and its activity are important for effective TNF-induced gene induction.

Conclusions

Multi-protein complexes are important entities of biological activity inside cells that serve to create functional diversity by organising the large number of different proteins into functional units. Many a time, when studying protein complexes rather than individual proteins, the biological insight gained has been fundamental, particularly in cases in which proteins with no previous functional annotation could be placed into a functional context derived from their molecular environment. Due to their position at the apex of signal transduction cascades, receptor signalling complexes that form at the cell membrane are often prominent examples of multi-protein complexes whose composition determines cellular fate and which are involved in the regulation of key biological processes.

To elucidate the composition of the native TNF-RSC following ligand binding we developed a modified tandem affinity purification (moTAP) technique. Using moTAP in combination with nanoLC tandem mass spectrometry we identified two novel components of the native TNF-R1-associated protein complex, HOIL-1 and HOIP. Together, HOIL-1 and HOIP were described to form a linear ubiquitin chain assembly complex (LUBAC) that attaches head-to-tail linked ubiquitin chains to target proteins [14]. LUBAC is thought to bind to NEMO and conjugate linear polyubiquitin chains onto specific Lys residues in NEMO's CC2-LZ domain in a Ubc13-independent manner [26], thereby influencing activation of NF-κB. The demonstration that LUBAC is recruited to the TNF-RSC in a stimulation-dependent manner not only increases the number of known E3 ligases required to regulate TNF-R1 signalling directly at the receptor level, but also provides the missing link between TNF binding and initiation of linear NEMO ubiquitination.

Using MEF cell lines deficient for main TNF-RSC components, we showed that LUBAC recruitment is abolished in the absence of TRADD, diminished in the absence of TRAF2, and almost undetectable in the absence of cIAP1 and cIAP2. Reconstitution experiments with knockout MEFs suggest that the catalytic activity of the cIAPs, but not of TRAF2, is essential for LUBAC recruitment to the TNF-RSC. The requirement of cIAP1's E3 activity suggested that ubiquitin may be involved in recruiting LUBAC. Indeed, both HOIL-1 and HOIP were capable of

binding linear and/or K63-linked ubiquitin chains, respectively. However, the interaction of HOIL-1 with ubiquitin was strongly increased in the presence of HOIP, indicating that LUBAC likely binds to polyubiquitin mainly via the zinc fingers of HOIP. Together, these data suggest a model where HOIL-1 and HOIP are recruited to the TNF-RSC via cIAPs-generated ubiquitin chains [2, 20, 36, 37].

Surprisingly, RIP1, despite being a prominent target of cIAP-mediated ubiquitin modifications [2, 20, 30, 32, 34], was not essential for HOIL-1 recruitment to the TNF-RSC. However, other proteins, for example, TRAF2 and cIAPs, have been described to be modified with polyubiquitin chains [3, 16, 27, 28, 39] and might therefore provide not only additional ubiquitin chains for recruitment of LUBAC but also alternatives to RIP1 as LUBAC-binding platform, especially in its absence.

As a sideline, our reconstitution experiments comparing wt and catalytically inactive mutants of cIAP and TRAF2, respectively, suggest that presence of a TRAF2 variant lacking E3 activity is sufficient for RIP1 ubiquitination to occur, whereas this modification cannot be detected in the absence of cIAP's catalytic activity. In line with several recent publications [2, 22, 31, 39], this indicates that TRAF2 may serve as an adaptor protein recruiting cIAPs to the TNF-RSC thereby bringing them in close proximity to RIP1 rather than as the E3 directly ubiquitinating RIP1.

Concerning the recruitment of LUBAC it is interesting to note that although HOIL-1 and HOIP can interact with NEMO [26], this interaction is not required for LUBAC to be recruited to the TNF-RSC. Contrariwise, our data indicate that the stimulation-dependent interaction of the IKK complex with the TNF-RSC depends on LUBAC. This is consistent with recent reports that NEMO selectively binds linear polyubiquitin chains via a UBAN (ubiquitin binding in ABIN and NEMO) motif [15, 18, 23].

The contribution of linear ubiquitin chains to efficient recruitment of the IKK complex to the TNF-RSC can also explain the observation that the ability of NEMO to bind linear ubiquitin chains is important for efficient TNF-induced NF-κB activation [18, 23]. However, our data indicate that this is not the only mechanism by which LUBAC influences TNF-signalling. LUBAC's activity not only promotes retention of NEMO in the TNF-RSC, but also retains cIAP1, TAK1, RIP1, and TRAF2 in the TNF-RSC, resulting in an overall stabilisation of the complex. So far, the mechanism leading to this stabilisation is not completely understood. However, the fact that not only NEMO but also other components of the TNF-RSC such as ABINs [15, 23] and IAPs [8] have been reported to bind linear ubiquitin suggests a model according to which linear ubiquitin chains generated by LUBAC provide an optimised scaffold for the binding of several constituents of the complex (Fig. 12.1). In this model initial recruitment of ubiquitin-binding components of the TNF-RSC, including LUBAC, depends on ubiquitin chains generated by cIAPs and possibly TRAF2. Once recruited, LUBAC attaches linear chains to NEMO [26] and, quite likely, also to other targets.

Due to their ability to strongly interact with these chains, NEMO as well as other proteins become more firmly interlocked with the complex. Sustained presence of cIAPs in the complex allows the generation of additional cIAP-generated ubiquitin

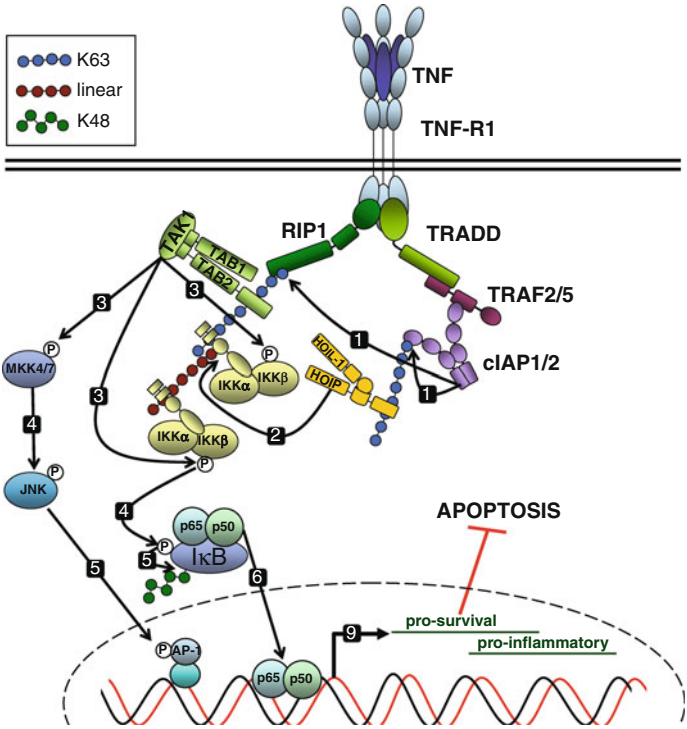

Fig. 12.1 A schematic of a working model for recruitment of LUBAC into the TNF-RSC. Following crosslinking of TNF-R1 by TNF, the adaptor protein TRADD is recruited to the death domain of TNF-R1. TRADD then builds a platform for recruitment of RIP1 and TRAF2/5 which in turn recruit cIAPs. cIAP1/2 ubiquitinate components of the TNF-RSC including RIP1 and themselves. These polyubiquitin chains then serve as a binding platform for LUBAC and the TAK/TAB complex as well as for initial recruitment of the NEMO/IKK complex. LUBAC is now able to linearly ubiquitinate NEMO, thereby providing an opportunity for additional NEMO/IKK complexes to bind to linear polyubiquitin chains. These interactions and binding steps bring components of the TNF-RSC into close proximity favouring further phosphorylation and ubiquitination events which lead to the activation of both NF-κB and MAPK cascades and finally to the transcription of both pro-inflammatory and anti-apoptotic genes

chains, thereby also stabilising the presence of those ubiquitin-binding proteins that prefer other linkage types including K63-linked over linear ubiquitin chains such as the TAK/TAB complex [13, 15, 17]. The retention of non-ubiquitin-binding components of the TNF-RSC may occur indirectly via their affinity to ubiquitin-bound interaction partners.

Hence, cIAP-generated ubiquitin chains act upstream of LUBAC-generated ubiquitin chains in initial complex formation and combination of both linkage types leads to enhanced stability of the TNF-RSC.

To dissect their different functions and to understand how these linkage types differ despite their three-dimensional structural similarity [15] will be important challenge for the future. LUBAC's central function and position in the TNF-RSC

upstream of the different signalling pathways emanating from TNF-R1 explains why not only the NF-κB pathway but also the activation of JNK is negatively influenced by the absence of LUBAC. Its position at the apex of TNF-induced signal transduction enables LUBAC to regulate signalling pathways emanating from the TNF-RSC by a multi-layered mechanism:

As mentioned above LUBAC may affect the activation of NF-κB by generating linear ubiquitin chains that lead to retention of the IKK as well as the TAK/TAB complex within the TNF-RSC. By prolonging their presence in the complex as well as by keeping them in close proximity, this allows for TAK1 to phosphorylate and activate IKK. In addition, binding of NEMO to linear ubiquitin chains induces not only an increase in the amount of recruited IKK but also a conformational change within NEMO [23] that may translate into an overall structural change of the IKK complex and finally to an activation of IKK that possibly qualitatively differs from the one that occurs in the absence of linear ubiquitin chains. A further possibility of regulation is added by the fact that NEMO is a direct target of LUBAC [26]. Ubiquitination of NEMO may again induce conformational changes leading to activation of IKK but may also serve as a binding platform for further NEMO molecules thereby favouring oligomerisation, trans-auto-phosphorylation, and possibly TAK1-independent activation of IKK complexes. In case of JNK, LUBAC may lead to an increase in signal intensity and duration by mediating an overall stabilisation of the TNF-RSC and thereby to the retention of proteins essential to the activation of the MAPK pathway such as TAK1 [33]. In addition a role for NEMO in effective TAK1-mediated JNK activation following stimulation with LPS, Interleukin-1β (IL-1β), or anti-CD40 has been described [21, 38]. Regulation mechanisms involving ubiquitin binding or ubiquitination of NEMO as described for NF-κB may therefore also apply to the activation of JNK. Finally, LUBAC's role in TNF-induced gene induction may also explain its inhibitory effect on TNF-induced cell death. Whether LUBAC regulates TNF-induced apoptosis solely by increasing the expression of pro-survival proteins or whether there are again multiple layers of regulation remains an open question to date. It will be interesting to investigate in future whether the formation, composition, and stability of the death-inducing complex II as well as other key steps in TNF-mediated cell death are affected by presence or absence of LUBAC.

To gain a deeper understanding of LUBAC in the context of TNF signalling, the identification of potential additional targets as well as the identification and more detailed characterisation of proteins able to bind linear ubiquitin chains will be critical. Since the recruitment of LUBAC increases the combinatorial complexity of ubiquitin modification within the TNF-R1 complex, it is an outstanding issue to determine which components of the TNF-RSC are modified by which type of ubiquitin chain. In addition it will be essential to investigate the functional implications of modification with or binding to the different types of polyubiquitin chains.

As ubiquitin ligases and polyubiquitin chains required for recruitment of LUBAC are not only present in the TNF-RSC but also in signalling induced by antigen receptors, other TNF-R superfamily members, IL-1 receptor (IL-1R)/Toll-like receptor

(TLR) 4, as well as NOD1 and NOD2 [1, 4, 10], it is possible that LUBAC also plays a role in the signal transduction triggered by other receptor–ligand systems. Investigation of this novel ubiquitin ligase in the context of these systems will be a fascinating area of research and is likely to provide insightful information on ubiquitin-dependent mechanisms of signal transduction.

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Chapter 13
Temporal Control of TNFα Signaling by Miz1

Anning Lin

The pro-inflammatory cytokine tumor necrosis factor (TNFα) regulates a wide range of biological activities, including inflammation, immune responses, apoptosis, and tumorigenesis mainly through its cytoplasm membrane receptor TNFR-1 [6, 7]. Engagement of TNF-R1 by TNFα induces the formation of the receptor complex 1, which is composed of TNF-R1-associated death domain protein (TRADD), TNF-receptor associated factor 2 (TRAF2) and TRAF5, receptor interacting protein 1 (RIP1), and two inhibitors of apoptosis cIAP1/cIAP2 [2, 4]. TRAF2/5 and RIP1 are involved in activation of multiple downstream effectors including c-Jun N-terminal protein kinase (JNK; also known as stress-activated protein kinase SAPK), p38, and the inhibitor of NF-κB kinase (IKK) complex. After dissociation from TNF-R1, complex 1 interacts with Fas-associated death domain protein (FADD) to form complex 2, which in turn recruits and activates caspases for cell death [1, 6].

The recruitment of complex 1 to TNF-R1 is a key step in TNFα-induced activation of JNK, p38, and IKK. How the activity of complex 1 is regulated is incompletely understood. Activation of the downstream signaling pathways by TNFα is also temporally controlled. For instance, while IKK is activated within a few minutes after TNFα stimulation, JNK1 activation takes 10–15 min to reach to its maximal activity. However, the underlying mechanism is unknown. Recently, we found that Miz1, a zinc finger transcription factor, also functions as a signal- and pathway-*s*pecific *m*odulator *o*r *r*egulator (SMOR) to selectively regulate TNFα-induced JNK1 activation [3]. Identified by mass spectrometry and yeast two-hybrid screening as a novel JNK-associated protein, Miz1 inhibits TNFα-induced activation of JNK1 but not p38, ERK, or IKK, and it does not inhibit activation of JNK1 by other stimuli such as UV or IL-1 either. This is because Miz1 is recruited to complex 1 to selectively suppress K63-linked polyubiquitination of TRAF2. Interestingly, upon TNFα stimulation, Miz1 itself undergoes K48-linked polyubiquitination and proteasomal degradation, thereby relieving its inhibition on TNFα-induced JNK1 activation. The removal of Miz1 from TNF-R1 complex 1 upon TNFα stimulation

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indicates that the recruitment of the adaptors to TNF-R1 may not be sufficient for their activation. It is likely that complex 1 needs to undergo the "de-repression" first, i.e., the removal of suppressors like Miz1, in order to be post-translationally modified before being able to activate the downstream effectors such as JNK1. Furthermore, the necessity of removing Miz1 for activation of JNK1 but not IKK may provide a temporal regulation of IKK and JNK activation to allow IKK to be activated first and JNK second. Further studies will determine the physiological significance of this temporal regulation. We also found that genetic disruption of Miz1 alleles augments TNFα-induced inflammatory response in macrophages and accelerates TNFα-induced cell death in mouse embryonic fibroblasts when NF-κB activation is blocked. Thus, Miz1 is a physiological relevant SMOR that selectively regulates TNFα-induced JNK1 activation for inflammation, or cell death when NF-κB activation is impaired.

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Part IV
Novel aspects of Signaling Activation by the TNF Family: *TNF Signaling and Gene Regulatory Networks*

Chapter 14
Large-Scale RNAi Screens to Dissect TNF and NF-κB Signaling Pathways

Marie Metzig, Dorothee Nickles, and Michael Boutros

Introduction

Signaling pathways are important for the maintenance of cell homeostasis. A complex network of biochemical signal cascades is responsible for the regulation of many processes that link cell proliferation, differentiation, apoptosis, and mediation of adequate immune responses [1]. Aberrant signal transduction has been implicated in a variety of human diseases, such as cancer, infection, and neurodegeneration. While significant advances have been made in the past years to understand signaling pathways, there are still many gaps in current models to understand principle mechanisms as well as in the identification of novel targets for therapeutic approaches. Our current knowledge about signaling pathways is mainly derived from biochemical experiments and classical genetic screens in model organisms. Improvements in genomic approaches now allow to dissect cellular pathways and their interconnection and provide new avenues to an analysis on a genome-wide scale to understand principle mechanisms underlying carcinogenesis and to identify potential novel targets for therapeutic intervention.

Sequencing of the human genome and the genomes of many model organisms opened the doors for new screening approaches. Large-scale RNA-interference (RNAi) screens have already shown to be a powerful tool in the further dissection of signaling pathways. The mechanism of RNAi is based on the introduction of double-stranded RNAs (dsRNA) leading to sequence-specific degradation of endogenous messenger RNA (mRNA) and gene silencing [2]. Since RNAi libraries have become available high-throughput RNAi-screens are a widely used approach to study signaling pathways in model organisms and human cells. In this article we focus on how large-scale RNAi screens can effectively be used for the characterization of the

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TNFα-induced NF-κB activation and help to identify potentially novel targets for therapeutic intervention.

TNFα-Induced NF-κB Activation

Tumor necrosis factor α (TNFα) belongs to the TNF super family consisting of at least 19 ligands and 29 different receptor types and is produced by many different cell types including macrophages, monocytes, lymphocytes, keratinocytes, and fibroblasts upon inflammatory stimuli [3, 4, 5]. TNFα signaling has a broad range of biological functions including the regulation of inflammation, survival, differentiation, and apoptosis. Signaling occurs via two distinct receptors, TNF receptor 1 (TNFR1) and TNFR2, respectively (Fig. 14.1). While TNFR1 is expressed in most tissues and binds soluble ligands, TNFR2 is restricted to hematopoietic cells and primarily activated by the membrane-bound form [6]. The biological function of TNFR2 remains not fully resolved, but there is increasing evidence for a modulatory role on TNFR1 signaling. Depending on cell type and environmental conditions stimulation of TNFR1 can result in two different responses, either expression of pro-apoptotic or pro-inflammatory genes, respectively. The latter one is more common and mediated by two major transcription factors, AP-1 and nuclear factor κb (NF-κB) [6].

The mammalian NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel, and RelB, which share an N-terminal Rel homology

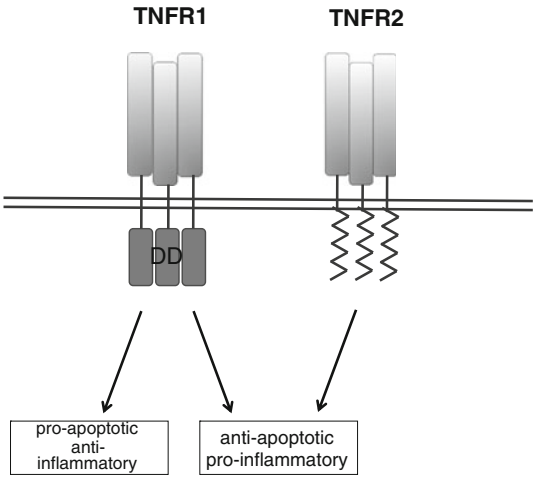

Fig. 14.1 Scheme of TNF receptor type 1 and 2 and their effects on programmed cell death and inflammation. Binding of TNFα causes trimerization of both transmembrane receptors TNFR1 or 2, respectively, leading to the recruitment of different signaling components. TNFR1 contains an intracellular death domain (CDD) and depending on cell type and environmental conditions signaling can either promote apoptosis or inflammation. In contrast, TNFR2 lacks a death domain and probably has a modulatory function for TNFR1 signaling

domain (RHD) crucial for DNA-binding and formation of homo- and heterodimers [7, 8]. Many different stimuli and signaling cascades can result in the activation of NF-κB, but most share the common principle of IκB kinase (IKK) complex inducing nuclear translocation of NF-κB proteins. TNFα binding to TNFR1 potently activates NF-κB via the classical or canonical signaling cascade (Fig. 14.2).

The physiological relevancy of TNFα-induced NF-κB signaling in the regulation of cellular processes such as cell proliferation, differentiation, apoptosis, tissue development, and inflammation is underlined by its evolutionary conservation. NF-κB signaling was first discovered to play an important role during embryonic development of *Drosophila melanogaster* [9]. Dorsal, the first identified homolog of NF-κB, is activated by Toll signaling, and its upstream components have been later also implicated in innate immune responses [10]. A second pathway leading to activation of Relish, another homolog of NF-κB in *Drosophila,* is termed Imd (RIP) pathway and strictly required for immune responses to gram-negative bacteria. Although less complex, Imd pathway contains many components structurally

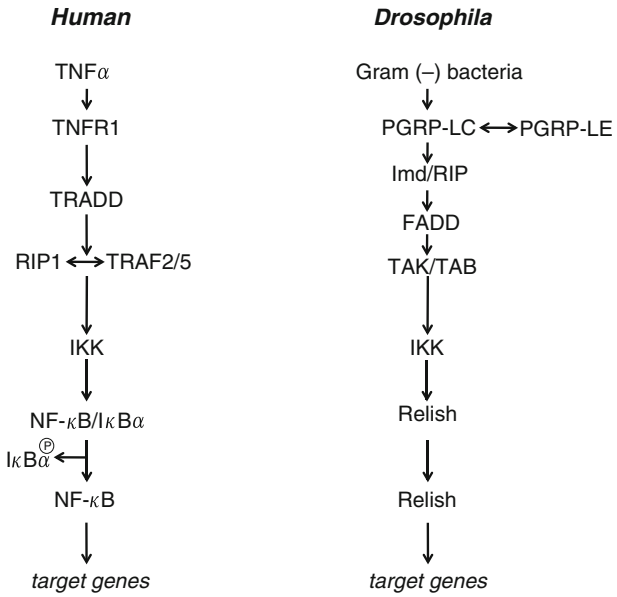

Fig. 14.2 Evolutionary conservation of TNFα and NF-κB signaling. (**a**) Mammalian TNFR1 pathway. When signaling is not active NF-κB proteins are bound to IκBα thereby kept inactively in cytoplasm. Upon TNFα binding to TNFR different signaling molecules such as TRADD, RIP1, and TRAF2/5 are recruited leading to the activation of IKK complex consisting of IKKα, IKKβ, and IKKy (Nemo). IKK induces the phosphorylation, ubiquitination, and proteasomal degradation of IκB proteins leading to the subsequent translocation of NF-κB proteins into the nucleus to activate target genes regulated by κB sites. (**b**) Imd/RIP pathway in *Drosophila melanogaster*. The evolutionary conserved counterpart of TNFα signaling in *Drosophila* is the Imd pathway, which is activated by gram-negative bacteria. Activation of the Imd/RIP pathway leads to nuclear translocation of Relish and regulation of target gene expression. The lack of genetic redundancy facilitates the analysis by RNAi and genetic approaches

similar to the mammalian TNFR1 signaling and thus was identified as the conserved equivalent in *Drosophila* (Fig. 14.2). Both Toll and Imd signaling pathways are commonly used as simplified models to study NF-κB signaling which can be dissected with many genetic tools that are available for model organisms.

TNFα/NF-κB Signaling: A Link Between Inflammation and Cancer

It is of key importance that secretion and processing of TNFα and TNFR1 signaling are tightly regulated. Augmented signaling can cause chronic inflammatory diseases like rheumatoid arthritis and Crohn's disease or result in septic shock [5]. We know today that chronic inflammation can also contribute to carcinogenesis. In fact, 15–20% of all human malignancies are estimated to be associated with chronic inflammation and infections [11, 12]. For instance, colorectal cancer is associated with colitis ulcerosa and lung carcinogenesis with chronic airway inflammation caused by tobacco. Examples of pathogen-driven inflammation promoting cancer are chronic hepatitis B and C infections as well as *Helicobacter pylori* infection; the first ones are associated with hepatocellular carcinoma and the latter is an important pathogenetic factor of gastric cancer. Conversely, cancer also drives inflammation, which is supported by the fact that not only host cells in tumor microenvironment, but also cancer cells themselves produce inflammatory cytokines [5].

However, these cytokines can also attract cytotoxic T cells that recognize and eliminate cancer cells leading to a reduction of the cancer mass.

This reciprocal relation between inflammation and cancer is also reflected in the opposed ways of how TNFα can affect carcinogenesis. About three decades ago TNFα has been named for its ability to cause hemorrhagic tumor necrosis in experimental models of cancer [5]. The mechanism of this anti-tumor effect was partly explained by a damage of tumor vasculature. In contrast, TNFα knockout mice turned out to be less susceptible to tumor development and elevated blood levels of TNFα were found to be associated with clinically poor prognosis, e.g., in patients with prostate cancer [13, 14]. Further studies showed that the tumor-promoting effects of TNFα such as cell transformation, progression of metastasis, and angiogenesis are mostly mediated by TNFR1 signaling and downstream activation of NF-κB [5]. In fact, in a variety of human solid tumors such as breast cancer, melanoma, pancreatic cancer, and colon cancer as well as in haematologic malignancies NF-κB proteins turned out to be aberrantly active [12, 15, 16]. The underlying mechanisms are multiple and can be due to dysregulated expression of endogenous pathway components or exogenous pathway stimulators such as TNFα.

However, independent on whether TNFα is considered to be a tumor promoter or inhibitor the TNFα/NF-κB pathway has been established as a promising target for therapy of chronic inflammatory diseases as well as human cancer [5]. Thus there is a critical need to understand its regulatory mechanisms and gain further insight into its role in carcinogenesis.

RNAi Screens to Dissect Signaling Pathways

RNAi is a conserved cellular process defining sequence-specific degradation of messenger RNA (mRNA) targeted by double-stranded (ds) RNA [17]. Described physiological function of RNAi are mediation of resistance against pathologic nucleic acids and the regulation of gene expression by the expression of short endogenous RNAs (miRNAs). Gene silencing using RNAi was first demonstrated in plants and *Caenorhabditis elegans*; feeding worms with dsRNA resulted in sequence-specific degradation of the corresponding mRNA and became a powerful tool for systematic approaches of functional genomics [18].

Whereas classical forward genetic screens are based on the creation, recovery, and mapping of genetic mutations, RNAi screens have the advantage that the sequence of a gene causing a certain cellular phenotype is known [19]. As RNAi libraries are commercially available, high-throughput RNAi screenings have become a widely used approach for loss-of-function analysis to study biological signaling pathways [2]. The first genome-wide cell-based RNAi screen identified genes with a role in cell growth and cell viability in *D. melanogaster* [20]. RNAi screening technique has also been applied to mammalian cells [21, 22]; in contrast to the use of dsRNA in *Drosophila* cell culture or *C. elegans* knockdown experiments, mammalian cells require the transfection of small-interfering RNAs (siRNAs) to circumvent the induction of anti-viral pathways [19, 23]. Currently, RNAi approaches are widely used to identify genes responsible for cancer proliferation or therapy resistance in human cells [24, 25].

Design Principles of Large-Scale RNAi Screens for TNFα/NF-κB

In general large-scale RNAi screens for the dissection of signaling pathways require a suitable RNAi knockdown library, a cell-based assay for monitoring changes in pathway activity and an appropriate method for data analysis [23]. Commonly used approaches for the detection of knockdown phenotypes are relatively simple readouts, such as luminescence and fluorescent reporters or cell survival, which are easily scalable for high-throughput screening and are often followed up by more complex phenotypic readouts, e.g., by automated microscopy.

To identify factors involved in signaling pathways that cause activation of target gene transcription, luciferase reporter assays are commonly used. Thereby, a gene encoding luciferase is fused to a promoter containing specific binding sites of the transcription factor of interest (e.g., NF-κB). As a result, activation of the signaling pathway is reflected by high luminescence while perturbation of the pathway by RNAi results in reduction of the luciferase signal (Fig. 14.3a). This system is applicable to almost any cell-based screen and has been broadly used for RNAi screens in cultured Drosophila cells as well as in human cell-based assays.

Besides classical genetic screens and biochemical experiments, RNAi screens significantly contributed to the understanding of the signaling pathway triggered

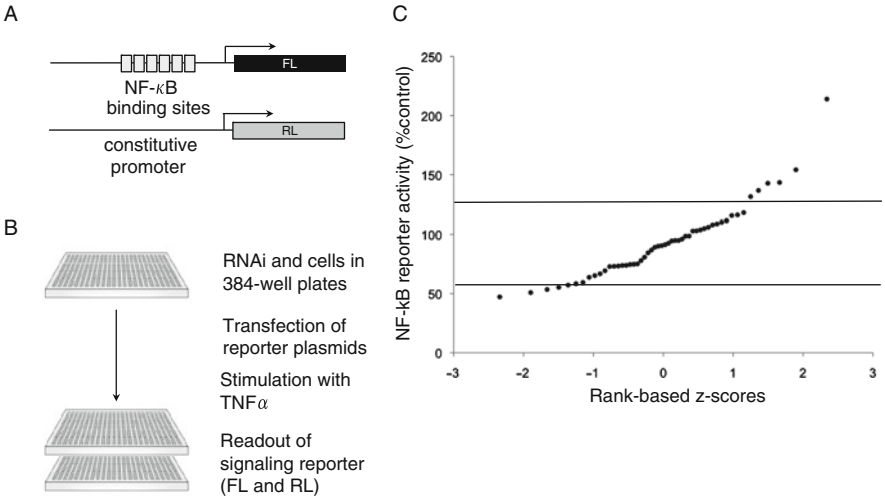

Fig. 14.3 Workflow of RNAi screen using high-throughput reporter genes. (**a**) Luciferase reporter system for TNFα signaling. A *Firefly* luciferase gene (FL) was fused to a promoter region containing NF-κB binding sites. Upon NF-κB translocation to the nucleus, FL is expressed which indicates that the signaling pathway is active and NF-κB target genes are induced. *Renilla* luciferase gene (RL) is constitutively expressed. RL expression is used as a control for cell viability and transfection efficiency. (**b**) RNAi screening procedure. Cells are transfected with siRNAs and reporter plasmids in 384-well plates. After a suitable stimulation period with TNFα FL and RL luciferase activity are measured by luminometer and correlated to pathway activity. (**c**) Limited siRNA screen for DUBs regulating TNFα signaling. Using the established protocol we performed a screen with a knockdown library comprised of siRNAs against 52 human DUBs. The plot shows NF-κB reporter activity after knockdown against rank-based *z*-scores. While most genes follow normal distribution only few top candidates show reduced (below 60%) or enhanced pathway activity (above 130%), respectively

by TNFα. For example, PGRP-LC [26], IAP2 [27], and novel receptors for phagocytosis were identified as key components of the Imd/RIP pathway by genome-wide RNAi screens in cultured *Drosophila* blood-like cells. In a screen in mammalian cells for novel regulators of TNFα-induced NF-κB activation amongst the family of deubiquitinating (DUB) enzymes in human cells, a short hairpin RNA (shRNA) knockdown library of 50 genes was screened for its effect on NF-κB activity [28]. Using this strategy cylindromatosis tumor suppressor gene (CYLD) was identified as a negative regulator of NF-κB activation by modulating TRAF2 ubiquitination. A combined in vitro and in vivo approach identified the Akirin protein family as an essential co-factor for NF-κB both in *Drosophila* and in mice [29].

As the ubiquitin system is expected to play an important role in regulation of TNFα-induced NF-κB activation, we recently screened a library containing siRNAs that target DUB enzymes in a human cell-based approach. The screening procedure is described in Fig. 14.3b. Gene-specific knockdown was performed using a pool containing four siRNAs with non-overlapping sequences. Human cells were

transfected with siRNAs and an NF-κB responsive *Firefly* luciferase reporter gene together with a constitutively expressed *Renilla* luciferase gene to normalize for cell viability and transfection efficiency. After a stimulation period with TNFα NF-κB responsive luciferase activity was measured by luminometer. The screen was carried out in two biological replicates and four technical replicates, respectively. Several previously uncharacterized DUBs were found that show significant up- or down-regulation of TNFα signaling (Fig. 14.3c). Subsequent re-tests and secondary assay systems in multiple cell lines are required to confirm the identified primary screening results [19]. In case of TNFα-induced NF-κB signaling alternative pathway read-out methods to monitor pathway activity can be used such as NF-κB localization studies or analysis of NF-κB dependent target gene expression by protein and mRNA measurements. Epistatic experiments may provide mapping information, e.g., by over-expression and simultaneous knockdown of known and the putative pathway components [27].

New Targets, New Drugs?

Since aberrant TNFα/NF-κB signaling is known to be involved in carcinogenesis as well as in chronic inflammation, the modulation of signaling activity is considered a promising strategy in pharmacological therapy of many human diseases. For instance, blocking TNFR1 signaling using anti-TNFα-antibodies is already widely applied in the treatment of patients with immune-mediated inflammatory diseases like rheumatoid arthritis and inflammatory bowel diseases [30]. With regards to cancer therapy two main approaches exist – either agonistic or antagonistic strategies – reflecting the opposed effects of TNFR1 signaling on cancer cells. Clinical trials of systemic TNFα application in cancer patients were rather disappointing because of negative side effects similar to endotoxic shock [5]. Today TNFα is clinically only used in the treatment of advanced soft tissue sarcoma and metastatic melanoma in a local form of application [31], but there are still efforts to increase safety of TNFα as a cancer therapeutic by development of molecular modifications or more targeted approaches like radio-inducible TNFα-expressing adenoviral vectors to reduce systemic toxicity [5].

In contrast, blocking TNFα signaling is considered to be an alternative powerful strategy for cancer therapy [5]. Neutralizing anti-TNFα-antibodies (e.g., Influximab) and soluble TNFR2 fusion proteins (Etanercept) have shown to inhibit cancer progression in experimental models of cancer as well as in clinical trials. Despite the initially promising results, the limitations of a non-targeted systemic TNFα/NF-κB blockage became apparent, which include immune suppression and increased susceptibility to infections as well as the potential enhancement of tumorigenesis [5]. One explanation for conflicting results in translating results from the bench to bedside is the insufficient knowledge about the regulatory networks that control and are controlled by TNFα/NF-κB signaling. Models of rather simple, linear abstractions of pathway architecture (as shown in Fig. 14.2) have to incorporate the temporal-spatial activity patterns and a more complete picture of cellular

components, which are involved. The identification of additional modules that regulate NF-κB signaling therefore remains an important field of research.

RNAi screening can also serve as a powerful tool in finding ways of more effective cancer therapy [32]. Systematic loss-of-function screens can identify genes, which are specifically dysregulated in cancer cells and thus might be promising targets for directed therapeutic approaches with less negative side effects. An example is the identification of CCDC50 as a candidate gene in an amplified region frequently found in chronic lymphatic leukemia and mantle cell carcinoma cells which is required for cell survival and NF-κB signaling [33]. Another example is a high-throughput RNAi screen for novel regulators of TNFα/NF-κB signaling showing that knockdown of CYLD, which is involved in familiar cylindromatosis, contributes to oncogenesis by the activation of NF-κB [28]. Using aspirin and derivatives, known inhibitors of CYLD, this effect could be reversed. These examples also demonstrate that RNAi screens to dissect druggable interacting pathways constitute a viable route toward defining new targets for known – or novel – drugs.

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Chapter 15
Pathogenic Role of IL-6 Combined with TNF-α or IL-1 in the Induction of Acute Phase Proteins SAA and CRP in Chronic Inflammatory Diseases

Kazuyuki Yoshizaki

Introduction

The fact that acute phase proteins, such as C-reactive protein (CRP) and serum amyloid A (SAA), are elevated during inflammation is known to nearly all medical practitioners. However, exact mechanism for induction of these acute phase proteins, both in vitro and in vivo, remains unknown. IL-6 induces inflammatory status in autoimmune diseases. Consequently, IL-6 blocking therapy with an anti-IL-6 receptor antibody (tocilizumab) for Castleman's disease (CD) [1, 2], rheumatoid arthritis (RA) [3], and juvenile idiopathic arthritis (JIA) [4] led to improvement in most clinical symptoms and laboratory findings. CRP and SAA levels in particular were reduced and completely normalized, even if another cytokines and chemokines were activated in patients with RA. On the other hand, single use of TNF-α blockers, such as infliximab or etanercept, reduced SAA and CRP levels in RA patients, but barely to within normal range [5]. In this study, to clarify the different pathologic roles of IL-6 and TNF-α or IL-1 in induction of SAA and CRP in chronic inflammatory disease, we used hepatoma-derived cell lines to analyze the difference between the IL-6 and TNF-α mechanism in vitro, and between IL-6 and TNF-α blocking therapy for RA in vivo.

Contribution of Cytokines to the Pathogenesis of RA

It has been suggested that cytokines contribute to the pathogenesis of RA, a chronic inflammatory disease. TNF-α, IL-1, and IL-6 were found to be functional in the affected inflammatory joints shown in Fig. 15.1, because these cytokines were produced in the joint, and blockage of these cytokines improved the symptoms and

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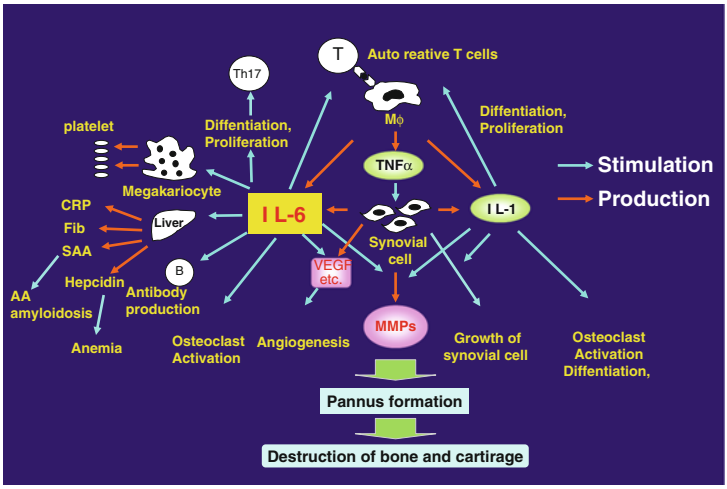

Fig. 15.1 Working hypothesis of pathogenic role of pro-inflammatory cytokines in RA. In inflammatory RA joints, TNF-α, IL-1, IL-6, and possibly other cytokines are produced and activated. These cytokines may contribute to the expression of abnormal findings both at joints and systemic symptoms

abnormal laboratory findings of RA. However, the exact roles of these cytokines remained speculative and largely unknown.

To clarify the pathogenic roles of cytokines for every abnormal in vivo finding, we analyzed the in vitro mechanism of the cytokine function followed by cytokine inhibition therapy for inflammatory disease to determine the effect of the therapy on the findings.

To ascertain the different clinical effects of IL-6 and TNF-α blockage, we first analyzed in vitro the mechanism of the induction of acute phase proteins, such as SAA and CRP, by IL-6 and TNF-α or IL-1 stimulation.

In Vitro SAA Induction Mechanism

To determine whether SAA induction was induced by pro-inflammatory cytokines, two hepatoma-derived cell lines, HepG2 or Hep3B, were stimulated with IL-6, TNF-α, and IL-1. Induction of SAA mRNA was detected with real-time RT-PCR. As shown in Fig. 15.2, a single stimulation with IL-6, but not with TNF-α or IL-1, significantly induced SAA mRNA, while double stimulation with IL-6 combined with TNF-α or IL-1 enhanced SAA mRNA induction synergistically. However, the stimulation with a combination of TNF-α and IL-1 had no effect. This finding suggests that, among the three cytokines, IL-6 was the pivotal factor while TNF-α and IL-1 were supplementary factors for the induction and augmentation of SAA mRNA [6].

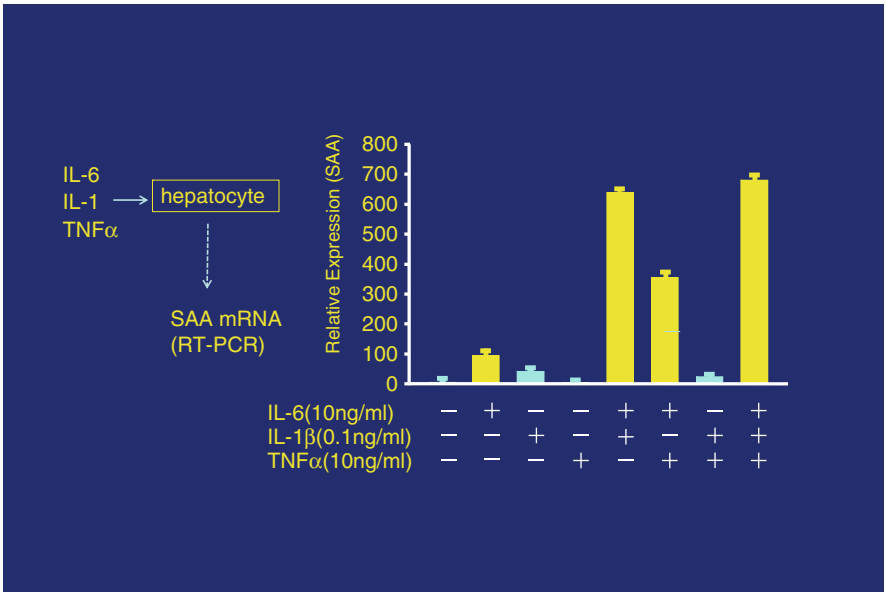

Fig. 15.2 Induction of SAA mRNA in hepatocytes stimulated with IL-6, IL-1, and TNF-α. A hepatoma-derived cell line, HepG2 or Hep3B, was stimulated with IL-6, IL-1, and/or TNF-α. SAA mRNA expression was assessed with real-time RT-PCR

We expected that stimulation with all three cytokines would enhance SAA induction even more, but no enhancement was observed compared to the double cytokine stimulation with IL-6 combined with TNF-α or IL-1 (Fig. 15.2).

This result indicates that the IL-6 signal transduction pathway is essential and different from that of TNF-α or IL-1, while TNF-α and IL-1 may use a common signal transduction pathway.

To prove this, we analyzed the transcriptional mechanism of SAA mRNA induction on the SAA promoter region, where the C/EBPβ response element (RE) and NF-kB RE, but not STAT3 consensus RE, are located (Fig. 15.3) [7].

We used several experimental methods for the analysis, including hyper-expression of transcriptional factors, introduction of a deletion mutant of the promoter region into cells, EMSA, super-shift assay, IP-western, and so on. The results of these experiments lead us to propose the model, shown in Fig. 15.4a, of transcription factors on the SAA promoter region for the induction and augmentation of SAA mRNA [8, 9].

Figure 15.4 shows the formation of the complex of transcriptional factors (right) and SAA production (left bar) by cytokine stimulation with (**a**) IL-6 combined with TNF-α or IL-1, (**b**) IL-6, (**c**) TNF-α or IL-1, and (**d**) without stimulation based on the results of in vitro experiments.

SAA induction was initiated by the activation of STAT3 stimulated by IL-6 as shown in Fig. 15.4b, followed by STAT3 induction and binding with activated NF-kBp65, which are essential for the augmentation of SAA mRNA expression

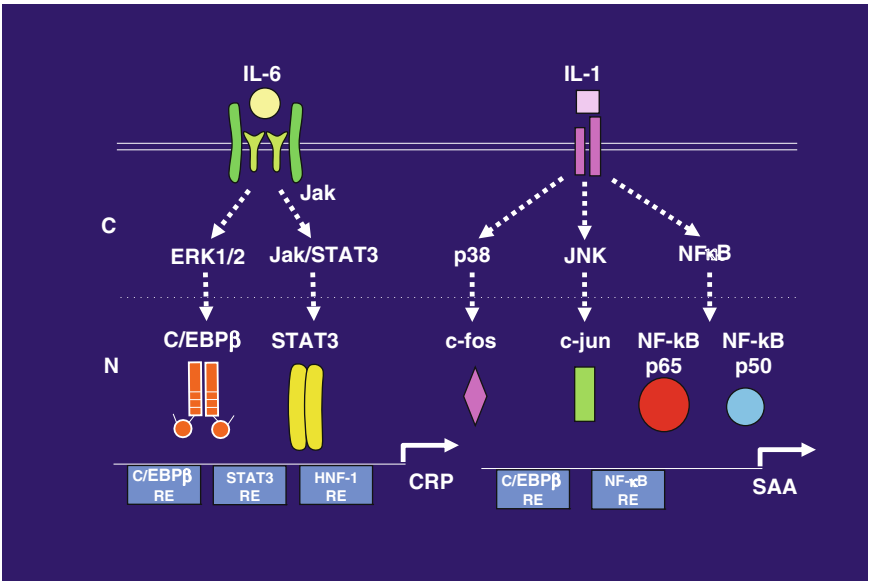

Fig. 15.3 Difference in IL-6 and IL-1 (or TNF-α) signal transduction pathways and comparison of response elements (RE) in CRP and SAA gene promoters. C/EBPβ RE, NF-kB RE, but not consensus STAT3 RE are located in the SAA promoter region, while C/EBPβ RE, consensus STAT3 RE, HNF-1 RE, but not NF-kB RE are located in the CRP promoter region

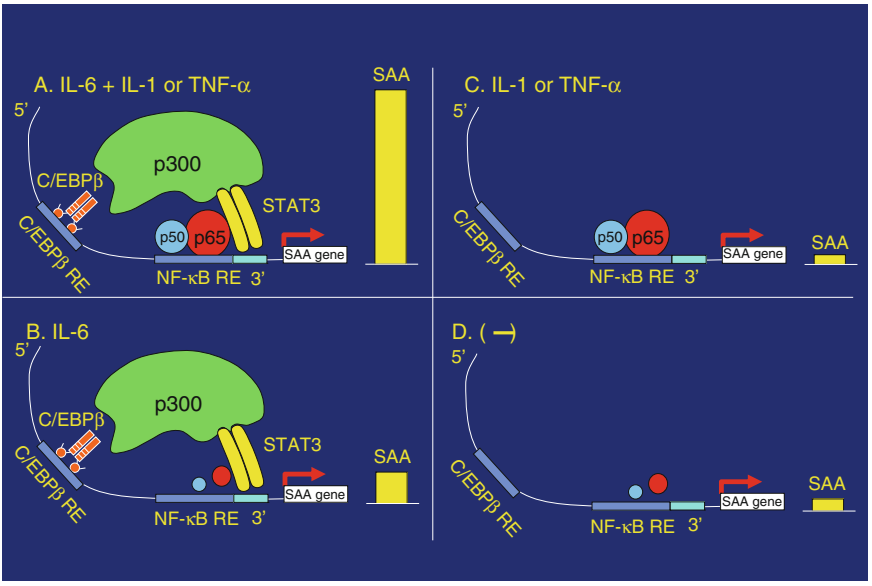

Fig. 15.4 Formation of transcription factor complex, comprising C/EBPβ, STAT3, NF-kB p65, p50, and co-factor p300, on the SAA promoter (*left*) and SAA mRNA induction (*right*) in hepatocytes. Hepatocytes were stimulated with (**a**) IL-6 combined with TNF-a or IL-1, (**b**) IL-6, (**c**) TNF-α or IL-1, and (**d**) no cytokines

(Fig. 15.4a). Even if NF-kB was activated with TNF-α or IL-1, however, SAA mRNA was not induced without STAT3 activation (Fig. 15.4c). NF-kB p65 without TNF-α or IL-1 stimulation was slightly activated by non-specific stimulators such as in vitro culture conditions, medium components, and so on (Fig. 15.4d).

This data clearly shows the functional difference between the role of IL-6 and TNF-α or IL-1 on the induction and augmentation of SAA expression, and this difference can be explained by the in vitro data for the different transcriptional mechanism of IL-6 and TNF-α or IL-1.

In Vitro CRP Induction Mechanism

It is known that CRP is also induced by pro-inflammatory cytokines involved in inflammation. To determine whether the CRP induction mechanism is different from that of SAA, the former was analyzed at the same level of promoter activity as that of SAA. The response elements (RE) of transcription factors in the CRP promoter region are different from those of SAA. NF-kB but not STAT3 consensus RE is present in the promoter region of the SAA gene, whereas STAT3 consensus RE but not NF-kB RE is present in the promoter region of the CRP gene [10]. Hepatocyte nuclear factor (HNF)-1 RE is located downstream of STAT3 RE (Fig. 15.3) [11].

Single stimulation with IL-6 but not IL-1 could induce CRP mRNA, while IL-6 combined with IL-1 augmented CRP mRNA induction synergistically as shown in Fig. 15.5 (left). This suggests that IL-6 is an essential factor and IL-1 is a supplementary factor.

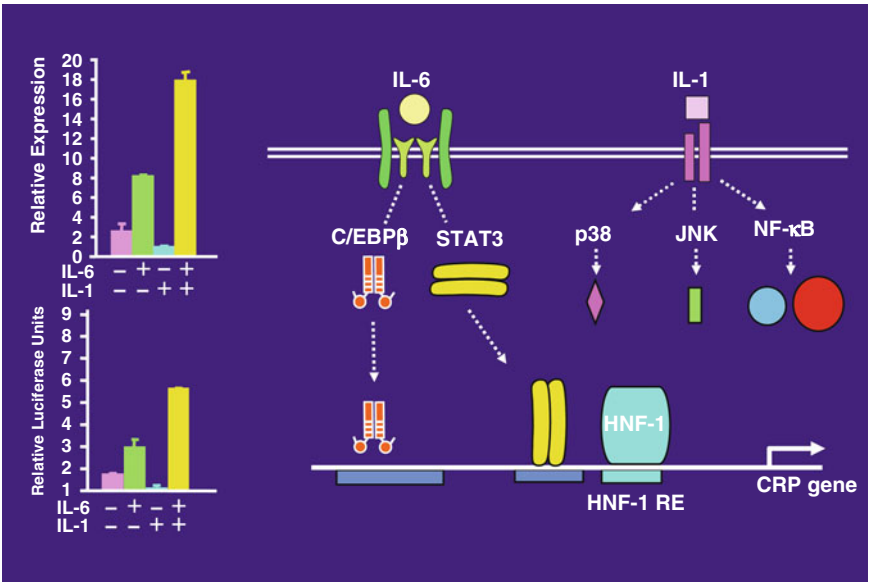

Fig. 15.5 Induction of CRP mRNA in hepatocytes stimulated with IL-6, IL-1, and IL-6+IL-1 (*left*). To make the contribution of transcription factors easy to identify, the IL-6 and IL-1 signal transduction pathways and the CRP promoter region are also shown in this figure

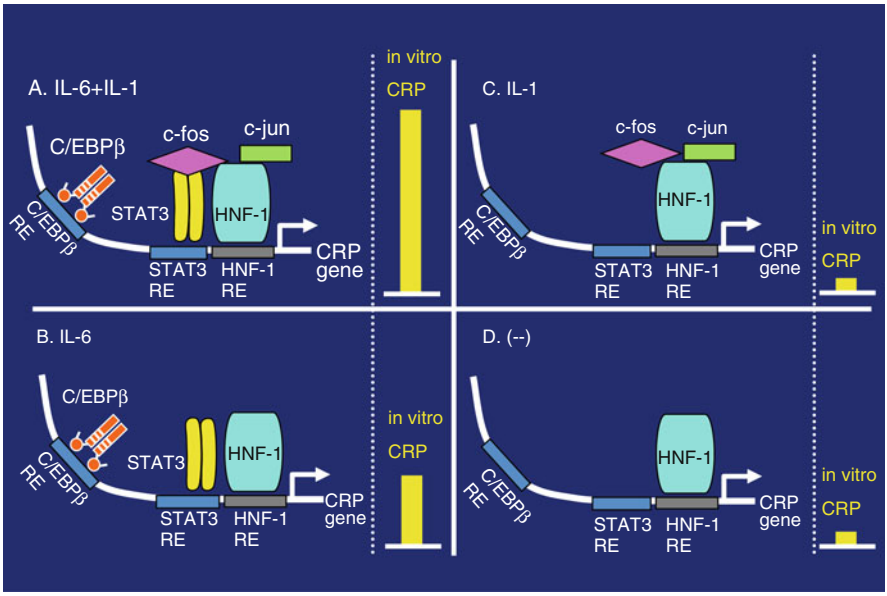

Fig. 15.6 Formation of transcription factor complex, comprising C/EBPβ, STAT3, HNF-1, c-fos, and c-jun on the promoter region (*left*) and CRP mRNA induction (*right*) in hepatocytes. Hepatocytes were stimulated with (**a**) IL-6+IL-1, (**b**) IL-6, (**c**) IL-1, and (**d**) no cytokines

To confirm this finding at the level of promoter activity, we used several examination methods for the analysis as we did for the SAA mRNA induction mechanism (Fig. 15.5, right).

To summarize, we propose the formation of a complex of transcription factors on the CRP promoter region for the induction and augmentation of CRP mRNA by (A) IL-6 and IL-1 stimulation, (B) IL-6, (C) IL-1, and (D) without stimulation, as shown in Fig. 15.6 [12]. STAT3 activation induced by IL-6 and followed by binding to HNF-1 was initiated upon the induction of the CRP gene (Fig. 15.6b). IL-1 induced c-fos activation followed by binding with a complex of activated STAT3 and HNF-1 augmented CRP mRNA induction (Fig. 15.6a). Although c-fos and c-jun were activated by IL-1, CRP mRNA was not induced because of inactivation of STAT3 without IL-6 (Fig. 15.6c) [13, 14]. Finally, HNF-1 was continuously activated and bound to HNF-1 RE.

Inhibition of SAA and CRP Production by IL-6 Blockade Both In Vitro and In Vivo

To identify the mechanism for cytokine-induced SAA and CRP production in vivo, we examined whether expression of SAA or CRP mRNA is inhibited in vitro by

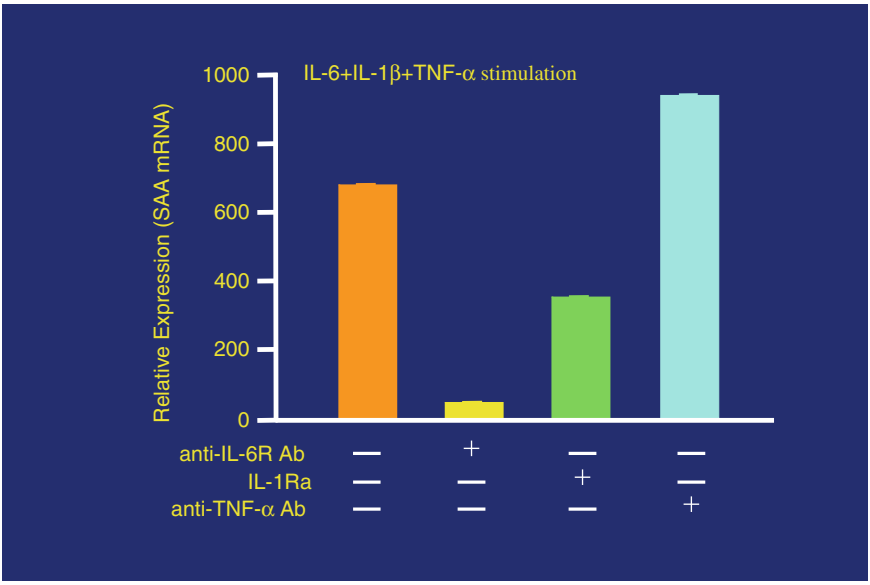

Fig. 15.7 Inhibition of SAA mRNA expression by each of the cytokine blockers in hepatocytes stimulated with IL-6, IL-1, and TNF-α. Anti-IL-6 receptor antibody, IL-1 receptor antagonist, and anti-TNF-α antibody were used as the respective cytokine blockers

IL-6 blockage, and whether serum SAA or CRP level is reduced in vivo by IL-6 blocking therapy.

A hepatoma-derived cell line was stimulated with IL-6, IL-1, and TNF-α to identify the in vivo inflammatory condition in RA in which at least three of the cytokines were activated. Next, the corresponding cytokine blocker was added to the culture. As seen in Fig. 15.7, only the IL-6 blocker, the anti-IL-6 receptor antibody (tocilizumab), almost completely inhibited the expression of SAA mRNA. However, the IL-1 blocker, the IL-1 receptor antagonist, achieved partial inhibition, while the TNF-α blocker, anti-TNF-α antibody, had no effect at all.[6]

Data obtained with the same analytical procedure used for the CRP experiment showed the same tendency (data not shown). These data confirmed the previous findings of the in vitro experiment involving SAA and CRP, that is, IL-6 is an essential cytokine and TNF-α or IL-1 is a supportive factor for the induction and augmentation of SAA and CRP. In addition, the efficacy of IL-6 blockade and TNF-α blockade for clinical treatment of RA is already known, since this IL-6 blockade induces not only reduction but also normalization of CRP and SAA levels. However, TNF-α blockade generated reduction, but without methotrexate it brought the levels barely to within normal range[5].

Pathogenic Role of IL-6 Combined with TNF-α or IL-1 on the Induction of SAA and CRP in RA Based on Evidence Obtained from In Vitro Experiments

Based on the evidence obtained from the in vitro experiment involving the SAA- and CRP-induction mechanism and clinical results for serum levels of SAA and CRP treated with IL-6 and TNF-α blockade therapy, we can suggest the pathogenic effect of IL-6 combined with TNF-α or IL-1 on induction and augmentation of SAA and CRP in RA, and the different pathogenic roles of IL-6 from TNF-α or IL-1 in RA. Figure 15.8 shows the proposed transcriptional mechanism and actual serum levels of SAA (left) and CRP (right) in RA treated with or without cytokine blockade (A, without treatment; B, with TNF-α blockade; and C, with IL-6 blockade).

The fact that SAA and CRP are elevated in inflammation is well known to practically all medical practitioners, but the induction mechanism of SAA and CRP in inflammation has remained unknown. Now, however, the findings of our study have made it known what the mechanism is of intracellular induction and augmentation of SAA and CRP by cytokines, such as IL-6 combined with TNF-α or IL-1, in chronic inflammatory diseases.

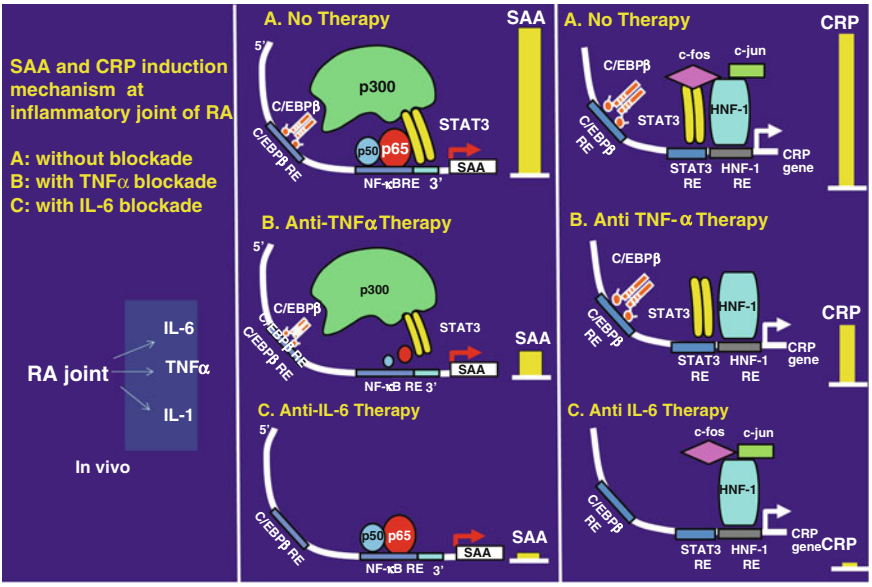

Fig. 15.8 Reduction of serum levels of SAA (*middle*) and CRP (*right*), and proposed formation of transcription factor complex on SAA and CRP promoters in RA patients treated with the cytokine blockers. RA patients were given (**a**) no therapy, (**b**) anti-TNF-α therapy, and (**c**) anti-IL-6 therapy

Furthermore, although the clinical effects of TNF-α and IL-6 blockage on RA therapy seem to be the same, IL-6 and TNF-α act on different pathogenic mechanisms in RA. This suggests, therefore, that these different cytokine blockers need to be used for different inflammatory diseases to obtain an adequate and satisfactory therapy.

We hope our analytical methods and procedures for identification of different results obtained in vitro and in vivo may lead in the near future to the elucidation of the definitive pathogenic role of cytokines in chronic inflammatory disease.

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Chapter 16
Understanding Life and Death at CD95

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Introduction

CD95 (APO-1/Fas) [21] is a member of the death receptor (DR) family, a subfamily of the TNF-R (tumor necrosis factor receptor) superfamily [2, 20]. Stimulation of CD95 with its natural ligand CD95L or with agonistic anti-CD95 antibodies, such as anti-APO-1, induces apoptosis in sensitive cells [34]. The signal transduction of CD95 starts with the formation of the death-inducing signaling complex (DISC), which is detected within seconds after receptor stimulation [19]. The DISC consists of CD95, the adaptor molecule FADD, procaspase-8a/b, procaspase-10, and c-FLIP$_{L/S/R}$(Fig. 16.1) [8, 21, 26, 30, 32].

After binding to the DISC procaspase-8a/b (p55/p53) undergoes autocatalytic processing, which leads to generation of active caspase-8 [24] (Fig. 16.1). In the course of procaspase-8a/b processing at the DISC the cleavage occurs at several Asp (D) residues between prodomain and small and large catalytic subunits. This results in the formation of the N-terminal cleavage products: p43/p41, the prodomain p26/p24, and the C-terminal cleavage products p30, p18, and p10 [13, 15, 30]. Active caspase-8 heteroteramer $p10_2$–$p18_2$ generated at the DISC triggers the apoptotic signal. Recently, it has been reported that cleavage products of procaspase-8 p30 and p43/p41 possess catalytic activity as well, which leads to apoptosis initiation [13, 15]. Hence, procaspase-8 processing at the DISC initiates apoptosis through generation of several catalytically active cleavage products of procaspase-8.

c-FLIP, also known as FLAME-1/I-FLICE/CASPER/CASH/MRIT/CLARP/Usurpin, is a well-described inhibitor of death receptor-mediated apoptosis. Five c-FLIP proteins have been characterized so far: three c-FLIP isoforms and two cleavage products [8, 9, 23]. Three c-FLIP isoforms include long (L), short (S), and Raji (R), e.g., c-FLIP$_L$, c-FLIP$_S$, and c-FLIP$_R$, respectively (Fig. 16.2). All three isoforms possess two DED domains and thereby bind to the DISC. c-FLIP$_L$ also contains catalytically inactive caspase-like domains (p20 and p12) (Fig. 16.2). The

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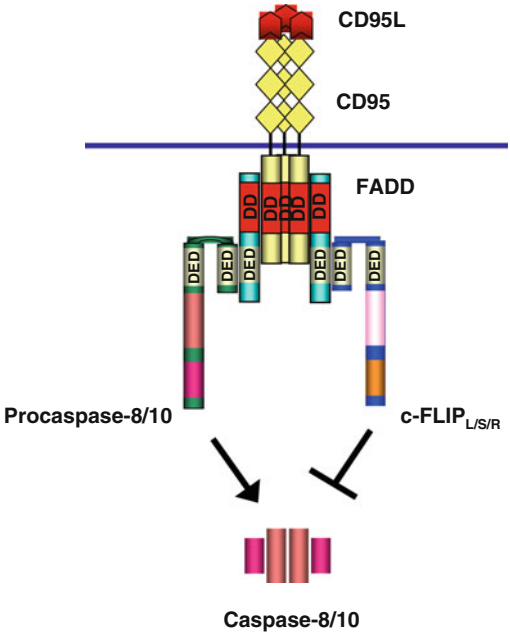

Fig. 16.1 CD95 death-inducing signaling complex (DISC). The DISC consists of CD95, (depicted in *yellow*), FAS-associated death domain, FADD (depicted in *light blue*), procaspase-8/procaspase-10 (depicted in *green*), and cellular FLICE-inhibitory proteins, c-FLIP (depicted in *blue*). The interactions between the molecules at the DISC are based on homotypic contacts. The death domain (DD) of CD95 interacts with the DD of FADD while the death effector domain (DED) of FADD interacts with the N-terminal tandem DEDs of procaspase-8, procaspase-10, and c-FLIP. DD are shown in *red* and DED are shown in *light yellow*

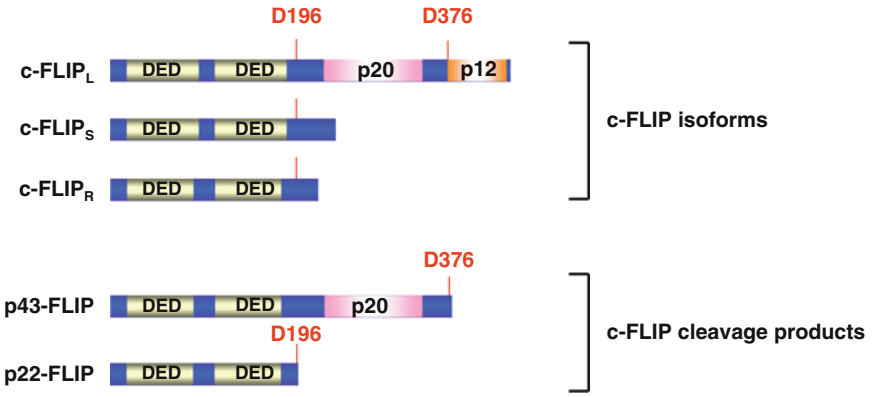

Fig. 16.2 The scheme c-FLIP proteins. c-FLIP isoforms and c-FLIP cleavage products are shown. DED (death effector domains) and caspase-like domains (p20 an p12) are indicated. D376 and D196 leading to the generation of p43-FLIP and p22-FLIP, respectively, are presented in *red*

two N-terminal cleavage products of c-FLIP, p43-FLIP, and p22-FLIP are generated as a result of cleavage at D376 and D196, respectively.

Recently, it has been reported that CD95L is not only a potent apoptosis inducer but can also activate multiple non-apoptotic pathways, in particular induction of transcription factor NF-κB [4, 22, 28]. The NF-κB family regulates expression of genes crucial for innate and adaptive immune responses, cell growth, and apoptosis. In most cells, the NF-κB dimer is sequestered in the cytosol by inhibitors of the κB protein (IκB), and its nuclear translocation can be induced by a wide variety of stimuli via activation of the IκB kinase (IKK) complex [11, 17]. IKK consists of two catalytic subunits, IKKα and IKKß, and a regulatory subunit, IKKγ/NEMO. Activation of the IKK complex leads to phosphorylation of IκB and its degradation via the proteosome. The NF-κB dimers can then translocate into the nucleus and bind to DNA to induce transcription of target genes. The exact molecular mechanism of NF-κB activation via CD95 has remained unclear [28].

It was shown that the main DED-containing proteins of the CD95 DISC, procaspase-8 and c-FLIP, play a prominent role in NF-κB activation [21]. c-FLIP N-terminal cleavage products p43-FLIP and p22-FLIP strongly induce NF-κB [9, 18]. p43-FLIP is generated by procaspase-8 at the DISC upon CD95 stimulation. p43-FLIP interacts with components of the TNFR-mediated NF-κB activation pathway, TNFR-associated factor 1 (TRAF1), TRAF2, and receptor-interacting protein (RIP), which together promote NF-κB activation [18]. p22-FLIP induces NF-κB by direct binding to the IKK complex [9]. p22-FLIP differs from p43-FLIP as p22-FLIP is generated by procaspase-8 activity in non-apoptotic cells without DR stimulation. Procaspase-8 can induce NF-κB not only via generation of p22-FLIP or p43-FLIP but also by direct binding to the CBM adapter complex formed upon TCR stimulation [33]. Thus, several studies indicate that the most prominent DED-proteins of the CD95 DISC, procaspase-8 and c-FLIP, play an important role both in CD95-induced apoptotic and in non-apoptotic pathways.

This complex interplay between components of the CD95 system leading to induction of non-apoptotic vs. apoptotic pathways has encouraged us to apply systems biology approach to understand life/death decisions at CD95. Contemporary models of apoptosis describe both intrinsic and extrinsic pathways. These models are mostly based on ordinary differential equations (ODEs) [3, 5, 7, 14, 16, 25, 29]. Modeling NF-κB activation has a long tradition. A number of mathematical models of NF-κB activation have been published describing the complex interplay of the various IκB subunits in NF-κB oscillatory activity [6, 12, 27]. Although pathways of CD95-mediated apoptosis and NF-κB activation have both been modeled to a considerable extent, no model of the crosstalk between CD95 apoptotic and non-apoptotic signaling has been developed so far. Therefore, we have decided to build a model of the crosstalk between these two signaling pathways.

The first step in setting up any model is defining its topology. To build the model of CD95-induced apoptosis and NF-κB, the signaling route from CD95 stimulation to NF-κB induction has to be defined first. In this study we describe experiments, dealing with the definition of the topology of the model, e.g., delineating the exact signaling pathway between CD95 DISC and NF-κB activation.

Results and Discussion

Engagement of CD95 results in caspase-dependent apoptosis [24] as well as the activation of NF-κB [4]. To establish an experimental setup for measuring the activation of both pathways we have analyzed CD95 signaling in HeLa cells stably overexpressing CD95 (HeLa-CD95). For the CD95 stimulation we have implied anti-APO-1 (anti-CD95) antibodies. Anti-APO-1 are agonistic antibodies, which were previously shown to induce both apoptosis and NF-κB activation [4, 34]. First, we characterized whether both signaling pathways are induced in HeLa-CD95 cells. Upon stimulation with 1500 ng/ml of anti-APO-1 antibodies we observed that HeLa-CD95 cells undergo apoptosis within 24 h (Fig. 16.3a). The observed cell death was caspase dependent. This was demonstrated using the caspase inhibitor zVAD-fmk (Fig. 16.3a). To test NF-κB activation in HeLa-CD95 cells upon stimulation with anti-APO-1 antibodies we performed NF-κB luciferase assays. HeLa-CD95 cells were first transfected with a plasmid that encoded an NF-κB-based luciferase reporter gene [1] and then stimulated with anti-APO-1 antibodies. Importantly, CD95 engagement led to the significant NF-κB induction (Fig. 16.3b). In this way, we demonstrated that both pathways, apoptosis and NF-κB, are induced in HeLa-CD95 cells. Thus, we used HeLa-CD95 cells as a model system for studying both pathways.

To get more insight into the mechanism of CD95-mediated NF-κB activation we investigated the question whether CD95-induced caspase activity is important for NF-κB activation. Activation of initiator procaspase-8 at the DISC triggers the caspase cascade, which involves effector caspases-3 and -7, which, in turn, cleave multiple substrates resulting in demolition of the cell. Effector caspase activity can be blocked by the pan-caspase inhibitor zVAD-fmk. Recently it has been reported that activity of the initiator procaspase-8 at the DISC could not be blocked by zVAD-fmk, even though procaspase-8 processing was strongly reduced [9, 15]. Therefore, application of the caspase inhibitor zVAD-fmk can be used to distinguish between initiator procaspase-8 activity at the DISC and downstream effector caspase activity.

Next, we treated HeLa-CD95 cells with the inhibitor zVAD-fmk prior to CD95 stimulation. CD95 signaling was analyzed using western blot analysis for procaspase-8 processing and IκBα phosphorylation as hallmarks of caspase and NF-κB activation, respectively (Fig. 16.4). In HeLa-CD95 cells without addition of zVAD-fmk, we detected phosphorylation of IκBα as early as 30 min after stimulation (Fig. 16.4, left side). Also at the same time point initial processing of procaspase-8 at the DISC, e.g., p43/p41, indicating procaspase-8 activity, could be detected (Fig. 16.4, left side). Thus, procaspase-8 activity at the DISC correlated in time with CD95-mediated phosphorylation of IκBα. Phosphorylation of IκBα reached a maximum after 60 min (Fig. 16.4, left side). This was also in accordance with an increased processing of procaspase-8, detected by an increase of p43/p41 and p18 cleavage products of procaspase-8 (Fig. 16.4, left side). Upon addition of zVAD-fmk processing of procaspase-8 could not be detected (Fig. 16.4, right side), which, however, does not exclude procaspase-8 activity as reported by Hughes et al. [15]. Interestingly, we observed that CD95-mediated phosphorylation of IκBα

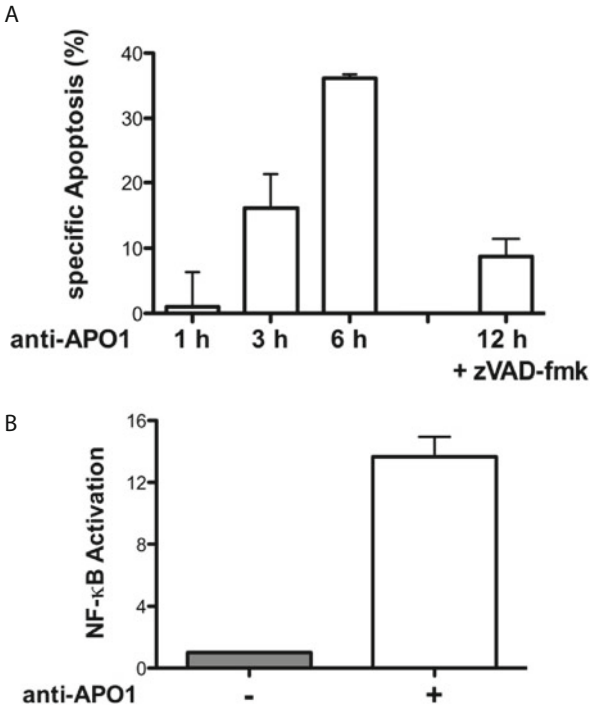

Fig. 16.3 CD95 engagement induces both apoptotic and NF-κB pathways in HeLa-CD95 cells. (**a**) HeLa-CD95 cells were stimulated with 1000 ng/ml of agonistic anti-CD95 antibodies for 1, 3, 6 h. Additionally HeLa-CD95 cells were stimulated for 12 h in the presence of 20 μM zVAD-fmk, which was followed by cell death analysis. (**b**) 1×10^5 HeLa-CD95 cells were transfected with a luciferase reporter plasmid (1 μg per well) and a Renilla-reporter plasmid (100 ng pro well). 24 h after transfection cells were stimulated with 1000 ng/ml of anti-CD95 antibodies. NF-κB-luciferase activity was determined 4 h after stimulation. The results represent the mean ± standard deviation of quadruplet cultures. Renilla transfection was performed to control and normalize transfection efficiency

occurs despite caspase inhibition (Fig. 16.4, right side). Phosphorylation of IκBα took place within 30 min after stimulation, which was similar to the kinetics of IκBα phosphorylation in zVAD-fmk-untreated cells. This shows that CD95-induced NF-κB activation does not depend on effector caspase activity. However, these data do not exclude involvement of procaspase-8 activity at the DISC in NF-κB induction.

Next, we tested whether p43-FLIP could induce NF-κB in HeLa-CD95 cells. To analyze NF-κB activation we used NF-κB luciferase assays. HeLa-CD95 cells were co-transfected with p43-FLIP and a luciferase reporter construct, which was followed by measuring luciferase activity after 24 h (Fig. 16.5a). These experiments showed that p43-FLIP is a strong inducer of NF-κB in HeLa-CD95 cells.

Since p43-FLIP is only generated after DR stimulation we have suggested that there is a direct connection to CD95-dependent NF-κB activation. We assume that

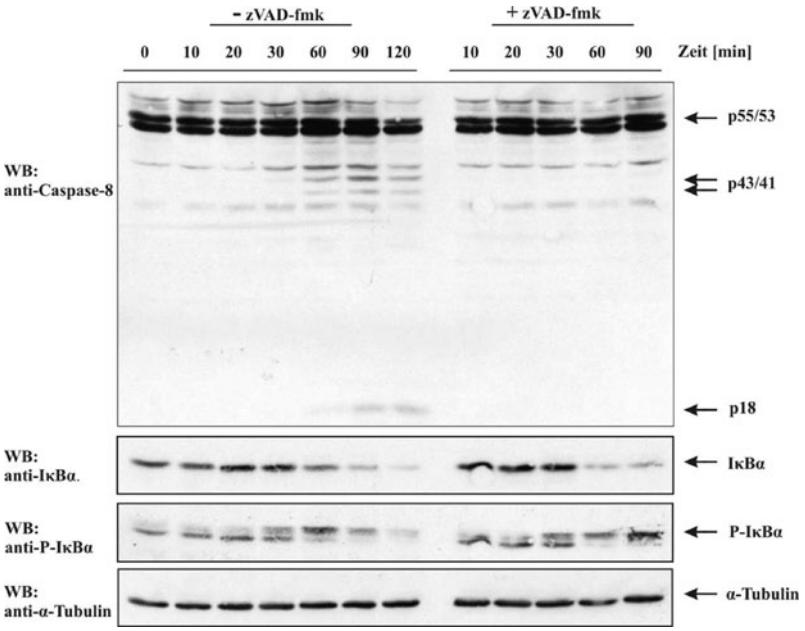

Fig. 16.4 CD95-mediated NF-κB pathway is independent of effector caspase activity. HeLa-CD95 cells were stimulated with 1000 ng/ml of agonistic anti-CD95 antibodies in the presence or absence of zVAD-fmk (20 μM). The cellular lysates were analyzed by western blot using antibodies against caspase-8 (C15 antibodies), IκBα and p-IκBα. Anti-tubulin western blot has been used as a loading control

CD95-mediated NF-κB activation involves CD95-induced procaspase-8 activity, and its only substrate c-FLIP$_L$. We suggest the following signaling route. Upon CD95 stimulation CD95 DISC is formed and procaspase-8 is activated at the DISC. p43-FLIP is generated from c-FLIP$_L$ by procaspase-8 activity and, subsequently, p43-FLIP induces NF-κB. To test this hypothesis, we analyzed if there is a correlation between p43-FLIP generation and IκBα phosphorylation in HeLa-CD95 cells upon CD95 engagement (Fig. 16.5b). Using western blot analysis we observed that the cleavage to p43-FLIP, detected 20 min after CD95 stimulation, is well-correlated with an increase in IκBα phosphorylation. After 40 min when all c-FLIP$_L$ molecules have been cleaved to p43-FLIP, IκBα phosphorylation reached its maximum. Thus, indeed appearance of p43-FLIP has demonstrated the direct correlation to induction of NF-κB.

Thus, we have tested our assumption on the connection between CD95 stimulation and NF-κB induction. It seems plausible that indeed p43-FLIP generated by procaspase-8 activity at the DISC induces NF-κB. Both DED-proteins procaspase-8 and c-FLIP are required for induction of apoptotic as well as non-apoptotic pathways. c-FLIP$_L$ can play anti-apoptotic role by inhibiting caspase-8 activation at

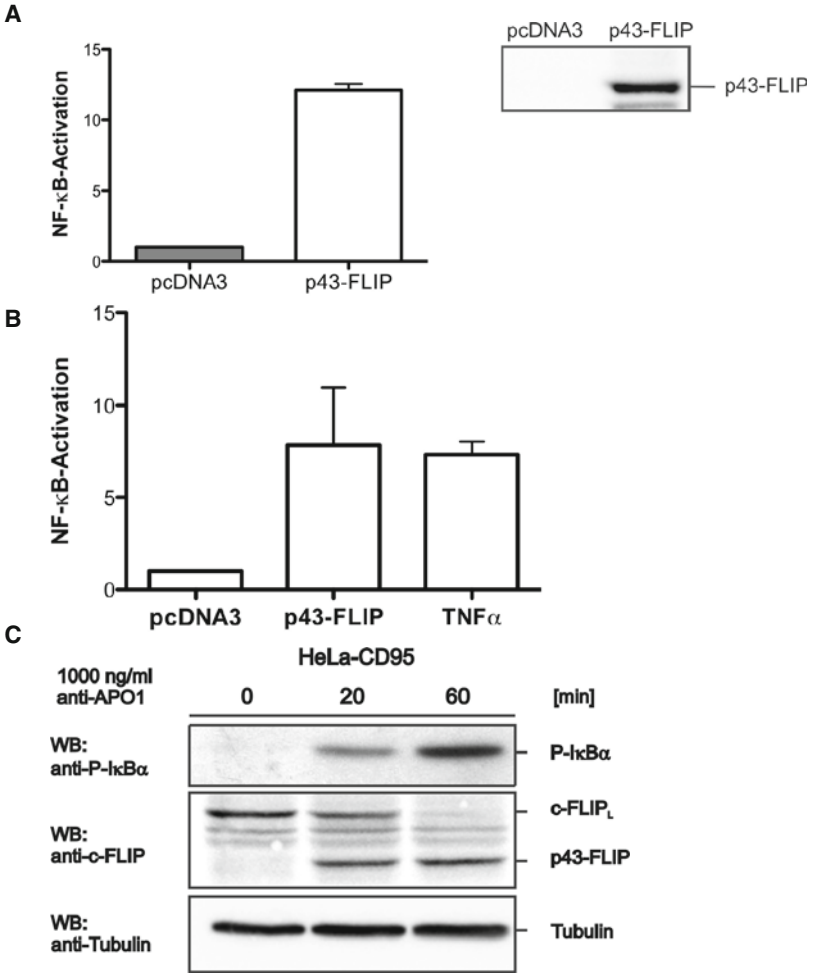

Fig. 16.5 p43-FLIP induces NF-κB in HeLa-CD95 cells. (**a**) 1×10^6 HeLa-CD95 cells were co-transfected with p43-FLIP (2 μg per well) and the luciferase reporter plasmid (2 μg per well). NF-κB-luciferase activity was determined 24 h after transfection. The results represent the mean ± standard deviation of quadruplet cultures. Renilla transfection was performed to control and normalize transfection efficiency. In addition, expression of p43-FLIP has been controlled by western blot using anti-c-FLIP NF6 antibodies. (**b**) 1×10^6 HeLa-CD95 cells were co-transfected with p43-FLIP (2 μg per well) and the luciferase reporter plasmid (2 μg per well). TNF stimulation (500 ng/ml) has been used as a positive control. NF-κB-luciferase activity was determined 24 h after transfection. The results represent the mean ± standard deviation of quadruplet cultures. Renilla transfection was performed to control and normalize transfection efficiency. In addition, expression of p43-FLIP has been controlled by western blot using anti-c-FLIP NF6 antibodies. (**c**) HeLa-CD95 cells were stimulated with 1000 ng/ml of agonistic anti-CD95 antibodies. The cellular lysates were analyzed by western blot using antibodies against c-FLIP (NF6-antibodies) and p-IκBα. Anti-tubulin western blot has been used as a loading control

the DISC and providing the basis for generation of p43-FLIP, which triggers NF-κB. In addition, when present at the DISC at low concentrations c-FLIP$_L$ has a pro-apoptotic role by accelerating caspase-8 activation at the DISC. Interestingly, regulation of life/death decisions at CD95 by procaspase-8 also has a dual role. Recruitment of procaspase-8 to the DISC, followed formation of active caspase-8 heterotetramer triggers the apoptotic pathway. This underlines the strong pro-apoptotic role of procaspase-8 in CD95-mediated signaling. From another side, procaspase-8 at the DISC generates p43-FLIP, which induces NF-κB. This shows a capability for an anti-apoptotic role of procaspase-8 at the DISC. Therefore, the balance between pro- and anti-apoptotic activities of procaspase-8 might have a major influence on the life/death decisions on CD95.

Understanding the complex balance between DED proteins of the DISC procaspase-8 and c-FLIP$_L$ and their role in the regulation of life/death decisions at CD95 is a current goal of our model of CD95-induced apoptosis and NF-κB activation. This model was built based on the topology of the CD95 signaling presented in this study. The aim of this model will be to understand the dynamics of DED proteins of the DISC and support the emerging paradigm in CD95 signaling that the DISC can act as a potent signal processor deciding between life and death.

Materials and Methods

Cell Lines

HeLa-CD95 was generated by selection with G418 according to standard protocols. HeLa-CD95 cells were maintained in DMEM (Life Technologies, Germany), 10 mM HEPES (Life Technologies, Germany), 50 μg/ml gentamycin (Life Technologies, Germany), and 10% fetal calf serum (Life Technologies, Germany) in 5% CO_2. 0.5 mg/ml of G418 were used to maintain HeLa-CD95. Transfections were done using FuGene 6 (Roche, Switzerland).

Antibodies and Reagents

Anti-IκBα, polyclonal antibodies were purchased from Santa-Cruz (USA) and anti-phospho-IκBα were from Cell Signaling (Germany). Anti-caspase-8 monoclonal antibody C15 (mouse IgG2b) recognizes the p18 subunit of caspase-8 [30]. Anti-FLIP monoclonal antibody NF6 (mouse IgG1) recognizes the N-terminal part of c-FLIP [31]. Anti-APO-1 (anti-CD95) is an agonistic monoclonal antibody (IgG3) recognizing an epitope on the extracellular part of CD95 (APO-1/Fas) [34]. Horseradish peroxidase-conjugated goat anti-mouse IgG1, IgG2a and IgG2b were from Southern Biotechnology Associates (Echingen, Germany). All chemicals used were of analytical grade and purchased from Merck (Darmstadt, Germany) or Sigma.

Analysis of Total Cellular Lysates

10^5 cells were either treated with indicated amounts of anti-CD95 for indicated periods of time at 37°C or left untreated, washed twice in 1 × PBS and lyzed subsequently in lysis buffer (30 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma, Germany), protease inhibitor cocktail, 1% Triton X-100 (Serva, Germany), and 10% glycerol). If pre-treated with zVAD-fmk, cells were pre-incubated for 30 min at 37°C with the indicated concentrations of zVAD-fmk prior to stimulation. Total cellular lysates were analyzed using SDS PAGE gels. Proteins were transferred to Hybond nitrocellulose membrane (Amersham Pharmacia Biotech, Germany), blocked with 5% non-fat dry milk in PBS/Tween (PBS plus 0.05% Tween 20) for 1 h, washed with PBS/Tween, and incubated with the primary antibody in PBS/Tween overnight at 4°C. Blots were developed with a chemoluminescence method according to the manufacturer's protocol (Perkin Elmer Life Sciences, Germany).

NF-κB Activation Assay

Twelve well-titer plates were seeded with 1×10^6 cells the day before transfection. Cells were transfected using the calcium phosphate method with various expression vectors, together with the NF-κB-driven luciferase reporter plasmid (1 μg) and a renilla plasmid for normalization (100 ng). Cells were washed with PBS 16 h after transfection and lyzed for 20 min at room temperature in 50 μl lysis buffer (Passive lysis buffer, Promega) followed by centrifugation (6500× *g*, 5 min) to sediment insoluble materials. Five microliters of cell lysates was mixed with 50 μl of the luciferase assay mixture (470 μM Beetle Luciferin (Promega), 1.07 mM $(MgCO_3)_4Mg(OH)_2 \times 5\ H_2O$, 20 mM *N*-Tris-(hydroxymethyl)-methylglycine, 2.67 mM $MgSO_4$, 100 μM EDTA, 33.3 mM DTT, 270 μM CoA(OAc), 530 μM ATP). Relative light units were measured with a Berthold duoluminomat (Bad Wildbad, Germany).

Cell Death Assay

Cells were plated and treated as indicated with anti-APO-1 for indicated periods of time and 1 μg/ml propidium iodide for 30 min. Cell death was assessed by propidium iodide uptake and quantified with a Cytomics FC 500 MPL flow cytometer (Beckman Coulter).

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Chapter 17
Unique Personalities Within the NF-κB Family: Distinct Functions for p65 and RelB in the Osteoclast

Deborah Veis Novack

The osteoclast (OC) is the resorptive cell of the skeleton responsible for both normal homeostasis and pathological bone loss. NF-κB is a critical signal for OC differentiation downstream of RANKL, and its global disruption protects mice from pathological bone loss. Differently from other TNF family members, RANKL activates both the classical NF-κB pathway, activating both p65 and cRel, and the alternative pathway, inducing expression and activation of RelB. In order to determine whether the classical and alternative NF-κB pathways have distinct roles in OC differentiation, we examined mice lacking p65, RelB, or cRel. p65 controls cell survival during a critical stage of commitment to the OC lineage by modulating JNK activation. Although JNK is required for activation of NFATc1, a critical differentiation factor, in the absence of p65, expression of JNK inhibitors MKP5 and Gadd45β is decreased, JNK is hyperactivated, and apoptosis ensues. Partial blockade of JNK or its downstream mediator Bid prevents apoptosis and allows OC formation. Only p65 transmits anti-apoptotic signals since p65–/– cells have intact cRel and RelB activation in response to RANKL, and cRel-deficient cells show normal survival and differentiation in vitro. Furthermore, overexpression of RelB in p65−/− precursors does not prevent apoptosis, confirming the unique role for p65 in OC survival.

Absence of RelB restricts OC differentiation, but without RANKL-induced apoptosis. Retroviral expression of RelB, but not p65, rescues the RelB−/− defect, again indicating that these subunits have distinct functions. Precursors lacking NIK, the upstream kinase in the alternative pathway, also have a non-apoptotic block in differentiation. Expression of either p65 or RelB in NIK−/− cells confirms that only RelB rescues differentiation.

Thus, p65 and RelB have critical but distinct roles in the OC.

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Distinct Kinetics for Activation of p65 and RelB in the OC Lineage

Although RANKL activates both the classical and alternative NF-κB pathways, the timing of nuclear translocation of p65 and RelB is distinct in OC precursors [1]. Peak p65 activation occurs after 15 min of RANKL treatment in these cells, followed by a return to baseline for several hours. At later times (1–3 days of RANKL), p65 is again found in the nucleus, but at low levels compared to the initial peak. In contrast, RelB is activated only after several hours of RANKL stimulation, and then reaches a sustained peak from 24 h, out to at least 72 h. These varied activation kinetics in OC precursors suggest that these subunits might have different roles in the OC lineage. We therefore examined mice lacking individual subunits to explore their function.

Role of p65 in OC Apoptosis

Soon after the identification of RANKL as the key osteoclastogenic cytokine, p65 was shown to be a downstream transcription factor, potently activated by RANKL, in OC precursors [2]. However, p65−/− mice have an embryonic lethal phenotype [3], and thus the specific role of p65 had remained unexplored. We used p65−/− mice on a TNF receptor1 (tnfr1)−/− background, to avoid the embryonic lethality [4]. However, these mice live only 2–3 weeks after birth, and for most studies we performed bone marrow transplants into irradiated WT hosts to generate p65−/− radiation chimeras. Radiation chimeras bearing p65+/+tnfr1−/− marrow served as controls for all such studies. p65 deficiency in bone-marrow derived cells led to decreased OC number and a severely blunted osteoclastogenic response to RANKL injection. In addition, p65−/− chimeras developed inflammation as well as p65+/+ controls in response to arthritogenic KRN serum but were protected from arthritis-related osteolysis.

We then explored the reason for the blunted OC responses in vivo and found that p65-deficient bone marrow macrophages (BMMs) undergo RANKL-induced apoptosis, and thus form OCs inefficiently in vitro [4]. However, blockade of apoptosis in p65−/− BMMs with the caspase inhibitor ZVAD-fmk restored OC formation and generated fully functional, resorptive OCs. In order to address whether this pro-survival role for p65 was specific, we confirmed that cRel and RelB responses were intact in p65−/− BMMs. We also found that RANKL did not cause apoptosis in RelB−/− BMMs. Furthermore, overexpression of RelB in p65−/− cultures did not rescue apoptosis or OC formation in response to RANKL. Thus, we concluded that p65 controls OC precursor survival but is not required for OC differentiation or function.

Because RANK, unlike TNFR1, lacks a death domain, the mechanism for RANKL-induced apoptosis was unknown. JNK had been identified as a mediator of TNF-induced apoptosis in p65-deficient cells and is activated by RANKL [5, 6].

We found that induction of two negative regulators of JNK, MKP5 and GADD45β, did not get upregulated by RANKL in p65−/− preOCs. JNK phosphorylation was also increased in these cells. Addition of the JNK inhibitor SP60025 at low doses during the first 2 days of culture rescued p65−/− OCs. One proposed effector of JNK-mediated apoptosis was Bid [7], which we were able to target with shRNA in p65−/− BMMs. Knockdown of Bid prevented apoptosis and rescued OC formation in the absence of p65. In summary, p65 has a central role in blocking a RANKL-induced pro-apoptotic pathway mediated by JNK, Bid, and caspase3, but is not required for OC differentiation.

Role of RelB in OC Differentiation

Several years ago, we were first to show that RANKL activates the alternative NF-κB pathway in OC precursors [8]. In this pathway, nuclear translocation of RelB requires processing of p100, which depends on NIK. In the absence of NIK, BMMs do not differentiate into OCs in response to RANKL and MCSF in vitro [8]. We hypothesized that unprocessed p100 blocked nuclear translocation of RelB in these cells. We were able to confirm this by mating NIK−/− and p100−/− mice, finding that deletion of p100 restores nuclear RelB and osteoclastogenesis [1]. Furthermore, expression of RelB, but not p65, rescued NIK−/− OC differentiation. We next turned to RelB−/− mice, and found that, similar to NIK−/−, they failed to form OC in vitro. Although RelB-deficient precursors expressed normal levels of RANK, induction of OC differentiation markers TRAP, Cathepsin K, DC-STAMP, and calcitonin receptor was weak or absent after RANKL treatment. The role of RelB in differentiation is specific, since p65 activation was normal in RelB−/− BMMs, and overexpression of p65 was unable to rescue OCs in RelB−/− cells.

Like NIK−/− mice, RelB−/− mice have only a small increase in bone mass, with a normal number of TRAP+ osteoclasts at baseline. In contrast, we had previously found that NIK−/− were resistant to stimulated osteoclastogenesis in the context of RANKL injection [8] and inflammatory arthritis [9]. We therefore wanted to determine if RelB−/− OCs were also unable to stimulate bone resorption in vivo. Using the osteolytic murine tumor cell line B16, we found that although tumors formed in bone following intracardiac injection, neither NIK−/− nor RelB−/− mice showed significant tumor-related osteolysis [1]. Thus, the alternative pathway, via RelB and NIK, is critical for OCs in vivo and in vitro.

cRel Is Not Required for Osteoclastogenesis

cRel is the third NF-κB subunit which, along with p65 and RelB, has a transactivation domain that would allow it to interact with distinct coactivators. In order to complete our survey of the effect of individual subunit deletions on OCs, we cultured cRel−/− BMMs, along with wild-type littermate controls, in M-CSF and RANKL. There was no difference in the number of OCs generated, or their ability to form actin rings and resorb when plated on bovine cortical bone. cRel-deficient

mice also had normal-appearing OCs in vivo, demonstrating that cRel does not have a unique role in OC differentiation.

Summary

Global blockade of NF-κB in the osteoclast (OC), affecting both classical and alternative pathways, prevents osteoclastogenesis and causes osteopetrosis in vivo [10, 11]. The classical pathway is activated rapidly in OC precursors, within minutes of RANKL exposure, but this activation is transient (Fig. 17.1a). In contrast,

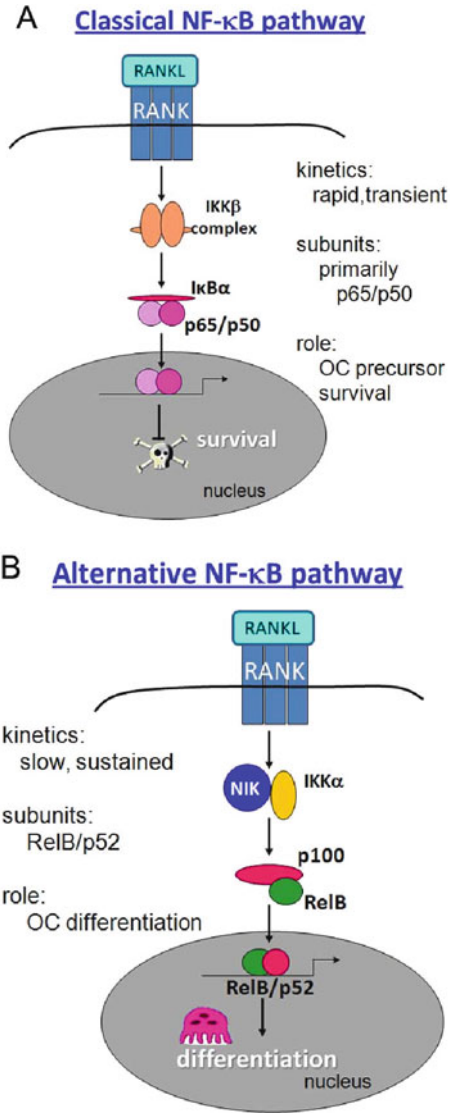

Fig. 17.1 NF-κB pathways in OCs. (**a**) In resting OC precursors, the classical pathway is off because p65/p50 NF-κB dimers are retained in the cytoplasm by IκBα. Binding of RANKL to RANK causes activation of the IKKβ complex within minutes, resulting in ubiquitination and proteosomal degradation of IκBα. This frees p65/p50 dimers to translocate to the nucleus and activate transcription of pro-survival genes that prevent apoptosis of OC precursors. (**b**) The alternative pathway is off in resting cells because RelB complexes are retained in the cytoplasm by p100, which has a C-terminal IκB domain. RANKL treatment activates NIK and IKKα, leading to proteosomal processing of p100 to p52, removing p100's IκB domain and allowing nuclear translocation of RelB/p52 dimers. The transcriptional targets of RelB promote QC differentiation. This pathway takes hours to activate but remains on for days

activation of the alternative pathway by RANKL takes up to 24 h to reach its peak but is sustained for days (Fig. 17.1b). We have shown that p65 is critical for survival of OC precursors during their RANKL-mediated differentiation but is not required for full OC differentiation or bone resorption [4]. Although cRel activation is induced by RANKL, loss of cRel does not impact osteoclastogenesis or survival. In contrast, RelB-deficient mice are resistant to stimulated osteoclastogenesis, and loss of RelB disrupts expression of OC differentiation genes in vitro [1]. Nevertheless, at baseline, RelB−/− mice have normal numbers of OCs and only slightly increased bone mass. Therefore, the alternative NF-κB pathway, mediated by NIK and RelB, plays a greater role in pathological bone loss than in normal bone homeostasis, and thus represents a promising target for the treatment of conditions such as postmenopausal osteoporosis, inflammatory osteolysis, and bone metastasis.

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Part V
Roles of the TNF Family in Infectious Diseases and Interrelationship of the TNF Family and Pattern Recognition Receptor Signaling: *Roles of the TNF Family in Infectious Diseases*

Chapter 18
Workshop Summary: Functions of the TNF Family in Infectious Disease

Michael Wortzman and Tania H. Watts

A session entitled, "Functions of the TNF family in infectious disease," was held at the 12th International TNF conference at El Escorial, Spain, April 28, 2009. The session highlighted the diverse and complex interplay between pathogens and the immune system and how TNF family members can contribute to both immune protection and immune pathology. Here we summarize some of the key findings and unifying themes that are discussed in more depth in the chapters that follow [1–6].

The Role of Soluble TNF, Membrane TNF, and Lymphotoxin in *Mycobacterium tuberculosis* Infection

Garcia et al. [1] review the importance of TNF in granuloma formation and bacteriocidal activities during mycobacterial infection. They point out that studies that test the role of TNFRs in *M. tuberculosis* (MTb) infection are complicated by the fact that lymphotoxin (LT) α as well as soluble and membrane-bound TNF can bind to TNFR1 and 2. Garcia et al. describe studies in which mice express only the membrane-bound form of TNF. Such studies reveal that mTNF can mediate some of the effects of sTNF and provide control of MTb during the acute phase of infection, but sTNF is required to maintain granulomas and for long-term control of disease. The use of TNF inhibitors for treatment of immune disorders such as rheumatoid arthritis and Crohn's disease has revealed an increased risk for reactivation or infection with MTb. The authors discuss how novel therapeutics that target soluble, but not membrane, TNF are showing promise in blocking inflammatory diseases in mouse models, while maintaining protection in acute *M. bovis* BCG infection. However, the authors point out that long-term studies with latent infection models are required to further evaluate this strategy.

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Interplay Between Heme and TNF in Severe Malaria

Gozzelino et al. [2] discuss the evidence for synergy between TNF and heme in causing tissue damage during severe malaria. The authors elaborate that heme is tightly controlled in the heme-binding pockets of hemoproteins during normal homeostatic conditions. However, if heme is released during pathological conditions, it can catalyze the production of free radicals and become cytotoxic. The authors suggest that severe malaria is triggered by hemolysis. During the blood stage of malaria, hemolysis leads to the release of hemoglobin to levels that overwhelm the normal scavenging mechanisms. In turn, hemoglobin can become oxidized to release free heme, which can sensitize non-hematopoietic cells to TNF-mediated programmed cell death, thereby contributing to disease pathogenesis and tissue damage. The mechanism by which heme sensitizes cells to TNF-induced death remains to be determined, but may involve c-Jun N-terminal kinase and reactive oxygen species.

TNF in Chagas Disease

Lannes-Vierra et al. [3] review the role of TNF in Chagas disease (CD), a vector-borne disease caused by the protozoan *Trypansoma cruzi*. Inflammatory cells, and potentially TNF, contribute not only to parasite control but also to the cardiac and digestive complications of CD, such that plasma TNF levels directly correlate with the severity of heart dysfunction in chronic chagasic cardiomyothapy (CCC). The authors discuss discrepant results from different studies on the link between single nucleotide polymorphisms (SNPs) in the TNF promoter and severity of CD in different populations. They argue that larger, better designed studies are required to determine genetic links between TNF SNPs and disease outcome in CD. The authors further explore the evidence that TNF contributes to the maintenance of CCC. Indeed, treatment of CCC in animal models with anti-TNF appears to ameliorate disease. On the other hand, treatment of a hamster model with soluble TNFR2 (Etanercept, which blocks TNFα and LTα) worsened disease in hamsters, suggesting that some TNFR signaling is required for protection. The authors propose that transient TNF at low levels is important in parasite control, but becomes damaging when it becomes deregulated due to persistent infection.

Subversion of TNF-Mediated Viral Control by Poxviruses

Alejo et al. [4] describe how production of soluble TNFR homologues is a common feature of poxviruses. The effects of the poxvirus encoded soluble TNFR family proteins include high affinity binding to TNF as well as sequestration of TNFR1. Another feature of the pox-encoded proteins CrmD and CrmB is their ability not only to bind TNF but also to bind simultaneously and with high affinity

to chemokines, thereby inhibiting both the establishment of the inflammatory state and the infiltration of leukocytes. The authors point out that there is limited data on the role of most of these vTNFRs on viral virulence in vivo. Here the mouse pox Ectromelia provides a useful model for in vivo analysis of this family of viruses. They find that deletion of the CrmD protein from Ectromelia attenuates virulence by six orders of magnitude in vivo, providing definitive evidence for the importance of the vTNFR as a virulence factor.

4-1BB/4-1BBL in Influenza and HIV Infections

Watts et al. [5] examine the role of the TNFR family member, 4-1BB and its TNF family ligand, 4-1BBL, in anti-viral CD8 T cell responses. During mild influenza infection, 4-1BB is only transiently expressed on lung CD8 T cells, and 4-1BBL is dispensable for initial CD8 T cell responses, although it is required later to sustain CD8 memory T cells. In contrast, during more severe influenza virus infection, 4-1BB expression is prolonged on the lung CD8 T cells and 4-1BBL is critical for keeping the CD8 T cells alive in the lung, allowing maximal viral clearance and mouse survival. Thus, the immune system may use inducible TNFR family members such as 4-1BB to prolong lymphocyte survival until the pathogen is cleared. Watts et al. also discuss how the 4-1BBL costimulatory pathway can contribute to a gain of function of CD8 T cells from chronically HIV-infected subjects ex vivo. A key question remains as to why this pathway is not sufficient to control HIV infection in vivo. In addition, Watts et al. review the critical role of TRAF1 in 4-1BB-induced survival signaling in CD8 T cells and provide a model describing the role of 4-1BBL and TRAF1 in the maintenance of CD8 memory T cells after antigen clearance.

A Lymphotoxin Pathway to Hepatocellular Carcinoma

Wolf et al. [6] review the role of the lymphotoxin (LT) receptor signaling pathway in inflammation and carcinogenesis. This pathway consists of the LTβ receptor (LTβR) and its ligand $LT\alpha_1\beta_2$. They elaborate that the role of LT signaling goes well beyond its well-established role in lymphoid organogenesis, contributing to inflammation during autoimmunity as well as in chronic infection. In a study now published in Cancer Cell [7] and presented at the 12th International TNF conference, the group of Heikenwalder identified the upregulation of the LT pathway in the liver during chronic infection of humans with hepatitis B or hepatitis C. They went on to investigate LT transgenic mouse models to show that chronic LT signaling in the presence of lymphocytes and the classical NFκB pathway leads to hepatocellular carcinoma. The authors discuss that LT is not an oncogene, but rather the LT signaling pathway promotes an environment favoring carcinogenesis following chronic viral infection of the liver. Interestingly, this pathway appears to be distinct from other non-viral liver diseases, which tend to have higher levels of TNF compared to

LT-induced inflammation. The finding that blockade with LTβR-Ig suppresses the chronic hepatitis driven hepatocellular carcinoma in the LT-transgenic model suggests a potential therapeutic use of LT signaling blockade in chronic viral hepatitis. The authors point out, however, that the role of LT in different cancer settings is complex and may depend on the particular cancer or model.

Emerging Themes

It is clear that TNF/TNFR family members play a prominent role in inflammatory conditions and in host defense against a variety of pathogens. The study of these interactions is complicated by the involvement of multiple organ systems, multiple cell types, and multiple receptor/ligand interactions. A still controversial area is the role of single nucleotide polymorphisms in affecting how individuals respond to particular infections. Both the TNF promoter as well as the gene for TNFR-associated factor 1, a signaling adaptor downstream of several TNFRs, including TNFR2 and 4-1BB, show SNPs in human [8, 9]. The potential role of such SNPs in controlling the immune response to infection and the balance between immunity and immunopathology remains to be more rigorously explored.

Interference with TNF signaling has shown great promise in blocking inflammatory diseases [10], but clearly more nuanced approaches are required in order to preserve the essential immune functions of TNF to prevent infectious complications, while avoiding the immune pathology that is associated with prolonged or high level TNF production. Novel therapies might well be informed by studying how viruses evade these pathways, for example, the ability of poxvirus CrmB protein to block both TNF and a chemokine receptor simultaneously. It is clear that complete blockade of TNF raises the infection risk [11], thus the ability to control the precise level and form of TNF over long periods of time, to simultaneously allow control of infection while avoiding immune pathology is an important area for further investigation. Understanding the synergies between TNF and other molecules involved in immune pathology in specific diseases, such as seen with heme in malarial infection [2], may also allow more specific strategies for particular situations.

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Chapter 19
Role of 4-1BBL and TRAF1 in the CD8 T Cell Response to Influenza Virus and HIV

Tania H. Watts, Gloria H.Y. Lin, Chao Wang, Ann J. McPherson, Laura M. Snell, and Laurent Sabbagh

Introduction

Activated T cells express a large number of signaling molecules, including both stimulatory and inhibitory members of the CD28/Ig superfamily as well as several members of the TNFR family. Costimulatory or coinhibitory receptors refer to receptors engaged with T cells during antigen-dependent responses that can either enhance or inhibit T cell activation. An interest in our laboratory over the last several years has been to understand the precise role for specific costimulatory receptors in the immune response and thereby to understand the need for so many receptor–ligand pairs in T cell activation. It is our view that the wealth of molecules modulating T cell activation allows fine-tuning and differential control of T cell immunity in different infectious disease contexts. Our work on the TNFR family member 4-1BB (CD137) and its ligand, 4-1BBL (CD137L), during different infectious processes illustrates that the precise role of this particular receptor/ligand pair may differ depending on the severity or duration of infection. This chapter provides a summary of work from our laboratory that was presented at the 12th International TNF meeting in El Escorial in April, 2009. For a more comprehensive review of the literature related to 4-1BB and related family members, the reader is referred elsewhere [1, 2, 3, 4, 5, 6].

Role of TRAF1 in 4-1BB Signaling

TRAF2 is important in transducing downstream signals from 4-1BB, including p38 MAPK and NF-κB activation [7–10]. However, until recently, the role of TRAF1 in 4-1BB signaling was not clear. TRAF1 differs from other TRAF family members in lacking the RING domain required for NF-κB activation [11]. TRAF1, when

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overexpressed, can block TRAF2 binding to TNFRs. Therefore, it was possible that TRAF1 acted as an inhibitor of TRAF2 [12]. TRAF1$^{-/-}$ mice were generated by Erdinyi Tsitsikov and T cells from these mice were found to be hyperproliferative to anti-CD3, and anti-CD3 pre-treated cells were also hyperresponsive to TNF, leading to the suggestion that TRAF1 is a negative regulator of T cell responses [13]. However, T cell-specific constitutive expression of a TRAF1 transgene delays the contraction of the CD8 T cell response to LCMV, suggesting a prosurvival role for TRAF1 in T cells [14]. Moreover, we had previously found that T cells from TRAF2$^{-/-}$ mice, or from mice expressing a dominant negative form of TRAF2, were also hyperresponsive to anti-CD3 and yet the T cells failed to respond to 4-1BB [7]. Therefore we hypothesized that T cells that develop in the absence of TRAF1 or TRAF2 may have a different threshold for antigen-receptor-dependent activation, hence their apparent hyperresponsiveness to anti-CD3 alone.

To test the role of TRAF1 in the response to 4-1BB signals in vivo, we crossed TRAF1$^{-/-}$ mice from Erdinyi Tsitsikov with the CD8 TCR transgenic line, OT-I, that encodes a TCR specific for H-2 K^{b} and ovalbumin 257–264 (SIINFEKL) [15]. We also took advantage of an agonistic antibody, 3H3, that is a potent stimulator of 4-1BB in vitro and in vivo [16, 17]. We co-injected mice intravenously with wild type or TRAF1-deficient OT-I T cells that could be distinguished by their differential expression of congenic markers. One day after injecting the T cells, we immunized the mice with an influenza virus that had been engineered to encode the SIINFEKL epitope in its neuraminidase stalk [18]. The immunization was done in the presence of anti-4-1BB agonistic antibody or control rat Ig antibody. We delivered the virus by the intraperitoneal route at low dose, which gives a relatively weak CD8 T cell response that is greatly augmented by inclusion of the anti-4-1BB agonistic antibodies. Strikingly, we found that when the T cells lacked TRAF1 they failed to expand in response to 4-1BB in vivo. We repeated the experiment after labeling cells with the fluorescent dye CFSE. The results showed that, regardless of the presence of TRAF1 or the anti-4-1BB agonist, the T cells divided equivalently in response to influenza virus. However, in the absence of anti-4-1BB or when T cells lacked TRAF1, the T cells failed to accumulate. This study showed that TRAF1 is critical for CD8 T cell survival during 4-1BB-dependent expansion in vivo [19]. Further analysis of in vitro activated CD8 T cells with or without TRAF1 showed that TRAF1 was essential for 4-1BB to activate the MAP kinase ERK. In turn, ERK was required for down-modulation of the proapoptotic molecule BIM [19]. Our previous work had shown that down-modulation of BIM by siRNA corrected the CD8 T cell survival defect observed with CD8 T cells lacking TRAF1 [20]. Thus 4-1BB-dependent ERK activation contributes to T cell survival via BIM modulation. One role of TRAF1 is to stabilize TRAF2 following 4-1BB signaling [19]. TRAF2 expression at the protein level is constant after 4-1BB signaling in WT T cells, but in TRAF1$^{-/-}$ T cells, TRAF2 protein levels decrease in the first 2 h [19]. However, proteasome inhibitors can prevent TRAF2 from disappearance in TRAF1-deficient T cells following 4-1BB ligation (Ann McPherson, unpublished). Work is in progress in our laboratory to determine if the main role of TRAF1 is to stabilize TRAF2 or whether it has additional direct signaling functions.

How Unique Are 4-1BB-Induced Survival Signals?

A number of costimulatory members of the TNFR family recruit TRAF2 and activate NF-κB. How then is 4-1BB unique? Is it just differential expression of 4-1BB relative to other TNFR family members that regulates its role in the immune response, or does it have different signaling properties compared to related TNFRs? To address this issue, our laboratory has focused on human CD8 T cell responses, using replication defective adenoviruses to deliver TNF family ligands to donor monocytes. We then use these monocytes to stimulate anti-viral recall responses of donor CD8 T cells. We previously showed that 4-1BBL is a potent costimulator of human influenza-specific CD8 memory T cells [21]. In contrast the TNF family ligand, CD70, which binds the TNFR family member CD27, was not as potent a survival factor for CD8 influenza specific T cells as 4-1BB [22]. CFSE labeling experiments showed that stimulation of human CD8 T cells with influenza peptide plus 4-1BBL or CD70 overexpressing monocytes led to a similar level of cell division and cytokine production by the CD8 T cells. However, greater numbers of CD8 T cells accumulated in 4-1BBL-stimulated as compared to CD70-stimulated cultures, indicating a greater survival role for 4-1BBL. 4-1BBL-stimulated T cells exhibited lower levels of the proapoptotic molecule BIM than CD70 stimulated cultures. Both CD27 [23] and 4-1BB [7–9] can recruit TRAF2 and can activate NF-κB. 4-1BB can also efficiently recruit TRAF1 [7–9], whereas evidence for recruitment of TRAF1 during CD27 signaling is lacking. Knockdown of TRAF1 by siRNA transfection of purified primary human CD8 T cells prior to stimulation resulted in increased BIM levels and lower CD8 T cell recovery after antigen plus 4-1BBL treatment. In contrast, TRAF1 knockdown had a minimal effect on CD70 stimulation of the T cells [22]. Thus 4-1BB and CD27 differ in their use of TRAF1 to modulate BIM levels and T cell survival.

Role of 4-1BBL and TRAF1 in Restoring Function in CD8 T Cells from Chronically HIV Infected Individuals

During chronic viral infection, CD8 T cells become progressively dysfunctional [24, 25, 27, 28, 29]. Our lab has shown that HIV-specific T cells from chronically infected donors require more than one costimulatory molecule for expansion ex vivo [30]. We found that 4-1BBL, when combined with CD80 or CD70, could induce the expansion and result in a gain of multifunctionality of CD8 T cells from chronically HIV-infected subjects whose starting T cell population was functionally impaired [22]. In contrast, the combination of CD70 and CD80 was relatively ineffective in this assay [22]. The key role of 4-1BB was to allow the T cells to survive through division and gain effector function. BIM levels were consistently lower in HIV-specific CD8 T cells expanded with 4-1BBL plus CD80 or CD70 compared to those expanded with CD70 plus CD80 [22]. Thus 4-1BB and TRAF1 are potentially important in control of chronic infection. TRAF1 is of particular interest in studies

of human T cell activation, because several recent studies have linked rheumatoid arthritis and other rheumatic diseases to single nucleotide polymorphisms (SNPs) in the TRAF1/complement factor 5 region [31–37]. It remains to be determined whether SNPs in TRAF1 affect the responses to TNFR signaling and thereby impact on the response of T cells to infection or contribute to autoimmunity.

Role of 4-1BBL in Mild Versus Severe Influenza Virus Infection

4-1BB is inducible on T cells upon antigen receptor signaling and therefore is expected to play a role on antigen-activated T cells. However, initial experiments with 4-1BBL-deficient mice showed that the absence of 4-1BBL had no impact on the initial primary expansion or contraction of the CD8 T cell response to influenza virus. The above studies were done with influenza delivered by a non-infectious intraperitoneal route, which results in minimal viral replication, but does induce a robust CD8 T cell response in the spleen [38]. As will be discussed below, under these conditions, 4-1BB is dispensable for initial T cell survival, but required at 3–6 weeks post infection for the antigen-independent maintenance of CD8 T cell memory [38, 39]. Why then would the immune system bother to express 4-1BB early after T cell activation if it is only needed much later? We reasoned that inducible costimulators such as 4-1BB might act to prolong the survival of T cells when infections become more severe. To test this, we analyzed the effect of 4-1BBL deficiency during a model of severe influenza pneumonia in mice, following intranasal infection with Influenza A/PR8. Under these conditions, we found that mice lacking 4-1BBL showed increased mortality compared to WT mice. Analysis of the cells in the lungs of mice with and without 4-1BBL showed that antigen-specific CD8 T cells were decreased in the lung of 4-1BBL-deficient as compared to WT mice following severe influenza infection. In contrast, other 4-1BB-expressing immune cells, such as CD4 T cells, dendritic cells, and NK cells, were found in similar numbers in the presence or absence of 4-1BBL [40]. Intranasal infection with a milder influenza strain, Influenza A/X31, did not show this defect in CD8 T cell numbers or increased mortality during primary respiratory infection of 4-1BBL-deficient mice. Influenza A/X31 virus is cleared by day 8 of infection, whereas the PR8 virus takes longer to clear, and 4-1BBL-deficient mice show a five-fold higher viral load at day 6–8 post-infection compared to PR8-infected WT mice [40]. At day 6 of the response, 4-1BB was found on T cells in the lungs of X31 or PR8-infected mice; however by day 8, 4-1BB was no longer detectable on lung CD8 T cells from the X31 infection, but persisted on the lung T cells from the more severe PR8 infection. Semi-quantitative PCR analysis showed that the ligand was induced in the lung by PR8, but not by the milder X31 virus [40]. Thus 4-1BB and its ligand are upregulated in the lung during severe influenza infection and protect the host from death by sustaining cytotoxic T cell survival to provide better viral control. In contrast, during milder infection, expression of 4-1BB and its ligand are not sustained in the lung, and this receptor ligand/pair is dispensable for viral control and mouse

survival. These studies suggest that the immune system may have acquired inducible TNFR family members such as 4-1BB to allow the immune system to prolong T cell survival with infections that take longer to clear.

Role of 4-1BBL and TRAF1 in T Cell Memory

As discussed above, 4-1BBL is not required for initial expansion of T cells in response to mild influenza infection. However, upon secondary infection, 4-1BBL-deficient mice have a decreased CD8 T cell response. Subsequent work revealed that this defect in secondary response is not due to a failure to expand upon restimulation or due to decreased "programming" of T cells early in the response, but is due to decreased maintenance of CD8 T cell survival in the absence of 4-1BBL [39–41]. We therefore asked how 4-1BBL could play a role in T cell survival after viral clearance, if 4-1BB is normally inducible by antigen receptor signaling. To rule out residual antigen presentation after the virus itself is cleared, we turned to an adoptive transfer model, in which TCR transgenic T cells are converted to memory-like T cells following stimulation with antigen and then IL-15. Transfer of CD8 memory T cells into 4-1BBL deficient mice revealed a survival defect of T cells when mice lacked 4-1BBL [39]. Our lab reported [39] that IL-15 can induce 4-1BB on memory $CD44^{hi}$ CD8 T cells in the absence of antigen. Subsequent work from Lieping Chen's lab [42], however, showed that anti-4-1BB could expand memory T cells in IL-15-deficient mice, suggesting that other stimuli may contribute to induction of 4-1BB in vivo. Our unpublished data show that IL-2, which like IL-15 is a member of the common gamma chain cytokine family, can also serve this function.

TRAF1 is critical in 4-1BBL-dependent memory T cell survival, as TRAF1-deficient memory CD8 T cells show impaired survival in the absence of antigen in vivo. Knockdown of BIM in the $TRAF1^{-/-}$ T cells corrects this defect, suggesting that 4-1BB-TRAF1-dependent down-modulation of BIM contributes to memory CD8 T cell survival [20].

As 4-1BB can be expressed on both T cells as well as other cells of the immune system, it was not entirely clear whether the effect of 4-1BBL on T cell memory was directly on the CD8 T cells or indirect via other cells. However, the finding that memory CD8 T cell survival was defective when TRAF1 was absent only in the CD8 T cells is consistent with a direct effect [20]. To more directly determine the cell types involved, we have used mixed bone marrow chimeras to show that 4-1BB on the αβ T cells is required for CD8 T cell memory (G.H.Y. Lin, manuscript in preparation).

The cell type involved in presenting 4-1BBL to the 4-1BB-expressing CD8 T cell to maintain memory remains incompletely defined. When 4-1BBL is absent on radioresistant cells, there is a defect in CD8 memory maintenance almost as large as the effect of complete 4-1BBL deficiency. In contrast, when 4-1BBL is absent on radiosensitive cells, there is only a small defect in CD8 T cell memory (G.H.Y. Lin, manuscript in preparation). Thus we propose that 4-1BBL in the bone marrow, perhaps on a stromal cell, is involved in the maintenance of CD8 T cell memory.

A Model for the Maintenance of CD8 T Cell Memory

Figure 19.1 shows our current model describing a hypothetical bone marrow "niche" that provides survival signals to CD8 memory T cells. A more complete discussion of this model can be found elsewhere [2]. We know that the bone marrow is a preferred site of homeostatic proliferation for CD8 T cells [43] and that IL-15 can contribute to this homeostatic proliferation [44] (reviewed in [45]). Our data show that in the absence of antigen, IL-2 or IL-15 can induce 4-1BB on CD8 memory T cells, which allows them to interact with 4-1BBL-expressing cells. In the absence of 4-1BBL, these memory CD8 T cells have a defect in survival. We have also shown that TRAF1 is crucial for 4-1BB-dependent CD8 memory T cell maintenance through the down-modulation of BIM. Therefore, our current model is that 4-1BB expressing CD8 memory T cells receive a TRAF1-dependent survival signal from a 4-1BBL expressing cell in the bone marrow. Though the precise cell type that provides 4-1BBL to maintain CD8 T cell memory remains undefined, most of

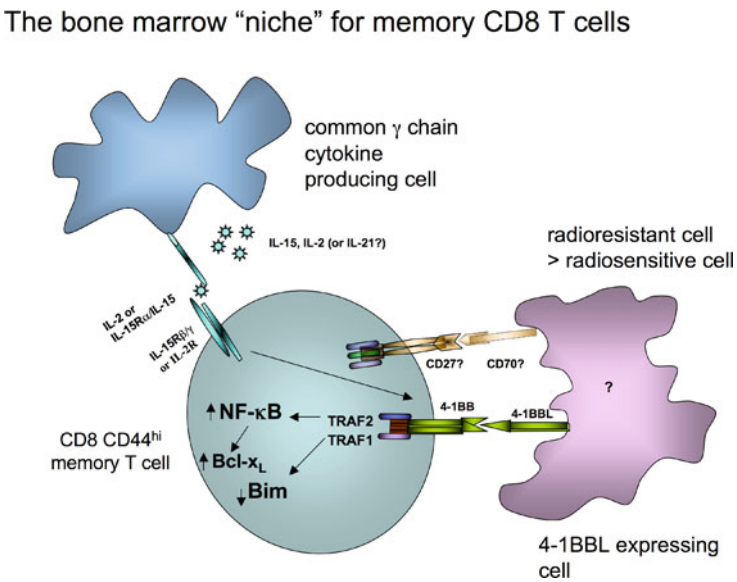

Fig. 19.1 A model for maintenance of CD8 memory T cells by 4-1BBL. We propose that a defined niche exists in the bone marrow where CD8 memory T cells meet cytokine producing cells as well as 4-1BBL-expressing cells. IL-15 or IL-2 can induce 4-1BB on memory but not naïve CD8 T cells in the absence of antigen. Other common gamma chain cytokines such as IL-21 might also contribute, but have not been tested. 4-1BB on the memory CD8 T cell then induces TRAF1-dependent survival signaling upon binding its ligand on an unidentified cell. Experiments with bone marrow chimeras suggest that the majority of this signal is provided by a radioresistant cell. Other TNFR family members may contribute to memory CD8 T cell maintenance in the bone marrow. CD27 is a prime candidate because it is highly expressed on memory CD8 T cells and we have found that for HIV-specific CD8 T cells 4-1BBL and CD70 can have synergistic effects [22]. This figure is adapted from [2]

the defect in T cell survival in 4-1BBL-deficient mice can be attributed to lack of 4-1BBL on a radioresistant cell (G.H.Y. Lin, unpublished).

Concluding Remarks

Our studies have shown that 4-1BB is an important survival molecule for activated and memory CD8 T cells following influenza virus infection and that TRAF1 plays a role in this survival signaling. The mechanism by which TRAF1 contributes to CD8 T cell survival is not fully understood. However, the finding that TRAF1 is overexpressed in many human cancers of B cell origin [46] and that TRAF1 polymorphisms have been linked to autoimmunity [31–37] makes this a pressing area for future research. We know that ex vivo, 4-1BBL can also contribute to the recovery of functional CD8 T cells from a non-functional starting population of CD8 T cells isolated from HIV infected individuals [22]. Thus 4-1BBL or anti-4-1BB agonists could provide a potential therapy for chronic infections such as HIV. However, too much stimulation through 4-1BB can lead to immune pathology, so care must be taken in applying 4-1BB agonists therapeutically [47]. It is not clear why the endogenous 4-1BB/4-1BBL signal is not sufficient to control viral load in most HIV-infected individuals. It will be of interest to determine whether polymorphisms in TRAF1 contribute to differential responses to cancer or viral infections in human populations, either by modifying their susceptibility to cancer or autoimmunity or by influencing their ability to control virus infection or respond to anti-4-1BB therapy.

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Chapter 20
Roles of Soluble and Membrane TNF and Related Ligands in Mycobacterial Infections: Effects of Selective and Non-selective TNF Inhibitors During Infection

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Introduction

Mycobacterium tuberculosis infection is a major health problem that predominantly affects developing countries and is associated with poverty and malnutrition [1, 2]. One-third of the global population is considered to be infected with *M. tuberculosis* mainly in a latent form which is difficult to be clearly diagnosed which can be reactivated after an alteration of the host immunity. Approximately 5–10% of infected individuals develop an active disease and the majority of healthy individuals are asymptomatic; the infection can remain in a latent form for many years and as long as the immune system can control bacterial dormancy [3]. However, alterations of the host immune system by different causes such as immunodeficiency due to HIV, malnutrition, complications of aging, and some genetic factors can favor a reactivation of latent tuberculosis infection. The use of TNF inhibitors for treatments of severe inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, and ulcerative colitis, has been associated with reactivation of latent tuberculosis and increased susceptibility to primary tuberculosis infection [4–8]. TNF possesses a broad range of activities required for maintenance of host immunity against mycobacterial infections. A non-redundant activity of TNF involves the development of functional granulomas and the activation of bactericidal mechanisms to control and eliminate intracellular bacilli and maintenance of latent infection. Although the majority of TNF activities have been attributed to the soluble TNF (solTNF) form, the transmembrane or membrane-bound TNF (mTNF) plays an important role in the control of acute tuberculosis infection. Other closely related TNF ligands such as lymphotoxin (LT) α and β play also a role in immunity to mycobacterial infections. This review deals with the protective roles of solTNF

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and mTNF and other closely related TNF ligands in immunity against mycobacterial infections, and novel therapeutic approaches for selective neutralization of solTNF reducing the risk of infections will be discussed.

Roles of TNF in Mycobacterial Infections

TNF is one of the first cytokines produced by alveolar macrophages after recognition of mycobacteria by Toll-like receptors (TLRs). TNF contributes to the development of granulomas which are highly organized new structures generated by cell-mediated immune responses to mycobacteria that involve the interactions of macrophages, dendritic cells (DCs), giant cells, T cells, B cells, γδT cells, neutrophils, and natural killer (NK) and NK T cells. The granuloma is an area of localized infection characterized by a high cellular activity associated with the production of many cellular mediators including cytokines and chemokines required for cell recruitment, cell circulation to lymph nodes, and development of bactericidal mechanisms. Cytokines and chemokines and the inflammatory response result in the regulated recruitment of inflammatory and immune cells. Bacilli are likely transported by DCs to the lung parenchyma and to draining lymph nodes to prime naïve T cells [9]. CD4 and CD8 T cells expressing IFN-γ, developing T helper 1 (Th1) immunity, and circulating monocytes are recruited to infectious sites and contribute to the granuloma maturation that should contain and destroy some of the bacteria thus preventing dissemination [10]. TNF, mainly released by macrophages and DC, is essential in this coordinated cellular process [11]. A simplified view of how mycobacteria activate cytokine and chemokine production and the important role of TNF in the development of bactericidal mechanisms and cell recruitment to granulomas is depicted in Fig. 20.1. As shown, TNF participates in host resistance in different ways: (1) activating macrophages and monocytes, (2) inducing chemokines and cell recruitment to infected sites, (3) activating bactericidal mechanisms of macrophages, such as the inducible nitric oxide synthase (iNOS or NOS2) producing NO, and apoptosis of infected phagocytes, (4) triggering T cell functions, and (5) synergizing with IFNγ to stimulate anti-mycobacterial mechanisms including autophagy [12].

Soluble TNF, Membrane-Bound TNF, and Lymphotoxins

TNF is produced as a cell membrane protein and the active form is a trimer. The 26 kDa precursor monomer protein is cleaved into 17 kDa monomers forming biologically active soluble trimers. Proteolytic cleavage is mediated by a cell membrane-bound metalloprotease(s), the TNFα converting enzyme (TACE) [13, 14] that also cleaves other membrane proteins including tumor necrosis factor receptor 2 (TNFR2) [15]. TNF as well as LTα (previously known as TNFβ) are cytokines that provide signals for secondary lymphoid tissue development [16–19]. TNF is

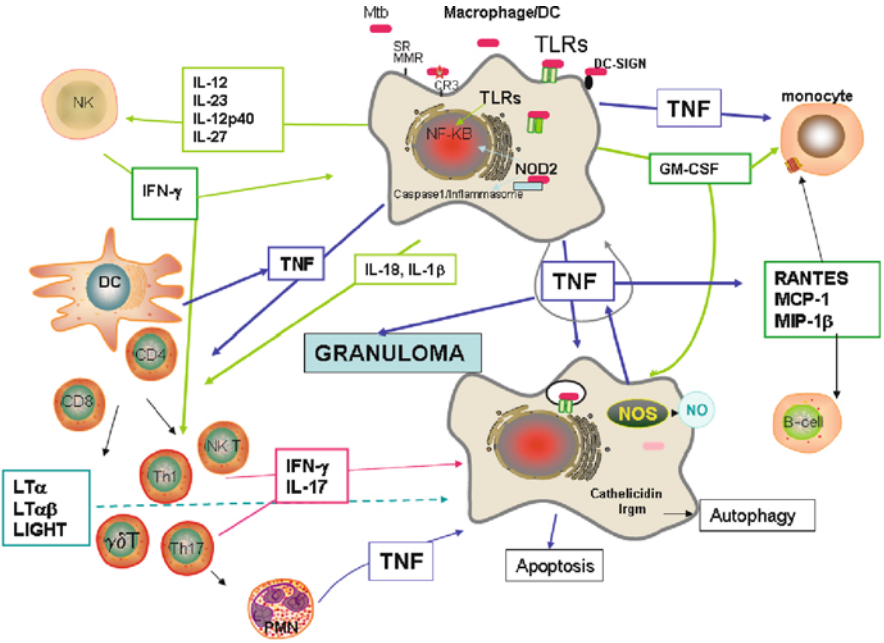

Fig. 20.1 TNF is induced by *M. tuberculosis* (Mtb) and *M. bovis* BCG infections. TNF is activated by immune-recognition of mycobacterial through TLRs and NOD2 in macrophages and dendritic cells. Phagocytic cells secrete TNF and other cytokines activating and recruiting monocytes and inducing the development of macrophage bactericidal mechanisms. IFN-γ and TNF are synergistic inducers of microbicidal activities such as NO generation, activation of Irgm involved in autophagy, and apoptosis inhibiting bacillus growth. TNF plays an important role in granuloma formation by participating to immune cell recruitment to infected sites

produced by monocytes/macrophages, neutrophils, T and B lymphocytes, NK, and many other cells, whereas LTα is secreted by T, B, and NK cells. LTα exists not only as a soluble homotrimer but also in the form of a heterotrimeric membrane molecule in association with a LTβ, forming LTαβ molecules that are found on activated lymphocytes [20, 21]. LTα1β2 is the predominant form, and LTα2β1 is a minor form (<2%) with no defined role [19]. As shown in Fig. 20.2, solTNF and mTNF exert their activities through two receptors which are expressed on the majority of cell types and tissues, 55 kDa TNF receptor 1 (TNFR1) (CD120a) and 75 kDa TNFR2 (CD120b) [22, 23]. LTα binds to TNFR1, to TNFR2, and to another receptor, the herpes virus entry mediator (HVEM). Heterotrimeric LTα1β2, the predominant form, interacts with LTβ receptor (LTβR) which plays an important role in lymph node development, splenic architecture, and lymphoid organ chemokine production required for the recruitment of DCs [24, 25]. LIGHT (lymphotoxin-like, shows inducible expression, competes with HPV glycoprotein D for HVEM, a receptor in T lymphocytes) is a member of the TNF/LT family of ligands, expressed by lymphocytes, monocytes, and DCs, that interacts with LTβR and HVEM and plays a role in co-stimulation of T cells [24, 26, 27].

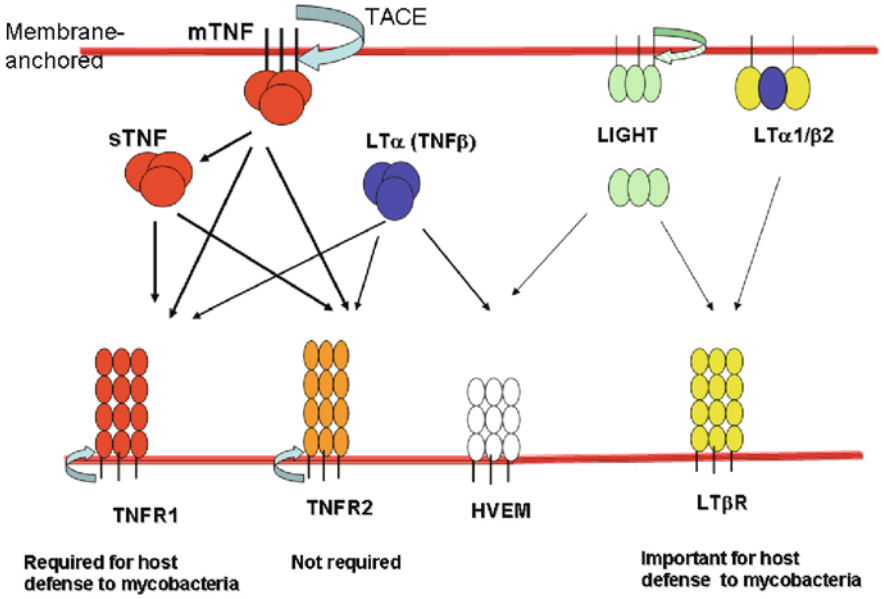

Fig. 20.2 TNF-LT ligands and receptors. Metalloproteases (mainly TACE) cleave the mTNF into the solTNF form (sTNF). TNFR1 and TNFR2 are also released from their membrane into soluble TNFR forms by metalloproteases (*open arrows*). Interactions between TNF-LT ligands and receptors are indicated by arrows. TNFR1, TNFR2, and LTβR trigger different intracellular signaling including NF-κB, JNK, MAPK, p38MAPK, AP-1, and caspase activation as reviewed [94–96]

Role of TNF in Experimental Animal Models of Tuberculosis Infection

A critical role of TNF for immunity to mycobacteria has been demonstrated using genetic mouse models of TNF inactivation and pharmacological approaches of TNF inhibition [28]. Several mouse models of infection have contributed to the analysis of the role of TNF during the course of mycobacterial infection. Intravenous infection with the vaccine strain *Mycobacterium bovis* BCG induces the formation of granulomas in lungs, liver, and spleen, and wild-type C57Bl/6 mice control and survive the infection. However, mice treated with anti-TNF antibodies showed impaired *M. bovis* BCG granuloma formation and increased bacillus proliferation in infected organs [29]. Sustained inhibition of TNF and LTα by transgenic expression of human TNFR1-Fc fusion protein resulted in fatal infection due to impaired granuloma formation and bacillus overgrowth [30, 31]. Double-deficient TNF/LTα mice were highly susceptible to *M. bovis* BCG infection, exhibiting a severely impaired immune response with reduced cell recruitment to granulomas and anti-mycobacterial functions [32, 33]. Expression of transgenic LTα in TNF/LTα double-deficient mice prolonged mouse survival after *M. bovis* BCG infection, showing that the role of TNF is non-redundant for resistance to avirulent

mycobacteria. Experimental infections with a virulent *M. tuberculosis* strain using different routes of infection in mice deficient in TNF, or in mice unable to use TNF by transgenic expression of TNF receptors or by administration of anti-TNF antibodies have shown alteration of granuloma formation associated with extensive lung necrosis and uncontrolled mycobacterial proliferation [30, 34–41]. Using TNF-deficient mice in a model of latent tuberculosis following an antibiotic treatment, arrest of the therapy was followed by a massive reactivation of the disease in TNF deficient mice but not in wild-type mice [42]. The predominant TNF receptor was shown to be TNFR1 for *M. bovis* BCG, *M. tuberculosis*, and *M. avium* resistance [34, 43, 44]. In contrast, TNFR2 appeared to play a minor role in mycobacterial host defense [45] (Fig. 20.2). A study using intra-vital microscopic observation has illustrated the dynamics of macrophages and T-cell interactions during liver *M. bovis* BCG granuloma formation and disintegration by anti-TNF treatment. This study has confirmed the essential role of TNF for maintenance and functionality of granulomas [46]. Interestingly, a recent study using *M. marinum* infection in a zebrafish model showed that TNF was not required for granuloma formation but plays an indirect role on granuloma integrity by limiting bacterial growth within macrophages and preventing their necrosis [47].

Membrane TNF Protects from BCG and Acute Tuberculosis Infections

Although the majority of TNF activities were at first attributed to the solTNF form, the specific activities of mTNF have more recently been elucidated. Evidence for a biological role of mTNF has been obtained in vitro as well as in vivo [48, 49–54]. An interesting mode of action of membrane-bound TNF is the induction of reverse signaling, which has been suggested for other members of the superfamily of TNF ligands, although the molecular mechanisms remain uncharacterized [55]. The activation of reverse signaling through mTNF was shown to trigger the protein kinase C pathway and to up-regulate E-selectin on activated human $CD4^+$ T cells [56, 57]. In vitro treatment with anti-TNF antibody activated intracellular signals through mTNF resulting in IL-10 production, cell proliferation, increased apoptosis, and cell cycle arrest of a human T-cell line [58].

In vivo, three different mTNF genetic mouse models have been evaluated for the protective role of mTNF during mycobacterial infections in the absence of solTNF. Olleros et al. investigated the resistance to *M. bovis* BCG and *M. tuberculosis* infections in transgenic mice expressing a mTNF ($TNF^{\Delta\text{-}12\text{-}10;\ \Delta\text{-}2+1;\ K11E}$) under the control of proximal TNF promoter on a TNF/LTα deficient background. Mice were totally resistant to *M. bovis* BCG infection but only partially protected by mTNF against *M. tuberculosis* [59, 60]. The protective activity of mTNF has been investigated in two different mTNF knock-in (KI) mouse strains that represent a major advance in terms of regulated expression of mTNF during experimental infection. *M. tuberculosis* infection of mTNF KI ($TNF^{\Delta 1\text{-}9,\ K11E}$) mice showed that mTNF can

substitute some of the activities of solTNF during the acute phase of infection but, during the chronic phase of the disease, solTNF appears to be necessary for resistance [61, 62]. *M. bovis* BCG infection in a second mTNF KI ($TNF^{\Delta 1\text{-}12}$) mouse strain showed partial control of the infection. These mice also exhibited protection during the acute phase but not in the chronic phase of *M. tuberculosis* infection whereas TNF-deficient mice rapidly died [63]. These data suggest that mTNF form confers protection during acute phase of *M. tuberculosis* infection while during the chronic phase solTNF appears to be required for long term immunity.

Lymphotoxins and LIGHT in Immunity to Mycobacterial Infections

The activity of LTα has been closely related to that of TNF since they share the same receptors. Experimental *M. bovis* BCG and *M. tuberculosis* infections in LTα KO mice which lack secondary lymphoid organs resulted in uncontrolled bacterial overgrowth, necrotic pulmonary lesions, and animal death [33, 64]. However, a more recently generated LTα KO mouse (neo-free LTα KO), which does not display altered TNF expression [65], called into question the previous studies on LTα KO mice in which TNF expression was decreased by alteration of the TNF locus. Mycobacterial infections of these new LTα KO mice will provide insights on the contribution of LTα in host defense mechanisms (Allie et al, J. Immunol. in press). LTα deficiency involves the lack of LTβ (LTα1β2) and the signaling through LTβR which also plays an important role in macrophage activation. Treatment of *M. bovis* BCG-infected mice with LTβR-Fc fusion protein, antagonizing LTβ and LIGHT, resulted in reduction of microbicidal macrophage activity and increased bacterial growth [66]. LTβR deficient mice, which lack Peyer's patches, colon-associated lymphoid tissues, and lymph nodes [67] showed uncontrolled *M. tuberculosis* infection with a delayed iNOS expression on macrophages forming granulomas [68]. In contrast, LIGHT deficient mice were not more sensitive to *M. tuberculosis* than wild-type mice indicating that direct cell contact interactions between lymphocytes bearing LTβ and monocytes/macrophages expressing LTβR are critical for the control of *M. tuberculosis* infection [68].

Neutralization of TNF and Risks of New Infection and Reactivation of Latent Tuberculosis

The large number of patients treated with TNF inhibitors has revealed an increased risk for opportunistic infections, most notably primary infection by *M. tuberculosis* as well as reactivation of latent tuberculosis which represent an important complication of such treatment [4–8, 69]. It has been shown that anti-TNF therapy decreases the frequency of CD4 T lymphocytes capable of producing IFN-γ upon antigenic activation. These effects can partially explain the increased incidence of tuberculosis

in patients treated with TNF inhibitors [70]. One study provides evidence that treatment of rheumatoid arthritis patients with etanercept impaired B cell function by reducing follicular dendritic networks, germinal center structures, and peripheral blood memory B cells which are not affected in patients treated with methotrexate [71]. A recent study has shown that anti-TNF antibodies reduced the population of CD8 effector memory T cells which mediate antimicrobial activity against *M. tuberculosis* by the expression of granulysin [72]. Many clinical studies are available today showing the association of anti-TNF therapies and the increased risk of primary and reactivated of tuberculosis. Clinical studies also point out that such complications may occur even after chemoprophylaxis for latent tuberculosis.

Strategies to Block TNF Activities

The extension of anti-TNF for use in the treatment of human inflammatory diseases other than rheumatoid arthritis, Crohn's, and psoriasis also increases the potential for complications associated with these therapies. Thus, new therapeutic strategies are required to attenuate the deleterious effects of total TNF blockade on the host immune system while maintaining the positive anti-inflammatory effects of TNF therapies. Given that this nonselective anti-TNF therapy has proven highly efficacious for severe inflammatory disease, the development of selective inhibitors of solTNF may therefore represent a promising future therapeutic strategy. A novel class of TNF inhibitors is available, known as dominant-negative (DN) TNF, that antagonize solTNF but not mTNF [73]. Recent studies have shown that these DN-TNF biologics are effective in reducing inflammation in mouse arthritis and Parkinson's disease models, yet in contrast to nonselective inhibition, do not suppress the resistance of mice to *Listeria monocytogenes* infection [74, 75]. We have analyzed the effects of a DN-TNF on host defense to *M. tuberculosis* and *M. bovis* BCG infections, and on protection against endotoxin-induced liver inflammation in *M. bovis* BCG-infected mice to study their influence during mycobacterial infections as well as their efficacy in preventing an acute inflammatory reaction. DN-TNF efficiently protected from endotoxin-mediated hepatotoxicity in *M. bovis* BCG-infected mice, while immunity against *M. tuberculosis* and *M. bovis* BCG infections was preserved, presumably by maintenance of physiological mTNF signaling [76]. Although long-term studies and murine latent tuberculosis are still required to better evaluate the effect of DN-TNF molecules, selective inhibition of solTNF seems to better preserve immune defenses compared with neutralization of both solTNF and mTNF. The different mechanisms of action of DN-TNF, etanercept, and anti-TNF antibodies are illustrated in Fig. 20.3 which shows that DN-TNF only inhibits solTNF signaling by subunit exchange with endogenous TNF, etanercept interacts with both solTNF and mTNF as well as with LTα and the minor form of LTαβ [77], and anti-TNF antibodies block mTNF and solTNF.

A study using a TNF virus-like particle-based vaccine that selectively targets solTNF has also shown protection from arthritis without inducing reactivation of

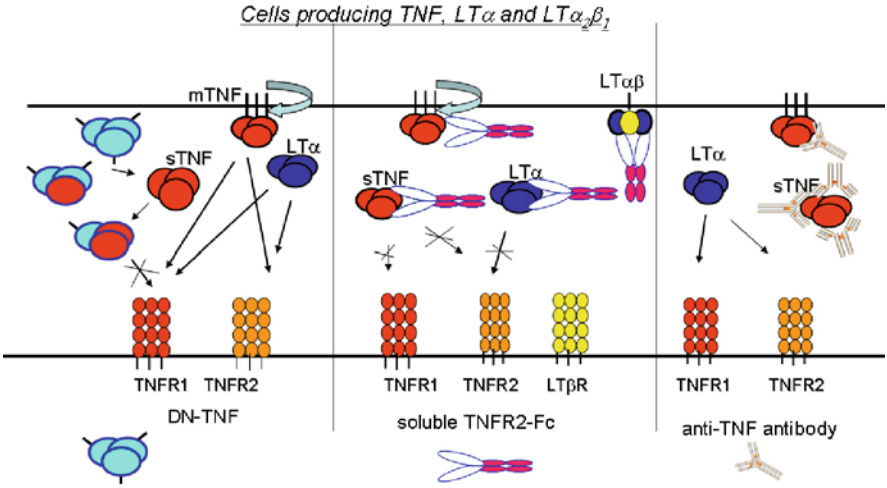

Fig. 20.3 Mechanisms of action of selective and non-selective TNF inhibitors. *Left*, DN-TNF, a mutated form of human solTNF (sTNF) with disrupted receptor binding interfaces, eliminates solTNF by a subunit exchange mechanism, but is unable to interact with mTNF, LTα, and LTα2β1. *Center,* solTNF, mTNF, LTα, and LTα2β1 (the minor form) can be neutralized by soluble TNFR2-Fc (e.g., etanercept) inhibiting interaction with corresponding receptors. *Right*, anti-TNF (e.g., infliximab, adalimumab, and golimumab) inhibits solTNF and mTNF. DN-TNF biologics inhibit solTNF receptor signaling mediating inflammation without suppressing mTNF and LTα protective immune responses through interaction with TNFR1 and TNFR2

latent tuberculosis [78]. Animals immunized with total TNF virus-like particles were highly sensitive to *M. tuberculosis* infection whereas mice producing antibodies against the N-terminus of TNF were not sensitive to the infection but were protected from arthritis. These differences were attributed to antibodies recognizing both mTNF and solTNF or interacting only with solTNF [78]. These data indicate that selective inhibition of solTNF may be a promising anti-inflammatory approach which does not suppress the immune response to acute or chronic tuberculosis infections.

New promising strategies of TNF inhibition designed at the level of both the TNFR and TNF molecules are being explored today. TNFR1 and TNFR2 contain an extracellular pre-ligand binding assembly domain (PLAD) which is distinct from the ligand binding domain. This region is necessary for the assembly of TNFR complexes in the absence of ligand and encourages trimerization upon activation by TNF [79]. A soluble form of PLAD derived from TNFR1 was shown to block TNF in vitro and inhibit arthritis in animal models [80]. Several human TNF variants with mutations corresponding to the six amino acid residues at the receptor binding site have been selected on the basis of high affinity for TNFR1 and absence of biological activity [81]. One of the TNF mutants showed inhibition of TNFR1 but not TNFR2 activities. This TNF mutant was able to protect mice in several hepatitis models including TNF/D-GalN and ConA [82, 83]. This

represents a new TNF inhibitor to be explored for its effects on infectious diseases. Finally, TNF inhibition by small-molecules has been reported [84]. This small molecule inhibited TNF–TNFR interaction by binding to intact biologically active TNF trimers and promoted subunit disassembly thereby inactivating the cytokine in vitro [84].

TNF and Chemokines in Mycobacterial Infections

Mycobacterial infections induce chemokine activation involved in migration of immune cells to the site of the infection thus favoring the development of granulomas [11, 85]. Chemokines are detected in bronchoalveolar lavage (BAL), lymph nodes, and blood from patients with active tuberculosis as reviewed [86].

M. tuberculosis infection of mouse macrophages activates chemokines such as MCP-1/CCL2, MIP-1α/CCL3, and MIP-1β/CCL4 and chemokine receptors CXCR3, CCR5, and CCR2 [87, 88, 89]. Deficiency in CCR2 (the receptor for MCP-1, MCP-3, and MCP-4) has confirmed its requirement for host defense against *M. tuberculosis* infection, as the majority of CCR2 deficient mice died early after infection and showed severe alteration of cell recruitment in infected lung [90]. In contrast, *M. tuberculosis*-infected CCR5 (the receptor for RANTES, MIP-1α, and MIP-1β) deficient mice were able to induce Th1 immune responses and to control the infection [91]. Surprisingly, mice deficient in CXCR3 (the receptor for IP-10, ITAC, and Mig) were found to be more resistant to *M. tuberculosis* than wild-type mice, suggesting that CXCR3 can attenuate the host immune response [92].

Deficiency of TNF during infection affects the recruitment of inflammatory and immune cells to granulomas in mouse models [36]. Studies in TNF-deficient mice showed that RANTES/CCL5, MCP-1/CCL2, and MIP-1β/CCL4 mRNA levels were reduced in TNF KO mice at early infection but were highly increased at 4 weeks of infection [37]. Dysregulation of circulating and pulmonary RANTES and MIP-1α was observed in TNF/LTα KO mice infected with *M. bovis* BCG [60]. Deficiency in TNF led to in vitro and in vivo reduced and/or delayed production of chemokines by pulmonary macrophages and aberrant granuloma formation [93]. Mice expressing a transgenic mTNF in a TNF/LTα KO background were able to activate RANTES and MCP-1α and developed bactericidal granulomas after *M. bovis* BCG infection [60]. These data suggest that solTNF and also mTNF play a role in the chemokine gradient of infected tissues determining efficient cell migration and control of infection.

Conclusion

TNF has an essential protective activity in latent and active tuberculosis infection in humans. Neutralization of TNF can have consequences on a non-diagnosed latent infection which can be reactivated and followed by an acute tuberculosis

infection. In addition, TNF inhibition renders patients more sensitive to new infections. Genetic and pharmacological animal data showing that inhibition of solTNF sparing mTNF is effective in abrogating inflammatory diseases and yet maintaining host defense activity may allow the improvement of anti-TNF therapy. These data suggest that the risks associated with total TNF inhibition might be reduced by the use of new compounds only blocking solTNF but sparing the protective effects of mTNF. A reduction in the infection risks associated with current anti-TNF drugs may allow the safer use of anti-TNF therapies in other inflammatory diseases.

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Chapter 21
Poxviral TNFRs: Properties and Role in Viral Pathogenesis

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Poxviruses

Poxviruses are a family of large irregularly shaped dsDNA viruses replicating exclusively in the cytoplasm of the infected cell that include a number of human and animal pathogens [13]. Most notorious amongst these is the fortunately eradicated variola virus (VARV), a highly specific and contagious human virus that caused smallpox, a disease with mortality rates of up to 30% that has caused major pandemics recorded as early as 1350 BC. Other poxviruses causing disease in humans include monkeypoxvirus (MPXV), an emerging zoonotic disease detected in African countries as well as in the USA with mortality rates of up to 10% [9], molluscum contagiosum virus, which causes benign tumorations mainly in children, and yaba-like disease virus that causes a mild, self-limiting illness. The prototypical member of this virus family is vaccinia virus (VACV) which was used as a live attenuated vaccine for the eradication of smallpox. Lately, recurrent human infections from circulating VACV-derived viruses adapted to animal hosts have been detected in India and Brazil, suggesting the potential for adaptation and re-emergence of these viruses. Additionally, poxviruses are currently under study for the development of vaccines and use in oncolytic therapies. Therefore, comprehension of the mechanisms of pathogenesis and a better understanding of the host immune modulation strategies used by poxviruses are essential.

Poxviral TNF-Binding Proteins

Concurrently with the first identification and molecular cloning of the human TNFRs in 1990 [11, 22, 27], Smith and colleagues identified a putatively secreted viral protein termed T2 in Shope fibroma virus, a poxvirus infecting rabbits, with

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similarity to the TNFR family. Indeed, they proposed that this could act as a soluble TNF or related cytokine receptor and that such a protein "would no doubt confer a selective advantage to the pathogen" [27]. Since then, most poxviruses have been shown to encode at least one predicted active secreted TNF-binding protein, which can be divided into two groups based on their primary sequences. A class of these TNF inhibitors termed viral TNFRs (vTNFRs) are homologues to the extracellular domain of host TNFRs, which are characterized by the presence of a variable number of copies of the cysteine-rich domain (CRD) that defines the TNFRSF. These include the mentioned M-T2 protein (and its orthologues present in other viruses of the Leporipoxvirus genus), four different proteins named cytokine response modifier B (CrmB), CrmC, CrmD, and CrmE, which are differentially expressed by members of the poxvirus family, as well as a viral homologue of the TNFRSF member CD30. A second class of secreted viral TNF-binding proteins have no amino acid sequence similarity to known cellular proteins and include the 2L protein of the yatapoxviruses and their swinepox and deer poxvirus orthologues.

With the exception of the viral CD30 homologue, which was shown to bind exclusively to cellular TNFSF8 and whose role in pathogenesis is not clearly defined [3, 15, 21], all other proteins mentioned were found to bind specifically and with high affinity to TNF and in some cases LT alpha of at least the species infected by the corresponding virus where they were derived from (see Table 21.1 for a succinct description of the main properties of these proteins).

The myxoma virus M-T2 protein is most similar to the cellular TNFR2. It contains four N-terminal CRDs, followed by a C-terminal domain that shows no similarity to cellular genes. This secreted protein was found to bind to rabbit TNF with high affinity through its four N-terminal CRDs and inhibit its activity in cell culture [23]. Additionally, M-T2 was described as a virulence factor during the development of myxomatosis in infected European rabbits. More recently, the intracellular pool of M-T2 was shown to have an important role, as it can inhibit TNF-mediated signaling through PLAD domain-mediated sequestration of cellular TNFR1, acting effectively as a dominant negative mutant that prevents virus-induced apoptosis independently of its TNF-binding capability [23–26]. Probably both activities act in concert in vivo to provide its, however, relatively minor role in pathogenesis.

The orthopoxviral CrmC and CrmE proteins are composed of only three CRDs each and, although most similar to the cellular TNFR2, have been found to bind to TNF but not LT. The possibility that VACV CrmE may act at the cell surface, by interacting with the cell membrane after secretion, as well as in solution has been raised [18]. The solution of the crystal structure of the VACV CrmE protein showed that it adopts a canonical TNFR fold. Although the structure of CrmE complexed with TNF was not reported, comparison of CrmE with the TNFR structure suggests that it binds to TNF using the 50s and 90s loops present on CRDs numbers 2 and 3, respectively. At the moment, this is the only member of the vTNFR family whose structure has been solved and it may be used as a model to understand the ways in which these proteins are able to bind to and inhibit their ligands. Thus, it has been proposed that the 90s loop is involved in fine-tuning ligand specificity and affinity,

Table 21. 1 Main properties of the poxviral TNF-binding proteins

Protein	Virus (virus family)	Known ligands	Role in vivo	Reference(s)
2L	Tanapox virus (yatapoxvirus)	TNF β2microglobulin	Unknown	[5, 16, 17, 24, 26, 28, 30]
T2	Shope fibroma virus, myxoma virus (leporipox virus)	TNF, LTα TNFR1	Virulence factor	
CrmB	VARV, MPXV, CPXV, CMPV, HSPV (orthopoxvirus)	TNF, LTα, chemokines	Anti-inflammatory, virulence factor	[1, 2, 8, 10, 14, 29]
CrmC	CPXV, VACV some strains only (orthopoxvirus)	TNF	Virulence factor	
CrmD	ECTV, CPXV (orthopoxvirus)	TNF, LTα, chemokines	Unknown	[2, 12]
CrmE	CPXV, VACV some strains only (orthopoxvirus)	TNF	Virulence factor	[18, 21]
vCD30	ECTV, CPXV (orthopoxvirus)	TNFSF8	Modulates Th1 responses in vivo, not major virulence factor during infection	[3, 15, 21]

whereas the 50s loop may determine the binding affinity of these viral receptors [9]. Finally, the CrmB and CrmD proteins share a domain structure similar to that of the M-T2 protein, with four N-terminal CRDs followed by a C-terminal domain unrelated to TNFRs. Both are secreted proteins and bind with high affinity to TNF and LT, preventing their biological activity in cell culture assays. Interestingly, these proteins were found to be able to bind to a reduced set of chemokines (chemotaxis-inducing cytokines) through their C-terminal domain, which has been named the SECRET domain (for Smallpox virus-Encoded Chemokine REcepTor) and inhibit their biological activity in cell culture [2]. The lack of amino acid sequence similarity of the SECRET domain with host chemokine receptors and other virus-encoded chemokine-binding proteins suggests that this domain represents a new protein structure able to bind chemokines with high affinity. The TNF and chemokine ligands were shown to be bound simultaneously through structurally independent domains of the VARV CrmB and ECTV CrmD proteins, which were proposed to act as immunomodulatory proteins with dual activities in vivo. Whether this property may be shared by the M-T2 protein remains to be addressed.

vTNFRs in Pathogenesis

Somewhat surprisingly given the importance of orthopoxviral infections in humans, the evidence on the contribution of orthopoxviral vTNFRs to virulence in vivo is scarce so far.

The CPXV CrmB protein was proposed to have anti-inflammatory activity in vivo using a chicken embryo chorioallantoic membrane infection model and the CrmB deletion mutant CPXV showed an approximately 50-fold reduced LD_{50} in intracranially infected mice [14]. However, the CPXV strain used still contained other active vTNFRs, impairing a clear attribution of TNF or chemokine inhibitory activities to the observed phenomena. Additionally, the model of intracranial infection used can hardly mimic the natural route of infection. More recently, expression of CPXV CrmB, VACV CrmC or CrmE proteins from a recombinant attenuated VACV was shown to increase the virulence using an intranasal mouse model of infection, but not an intradermal infection model. Deletion of the VACV CrmE, but not CrmC, did similarly attenuate viral infection in mice using the intranasal model [18]. This evidence is limited to models that required high virus doses to cause disease and the effects observed are relatively minor. Lastly, no description of the possible role of the CrmD protein in vivo has been published yet. Therefore, we believe that better in vivo models may be necessary for the study of the contribution of vTNFRs to viral pathogenesis and immune regulation.

Ectromelia Virus

Ectromelia virus (ECTV) is an orthopoxvirus that has host specificity for the mouse and is genetically very similar to VACV, VARV, and MPXV [7]. Susceptible strains of mice infected with ECTV develop mousepox that, like smallpox, is a severe disease with high mortality and infectivity rates, and constitutes an excellent model to study smallpox. The fact that infection with ECTV in susceptible strains of mice rapidly becomes systemic when inoculated via the subcutaneous or intranasal route has been exploited not only as a tool to investigate the pathogenesis and immunology of poxvirus infections, but also as a model of generalized virus infections, genetic resistance to disease, and viral immunology [7].

Previous data suggested an important role for TNF-induced signaling in mousepox pathogenesis, as transgenic-resistant mice lacking functional TNFR1 and TNFR2 became susceptible to ECTV, with highly increased viral titers in the main target organs, liver and spleen, of the infected mice [20]. Moreover, treatment of susceptible BALB/c mice with murine TNF was able to hinder ECTV replication and mortality to some extent [4]. Importantly, ECTV encodes only one active vTNFR, the CrmD protein, which is expressed as a secreted protein at late times postinfection. VARV, the causative agent of human smallpox, does similarly encode only one secreted vTNFR predicted to be active, the CrmB protein. As mentioned before, both proteins have been characterized and shown to have similar binding properties

in vitro. For these reasons, ECTV constitutes an excellent model for the study of the role of vTNFRs in the pathogenesis of orthopoxviral infections.

To address this issue, we have constructed a recombinant ECTV lacking the gene encoding CrmD and studied its virulence in vivo. Experiments performed in cell culture confirmed that this deletion mutant did not express any detectable soluble TNF-binding activity and that deletion of CrmD did not alter the replication capacity of the virus. When infected into susceptible BALB/c mice, however, the CrmD deletion mutant was found to be severely attenuated, with an LD_{50} reduced by at least six orders of magnitude. Moreover, absence of CrmD was accompanied by a strong and quick inflammatory response at the initial infection site and reduced viral spread to the main target organs, spleen and liver. This identifies CrmD as one of the main determinants of mousepox virulence in vivo, suggesting that the related CrmB protein found in VARV might be of similar importance during the development of human smallpox. Interestingly, the TNF-regulated response in macaques experimentally infected with VARV was impaired as compared to infections with other viruses [19], suggesting an active viral TNF-blocking mechanism which may be mediated by the CrmB protein, amongst others.

Given that CrmD is able to block both TNF and chemokines simultaneously, we propose a working model (see Fig. 21.1) in which blockade of TNF hinders the establishment of a proinflammatory state locally, while chemokine blockade further impairs the ensuing recruitment of leukocytes to the initial infection site. This may allow enhanced replication and spread of the virus through the infected host by preventing the establishment of an adequate antiviral immune response in vivo.

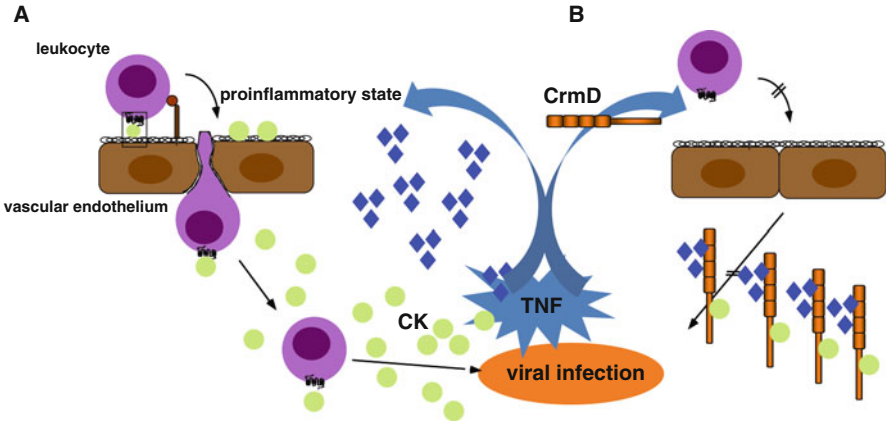

Fig. 21.1 Proposed working model for the CrmD protein. (**a**) In response to viral infection, TNF and other proinflammatory cytokines are produced. This proinflammatory state promotes the expression of chemokines (CK) that will recruit leukocytes to the site of infection to eliminate or control the incoming virus. (**b**) In the presence of the secreted TNF and CK inhibitory protein CrmD expressed by ECTV infected cells, this reaction is blocked in two steps by first impairing the establishment of a proinflammatory state and later directly preventing leukocyte migration

To further understand the molecular mechanisms of CrmD activity during infection, we are currently studying in greater detail the structural determinants of CrmD interactions with its TNF/LT and chemokine ligands. Our aim is to generate recombinant ECTV expressing mutant versions of the protein binding to only one of its known ligands at a time. This will allow us to determine the individual contribution of these cytokines in the establishment of the antiviral response. This approach will also help us to understand the structural basis of the CrmD–ligand binding that could be used as a model to study molecularly the TNFSF–TNFRSF interaction, since surprisingly, despite the importance of these cytokines in the immune response, there are few data about the structural features of the ligand–receptor complexes.

Conclusions

In this study, we demonstrate that CrmD is an essential virulence factor, as deletion of CrmD from ECTV resulted in an avirulent virus that induced an acute early inflammatory response at the initial site of infection but did not establish a fatal disease. This is one of the most dramatic attenuation phenotypes described in poxviruses after inactivation of a viral immune modulatory activity and demonstrates a critical role of TNF and a reduced set of chemokines in antiviral defence. Moreover, this unique experimental model of virus infection will allow us to dissect in vivo the relative contribution of TNF and chemokine activities to the establishment of an adequate immune response.

Anti-TNF therapy, based on either monoclonal antibodies or soluble TNFRs, is available in the clinic as an efficient way of blocking adverse inflammatory responses. A better knowledge of the strategies used by viruses to modulate TNF activity during infection may uncover new anti-TNF strategies that could be applied to the clinic. The use of soluble versions of TNFRs by viruses confirms an adequate strategy used in the clinic. A better understanding of the function of the different vTNFRs in vivo may help us to improve even further the potency of soluble TNFRs in blocking adverse inflammatory responses in a number of human disease conditions.

The investigations described here on viral strategies to modulate the activity of TNF illustrate how a better understanding of the interaction of viruses with the host immune system may provide information relevant to viral pathogenesis and suggest new strategies of immune modulation for the treatment of human disease.

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Chapter 22
Heme Sensitization to TNF-Mediated Programmed Cell Death

Raffaella Gozzelino and Miguel P. Soares

Abbreviations

Fe	iron
JNK	Jun N-terminal kinase
NF-κB	nuclear factor kappa B

Introduction

Heme, that is, iron (Fe) protoporphyrin IX, exists as a prosthetic group of hemoproteins, where it plays a critical role in several biologic functions, including some that are essential to support life under aerobic conditions (reviewed in [1–3]). Presumably, heme prosthetic groups are part of a highly evolutionarily conserved strategy that allows for the incorporation of Fe atoms into the tertiary structure of proteins, i.e., hemoproteins. By doing so, hemoproteins acquired the ability to exchange electrons with a variety of ligands, including the gasotransmitters (O_2), nitric oxide (NO), and possibly hydrogen sulfide (H_2S) [4].

As an electron exchanger, Fe-heme can catalyze the production of free radicals through the Fenton chemistry [5]. Under homeostasis, this potentially hazardous effect is tightly controlled by the amino acids present in the "heme pockets" of hemoproteins. While efficient at preventing the pro-oxidant effects of prosthetic heme groups, this control mechanism fails once hemoproteins undergo conformational modifications and release non-covalently bound heme. The "free heme" produced in this manner can catalyze, in an unfettered manner, the production of free radicals and therefore act as a potent cytotoxic agonist (reviewed in [1, 2]). The term free heme is used hereby to refer to heme not contained within the heme pockets of hemoproteins. This does not preclude its association with proteins or lipids, but in a manner that does not control its pro-oxidant activity.

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While the cytotoxic effects of free heme are well established [6, 7] its pathophysiologic role, if any, has remained elusive (reviewed in [1, 8, 9]). We have recently demonstrated that free heme can synergize with a number of pro-inflammatory agonists, including tumor necrosis factor (TNF), to induce programmed cell death [10]. We will argue that this cytotoxic effect might contribute to the pathogenesis of several immune-mediated inflammatory diseases [8], as illustrated hereby for malaria (reviewed in [9]).

Malaria

Malaria, the disease caused by *Plasmodium* infection, is endemic to many regions of the globe; affecting millions of individuals, mainly in subtropical countries were it remains a major cause of morbidity and mortality [11, 12]. The life cycle of *Plasmodium* is initiated in its human host upon the bite of an infected *Anopheles* mosquito, which injects *Plasmodium* sporozoites into the blood stream [12]. Within minutes, *Plasmodium* sporozoites can reach the liver, infecting hepatocytes [13], where they replicate extensively giving rise to the so-called liver stage of infection (reviewed in [14]). This initial stage of the infection is clinically silent, most likely based on the expression of protective genes [15] by the infected host [16]. Eventually, *Plasmodium* replication within hepatocytes produces merozoites that are released into the blood stream, infecting red blood cells and thus initiating the "blood stage" of infection [12]. Within the red blood cell compartment, *Plasmodium* manages to evade host adaptive immunity, that is, T and B cells, presumably because red blood cells do not express high levels of major histocompatibility antigens, which are strictly required to present antigens to T cells. While relatively efficient, this evasion strategy presents some major limitations, revealed by the appearance of the clinical signs of malaria.

The clinical signs of malaria can range from cycles of fever episodes to much more severe outcomes that include respiratory distress, pulmonary edema, circulatory collapse, abnormal bleeding, jaundice (reflective of hepatic failure), hemoglobinuria, severe anemia, convulsions, prostration, and/or impaired consciousness [17] (reviewed in [12, 18]). Severe malaria defines the occurrence of one or more of these clinical signs in individuals with no other apparent cause of disease aside from *Plasmodium* infection [17] (reviewed in [18]). The clinical course of severe malaria is often fulminant, with impaired consciousness, respiratory distress, and severe anemia being the major predictors of death (reviewed in [18–20])). The cellular and molecular mechanisms underlying the pathogenesis and outcome of severe malaria remain elusive, probably explaining the lack of efficient treatment against this disease. We propose that severe malaria is a disease triggered by hemolysis and in particular by the free heme released from oxidized hemoglobin.

A Central Role of Free Heme in the Pathogenesis of Severe Malaria

Plasmodium replication inside red blood cells leads unavoidably to hemolysis and thus to the release of hemoglobin into circulation (reviewed in [21]). There are several protective mechanisms against the deleterious effects of cell-free hemoglobin. Probably the most prevalent relies on haptoglobin (Hp; human, P00738), a plasma protein that binds cell-free hemoglobin with high affinity (K_M~2.10^{-11}–10^{-12} M) and allows for subsequent disposal of hemoglobin by hemophagocytic macrophages, thus preventing hemoglobin accumulation in plasma [22]. However, once the scavenging capacity of haptoglobin is overwhelmed, such as during the blood stage of *Plasmodium* infection, cell-free hemoglobin does accumulate in plasma [23] (reviewed in [24]). When exposed to reactive oxygen species, ferrous (Fe^{2+}) hemoglobin is oxidized into (Fe^{3+}) methemoglobin, releasing its heme prosthetic groups and therefore producing free heme [23, 25] (see figure 22.1). Extracellular free heme can be "transferred" into several plasma proteins [26], including, hemopexin [27], albumin [28], α_1-microglobulin [29], and lipoproteins [30]. Among these, hemopexin (Hpx; human; P02790) binds free heme with the highest affinity (K_d<10^{-12} M) and as such is thought to play a central role in preventing the deleterious effects of extracellular free heme [27, 31]. As for haptoglobin, the scavenging capacity of hemopexin can also be overwhelmed during the blood stage of *Plasmodium* infection, thus leading to the accumulation of free heme in plasma.

When released into circulation, free heme is thought to target preferentially vascular endothelial cells where it acts in a cytotoxic manner [6]. How heme gains access to the intracellular compartment of endothelial or other cell types is not clear (reviewed in [1]). Based on its hydrophobicity, free heme can intercalate itself into the phospholipids of the plasma membrane and presumably in this manner gain intracellular access. However, this notion is challenged by the demonstration that free heme gains intracellular access via an active mechanism, driven by several evolutionary conserved heme transporters (reviewed in [1, 32, 33]).

We found that free heme can sensitize a variety of non-hematopoietic cells to undergo programmed cell death in response to pro-inflammatory and/or cytotoxic agonists [10] (reviewed in [1]) (see figure 22.1). Presumably for this reason, free heme plays a pivotal role in the pathogenesis of severe malaria, as demonstrated initially for experimental cerebral malaria in mice [9, 23], a neuroinflammatory syndrome that resembles human cerebral malaria [19] (reviewed in [9]). Free heme also plays a critical role in the pathogenesis of non-cerebral forms of severe malaria in mice [10], such as those associated with hemolytic anemia, hepatic damage, and eventually multi-organ failure [10]. The pathogenic effect of free heme seems to synergize with that of TNF, a cytokine that plays a critical role in the pathogenesis of both cerebral [34, 35] and non-cerebral [36] forms of severe malaria.

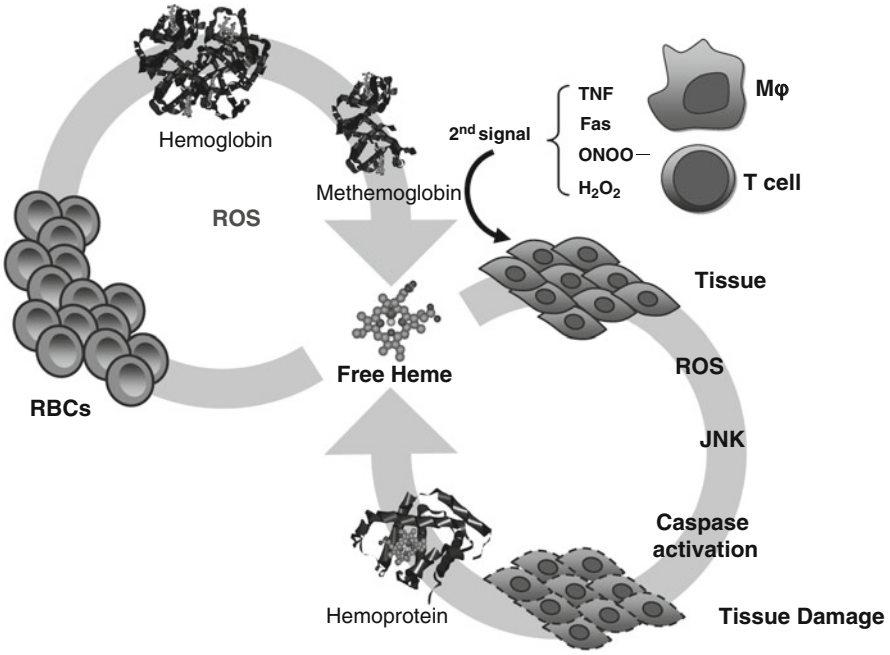

Fig. 22.1 Heme sensitization to programmed cell death. Disruption of red blood cells (RBCs) leads to hemolysis and thus to the release of cell-free hemoglobin into circulation. During inflammatory conditions cell-free hemoglobin is oxidized into methemoglobin, an effect associated with the release of its heme prosthetic groups. Free heme fosters, in turn, the production of reactive oxygen species generating a positive feed-back loop which promotes further RBCs lysis [45]. Once released into circulation free heme can sensitize non-hematopoietic cells (tissue) to the induction of irreversible oxidative stress in response to a "second signal" produced by innate or adaptive immune cells, e.g., TNF, Fas, ONOO–, H_2O_2. Moreover, free heme primes cells to activate JNK in a sustained manner leading to caspase-dependent programmed cell death by apoptosis. Widespread apoptosis might result in further release of intracellular hemoproteins, i.e., myoglobin, thus promoting further accumulation of free heme and inducing a positive feed-back loop, which amplifies free heme sensitization to programmed cell death

Briefly, we found that when infected by *Plasmodium* (i.e., *chabaudi chabaudi*), DBA/2 mice present high plasma concentrations of TNF as well as free heme [10]. Moreover, infected mice proceed to develop a lethal form of liver failure, characterized by widespread hepatocyte programmed cell death [10]. Neutralization of circulating TNF or free heme is sufficient to protect hepatocytes from undergoing programmed cell death and to suppress the development of liver failure in these mice [10]. This suggests that free heme synergizes with TNF to trigger tissue damage, ultimately compromising host viability [37]. We found that this is indeed the case in that free heme can potentiate the cytotoxic effects of TNF, acting as a potent catalyst of programmed cell death.

Free Heme Sensitizes Cells to Undergo TNF-Mediated Apoptosis

TNF is a pleiotropic cytokine that has been functionally implicated in the pathologic outcome of a variety of infectious diseases (reviewed in [38, 39]), including malaria [34]. While the pro-inflammatory effect of TNF can be readily observed under a number of experimental conditions, its cytotoxic effect is more elusive [40] (reviewed in [38, 41]). Engagement of the TNF receptors 1 and 2 can activate several signal transduction pathways leading to programmed cell death [40, 41]. However, this cytotoxic effect appears to be constitutively repressed through the expression of a series of immediate early TNF-responsive genes [15, 38, 40]. Expression of these cytoprotective genes can be induced by a variety of signal transduction pathways that share, as a common feature, their ability to activate the nuclear factor kappa B (NF-κB) family of transcription factors [42]. Once NF-κB activation is repressed, the cytotoxic effects of TNF become apparent, demonstrating that NF-κB activation limits the cytotoxic effects of TNF [42]. Since NF-κB activation is a common denominator of most, if not all, immune-mediated inflammatory conditions it is difficult to conceive how the cytotoxic effects of TNF would promote the pathogenesis of those diseases. One possibility would be that this cytotoxic effect requires the presence of one or several additional cytotoxic molecules that would override the cytoprotective effect of NF-κB-dependent genes. We found that free heme acts in such a manner, thus providing a possible mechanism underlying the cytotoxic effects of TNF during the pathogenesis of immune-mediated inflammatory conditions, such as malaria [10, 23, 34].

Most primary cells or cell lines do not undergo programmed cell death when exposed in vitro to free heme (5–40 μM), the same being true for TNF (5–100 ng/ml). However, when exposed to free heme in the presence of TNF, primary cells as well as immortalized cell lines undergo programmed cell death, as demonstrated using hepatocytes [10] (reviewed in [1]). This cytotoxic effect occurs within 4–24 h and acts in a dose-dependent manner in that increasing the concentration of either free heme (5–40 μM) or TNF (5–100 ng/ml) results in more programmed cell death [10]. While free heme can sensitize several cell types to undergo TNF-mediated apoptosis, e.g., hepatocytes, oligodendrocytes, and β-cells of the pancreas, this cytotoxic effect is not observed in all cell types, as demonstrated for vascular endothelial cells [10] (reviewed in [1]). Considering that in the event of inflammatory hemolytic conditions vascular endothelial cells are probably the first cells to be confronted with free heme, this might explain why these diseases do not necessarily lead to loss of vascular integrity and disseminated microvascular hemorrhage.

Induction of programmed cell death by free heme and TNF occurs most probably via apoptosis, as demonstrated in hepatocytes by the occurrence of chromatin condensation and nuclear shrinking as well as by the processing of pro-caspase-3 zymogen into its active cleaved form, two hallmarks of apoptosis [43, 44]. Moreover, pharmacologic inhibition of caspase activation suppresses the cytotoxic effect of heme plus TNF, further supporting the notion that heme sensitizes cells

to undergo TNF-mediated apoptosis. Although prevalently apoptotic, we cannot exclude the contribution of a necrotic pathway in this process.

The mechanism via which free heme sensitizes cells to undergo TNF-mediated apoptosis remains to be fully established. Pharmacologic inhibition of mRNA and/or protein synthesis fails to interfere with heme plus TNF-driven cytotoxicity, thus suggesting that free heme acts independently of newly expressed proteins to sensitize cells to apoptosis. Instead, free heme acts as a potent pro-oxidant, catalyzing the production of reactive oxygen species in response to TNF, presumably via the participation of its Fe atom in the Fenton chemistry (see figure 22.1). This notion is supported by several independent observations in hepatocytes. First, heme plus TNF lead to the accumulation of high levels of intracellular reactive oxygen species as well as lipid peroxidation derivatives, at concentration in which neither heme nor TNF alone yield the same effect [10]. Second, anti-oxidants such as *N*-acetyl cysteine or butylated hydroxyanisole suppress the cytotoxic effects of free heme plus TNF [10]. Third, protoporphyrin IX, a molecule with identical molecular structure to heme but lacking its Fe atom, fails to promote TNF-mediated apoptosis (R. Gozzelino, unpublished observation).

The mechanism via which the pro-oxidant effect of free heme sensitizes cells to undergo TNF-mediated apoptosis relies functionally on the activation of a signaling transduction pathway involving the c-Jun N-terminal kinase (JNK) (R. Gozzelino, unpublished observation) (see figure 22.1). This notion is supported by several observations, again in hepatocytes. First, the anti-oxidants *N*-acetyl cysteine and butylated hydroxyanisole suppress the activation of JNK in response to heme plus TNF (R. Gozzelino, unpublished observation). Second, pharmacologic or genetic inhibition of JNK expression suppresses the ability of free heme to sensitize cells to undergo TNF-mediated apoptosis (R. Gozzelino, unpublished observation). Third, inhibition of JNK expression reduces the production of reactive oxygen species in response to heme plus TNF, an effect that is sufficient to protect against apoptosis (R. Gozzelino, unpublished observation).

Concluding Remarks

In addition to reveal the pro-apoptotic effect of free heme our data provide a functional link between free heme [23] and TNF [34] in the pathogenesis of severe malaria. While speculative at this point, we believe that the same mechanism might operate in the pathogenesis of other immune-mediated inflammatory diseases. If this proves to be the case, then targeting free heme might be used therapeutically to suppress the pathogenesis of not only severe malaria, but also that of other immune-mediated inflammatory diseases.

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Chapter 23
TNF-α and TNFR in Chagas Disease: From Protective Immunity to Pathogenesis of Chronic Cardiomyopathy

Joseli Lannes-Vieira, Isabela Resende Pereira, Nathália Ferreira Vinagre, and Lucia Elena Alvarado Arnez

Introduction

American trypanosomiasis or Chagas disease (CD), discovered in 1909 by the Brazilian physician Carlos Chagas, is a vector-borne complex disease caused by the haemoflagellate protozoan parasite *Trypanosoma cruzi* [1]. One hundred years after its discovery, CD is still epidemiologically relevant, afflicting 12–14 million neglected individuals in 18 endemic countries in Latin America, as well as in non-endemic countries in North America, Europe, and Asia [2, 3]. In the last three decades, governmental initiatives targeting the main vector in endemic areas resulted in successful decline of the incidence of acute infection. For instance, in Brazil, the numbers of acute infection dropped from more than 100,000 new cases/year during the 1980s to less than 500 new cases/year during 2001–2006 [2, 4]. However, the current epidemiological data indicate that we must devote our efforts and agenda to the implementation of sustainable policies for CD control. These measures should incorporate guaranteeing access to the trypanocidal treatment and therapies required by patients, particularly pregnant women and children, developing new drugs and therapeutic strategies, identifying progression markers, as well as elucidating pathogenic mechanisms leading to the distinct clinical forms of CD [3, 4].

Following infection with *T. cruzi*, most individuals generate effective immune response in the acute phase, control the parasite, and become asymptomatic, remaining in the indeterminate form of CD. On the other hand, parasite persistence associated with imbalance of effector functions or cytokine production sometimes leads to an exacerbated immune response capable of mediating damage in target tissues. This can result in the chronic clinical forms of CD, the digestive form (megaoesophagus and/or megacolon) in 5–10% of patients, and the chronic

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chagasic cardiomyopathy (CCC), the most severe form of the disease, afflicting 30–40% of patients [5, 6].

Although heart invasion by inflammatory cells contributes to parasite dissemination control in the acute phase of *T. cruzi* infection, these cells seem to be also involved in CCC formation, characterised by chronic myocarditis with prominent fibrosis and organ dysfunction [5–8]. The chronic inflammatory heart infiltrates, which are not directly related to the scarce parasite, are mainly composed of mononuclear cells, lymphocytes, and macrophages [9–11]. Among the lymphocytes, $CD8^+$ T cells outnumber $CD4^+$ T cells in the focal and diffuse heart infiltrates of CCC patients [11, 12] and chronically infected experimental models [13]. The molecular pathways governing the development of $CD8^+$ T cell-enriched myocarditis are not yet identified. In past years, growing evidence has suggested that the TNF-α/TNFR-mediated pathway is involved in *T. cruzi* control in acute infection [14, 15] but might also play a pivotal role in myocarditis formation and, consequently, in CCC development [16–18]. Therefore, understanding how TNF-α/TNFR-mediated immune response controls parasite growth and lead to heart damage may provide insights into the design of new therapeutic strategies that can improve the prognosis of CD patients.

TNF-α Polymorphisms in Chagas Disease

The factors that determine the outcome of the clinical patterns of CD most certainly depend on the interactions among the genetic makeup of the parasite, the host immunogenetic background, and environmental factors [6, 19]. The genetic variation within *T. cruzi*, which results in different parasite lineages with distinct biological behaviour and biochemical characteristics, does not uniquely determine the clinical evolution to the cardiac or digestive forms of CD [19]. Furthermore, the development of CCC in only 30–40% of *T. cruzi*-infected individuals and the tendency for familial aggregation [20] suggest that host genetics may be involved in host susceptibility.

TNF-α plasma levels directly correlate with the degree of heart dysfunction in CCC patients [21–23], suggesting the participation of TNF-α in the formation of chronic heart inflammation and cardiac injury. Potential differences between groups of asymptomatic and CCC patients, and between healthy and infected individuals, could be related to *TNF* gene variations. Most studies on *TNF* gene variations have analysed the frequencies of single nucleotide polymorphisms (SNPs) or microsatellite alleles. The results concerning *TNF* polymorphisms are rather conflicting in CD. The study of *TNF* promoter polymorphisms (-308, -244 and -238) in a small group of Peruvian patients showed no difference in genotype frequencies of these SNPs between cases and controls or between asymptomatic and CCC individuals [24]. However, the frequency of the *TNF* -308A allele, which results in a twofold greater level of transcription than the *TNF* -308G allele [25], was increased in Mexican chagasic patients compared to non-infected individuals, while CCC patients also presented a higher frequency of the A allele compared to asymptomatic individuals

[26], suggesting that the *TNF* -308A allele could be associated with susceptibility to CCC.

Interestingly, in 42 Brazilian chagasic patients with ventricular ejection fractions ≤ 40%, the analysis of the frequency of the *TNF* -308A and microsatellite *TNFa2* allele, which also determine a high TNF-α production [27], showed that patients expressing these alleles have a shorter lifespan compared to patients with other alleles [28]. Furthermore, 12 *TNF* microsatellite haplotypes were associated with susceptibility to, and one with protection against, CD symptom development; some of these haplotypes contained alleles that were individually associated with the disease. These results indicate that the chromosomal region encompassing the TNF locus plays an important role in CD development [29]. However, a more recent study involving 80 asymptomatic and 160 cardiopathic patients, who were matched by age and geographic area, analysed the same alleles with reference to the progression of CCC and grouped the cardiopathic patients according to the dysfunction of the left ventricle into severe CCC, moderate CCC, and without any cardiac dysfunction. In this case, neither *TNF* -308A nor *TNFa2* polymorphisms were associated with the development of heart disease or progression to severe forms [30]. Along the same lines, -22C and -348C *BAT1* gene variants are risk factors for the development of CCC in the Brazilian population [31], suggesting that these variants, proposed to result in less effective control of pro-inflammatory cytokine production (e.g. TNF-α and IL-6), might contribute to the high production of these cytokines in CCC patients. Furthermore, polymorphisms in lymphotoxin α, another TNF family member, predispose individuals to CCC [32]. Such studies suggest that genetically determinate immunological imbalances involving TNF family members and regulators might contribute to the outcome of the different clinical forms of CD, particularly to CCC severity. However, the differences in the experimental approaches do not allow a meta-analysis of the already published data that could define associations among the polymorphisms tested. Therefore, to clarify the apparent controversial participation of gene polymorphisms of the TNF family members, receptors, and regulators in CD pathogenesis, further appropriately designed studies are required.

TNF-α in *Trypanosoma cruzi* Immunity

Increase in the frequency of TNF-α-expressing and TNFR1 (p55/60)-bearing splenocytes was detected in C57BL/6 mice acutely infected with the low-virulence *T. cruzi* Colombian strain [18]. TNF-α production was previously shown to be enhanced in *T. cruzi*-infected mice in association with susceptibility to infection revealed by parasitaemia levels [33]. Several *T. cruzi*-derived molecules, including glycosylphosphatidylinositol-anchored mucins and DNA, have been shown to trigger the production of pro-inflammatory cytokines, including TNF-α involving TLR-mediated or MyD88-dependent pathways [34]. TNF-α synthesis can also be controlled by different intrinsic stimuli such as hormones, nitric oxide (NO), cytokines, and other inflammatory mediators [35]. As several of these molecules are

up-regulated in *T. cruzi* infection [36, 37], the molecular pathway leading to TNF-α overproduction in CD remains to be defined.

Based on in vitro experiments showing that recombinant TNF-α inhibited intracellular multiplication of *T. cruzi* in mouse peritoneal macrophages [38], the biotherapeutic potential of rTNF-α was tested in vivo, revealing aggravation of infection and accelerated mortality in a dose-dependent manner [39]. In fact, TNF-α has been implicated in cachexia occurring during the acute infection [40] and in splenic necrotic changes associated with severe *T. cruzi* infection [41]. In this context, we recently demonstrated that the TNF/TNFR1 pathway plays a role in macrophage hyperactivation in *T. cruzi* infection, up-regulating TLR4 expression and sensitising the infected animals to LPS-induced shock [42]. On the other hand, action on TNFR1 is required for specific IgG, but not IgM, antibody production during *T. cruzi* infection [43]. Furthermore, signalling via TNFR1, but not TNFR2 (p75/80), plays a crucial role in *T. cruzi* growth control during experimental acute infection. Signalling via TNFR1 controls parasite uptake, NO and chemokine production, as well as cell mobilisation to sites of infection [15]. In fact, previous studies adopting sTNFR1-FcIgG3 transgenic and TNFR1$^{-/-}$ mice revealed that TNFR1 signalling is required for parasite burden control during *T. cruzi* acute infection [44]. TNFR1$^{-/-}$ mice, which are highly susceptible to *T. cruzi* infection, survived the acute infection when treated on days 10–17 post-infection with a subcurative dose of the trypanocide drug Benznidazole; subsequently, they exhibited reduced myocarditis during the chronic phase. This was in contrast to IFN-γ-deficient mice, which succumbed shortly after treatment cessation with intense parasitaemia, parasitism, and inflammation [18, 45]. Importantly, TNFR1 is crucial for the development of *T. cruzi* splenomegaly [18], which has been linked to aberrant B and T cell activation [46], especially CD8$^+$ T cell dysregulation [13, 47]. CD8-enriched *T. cruzi*-elicited myocarditis is dependent on the differential activation of CD8$^+$ T cells after infection [13]. Interestingly, reduction in myocarditis without parasite burden was associated with a depletion in CD8$^+$ T cells in *T. cruzi*-infected TNFR1$^{-/-}$ mice, compared to C57BL/6 [18], corroborating prior data showing decreased inflammation in skeletal muscles of TNFR1-deficient mice [43]. Notably, TNFR1 signalling is required for up-regulation of the cell adhesion molecule ICAM-1, which contributes to myocarditis [48], in heart endothelial cells during *T. cruzi* infection [20]. Altogether, the above discussed data support the idea that, although important, TNF/TNFR1 signalling is not essential for the control of parasite dissemination. Nevertheless, TNF-α might have detrimental effects, contributing to immunological abnormalities and the development of inflammatory processes during *T. cruzi* infection.

Beneficial Effects of TNF-α Blockade in *Trypanosoma cruzi* Infection

Cytokines produced in the heart tissue of *T. cruzi*-infected individuals during the initial immune response might influence subsequent immune reactions and

clinical outcome in CD. TNF-α is detectable in inflamed hearts of CCC patients [16] and is produced by T cells derived from endomyocardial biopsies [17], suggesting that TNF-α might be involved in the maintenance of chronic CD8-enriched myocarditis. Considering TNF-α sources in the cardiac tissue, this cytokine is also produced by macrophages and cardiomyocytes, [49, 50] which, in an autocrine or paracrine manner, might stimulate infected cells to release NO and control *T. cruzi* growth [51]. In addition, TNF-α might also stimulate heart-infiltrating macrophages or cardiomyocytes to produce CC-chemokines, such as CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES [15, 51], contributing to inflammatory cell recruitment. Furthermore, in experimentally induced acute and chronic *T. cruzi* infections, the intensity of the inflammation is directly related to CCL3, CCL4, and CCL5 status of the cardiac tissue, occurring in a TNF-α-enriched milieu [52, 53]. Up-regulation of CCR5, a CC-chemokine receptor, particularly by $CD8^+$ T cells, occurs during *T. cruzi* infection and is implicated in the predominance of $CD8^+CCR5^+$ cells in the heart tissue of *T. cruzi*-infected animals [52, 54]. Increased CCR5 expression by $CD8^+$ T cells was detected after in vitro stimulation with *T. cruzi* antigens [54], demonstrating a central role of the parasite in CCR5 modulation. In this context, we have recently demonstrated that signalling via TNFR1 is crucial for $CD8^+CCR5^+$ differentiation, $CD8^+CD44^+CD62L^{-/low}$ compartmentalisation, and $CD8^+$ T cell-enriched myocarditis during *T. cruzi* infection [18]. Furthermore, anti-TNF-α antibody (Infliximab, Remicade®) treatment of *T. cruzi*-infected mice resulted in decreased frequency of $CD8^+CCR5^+$ T cells in the peripheral blood and spleen and was associated with reduced heart inflammation, fibronectin deposition and cardiomyocyte damage [18]. Thus, interfering with the biological effects of TNF-α using Infliximab, an antibody that blocks soluble and membrane-bound TNF-α [50, 55], led to CCR5 down-modulation and decreased heart inflammation, without affecting parasite control [18]. Thus, it seems that the TNF/TNFR1 signalling pathway plays a crucial role in inflammatory cell extravasation into cardiac tissue by controlling the expression of CCR5, a molecule involved in the establishment of *T. cruzi*-elicited acute and chronic heart tissue inflammation and damage [52, 53]. Additionally, short treatment with Infliximab diminished chronic myocarditis in *T. cruzi*-infected rats, without evidence of parasite reactivation [56]. Those data corroborated our recent data indicating that Infliximab administration to chronically infected mice does not disrupt specific IFN-γ production by $CD8^+$ T cells (Vinagre et al., in preparation). Interestingly, in Verapamil-treated *T. cruzi*-infected mice, in situ modulation of TNF-α and IL-1β correlated with reductions in heart fibrosis and inflammation [57]. Recently, TNF-α down-modulation through pentoxifylline decreased necrotic areas in the spleen during acute *T. cruzi* infection [58]. Additionally, pentoxifylline administration during the chronic *T. cruzi* infection partially reversed the cardiac electric abnormalities, preserving IFN-γ production by specific $CD8^+$ T cells and avoiding infection reactivation (Pereira et al., in preparation). However, the participation of TNF-α in this process can be challenged [50] and remains to be proved. Altogether, these findings reinforce the hypothesis that TNF-α blockade might exert a beneficial effect during *T. cruzi* infection by modulating heart fibrosis or fibrogenic inflammation.

Conversely, treatment with Etanercept (soluble human TNFR2/p75 that binds TNF-α and lymphotoxin α) aggravated CCC in hamsters, suggesting that the absence of TNF signalling might be deleterious to the failing heart in CD-related cardiomyopathy [59]. This apparent paradoxical effect of TNF-α blockade in *T. cruzi* infection emphasises the need for a greater understanding of the modes of action and the limiting factors of emerging novel therapeutic tools that target TNF [56]. TNF-α is involved in cytoprotective signals that prevent and/or delay the development of cardiomyocyte apoptosis [60]. However, it is clear that prolonged exposure to high levels of TNF-α has deleterious effects on cardiac function [61]. In fact, chronic chagasic patients with left ventricular dysfunction had twofold higher TNF-α levels than patients without ventricular dysfunction [21]. In addition, cumulative and complementary effects of TNF-α and NO levels correlate with adverse prognosis in chagasic patients [22], suggesting that susceptibility to severe chagasic chronic cardiomyopathy is a multi-factorial process in which TNF-α plays a protagonistic role.

Conclusions and Future Avenues

The literature suggests that genetic heterogeneity in infectious disease susceptibility relies on diverse genes that mediate immune response. In view of this, the study of

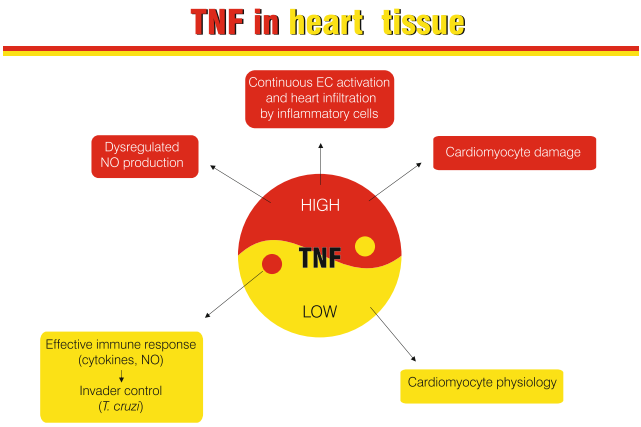

Fig. 23.1 Hypothetical mode of action of TNF-α in the cardiac environment under physiological (low TNF-α concentration – *yellow*) and pathological (high TNF-α concentration – *red*) conditions. In the physiological condition, TNF-α is produced by cardiomyocytes and cardiac macrophages [49, 50, 60]. During acute infection with *T. cruzi*, a localised and transient increase in TNF-α production by cardiomyocytes or infiltrating inflammatory cells might lead to cytokine and NO release, and parasitic control, resulting in restoration of the physiological status. However, if the invader persists and/or TNF-α production is dysregulated, high local TNF-α concentration might lead to cardiomyocyte damage, dysregulation of NO production [22], persistent endothelial cell (EC) activation, and heart infiltration by inflammatory cells (T cells and macrophages), perpetuating chronic inflammation and, consequently, cardiomyopathy [18, 61]

additional and functionally relevant SNPs in TNF family members and TNF regulator genes might shed light on the role of TNF-α in CD susceptibility and clinical outcomes. Moreover, the biological roles played by TNF-α (and other TNF family members such as lymphotoxin α) in *T. cruzi*-elicited cardiomyopathy deserve to be further explored for the development of rational therapeutic strategies, such as immunoregulators associated with trypanocidal drugs. In this context, our results suggest that the detrimental effects of TNF-α in *T. cruzi* infection might be determined by the level and duration of TNF-α production. Therefore, modulation (but not complete abrogation) of TNF-α production or its biological action might keep parasite dissemination under control, while impairing the development of harmful myocarditis and cardiomyocyte lesions during *T. cruzi* infection (see Fig. 23.1). Certainly, many details must be elucidated before the participation of TNF-α in chronic chagasic cardiomyopathy is fully understood. Once this is accomplished, accessibility to novel therapeutic strategies, including adoption of immune reagents such as antagonists and blocking antibodies, will hopefully be made available to all patients, even those individuals afflicted by poverty and neglected diseases.

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Chapter 24
Lymphotoxin's Link to Carcinogenesis: Friend or Foe? From Lymphoid Neogenesis to Hepatocellular Carcinoma and Prostate Cancer

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Lymphotoxin: Past and Present

It was only in the late 1960s that lymphotoxin (LT) was described and denoted as a "cytotoxic factor." Isolated lymph node cells from rats showed cytotoxic effects on syngeneic or allogeneic fibroblasts in the presence of specific antigens [84, 85]. Besides, in vitro stimulation of murine lymphocytes led to secretion of these earlier described cytotoxic factors into the culture medium, capable of killing various cell types (e.g., *L* cells) [37, 38]. LT was later isolated from stimulated lymphocytes and characterized in various species including mouse, guinea pig, and human [51, 95] and defined as a "lymphokine" being a non-antibody mediator of cellular immunity generated by lymphocyte activation [36]. In 1984, after purification of the proteins to homogeneity, determination of the amino acid sequence and cloning of the cDNA were accomplished [1, 2, 39]. These studies revealed LT's close relationship to TNFα, leading to a renaming of LT into TNFβ. Later, TNFβ was called LTα or TNFSF1B (for TNFSF member 1B), and subsequently LTβ (TNFSF3) was described. Of note, besides their biochemical similarities LTα, LTβ, and TNF (TNFSF1A) are found in close proximity on genomic level, within the major histocompatibility complex region on mouse chromosome 17 (humans: chromosome 6) [71].

From Toxicity to Function

Under physiological conditions LTα and LTβ are expressed by activated T, B, NK, and lymphoid tissue inducer cells. Like most of the TNF superfamily ligands, LTβ is a type II transmembrane protein (intracellular N-terminus), whereas LTα is soluble, but can be recruited to the cell membrane to form heterotrimeric complexes

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with membrane-bound LTβ. Consequently, LT can also exist as membrane-bound heterotrimers ($LT\alpha_1\beta_2$ or $LT\alpha_2\beta_1$). As secreted homotrimeric molecules, LT ($LT\alpha_3$) signals via TNFR1 (p55), TNFR2 (p75), and herpes virus entry mediator (HVEM). As membrane-bound heterotrimers, LT ($LT\alpha_1\beta_2$ and $LT\alpha_2\beta_1$) is believed to mainly trigger lymphotoxin β receptor (LTβR) [94], mostly expressed on stromal and parenchymal cells. Activation of LTβR and TNFR1 can activate both the canonical and non-canonical NFκB-signaling pathway [94], leading to the regulation of a variety of downstream targets, involved in apoptosis, cell survival, cell proliferation, inflammation, and homeostasis [47]. With the exception of LTβ, all TNF superfamily ligands form non-covalent, self-assembling homotrimers (or multimers of higher number [9]) resulting from an approximately 20–30% amino acid homology in their interacting protein interfaces. The receptor specificity of the approximately 40 TNFSF members is guaranteed by little sequence similarity of their external surfaces. However, in the case of TNF and LTα, the homology of about 30% in their primary sequence leads to a conserved motif in tertiary and quaternary structure being sufficient for the binding of both molecules to the same receptors (TNFR1 and TNFR2).

Lymphotoxin, Lymphoid Neogenesis, and Lymphoid Microarchitecture

Evidence that LT is involved in the development of lymphatic tissues was first provided by generation of knockout mice. $LT\alpha^{-/-}$ mice (deficient in $LT\alpha_1\beta_2$, $LT\alpha_2\beta_1$ and $LT\alpha_3$) lack Peyer's patches (PPs) as well as peripheral and mesenteric lymph nodes (pLN; mLN) [23]. However, according to Fu, Banks, and colleagues a certain number of $LT\alpha^{-/-}$ mice develops mLN-like structures in 1–20% of all investigated mice. Further, $LT\alpha^{-/-}$ mice lack an organized splenic microarchitecture, germinal centers, follicular dendritic cell (FDC) networks, compartmentalization into T- and B-cell areas, display size reduction of white pulp follicles and are devoid of a distinct marginal zone [23, 76].

Generation of $LT\beta^{-/-}$ mice elucidated the distinct roles of LTα and LTβ in lymphoid organogenesis [52]: $LT\beta^{-/-}$ mice (devoid of LTβ, $LT\alpha_2\beta_1$, $LT\alpha_1\beta_2$) continue to express $LT\alpha_3$ and exhibit a phenotype similar to $LT\alpha^{-/-}$ mice – except that cervical lymph nodes (cLNs) and mLNs are retained and that the splenic microarchitecture is somewhat less disturbed [73]. These data also suggested that LTα can function independently from LTβ during the development of lymphoid organs.

Generation of $LT\beta R^{-/-}$ mice helped to define the role of LTβR in the formation of secondary and tertiary lymphoid tissues and microarchitectural organization and elucidated whether these processes require homotrimeric or heterotrimeric LT [32]. $LT\beta R^{-/-}$ mice lack mLNs, cLNs, inguinal LNs, as well as PPs. At the putative LN locations cavernous spaces with endothelial linings could be found, indicating that LN development is completely arrested [32]. Spleen sections of $LT\beta R^{-/-}$ mice show marked alterations in splenic microarchitecture, lack of a marginal zone,

germinal centers, FDCs, and organized follicles. Thus, it can be concluded that LTβR signaling is crucial for the organogenesis of the peripheral LN system and appears to be essential for the correct development of the gut-associated lymphatic tissues.

Notably, similar to $LT\alpha^{-/-}$ mice, $LT\beta R^{-/-}$ mice show inflammation of the liver, lung, pancreas, and kidney mainly composed of activated lymphocytes. It is believed that this possibly reflects a state of autoimmunity or an in vivo situation in which immune cells have started to colonize new niches in non-lymphoid organs due to the lack of various secondary lymphoid organs [16, 32, 69, 76].

The generation of LTβR-Ig, a fusion decoy protein which enables a functional inhibition of LTβR signaling, helped to study the role of surface LTαβ complexes in the development of peripheral lymphoid organs. By varying the gestational day of LTβR-Ig injection, the genesis of LNs and PPs appeared to proceed in an anterior-to-posterior order (mesenterics, brachials, axillaries, inguinals, and poplietals, PPs) [82]. Postgestational LTβR signaling during the first 6 weeks after birth is shown to be critical for the development of intestinal lamina propria B cells and isolated lymphoid follicles of the intestine [59].

As TNFα, $LT\alpha_3$ binds to TNFR1 and TNFR2, both of which show common but also distinct activities depending on the cell type in which they operate. TNFR2 signaling has been mainly implicated in lymphocyte proliferation, whereas TNFR1 is generally known to mediate apoptosis and induction of the canonical NFκB pathway [26, 75, 76]. $TNFR1^{-/-}$ or $TNFR2^{-/-}$ mice had not been recognized to manifest defects in lymphoid tissue structure [26, 76] and primary and secondary lymphoid tissues were phenotypically indistinguishable from wild-type littermates. However, mature FDC networks and germinal centers were undetectable in $TNFR1^{-/-}$ but present in $TNFR2^{-/-}$ spleens [27]. In fact, LNs appear to form with normal distribution even in mice with targeted ablation of both TNFR1 and TNFR2 [30]. This suggested that disturbed LN and PPs formation in $LT\alpha^{-/-}$ mice was not mediated by ablation of signals through the homotrimeric ligand TNFR1/TNFR2 arm, but might rather represent failure of signaling through the LTβR arm [30].

$TNF\alpha^{-/-}$ mice were independently generated by several groups. Not all reported phenotypes were consistent with each other. In contrast to $LT\alpha^{-/-}$ and $TNFR1^{-/-}$ mice, LNs and PPs could readily be detected in one TNF knockout mouse line, suggesting that TNF is not necessary for lymphoid organogenesis [75]. However, data published by Kuprash and colleagues [55] support the role of the TNFα/TNFR1 pathway in PPs development, since their knockout line does not exhibit LT-specific defects (e.g., lack of lymph nodes or marginal zone), but nevertheless completely lacks PPs.

Lymphotoxin and Inflammation

A major role of LT is implied in inflammation and generation of tertiary lymphoid organs (TLOs), which are sites of lymphoid neogenesis induced by various stimuli (e.g., infection) and which are not restricted to specific developmental

windows or anatomic locations. For the induction of TLOs LT activates endothelial cells for the expression of adhesion molecules (e.g., V-CAM, I-CAM) [21, 22] and regulates and induces expression of homeostatic and a variety of pro- and anti-inflammatory chemokines (e.g., CXCL13, CCL19, CCL21, CCL5, CCL2, CXCL10) [5, 10, 24]. TLOs or lymphoid neogenesis can also be found in various human autoimmune diseases (e.g., rheumatoid arthritis, Sjögren's syndrome, myasthenia gravis, Graves' disease, chronic obstructive pulmonary disease, Hashimoto thyroiditis, ulcerative colitis). These TLOs also display LT upregulation, expression of chemokines, and adhesion molecules (e.g., CXCL13, CCL21; PNAd) and show cells or structures known to depend on LT signaling (e.g., FDCs; high endothelial venules) [10, 24]. Further, LT upregulation and TLOs can also be found in pathogen-induced chronic inflammation (e.g., hepatitis B and C virus-induced chronic hepatitis, *Helicobacter pylori*-induced gastric inflammation, *Borrelia burgdorferi*-induced lyme disease) [24, 43].

LT's effects to induce cell adhesion and chemokine expression have not only be examined in vitro [21, 22] but several transgenic mouse models were generated to investigate the role of TNFSF members and its target genes in the context of lymphoid neogenesis, development of autoimmune diseases and inflammatory disorders. Transgenic expression of LTα or LTαβ induced chronic insulitis and glomerulonephritis, when driven ectopically under the control of the rat insulin promoter II (*RIP*; active in pancreatic islets of Langerhans, the proximal tubuli of the kidney and in skin) [25, 46, 53, 77]. RIPLT transgenic mice display perivascular accumulation of mononuclear cells ($CD4^+$, $CD8^+$ T cells, $B220^+$ B cells, $F4/80^+$ macrophages, $CD11c^+$ dendritic cells), FDC networks, and germinal centers in kidney and pancreas [46, 53, 77]. Although insulitis was found in the case of many but not all islets, RIPLTα mice did not progress to diabetes until the age of 8–12 months [78]. Notably, in RIPLTα kidneys pathological changes were observed, including mesangial proliferation and mesangiolysis within the glomeruli [25, 46, 53, 77].

Infiltrates in both RIPLT transgenic kidneys and pancreata contain specialized blood vessels called high endothelial venules (HEVs) [25]. HEVs are postcapillary vessels of secondary lymphoid organs composed of endothelial cells with a characteristic cuboidal morphology. Lymphocytes selectively adhere to and migrate across HEVs to initiate immune responses [48, 49]. In addition, HEVs were also observed in chronically inflamed non-lymphoid tissues [24] and a role of LTβR signaling as a requirement for the homeostatic control of HEV differentiation and function was described [11].

The fact that lymphoid neogenesis and TLO formation are induced not only by ectopic expression of TNF/LT family members but also by chemokines such as CCL21, CCL19, CXCL13 is very intriguing [14, 28, 63, 64, 68].

Although, inflammation-induced tissue destruction was detected in several of these models over time (e.g., kidney of RIPLTα mice [46]), the effects were rather mild, when compared to the strong degree of inflammation. Recently, we generated mice expressing LTα and LTβ under the control of the distal leukocyte-specific protein tyrosine kinase (lck) promoter, resulting in LT expression on developing

thymocytes within the thymus and on peripheral T cells in various lymphoid organs [45]. In this case transgene expression on thymocytes led to the activation of thymocytes, accelerated thymic involution, aberrant T cell development, and an altered thymic microarchitecture. Thymic involution, caused by massive apoptosis of thymic epithelial cells, was rescued by the removal of either TNFR1 or LTβR in the stromal compartment, but not by depletion of overall TNFR2, LTα, or LTβ expression. Therefore, ectopic thymic LTαβ expression induces toxicity on thymic stromal cells, which is mediated by two non-redundant pathways: $LT\alpha_3$ signaling through TNFR1 and $LT\alpha_1\beta_2$ signaling through LTβR.

LTβR Signaling in Health and Disease

Pleiotropic functions are attributed to LT – in health and disease [90] – which in some instances can go beyond the well-documented role of LT in the development and maintenance of lymphoid organs. This includes, e.g., an important role of LT signaling in the acceptance of donor stem cells, controlling cuprizone-induced demyelination, the progress of experimental autoimmune encephalomyelitis (EAE), the control of lipid metabolism or liver regeneration [34, 58, 67, 80, 91].

If absent or deregulated, LTβR signaling can lead to unexpected deficiencies or can even become injurious. As mentioned above deregulation of LTα and LTβ expression and aberrant LTβR signaling can be found in various human autoimmune disorders as well as chronic inflammatory disorders induced by pathogens [10, 24, 33]. In line, studies with different mouse models revealed an important role of LT signaling in the development of atherosclerosis [35, 88] and a polymorphism in the LTα gene was found to be involved in the susceptibility toward myocardial infarction and the extent of myocardial atherosclerosis in humans [56]. Also for the pathogenesis of other autoimmune diseases, such as rheumatoid arthritis (RA), an involvement of LTβR signaling was discovered [29]. Blocking of LTβR signaling by using a LTβR–Ig fusion protein could prevent the onset of collagen-induced arthritis (CIA) or reduce its severity depending on the time point of LTβR–Ig treatment [29]. Based on these results, clinical trials on patients suffering from RA have been initiated by Biogen IDEC.

Recently, targeted depletion of $LT\alpha^{hi}T_H1$ and T_H17 cells with a monoclonal antibody against LTα could inhibit autoimmune disease in mouse models for delayed-type hypersensitivity, EAE as well as CIA, reflecting a correlation between $LT\alpha^{hi}T_H1$ and T_H17 cells and disease progression in the respective models [15]. The above data indicate that not only suppression of LTβR signaling itself but also elimination of cells expressing high levels of LT can be beneficial.

NFκB, Chronic Inflammation, and Cancer

Only recently, inflammation was added as the seventh hallmark to carcinogenesis [17], being generally accepted to be on eye level with self-sufficiency in growth

signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evasion from apoptosis, as defined by Hanahan and Weinberg [42]. The concept of a potential link between cancer and inflammation exists since the second half of the nineteenth century when Virchow and others made the observations that tumors often arise at sites of chronic inflammation. Today, we know that inflammatory cells are very often present in tumor biopsies [7] and we just start to understand the cellular and molecular inflammatory mediators (e.g., immune cells; cytokines) driving carcinogenesis and why in some instances immune cells can be pro- or anti-carcinogenic.

It became more and more obvious that chronic inflammation can provide a microenvironment consisting of various mediators such as cytokines, chemokines, and prostaglandins, possessing tumor-promoting effects by enhancing cell proliferation, cell survival, angiogenesis, and tumor cell migration [66]. In line, it was shown that tumors can contain immune cells, secreting, e.g., TNFα, IL1β, IFNγ, IL6, IL8, ROS, growth factors, and matrix-degrading enzymes which unexpectedly can promote DNA damage, cell proliferation leading to pre-neoplastic lesions or cancer [19, 20, 86].

One key pathway integrating these pro-inflammatory signals is activated by the transcription factor NFκB, whose role is well described in inflammatory and innate immune responses [93]. Two different effector pathways have been described: the canonical NFκB pathway, which is usually triggered in response to microbial and viral infections as well as pro-inflammatory cytokines. This leads to activation of the IKK (α, β, γ) complex and degradation of IκB inhibitors enabling the translocation of transcription factors RelA, cRel, and p50 [47]. The "alternative pathway", which represents the second described NFκB pathway is independent of IKKβ but depends on the NFκB-inducing kinase (NIK) and IKKα [87]. Phosphorylation of IKKα by NIK results in the phosphorylation and processing of p100 leading to nuclear translocation of RelB/p52 dimers. Both, the canonical and the non-canonical pathways lead to the transcription of target genes being involved in the mediation of various biological functions [47].

Despite the well-characterized involvement of NFκB signaling in immunity and inflammation [93], the role of this signaling in cancer development and progression is only bit by bit revealed. Only recently, two independent studies showed NFκB-dependent activation as a molecular link between inflammation and cancer. Using a colitis-associated cancer model, Greten and colleagues show that although the absence of IKKβ in intestinal epithelial cells does not decrease inflammation it leads to a dramatic decrease in tumor incidence without affecting tumor size [40, 41]. Moreover, NFκB signaling was shown to drive the development of hepatocellular carcinoma in mdr2$^{-/-}$ mice [79]. In line, in another study constitutive NFκB activity was found in diffuse large B-cell lymphoma indicating a role for this signaling pathway as a primary pathogenic event in lymphomagenesis [18].

On the other hand, a controversial role of NFκB signaling was described in mice lacking IKKβ specifically in hepatocytes [65], which display an increased HCC formation after DEN application, suggesting a protective, anti-carcinogenic function of canonical NFκB signaling under the conditions of chemically-induced

carcinogenesis. Interestingly, mice lacking IKKγ (also called NEMO) specifically in hepatocytes develop steatohepatitis and HCC [61]. Therefore, the role of NFκB signaling in liver cancer development most likely depends on the mouse model and the type or degree of liver inflammation and injury [92].

Lymphotoxin and Its Link to Cancer

As if the biological functions of LT are not multifaceted enough, only recently various studies were published suggesting LTβR signaling to be involved in cancer development. In a first study, LTβR activation on tumor cells by activated host lymphocytes was described to be capable of initiating a novel pro-angiogenic pathway leading to organized tumor tissue development [44]. Lukashev and colleagues [62] demonstrated that agonistic anti-LTβR monoclonal antibody (mAb) CBE11 inhibits tumor growth in xenograft models and potentiated tumor responses to chemotherapeutic agents. Further, within this study the authors demonstrated in a syngeneic colon carcinoma tumor model that treatment of tumor-bearing mice with a murine agonistic antibody against LTβR caused increased lymphocyte infiltration and tumor necrosis. Finally, CBE11 treatment decreased tumor size and/or improved long-term animal survival in two out of six independent orthotopic xenografts derived from surgical colorectal carcinoma samples. Therefore, targeting LTβR with agonistic mAbs might offer a novel approach for treating a significant percentage of colorectal and potentially other cancer types [62].

Furthermore, an activating mutation in the LTβR gene was described to result primarily in constitutive activation of the non-canonical NFκB pathway, which is involved in the pathogenesis of multiple myeloma [50]. In a very recent study on nasopharyngeal carcinoma (NPC), a distinct type of head and neck cancer, LTβR was demonstrated to be frequently overexpressed in primary NPC tumors, concomitant with an amplification of chromosome 12p13.3 [74]. In vitro studies in nasopharyngeal epithelial cells revealed increased NFκB activity and cell proliferation due to LTβR overexpression. Addition of $LT\alpha_1\beta_2$ led to an increased cell proliferation of nasopharyngeal epithelial cells and siRNA knockdown of LTβR expression showed a growth inhibition of the NPC tumors with 12p13.3 amplification [74], further strengthening a potential oncogenic role of LTβR signaling in this cancer type.

A further link between LT and cancer but in the context of a different mechanism was recently proposed by Zhou and colleagues [96]. The researchers investigated a non-antigen-based cancer immune prevention strategy using the transgenic adenocarcinoma of the mouse prostate model (TRAMP) which expresses the SV40 T antigen (Tag) controlled by rat probasin regulatory elements and which spontaneously develops prostate cancer with 100% penetrance. Targeted mutation of the LTα gene prevented efficient negative selection of tumor-reactive T cells, reduced cancer incidence by approximately 50%, significantly reduced the size of the investigated prostate cancers and almost completely ablated metastases. Interestingly,

treatment with LTβR–Ig interrupted clonal T-cell deletion, reduced the size of the primary cancer, and completely prevented further metastasis, demonstrating the value of non-antigen-based immune prevention for individuals with genetic predisposition to cancer [96]. These data indicate that in contrast to the above-described mechanisms, blocking of LTβR signaling could also be involved in supporting a tumor-reactive T-cell response. Notably, LT's involvement in cancer is not universal to all cancer types or signaling pathways leading to cancer. In a very instructive study Kuprash and colleagues [54] showed that neither lack of TNF nor LTα was shown to reduce tumor burden in the background of p53 deficiency.

LTβR Signaling in the Liver

In the liver LT has been demonstrated to directly act on hepatocytes which physiologically express high levels of LTβR but little LT [12, 43]. T-cell-derived LT and LIGHT signaling to hepatocytes was demonstrated to control lipoprotein homeostasis [58]. In addition, LTβR signaling was shown to be important for liver regeneration through T-cell-derived LT expression [91] and Ruddell and colleagues [83] demonstrated that LTβR signaling regulates hepatic stellate cell function and wound healing. Thus, hepatic LTβR signaling controls liver homeostasis, in both health and disease.

It has recently been demonstrated that pharmacological inhibition of LTβR signaling reduces virus-, bacteria- and concavalin A-induced liver injury [3, 4, 81]. An involvement of LTβR signaling in the host response to liver infection was partially studied in the case of hepatitis B (HBV) and hepatitis C viruses (HCV) in vivo and in vitro. Increased LTβ expression was found in livers from HCV-infected patients especially not only in liver progenitor (oval) cells but also in small portal hepatocytes as well as immune cells [60]. Besides, LTα was shown to be upregulated as a consequence of hepatitis B virus X protein expression in liver cell lines [57] and siRNA knockdown of various components of the LTβR signaling pathway (e.g., LTβ; Rel A) was shown to interfere with HCV replication in vitro [72]. Indeed, several reports point toward an interaction of the HCV core protein with the LTβR, leading to the modulation of the LTβR signaling pathway [13, 70, 97].

Hepatic LTβR Signaling and Hepatocellular Carcinoma

More recently our group discovered a causal involvement of LTβR signaling in the development of chronic hepatitis and hepatocellular carcinoma (HCC). Patients suffering from HBV- or HCV-induced chronic hepatitis or HCC displayed a strongly increased hepatic expression of LTα, LTβ, and LTβR [43]. Moreover, TNFR1 expression was drastically upregulated, whereas TNF was found to be only slightly elevated in Virus-induced hepatitis on mRNA level. This strong upregulation of components of the LTβR signaling pathway was independent of the degree of

liver inflammation, fibrosis, patient age, gender, type of virus infection, or HCV genotype. Surprisingly, various other liver diseases with non-viral etiology, such as alcoholic steatohepatitis, cholestasis, primary biliary cirrhosis, steatosis, focal nodular hyperplasia, Wilsons's disease, and other liver disorders, showed significantly lower levels of LTα, LTβ, and LTβR mRNA expression [43]. This was irrespective of whether these diseases are linked to inflammation or not. However, non-viral liver diseases showed significantly higher expression levels of TNF. Notably, upregulation of LTβR, LTα, and LTβ transcripts was also detected in non-virus-related HCC, which could stem from activated, tumor-infiltrating lymphocytes and/or from neoplastic hepatocytes that have upregulated LT, possibly in response to IL6. Indeed, it was demonstrated that HCC-derived cell lines express IL6 [6] leading to increased LT levels [89].

But which cell type is responsible for the elevated expression of LT in chronic HCV-infected patients? As mentioned above, under physiological conditions LT is mainly produced by lymphocytes. However, infection of Huh-7.5 cells with fully infectious, cell culture-derived HCV also leads to elevated levels of LTα, LTβ, LIGHT, and LTβR transcripts 48 and 72 h post-infection. Therefore, we speculated that LT expression in HCV-infected livers could stem from lymphocytes as well as hepatocytes. Thus, to identify the cellular source of LTα, LTβ, and LTβR mRNA expression in human HCV-infected livers, cells were collected from HCV-induced hepatitis and HCC. Liver cells were sorted according to their CD45 surface expression, resulting in CD45-enriched (T, B cells; monocytes, macrophages/Kupffer cells; dendritic and NK cells) or CD45-depleted (hepatocytes, oval cells, bile duct epithelial and endothelial cells) fractions. As expected, $CD45^+$ lymphocytes showed elevated expression of LTα and LTβ, in both HCV-induced hepatitis and HCC. However, also the $CD45^-$ cell fraction, showed an elevation in LTα, LTβ, and LTβR mRNA expression in liver tissues from HCV-induced hepatitis and HCC when compared to healthy liver controls. These results could be corroborated by immunohistochemical analysis for LTβ.

To determine whether sustained hepatic LTβR signaling is causally linked to chronic hepatitis and liver cancer development, we analyzed two transgenic mouse lines that express LTα and LTβ in a liver-specific manner (*tg*1223 mice further denoted as AlbLTαβ mice). At an age of 3 months, no macroscopic differences between AlbLTαβ transgenic mice and transgene-negative littermates were visible; however, on mRNA level, expression of various transcripts including chemokines, genes involved in early growth response, cholesterol metabolism, and immediate early response (e.g., *c-Fos, Jun-b, Socs-3*) was significantly elevated. In contrast, genes involved in cell cycle control, histone modifications, and cell metabolism were significantly downregulated and were already significantly altered (Fig. 24.1a). From an age of approximately 6 months on, transgenic mice developed hepatitis characterized by infiltrating B and T cells, macrophages, hepatocyte and oval cell proliferation (Fig. 24.1b). This led to liver damage as documented by elevated serum transaminase (ALT/AST) levels as well as enhanced production of cytokines/chemokines such as IL6, IL1β, IFNγ, and TNF. The incidence of chronic hepatitis reached 100% in 9-month-old mice, with infiltrates progressing in size over

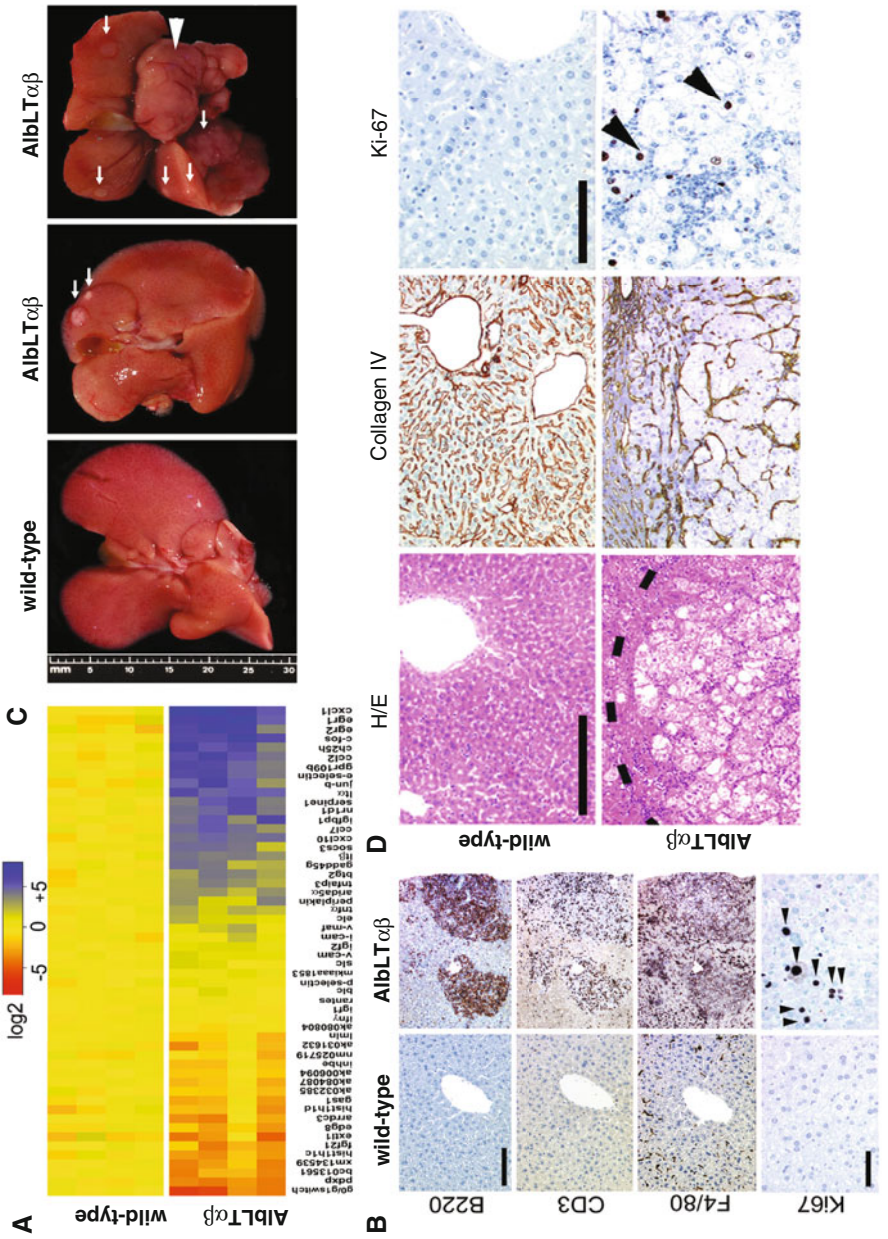

Fig. 24.1 (continued)

time. Remarkably, in approximately one-third of the mice at an age of ≥12 months the HCC development can be detected (Fig. 24.1c, d). Tumors in this mouse model are characterized by the expression of gp73 (GOLPH2), alpha-fetoprotein (AFP), and glutamine synthetase. Furthermore, aCGH analysis revealed that HCC derived from independent mice display different patterns of chromosomal aberrations. HCC within one and the same mouse were monoclonal, suggesting intrahepatic spread.

But is LT itself an oncogene that causes HCC development and is the triggering of LTβR signaling sufficient to cause inflammation-induced carcinogenesis?

To address these questions, AlbLTαβ mice were intercrossed with various knockout mice: (i) TNFR1$^{-/-}$ mice, to investigate whether in the absence of TNFR1 signaling would prevent inflammation-induced HCC formation in AlbLTαβ mice. (ii) RAG1$^{-/-}$ mice, which lack mature B and T cells, and (iii) to IKKβ$^{\Delta hep}$ mice, which are devoid of NFκB signaling specifically in hepatocytes, to investigate the role of lymphocytes and NFκB signaling in the development of inflammation-induced HCC formation. Surprisingly, AlbLTαβ/RAG1$^{-/-}$ or AlbLTαβ/IKKβ$^{\Delta hep}$ mice were devoid of chronic hepatitis or carcinogenesis, suggesting that lymphocytes and NFκB signaling – at least in this mouse model – are tumor promoting. Further, these results suggested that LT is not an oncogene but rather induces a pro-carcinogenic environment (Fig. 24.2). Backcrossing experiments to TNFR1$^{-/-}$ mice did not alter HCC incidence, suggesting that HCC formation in AlbLTαβ mice does not depend on pathways involving TNFR1.

We further sought to indentify the major LT-responsive liver cell type by using IKKβ$^{\Delta hep}$ mice. IV. administration of TNF into IKKβ$^{\Delta hep}$ mice did not cause p65 translocation in hepatocytes but upregulated NFκB target genes, presumably through TNF-activated NPC. In contrast, treatment with an agonistic LTβR antibody (3C8) in IKKβ$^{\Delta hep}$ mice neither induced nuclear p65 translocation in hepatocytes or NPC nor upregulation of selected NFκB target genes. Therefore, hepatocytes but

Fig. 24.1 Development of chronic inflammation and hepatocellular carcinoma in mice expressing LTα and LTβ under the control of the albumin promoter. (**a**) Real-time PCR analysis for mRNA expression in liver of candidate genes at the age of 3 months. Data are presented in a log 2 scale (*blue*: upregulated; *red*: downregulated). *Rows* indicate individual mice; *columns* represent particular genes. Each data point reflects the median expression of a particular gene resulting from three to four technical replicates, normalized to the mean expression value of the respective gene in C57BL/6 livers. (**b**) Immunohistochemical analysis of representative 9-month-old C57BL/6 and AlbLTαβ livers. B220^{+} stained B cells, CD3^{+} T cells, F4/80^{+} macrophages, Kupffer cells, and A6^{+} oval cells (scale bar: 150 μm). Ki67^{+} proliferating hepatocytes (*arrow heads*) and inflammatory cells are indicated (scale bar: 50 μm). (**c**) Macroscopy of C57BL/6 (*left panel*) and AlbLTαβ livers at the age of 12 (*middle panel*) and 18 months (*right panel*). *White arrows* indicate tumor nodules. *White arrowhead* indicates a liver lobe completely affected by HCC. Scale bar size is indicated. (**d**) Histological analysis of livers derived from C57BL/6 and AlbLTαβ mice. *Dashed line* depicts the HCC border. Collagen IV staining highlights the broadening of the liver cell cords and loss of collagen IV networks indicative of HCC in AlbLTαβ mice (scale bar: 200 μm). High numbers of Ki67^{+} proliferating hepatocytes (*arrow heads*) are only found in AlbLTαβ HCC (*right column*; scale bar: 100 μm). Adapted from Haybaeck et al. (2009)

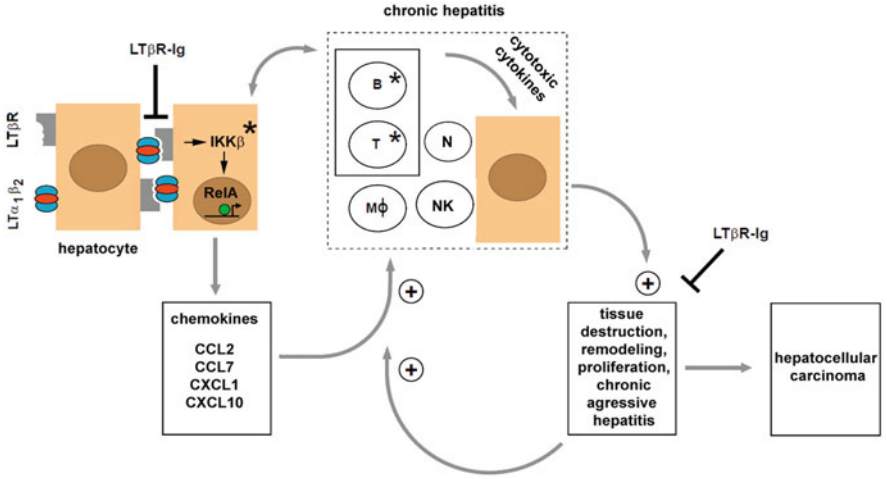

Fig. 24.2 Scheme of chronic inflammation-induced liver carcinogenesis in mice expressing LTα and LTβ under the control of the albumin promoter. Transgenic hepatocytes (*brown*) express LTα, LTβ and induce chemokine production (e.g., CCL2, CCL7, CXCL1, CXCL10) in the presence of IKKβ and intrahepatic lymphocytes. Chemoattraction and activation of myeloid cells and lymphocytes expressing particular chemokine receptors (e.g., CXCR3, CXCR2, CCR2, CCR1) cause hepatitis: CXCL10 attracts $CXCR3^+$ T and NK cells; CXCL1 attracts $CXCR2^+$ T, B cells, and neutrophils; CCL2 attracts $CCR2^+$ macrophages; and CCL7 attracts $CCR1^+$ monocytes. Activated, infiltrating immune cells secrete cytotoxic cytokines (e.g., IL6, IL1β, TNFα, IFNγ, LTαβ) that cause tissue destruction, hepatocyte proliferation, cell death, and tissue remodeling. In such an environment, hepatocytes are susceptible to chromosomal aberrations leading to HCC. Tissue destruction and remodeling supports the infiltration of activated inflammatory cells (e.g., myeloid cells) leading to a feed-forward loop toward chronic aggressive hepatitis. *Asterisks* indicate that genetic depletion of those components (IKKβ; T and B cells) blocks chronic hepatitis development and HCC. Blocking LTβR signaling with LTβR–Ig in 9-month-old AlbLTαβ mice reduces chronic hepatitis incidence and prevents HCC. **+**, indicates the fortification of a described process; ⊣, indicates the suppression of a described process. The transcription factor RelA is schematically depicted as a *green circle*, inducing transcription of NF-κB target genes (e.g., chemokines) (*arrow*). B, T, B and T cells; MØ, macrophages; N, neutrophils; NK, NK cells. Adapted from Haybaeck et al. (2009)

not NPC are likely to be the major liver cells integrating LT signaling. Additionally, upon 3C8 treatment, $IKK\alpha^{AA/AA}$ livers upregulated selected NFκB target genes similar to C57BL/6 mice. Therefore, the absence of IKKα in both, hepatocytes and NPC, still allows NFκB target gene expression upon 3C8 treatment, suggesting the involvement of the classical NFκB pathway in LTβR-induced hepatic signaling.

Treatment of 9-month-old AlbLTαβ mice suffering from chronic hepatitis with LTβR–Ig completely suppressed chronic hepatitis-driven HCC formation and significantly reduced the incidence of chronic hepatitis compared to untreated mice, suggesting that long-term suppression of LTβR signaling reduces the incidence of chronic hepatitis and the subsequent transition toward HCC (Fig. 24.2). Our results show that LT signaling is critically involved in hepatitis and subsequent HCC development. Blocking LTβR signaling might become a beneficial therapeutic

approach in the context of liver diseases displaying sustained hepatic LTβR signaling.

The above-described results convincingly demonstrate an participation of LTβR signaling in carcinogenesis, however, involving various mechanisms and biological functions in the diverse in vivo paradigms tested. It will be an important future task to investigate and reveal in detail the mechanisms underlying the different effects of induced LTβR signaling in the various cancer models (Fig. 24.3). What could be the reason(s) for the fact that increased LTβR signaling could in some instances generate a pro-carcinogenic environment [43] and in other paradigms lead to tumor reduction [62]? Apparently, this might have something to do with the tumor type investigated as well as the organ in which the tumor has developed. In this regard, it is also important to note that, e.g., transgenic mice, expressing LT in other organs than liver have so far not been reported to display inflammation-induced carcinogenesis, although most of these models show strong signs of inflammation. It is very possible that tumorigenesis develops with low frequency at late stage of life in these animals (similar to AlbLTαβ mice) and that this has escaped the attention of the experimentators. However, it is also possible that a highly regenerative, proliferative environment (e.g., like in liver) is needed to induce inflammation-induced carcinogenesis in the context of deregulated LTβR signaling.

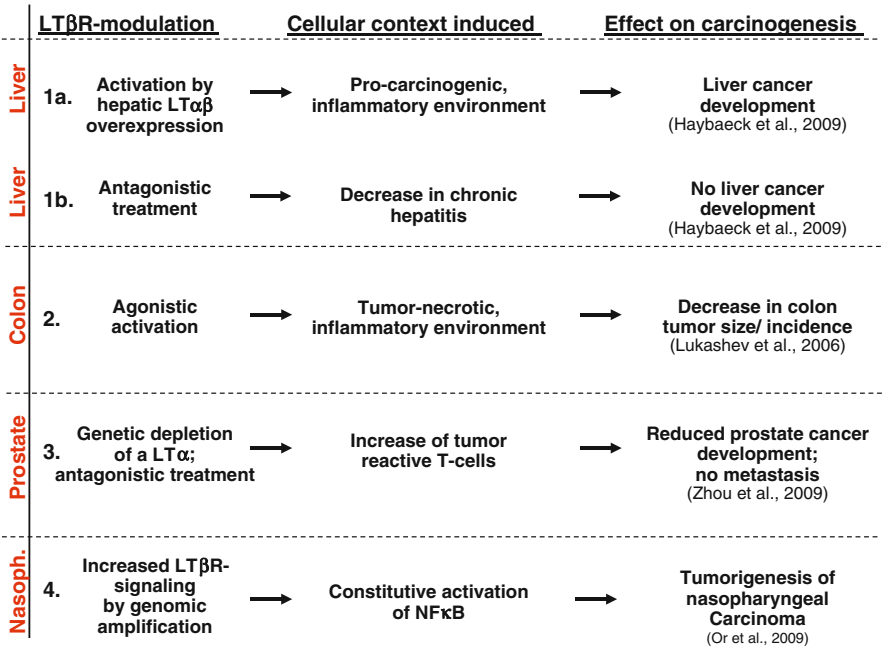

Fig. 24.3 Scheme summarizing the current paradigms of LT signaling in carcinogenesis. The various paradigms describing LT's involvement in carcinogenesis are summarized in this figure as published in [43, 62, 74, 96]. Nasoph, nasopharynx

On the other hand, data by Lukashev and colleagues [62] clearly indicate that in a syngeneic colon tumor model, agonistic LTβR antibody treatment induces lymphocyte infiltration and tumor necrosis. One proposed mechanism might be the induction of pro-inflammatory chemokines, functioning as chemoattractants for T, NK, and dendritic cells, contributing to tumor destruction.

Treatment with LTβR–Ig in a prostate cancer model (TRAMP) interrupted clonal T cell deletion, reduced the size of the primary cancer, and completely prevented further metastasis [96] – suggesting an important role of LT in controlling anti-tumor T cell selection.

Recent data of various laboratories indicate that there is still much more to be learned about the link between LT signaling and cancer, at the site of carcinogenesis as well as at the site where the host's adaptive immune response is triggered against cancer. Exciting times are ahead of us, verifying the utility of LTβR antagonists and agonists in the various different clinical settings.

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Part VI
Roles of the TNF Family in Infectious Diseases and Interrelationship of the TNF Family and Pattern Recognition Receptor Signaling: *Interrelationship of the TNF Family and Pattern Recognition Receptor Signaling*

Chapter 25
Anti-inflammatory Functions of Caspase-8

Akhil Rajput, Tae-Bong Kang, Konstantin Bogdanov, Jin-Chul Kim, Tehila Ben-Moshe, Andrew Kovalenko, and David Wallach

Involvement of Signaling Proteins Activated by TNF/NGF Family Members in Signaling Pathways Activated by Other Inducers

One of the most important contributions of the TNF field of study to biological research was the discovery of two new groups of signaling proteins: the TRAF family of ubiquitin ligases [1] and proteins containing the death fold, a group of evolutionarily related motifs of which the first to be discerned, the death domain, occurs in the intracellular domains of the death receptors and in adapter and signaling proteins that bind to them [2–6]. Knowledge gained through the study of these two groups of proteins has made pivotal contributions to our understanding of the mechanisms by which the TNF family exerts its numerous cellular effects. In addition, however, it has contributed much to our knowledge of other molecular processes. In the very first studies of the death domain it was already acknowledged that the same domain also exists in proteins whose function seems unrelated to that of the TNF family. Later studies revealing the existence of additional motifs of the death-fold group (the death effector, CARD, and Pyrin motifs) disclosed an even wider spectrum of proteins containing this fold, only some of which participate in the signaling activities induced by the TNF family. Likewise, studies of the TRAF domain, while disclosing the identity of a number of TRAF proteins that contribute in a variety of ways to signaling by the TNF family, also revealed the existence of one such protein (TRAF4), which seems not to be involved in this signaling at all [7]. Later studies revealed a whole family of protein motifs related to the motif that defines the TRAF family (the MATH domain), which occurs in many additional proteins, most of which have little to do with the function of the TNF family [8].

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The TRAF proteins that associate with receptors of the TNF/NGF family and participate in their signaling are now also known to mediate signaling by various other inducers, including the TIR domain proteins (receptors of the Toll-like receptor (TLR) and interleukin (IL)1 families, and adapters that bind to them), the RIG-I/MDA5 complex, and IRE1, an endoplasmic reticulum protein that serves as a sensor in the unfolded protein response [9]. The cellular functions to which TRAF proteins contribute in these cases are heterogeneous. However, most of these activities share a common denominator in that, as in the case of their functioning in response to the TNF family, those additional functions of the TRAFs serve mainly to mediate the response of cells to stress or pathogens. The mechanisms by which the TRAF proteins mediate these additional functions are also similar to those underlying their response to the TNF family. In both cases, they act either as ubiquitin ligases or as adapters that bind other ubiquitin ligases (such as the cIAPs) or protein kinases.

Knockout and transgenic studies of the two proximal death-fold-containing signaling proteins in the cell death pathway activated by the TNF family, the adapter protein FADD/MORT1 and the protease caspase-8, suggested that these proteins also serve functions distinct from those by which they were initially identified. Knockout of both resulted in the death of mice in utero in a manner that seemed independent of their roles in death induction or of any other function of the TNF family. Additional studies, both in transgenic mice and in cultured cells, further indicated that these proteins serve various functions unrelated to the signaling that they mediate in response to ligands of the TNF family [10]. However, in contrast to the relatively clear information about the cellular functions that TRAF proteins mediate in response to inducers other than members of the TNF family, and the detailed knowledge of the composition of signaling complexes and the molecular functions to which the TRAF proteins contribute in these cases, the exact nature of the additional functions of FADD/MORT1 and caspase-8 has not been clearly defined. It is evident that these functions are distinct from the induction of death by these proteins in response to receptors of the TNF/NGF family, and that they reflect involvement of these proteins in physiological functions of living cells. It is not clear, however, whether these functions involve enhancement of cell growth, protection from death, or regulation of differentiation. Moreover, information about the signaling complexes and mechanisms that mediate these effects of FADD and caspase-8 is also limited and in part controversial.

Inflammatory Processes Triggered by Caspase-8 Deficiency

To gain further insight into the identity of the various cellular functions to which caspase-8 contributes and the mechanisms by which it does so, we generated the following kinds of transgenic mice: (i) mice expressing a BAC transgene of the caspase-8 gene locus in which the active-site cysteine in caspase-8 was mutated

(BAC C362S); (ii) mice expressing a similar BAC transgene in which the aspartic acid residue upstream of the site of initiation of caspase-8 self-processing was mutated (BAC D387A); (iii) a series of mouse strains in which caspase-8 was conditionally deleted in specific tissues.

Mice that expressed the enzymatically inactive caspase-8 allele (BAC C362S) in addition to their two endogenous caspase-8 alleles appeared normal in all respects. However, when these mice were mated with mice possessing a knockout caspase-8 allele, those offspring that had only one wild-type allele in addition to their BAC C362S allele were found to be extremely sick. Soon after birth they developed inflammation in various internal organs as well as in the skin (Fig. 25.1 and [11]). The fact that the development of this inflammation depended on the ratio of the BAC C362S and wild-type caspase-8 alleles suggested that the pathology reflected a dominant-negative effect of enzymatically inactive caspase-8 on the function of the enzymatically competent wild-type molecules.

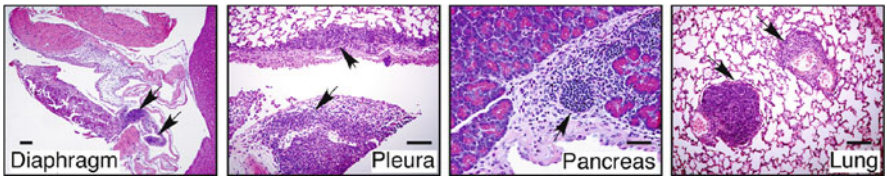

Fig. 25.1 Ubiquitous chronic inflammation in mice expressing an enzymatically inactive *caspase-8* transgene. Tissue specimens of mice with one wild-type and one BAC C362S caspase-8 allele demonstrate multifocal cellular inflammatory infiltration in diaphragmatic parietal pleura, lung visceral pleura, and interstitium of pancreas and lung (*arrows*). Scale bar, 100 μm (from [11])

In contrast to the dramatic pathological impact of the expression of enzymatically inactive caspase-8 allele, expression of the 'non-cleavable' BAC D387A allele did not seem to have any detrimental effect on the mice. As reported in detail elsewhere, cells of mice that expressed only this allele (after deletion of both wild-type alleles as a result of mating with mice with the knockout allele) exhibited none of the functional changes ascribed to elimination of any of the known non-apoptotic functions of caspase-8. However, these cells were highly resistant to induction of death by ligands of the TNF family, indicating that caspase-8 processing is required for such death induction [12]. And yet, as mentioned above, the mice appeared normal in all respects. These two findings – that expression of an enzymatically inactive caspase-8 allele in addition to an active one prompts severe inflammation in various organs, while replacement of the normal caspase-8 allele in mice with one that cannot be cleaved has no such effect even though this allele is incapable of mediating death induction – indicate that (i) caspase-8 has an anti-inflammatory effect; (ii) this effect depends on the enzymatic function of caspase-8 but not on its self-processing; and (iii) this functional role of caspase-8 is mechanistically distinct from its apoptotic role. These findings also implied that although caspase-8 is required for normal murine embryonic development, it is not its apoptotic function but some other activity of this enzyme that serves this role.

Studies of the functional consequences of the deletion of caspase-8 in various specific tissues provided further evidence for this enzyme's anti-inflammatory role. The first such indication came from mice in which caspase-8 had been specifically deleted from the hepatocytes. This deletion, while apparently not affecting the normal physiology of the liver, resulted in significant reduction of the burst in liver cell growth observed following partial hepatectomy. Paradoxically, however, in certain mouse strains the eventual extent of liver growth after the operation was not decreased as a result of caspase-8 deficiency in the hepatocytes, but rather enhanced. Careful examination revealed that the hepatocytes of these mice, despite initially exhibiting a reduced rate of growth, later grew more rapidly than the hepatocytes of normal mice. This delayed growth enhancement was found to occur as a consequence of an inflammatory process triggered by partial hepatectomy in the caspase-8 deficient but not in the normal liver [13].

In the liver, inflammation as a result of caspase-8 deficiency occurred only after a triggering event (partial hepatectomy). In some other tissues, however, the mere absence of caspase-8 suffices to prompt spontaneous initiation of inflammation. This was observed most dramatically after deletion of caspase-8 in the epidermis.

Exploring the Mechanism for a Skin Inflammatory Disease Prompted by Caspase-8 Deficiency in the Epidermis Revealed an Inhibitory Effect of Caspase-8 on Signaling for IRF3 Activation

Mice in which caspase-8 was specifically deleted in the basal layer of the skin epithelium appeared normal at birth. However, about 3 days later they started to develop cutaneous inflammation that progressed rapidly, eventually resulting in death. The inflammation was reflected in accumulation of macrophages, eosinophils, and granulocytes in the dermis and in aberrant expression of differentiation-associated proteins in the epidermis (Fig. 25.2 and [11]).

In seeking the mechanism underlying this inflammation, we found that it was not decreased when the macrophages accumulating in the dermis were eliminated by injection of clodronate-loaded liposomes. Moreover, it was only slowed down but not prevented on a TNF null background. (In these respects it differed from a similar pathology observed in mice in which components of the canonical NF-κB pathway are deleted; the latter pathology was found to fully depend on TNF as well as on the accumulation of macrophages in the dermis [14, 15]. These differences suggest that the mechanisms for the pathology in the two cases differ.) The skin inflammation was not decreased in the combined absence of IL1α and ILβ, nor was it decreased in mice deficient in either MyD88 or TRIF, the two main adapter proteins for signaling by the TLRs. The latter finding suggested that although the skin pathology starts after birth, its triggering cannot be ascribed to the fact that the mice become exposed postnatally to extraneous activators of TLR (e.g., bacterial endotoxin). Indeed, on applying gene array analysis to scrutinize molecular changes in the inflamed skin

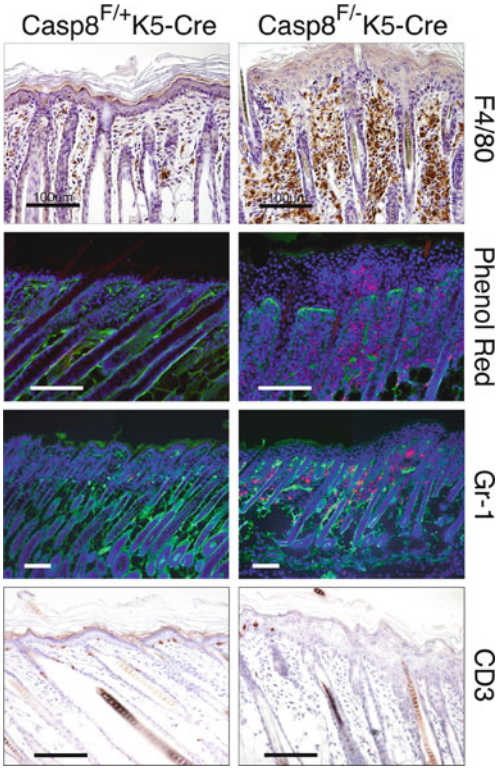

Fig. 25.2 Epidermis-specific knockout of caspase-8 triggers a fatal chronic skin inflammation. Staining for macrophages (anti-F4/80 antibody), eosinophils (phenol red uptake), granulocytes (anti-Gr-1 antibody), and T lymphocytes (anti-CD3 antibody) is seen in skin sections from mice whose skin expresses caspase-8 (*Casp-8$^{F/+}$K5-Cre* mice) and from those in which it does not (*Casp-8$^{F/-}$K5-Cre* mice) at P7. Scale bar, 100 μm (from [11])

we found that although visible changes in the mouse skin architecture were discernible only by about 3 days after birth, enhanced transcription of genes encoding certain inflammatory proteins in the epidermis started much earlier; some of the transcripts were already upregulated at 1 and even at 2 days prenatally. This finding suggested that some endogenous component(s) of the epidermis are responsible for the induction of inflammatory genes in it. To gain a clue to the nature of this inducer, we applied in situ RNA hybridization to identify the site within the epidermis in which upregulation of the inflammatory genes occurs. Although the deletion of caspase-8 in the epidermis is triggered in the proliferating basal keratinocytes, we found that upregulation of the inflammatory genes does not occur until the cells of the basal layer commit to terminal differentiation and reach the granular layer, the region in the epidermis at which the cornification process is initiated. This finding suggested that the trigger of inflammation is some endogenous mediator(s) formed in association with the cornification process.

Many of the inflammatory proteins that we found to be upregulated in the caspase-8-deficient epidermis are known to be induced by interferon (IFN). Since we observed no increase in the expression of IFN genes themselves during the first few days after birth, we examined the state of the interferon regulatory factor (IRF)

proteins, transcription factors that mediate part of the IFN response, to determine whether the observed upregulation occurs as a direct consequence of IRF activation. Expression of IRF7 was barely detectable in the mouse epidermis. IRF3, on the other hand, could easily be discerned, and we found that as early as 1 day after birth its phosphorylation was significantly enhanced in the caspase-8-deficient epidermis. TBK1, a protein kinase known to mediate phosphorylation and activation of IRF3, was also found to be hyper-phosphorylated in the caspase-8-deficient epidermis (Fig. 25.3 and [11]).

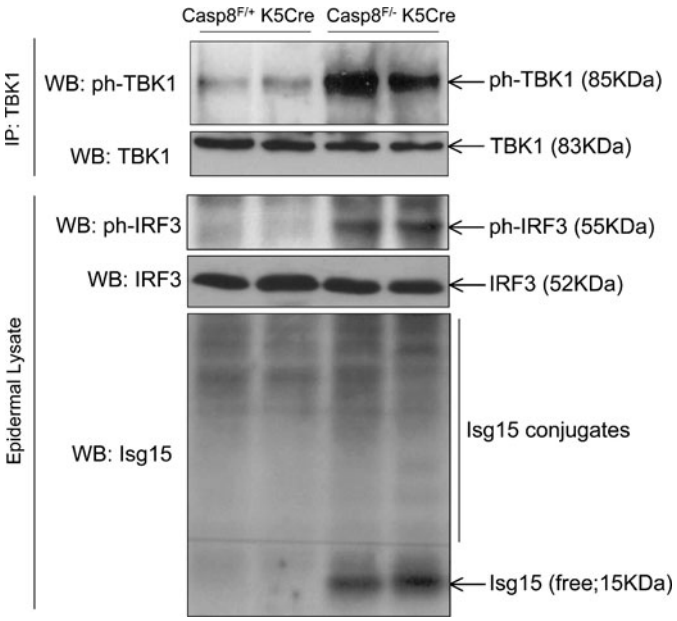

Fig. 25.3 IRF3 is constitutively activated in the caspase-8-deficient epidermis. The figure depicts Western blot analysis of epidermal lysates from two *Casp-8*$^{F/+}$*K5-Cre* and two *Casp-8*$^{F/-}$*K5-Cre* littermates at P3 for phosphorylation of TBK1 (ph-TBK1, enriched by immunoprecipitation with anti-TBK antibody) and of IRF3 (ph-IRF3), as well as for expression of IRF3 and the IRF3-induced protein Isg15 and for conjugation of Isg15 to the cellular proteins (from [11])

Since the TLRs, which are known to mediate activation of IRF3 in response to extracellular inducers, appeared not to be involved in the initiation of inflammation in our mice, we surmised that this activation is induced by intracellular agents. One intracellular trigger previously shown to activate IRF3 is the accumulation of fragmented cellular DNA [16]. In assessing the response of cultured keratinocytes to transfected DNA, we found that in caspase-8-deficient cells, activation caused by DNA transfection occurs more effectively than in keratinocytes expressing the enzyme [11]. This finding raised the possibility that the generation of inflammatory mediators in the caspase-8-deficient epidermis occurs by the following mechanism: (i) accumulation of fragmented DNA, and perhaps also of other components of the

dying keratinocytes in the granular layer, triggers signaling by intracellular pattern response receptors; (ii) this response is normally subdued by caspase-8 and when the enzyme is absent the response is heightened, resulting in massive generation of inflammatory mediators; and (iii) generation of inflammatory mediators such as TNF induces secondary changes in the epidermis that further perpetuate inflammation.

Regulation of RIG-I Signaling by Caspase-8

The best-studied mechanism of activation of responses to intracellular nucleic acid is that mediated by the RIG-I signaling complex. RIG-I is a cytoplasmic receptor for ribonucleic acid. Once activated, it binds to the mitochondria-associated CARD domain containing the protein MAVS/VISA/CARDIAC. The latter then activates the protein kinases TBK1 and IKKe, which in turn phosphorylate and thus activate IRF3 and IRF7. Triggering of the RIG-I complex also leads to activation of NF-κB. Although the RIG-I complex cannot be directly activated by DNA, recent studies have shown that it does mediate part of the cellular response to the accumulation of foreign (or fragmented endogenous) DNA. This occurs as a result of Pol III-mediated transcription of the DNA to RNA, which in turn binds to RIG-I and activates it. Unpublished findings in our laboratory have shown that in various cells, deficiency of caspase-8 results in a significantly enhanced activation of IRF3, not only by desoxyribonucleic acid but also by Sendai virus and by transfected double-stranded RNA, two direct activators of RIG-I. We found, moreover, that in response to these inducers, caspase-8 is recruited to the RIG-I complex.

Epilogue

Our findings suggest that, apart from its role as a crucial mediator of apoptotic cell death in response to ligands of the TNF family, caspase-8 also serves to restrict the innate immune response, and that at least one of the mechanisms by which it does so is by associating with the RIG-I signaling complex and subduing part of its signaling activities. The induction of apoptosis in pathogen-afflicted cells also results in restriction of the innate immune response, first by eliminating the pathogen itself, whose release from the infected cells might trigger such a response and second through anti-inflammatory signals that are known to be generated by apoptotic cells. Thus, restriction of the innate immune response is a common consequence of at least part of the variety of cellular effects of this enzyme. Such a role for a caspase is not surprising in view of the fact that an entire subgroup of this protease family has clearly been destined to control inflammation, though in a positive manner, by mediating the processing of precursors of inflammatory mediators such as IL1.

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Chapter 26
Endogenous Ligand-Induced Activation of TLR4 in Pre-metastatic Phase Is Both Downstream and Upstream of TNF Signaling

Yoshiro Maru

Discovery of Endogenous Ligands for TLR4 in Pre-metastatic Milieu

Recently accumulating information suggests the existence of endogenous TLR4 candidate including hyaluronan fragments, HMGB1, Tenascin, saturated fatty acids, S100A8, etc. (reviewed in [1]). We have added one more candidate SAA3 (serum amyloid A3) [2]. In our experimental metastasis assay, we subcutaneously inject tumor cells that never reach the lungs, which we call pre-metastatic lungs (pre-metastatic phase) [3]. Then we purposely inject labeled tumor cells via the tail vein and count their numbers in the lungs (metastatic phase). A cDNA microarray screening for up-regulated genes in pre-metastatic lungs between tumor-bearing and non-bearing mice gave both S100A8 and SAA3 in the top 50 genes [2, 4]. Surface plasmon resonance analysis of TLR4/MD-2 complex purified from baculovirus and S100A8 or SAA3 purified from mammalian cells provided direct evidence for binding. TLR is a well-studied pattern recognition receptor working as a sensor for bacterial endotoxin or lipopolysaccharide (LPS). S100A8 was initially identified in the synovial fluid of rheumatoid arthritis patients and is one of the members of well-conserved EF-hand Ca2+-binding protein family [5, 6]. SAA3 belong to the SAA family of acute phase reactant [7]. SAA1 and SAA2 are generated in liver in response to inflammation and integrated into HDL (high density lipoprotein). SAA3 is produced extra-hepatically, such as in myeloid cells and endothelial cells. We have shown that S100A8 induces SAA3 expression with promoter activation by two- to fourfolds [2].

The discovery of the endogenous ligands for TLR4 raises a fundamental issue of what their physiological functions are in homeostasis, especially in lungs. TLR4-null mice have been found to manifest emphysema [8]. Lung resident epithelial cells

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as well as macrophages defend against constant assaults by air-borne pathogens by producing ROS (reactive oxygen species). S100A8 has been shown to induce iNOS (inducible nitric oxide synthase) whose product NO plays an essential role in the onset and progression of chronic obstructive pulmonary disease (COPD). Given that LPS has been shown to induce differentiation of GMP (granulocyte myeloid precursor) in hematopoietic system and release of myeloid cells from bone marrow [9], both S100A8 and SAA3 may have similar roles in a homeostatic sense (Fig. 26.1). In other words, even in the absence of exogenous pathogens, leukocytes might persistently stand sentinel over the invasion by going back and forth between luminal and abluminal sides of both airways and vessels. S100A8 was shown to regulate cytoskeleton in transendothelial migration of leukocytes [10]. Numbers of CD11b-positive BMDC (bone marrow-derived cell) are increased in tumor-bearing mice. This mimics pneumonia in which the homeostasis is destroyed

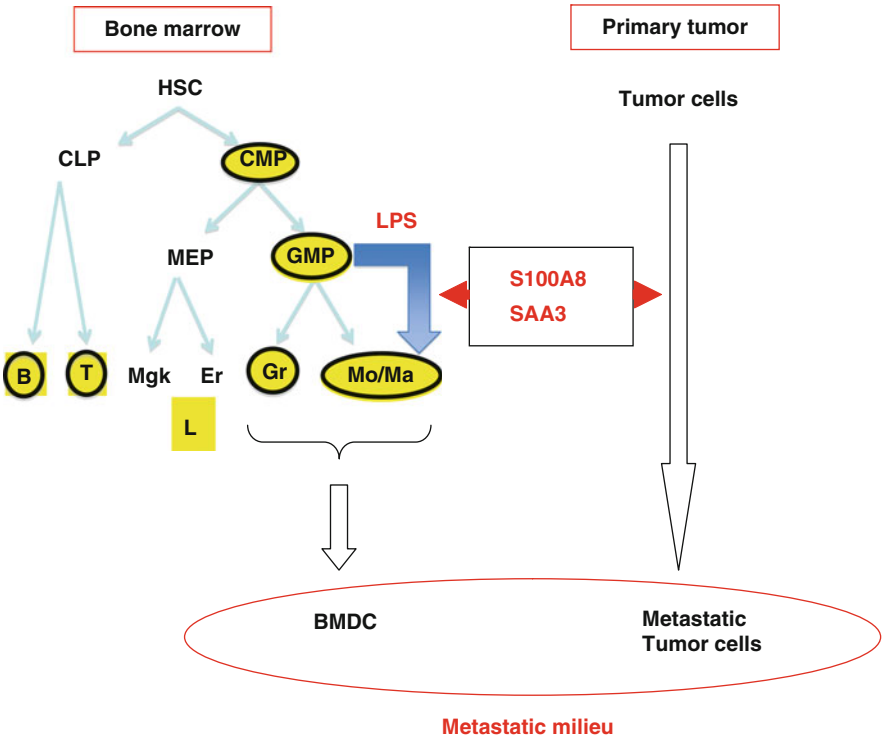

Fig. 26.1 LPS induces differentiation of GMP by skipping cytokines and promotes myeloid cell release from bone marrow. In metastatic milieu, recruitment of BMDC and tumor cells may be driven by the endogenous TLR4 agonists S100A8 and SAA3. HSC, hematopoietic stem cell; CMP, common myeloid precursor; GMP, granulocytic myeloid precursor; Gr, granulocyte; T and B, lymphocyte; L, leukocyte; BMDC, bone marrow-derived cell

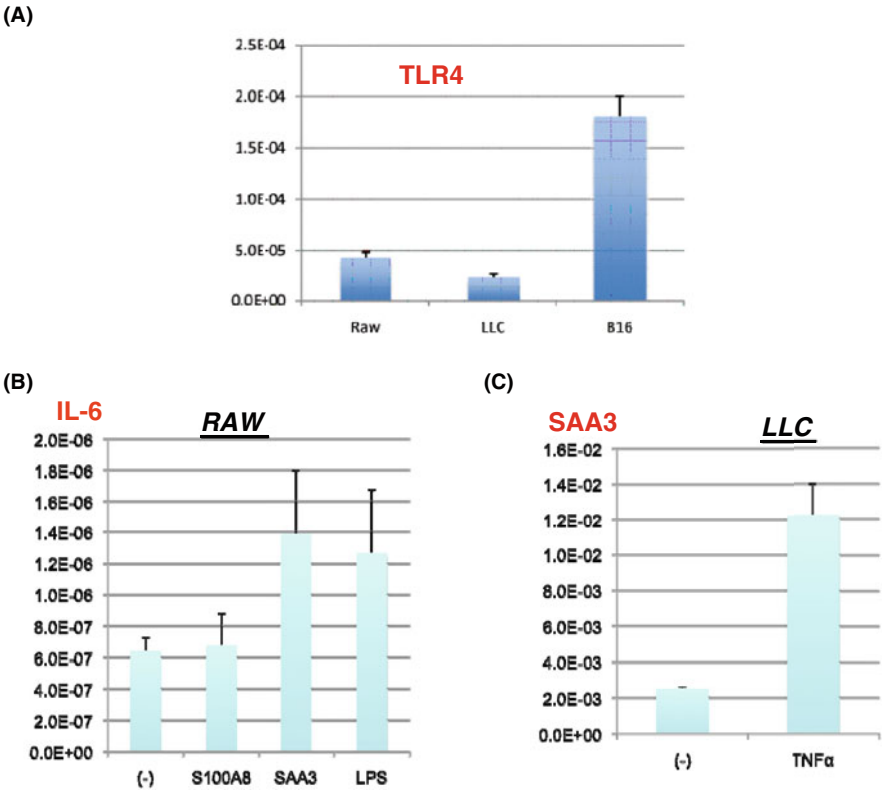

Fig. 26.2 qPCR analysis of TLR4 mRNAs in Raw, LLC, and B16 cells (**a**), of IL-6 mRNAs in Raw cells that were stimulated by mock (−), S100A8, SAA3, and LPS (**b**), and of SAA3 mRNAs in LLC that were stimulated by mock (−) and TNF (**c**)

by microbial invaders. However, the pre-metastatic lungs lack invaders whether exogenous microbes or endogenous tumor cells. Therefore I propose to call it 'homeostatic inflammation' [1]. S100A8 and SAA3 showed chemotactic activity for TLR4-expressing cells such as macrophages and tumor cells (Fig. 26.2a).

The Complex Paracrine Modes in TNFR and TLR4 Crosstalks

The pathological phenotypes seemingly and dramatically differ in disorders with increased serum levels of TNF. The most striking circumstances are cachexia and obesity. Cachexia originates from the classical theory of the body fluid by Hippocrates and cachexin is another name of TNF. Clinical manifestation of

tumor-associated cachexia is emaciation by atrophy of adipose tissue and skeletal muscle with elevated TNF and IL6 in the serum [11]. Anti-IL6 blocking antibody was shown to ameliorate cachexia in tumor-bearing mice [12]. Given that muscle-specific activation of IKKbeta in the NFkB pathway, the major signaling in TNF, resulted in prominent muscle wasting [13], TNF plays an essential role in cachexia. An increased lipolysis underlies the atrophy. Interestingly, serum levels of TNF also correlate well with body mass index and triglyceride in obese adolescents [14]. An increased release of fatty acid, which is independent of hormone-sensitive lipase, is due to TNF derived from activated macrophages in adipose tissue. A vicious cycle is assumed between fatty acid-TLR4 signaling to produce TNF in infiltrating macrophages and TNF-stimulated hypertrophic adipocytes that release fatty acid [15]. A recent report suggests serum levels of SAA1 and SAA2 that are secreted by hepatocytes are associated with adipocyte size and macrophage infiltration, which may establish another adipocyte–macrophage crosstalk [16].

TNF was originally identified as an endotoxin-induced serum factor in macrophages that causes necrosis of tumors and therefore presumed to be responsible for suppression of tumor cells by activated macrophages [17]. A variety of molecules that usually reside in the cytoplasm or nucleus are released form necrotic tumor cells including S100A8, HMGB1, Hsc70, uric acid, nucleic acids, etc., some of which are proposed endogenous ligands for TLR4 [1]. Thus necrosis of tumor, whether chemotherapy-induced or spontaneous due to relatively less nutrition or oxygen supply, could make a paracrine loop, and the persistent secretion of TNF may result in cachexia. As in the case of LPS, S100A8 stimulates expression and secretion of TNF in mouse bone marrow cells [18]. In our metastasis experiment, up-regulation of S100A8 in lungs requires TNF in vivo and TNF in turn induces S100A8 expression in organ culture of lungs [4]. We have also shown that SAA3 can activate NFk-B and promote production of both TNF (Ishibashi and Maru, unpublished results) and IL6 (Fig. 26.2b) in mouse macrophage cell line Raw cells. TNF in turn can stimulate LLC cells to generate SAA3 (Fig. 26.2c). Importantly, this ping-pong mechanism between TLR4 agonist and TNF can be established only when the TLR4 agonist is of endogenous origin.

The triggering mechanisms of preparation of pre-metastatic microenvironment may be TNF or other growth factors produced from primary tumor as an initial event. Then TNF stimulates the paracrine system of S100A8–SAA3 in the lungs [2]. Since both of the endogenous TLR4 agonists can drive mobilization of TLR4-expressing myeloid cells from bone marrow, the paracrine field expands by recruiting them. SAA3 and S100A8 can also induce expression of TNF in the lungs (Tomita and Maru, unpublished results). Thus the pre-metastatic lungs can dispense with TNF that is initially required for triggering the paracrine system [3].

Then what are the mechanisms by which recruited tumor cells stay in the lungs and achieve re-growth. VEGF has been thought to be responsible for it by stimulating tumor cells that express VEGFR1 and/or promoting angiogenesis [19]. However,

anti-VEGF therapy alone has failed to exert prominent effects on metastasis. We suppose that gradient of chemokines such as CXCL12 (SDF-1) or the endogenous TLR4 agonists could potentially mobilize tumor cells from primary site but their direct biological effects are not sufficient for metastasizing tumor cells to stay and start re-growth. Expression of extracellular matrix like fibronectin in lung fibroblasts has been proposed to make BMDC that carries α4β1 integrin (fibronectin receptor) stay in the pre-metastatic microenvironment [20].

Based on the SAA3–TNF crosstalk, we assume a possible mechanism for the tumor cell recruitment in the pre-metastatic lungs. Ephrin-A1 has been initially identified as a TNF-inducible gene in human umbilical endothelial cells [21]. It is a GPI-anchored membrane ligand for Eph family of receptor tyrosine kinase including EphA1 through EphA8 [22]. We have reported that the prototype of the family EphA1 was expressed in a variety of tumors of epithelial origin [23]. EphA2 was shown to be expressed in endothelial cells and be involved in metastasis [24, 25]. We have shown that expression of both EphA1 and PAI-1 (plasminogen activator inhibitor-1) can be induced by an oxidative stress responsive transcription factor ATF3 [26, 27]. ATF3 has been proposed to give a negative feedback on the TLR4-mediated and NFk-B-dependent promoter activation of cytokines such as IL-6 by recruiting HDAC1 that modulates the promoter activity [28]. We presume that once SAA3 is produced in pre-metastatic lungs, it stimulates TLR4-expresing lung cells such as resident macrophages and lung epithelial cells to secrete TNF, which then binds TNFR on endothelial cells. Both S100A8 and SAA3 can also induce ephrin-A1 expression in endothelial cells (Fig. 26.3a). Expression of ephrin-A1 is induced in TNF-activated endothelial cells, which could cause cell–cell contacts with EphA-expressing endothelial cells. Once tumor cells reach the lungs, they may make cell–cell contacts with endothelial cells through the tight EphA–ephrin-A1 interactions. They may also be stimulated by TNF that is produced in the pre-metastatic microenvironment. SAA3 generation in the TNF-stimulated tumor cells could make a paracrine loop and activate both TLR4-expressing myeloid cells and endothelial cells (Fig. 26.3b).

Conclusion

The discovery of the endogenous TLR4 ligand candidates has enabled the mechanistic explanation of TLR4-TNFR crosstalks in metastasis. However, it still remains to be uncovered how TLR4 discriminates endogenous versus exogenous ligand-induced signaling and subsequent biological consequences. In addition the roles of NFk-B and ROS in the intersection between TLR4 and TNFR signaling also need further study.

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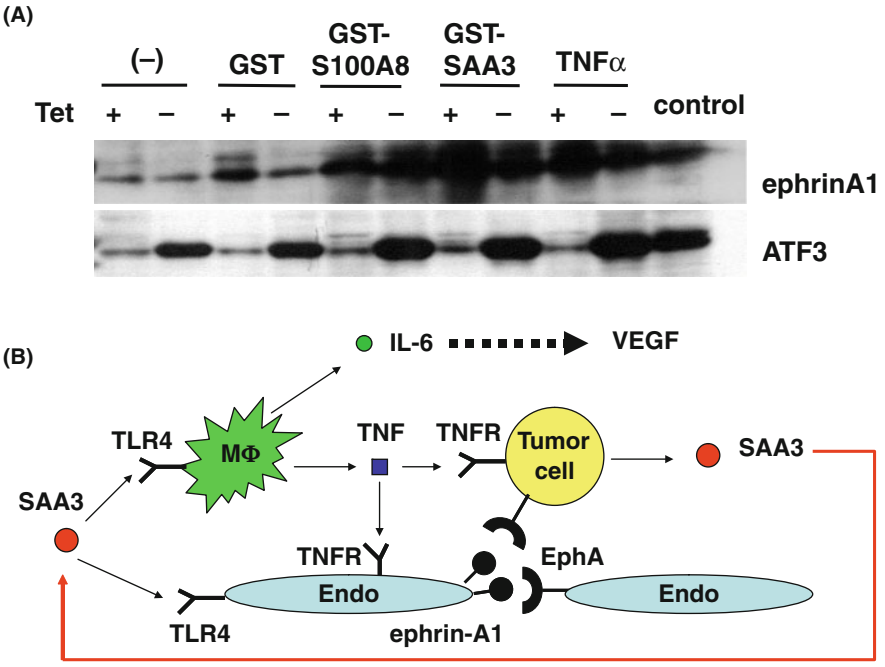

Fig. 26.3 (**a**) NP31 endothelial cells that express ATF3 under the regulation of tetracycline (tet)-inducible system [26] were cultured with (+) or without (−) tet in the presence or absence of stimuli by mock (−), GST, GST-S100A8, GST-SAA3, and TNF. Control indicates transiently transfected 293 cells with ephrin-A1 (*upper panel*) and ATF3 (*lower panel*) and anti-ephrin-A1 (*upper panel*) and anti-ATF3 (*lower panel*) immunoblotting. (**b**) A proposed paracrine loop of SAA3-TNF in metastatic microenvironment. Both TNF and SAA3 induce expression of ephrin-A1 that binds EphA expressed in both endothelial cells as well as a variety of tumor cells

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Part VII
Novel Aspects of Immune Regulation by the TNF Family

Chapter 27
Introductory Words About TL1A/DR3

Stephan R. Targan

TL1A, a protein member of the tumor necrosis factor superfamily 15 (*TNFSF15*), signaling through its receptor DR3, has been defined as a master regulatory cytokine that plays a key role in human intestinal inflammation. Recent studies have also defined a critical role for TL1A in the pathogenesis of mouse experimental autoimmune encephalomyelitis (EAE), models of allergic lung inflammation and human rheumatoid arthritis. The initial discovery of TL1A has given way to subsequent genetic, human, and animal investigation at the bench and will reach the bedside in the form of a clinical trial in 2011–2012. Furthermore, *TNFSF15* and TL1A fit superbly into the personalized medicine paradigm, in which the combination of genetic, biologic, and micro-environmental information may well combine to inform the design of a therapeutic for the subgroup of Crohn's disease patients that will be uniquely likely to benefit.

TL1A was first cloned in 2002 at Human Genome Sciences [1]. It is thought to be involved in the inflammatory process of both rheumatoid arthritis and atherosclerosis [2]. However, TL1A seems to exert its effect most profoundly in the gut [3–7]. In vitro studies have shown that TL1A is produced by human umbilical vein endothelial cells (HUVEC), as well as by T cells, monocytes/macrophages, and dendritic cells (DC) [3, 5, 8–10]. TL1A enhances the induction of IFN-γ expression in human DR3+ T and natural killer cells when combined with IL-12/18 [3, 4, 5]. TL1A is a very potent enhancer of IFN-γ production in that as little as 100 pg/mL is capable of inducing a 5–10 fold increase in IFN-γ levels from IL-12/IL-18-activated human T cells [3]. Co-activation is most evident for CD4+/CCR9+ T cells which are enriched in the lamina propria and intraepithelial lymphoid compartment of the small intestine [11–13]. These T cells are also enriched in the peripheral circulation of patients with CD and celiac disease. In addition, peripheral CD4+/CCR9+ T cells have been shown to express surface membrane TL1A, which appears to

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co-stimulate IFN-γ production independent of, but in synergy with, IL-12 and IL-18 [14]. We have shown that TL1A production is induced by the FcγR signaling pathway [15] and by enteric microorganisms in APCs [16]. Microbial activation of TL1A plays important role in modulating the adaptive immune response. In autologous monocyte-T cell cocultures, TL1A production by monocytes potentiates IFN-γ production by $CD4^+$ T cells [14–16]. Therefore, TL1A expressed in gut-associated T cells and monocytes/monocyte-derived DC is important in enhancing Th1 responses.

In a murine model of chronic colitis, administration of neutralizing TL1A antibodies attenuated colitis via an effect on Th1 and Th17 responses which suggests that TL1A is a central regulator of intestinal inflammation during colitis [17]. Further results showed the important role of TL1A alone and with IL-23 in enhancing Th17 cytokine expression [17]. In line with our observations of a role for TL1A in the generation of Th17 responses, a recent report using TL1A deficient mice shows that $TL1A^{-/-}$ DC exhibited a reduced capacity to induce Th17 differentiation [18]. Several publications demonstrated that the differentiation of human Th17 cells is distinct from that in mice in that different cytokines are required for optimal Th17 induction. While TGF-β and IL-6 initiate TH17 differentiation in mice and IL-23 is believed to support Th17 survival and expansion in humans [19–22], the role of TGF-β is still controversial in human Th17 differentiation [23–28]. For the differentiation of human Th17 cells either a combination of IL-1β and IL-6 or TGF-β and IL-21 is required. The role of IL-23 in the differentiation of human Th17 cells is also controversial [24–27]. However, we were able to demonstrate that FcγR stimulation of monocytes leads to the concomitant induction of TL1A and TGF-β, IL-6, IL-23, and IL-1β, a cytokine milieu that has been shown to foster the development of Th17 cells ([28], and references therein). We found that TL1A enhances Th17 differentiation from naive human CD4+ T cells and enhances IL-17 production of committed CD45RO+CCR6+ human Th17 cells.

Accumulating evidence indicates that the TL1A/DR3 signaling pathway is important in CD mucosal inflammation. Unlike any other known CD-associated gene, *TNFSF15* variants have been found in all ethnic groups studied. Interestingly, however, the associations vary among the cohorts in terms of diagnosis and conferred risk. A recent GWAS revealed a significant association of genetic variants of the *TNFSF15* gene with CD in a large cohort of Japanese patients, in several European cohorts [29, 30], in US Jewish patients [31], and combined data from the NIDDK IBD Genetics Consortium, Belgian-French IBD Consortium, and the WTCC [32]. Haplotypes A and B are associated with susceptibility in non-Jewish Caucasian CD and UC. In addition, *TNFSF15* haplotype B is not only associated with risk, but also with severity in Jewish CD [28, 31, 33]. We recently discovered that, in addition to CD, variants in the *TNFSF15* gene are also associated with both Jewish and non-Jewish severe UC needing surgery. Moreover, monocytes from Jewish patients carrying the risk haplotype B express higher levels of TL1A in response to FcγR stimulation [28]. These results show that CD-associated *TNFSF15* genetic variations contribute to enhanced induction of TL1A that may lead to an exaggerated Th1 and/or Th17 immune response, resulting in

severe, chronic mucosal inflammation. Further work is needed to determine the exact disease-associated variants within this gene that will explain the complicated genotype/functional correlations that lead to more severe inflammation.

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Chapter 28
Introductory Words About TWEAK/Fn14

Linda C. Burkly

In the past decade, the cytokine tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor FGF-inducible molecule-14 (Fn14) have emerged as a ligand/receptor pair of the TNF superfamily that is prominently featured in the physiological and patho-physiological remodeling of tissues [1, 2].

TWEAK is considered to be primarily a soluble cytokine expressed by all leukocytes, including monocytes/macrophages, natural killer cells, dendritic cells, and activated T cells, and can also be expressed by cell types that make up the tissues, including endothelial cells. Fn14, a type I transmembrane protein, is expressed at relatively low levels in normal healthy tissues and universally and dramatically upregulated in response to a variety of stresses on epithelial and mesenchymal cell types, including endothelial cells, that make up the tissue structures. TWEAK can stimulate a variety of seemingly diverse responses through Fn14 upregulated in injured and diseased tissues, including proinflammatory responses, angiogenesis, cell survival, proliferation and death, and progenitor responses. Amongst these well-documented activities, the proinflammatory activity of TWEAK includes the production of cytokines, chemokines, and matrix metalloproteinases. TWEAK thereby effectively induces innate immune responses contributing to remodeling in the tissue microenvironment.

A growing body of evidence supports the view that TWEAK signals through upregulated Fn14 to orchestrate multiple activities, resulting in diverse outcomes depending on the context. In health, TWEAK/Fn14 signaling coordinates acute inflammation, angiogenesis, progenitor cell expansion, and other activities to promote proper tissue regeneration. In contexts of autoimmune and inflammatory diseases, prolonged TWEAK/Fn14 signaling promotes chronic inflammation, pathological angiogenesis, cell death leading to tissue destruction, and pathological remodeling, and potentially impedes endogenous repair mechanisms by promoting the expansion of progenitor cells and inhibiting their differentiation [3].

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Novel aspects of innate immune regulation and tissue remodeling by the TWEAK/Fn14 pathway were presented and discussed. M. Pellegrini presented the Fn14 structure and remarkable species conservation of the TWEAK/Fn14 pathway, thereby strongly implying its functional importance. A novel role in tissue remodeling in health was reported by A. Sanz in that after surgical removal of one kidney, TWEAK signals through Fn14 upregulated in the tubular epithelial cells of the remaining kidney to promote compensatory organ growth and thereby adapt to the surgical tissue loss [4]. There was much interest in the nature of the signals that induce Fn14 expression in this context, though they are presently unknown. A novel physiological role in tissue remodeling was also shown in the studies of bone biology by G. Atkins, who reported that TWEAK promotes osteoblast progenitor cell expansion and inhibits their differentiation as measured by reduced mineralization through the induction of sclerostin [5]. A key question addressed was how TWEAK interplays with its older sibling, TNFα, as both are expected to be present in an inflammatory milieu. While TWEAK and TNFα are similar with respect to their pleiotropic activities, they have distinct receptors and signaling capabilities; TNFα induces canonical NFκB activation through TNFR1 and TNFR2 while TWEAK induces prolonged NFκB activation through Fn14, with biphasic activation of the canonical and noncanonical pathways [6]. G. Atkins showed that, in contrast to TWEAK, TNFα promotes mineralization; however, this effect is antagonized by TWEAK through their synergistic induction of sclerostin. The question of whether TWEAK can regulate TNFα signaling was also explored by M. Ehrenschwender who reported that in TWEAK-primed tumor cell lines, TRAF2/cIAP depletion regulates TNFα/TNFR1 signaling, reducing TNFα-mediated canonical NFκB activation and promoting cell death [7]. These results are in good agreement with other studies of TNFα crossregulation by TWEAK priming [8], as well as priming by CD40L/CD40, another noncanonical NFκB-activating pathway, as reported by D. Vucic in Session 8 of this conference. Thus, intersection of the TWEAK and TNFα pathways may affect biological outcomes in contexts of inflammation and cancer. Addressing the role of the TWEAK/Fn14 pathway in inflammatory disease, L. Burkly reported attenuation of intestinal damage in the model of TNBS-induced acute colitis, elucidating a novel role of TWEAK in promoting innate immunity by signaling through Fn14 upregulated on intestinal epithelial cells. Burkly further demonstrated that TWEAK regulates epithelial repair, promoting their apoptosis after γ-irradiation injury, another example of how the TWEAK/Fn14 pathway regulates tissue remodeling, and perhaps an added mechanism through which TWEAK/Fn14 promotes epithelial damage in colitis. The question as to whether these TWEAK-induced effects were TNF-dependent is currently under investigation.

In summary, this session elucidated novel roles of TWEAK in tissue remodeling of tissues in contexts of health and disease, and involving TWEAK induced innate inflammatory responses in the tissue microenvironment alone or via crossregulation of TNFα responses. Future studies are needed to further enlighten our understanding of TWEAK's interplay with TNFα, as well as other cytokines in the inflammatory milieu.

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Chapter 29
Insights into TL1A and IBD Pathogenesis

David Q. Shih, Kathrin S. Michelsen, Robert J. Barrett, Eva Biener-Ramanujan, Rivkah Gonsky, Xiaolan Zhang, and Stephan R. Targan

Introduction

Immune responses in the intestinal compartment are tightly regulated to tolerate the heavy antigenic load characteristic of the gut (reviewed in [36]). Studies in humans and mice demonstrated that perturbations of both the innate and adaptive immune system (Table 29.1) can result in mucosal inflammation and pointed to a complex, multi-gene pathology (reviewed in [36, 37]). CD and UC are two main forms of IBD that are chronic, relapsing inflammatory disorders of the gastrointestinal tract. Although the exact pathogenesis of IBD remains uncertain, it is thought that IBD is caused by a dysregulated immune response to enteric microflora in genetically susceptible hosts [36, 37].

The tumor necrosis factor (TNF) family of cytokines consists of type II transmembrane proteins and has roles in diverse biological functions such as proliferation, differentiation, and gene activation [1]. Binding of TNF to its respective receptors initiates signal transduction pathways that activate NF-κB and mitogen-activated protein (MAP) kinase family such as C-Jun N-terminal protein kinase [1]. A primary function of TNF superfamily molecules is to initiate a pro-inflammatory response and its perturbation participates in the pathology of many autoimmune and inflammatory diseases including IBD [9]. Targeting soluble TNF and TNF expressing T cells can benefit a subset of CD [40] and UC patients [31]. However, the partial success of blocking TNF in IBD emphasizes the complexity of mucosal immune regulatory mechanisms and prompted investigation of other ligands in the TNF family.

Of the TNF members, TL1A has emerged as an important mediator of gut inflammation. TL1A was first cloned in 2002 at Human Genome Sciences and its expression is increased in inflamed tissues [19]. TL1A binds to death domain receptor 3 (DR3, TNFRSF25) and co-localizes to antigen presenting cells (APC) and T

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Table 29.1 Cytokine profile in inflammatory bowel disease

Cytokine	Crohn's disease	Ulcerative colitis	Reference	Cytokine	Crohn's disease	Ulcerative colitis	Reference
Innate immune response				Adaptive immune response			
IL-1β	I	I	[30]	IL-5	N	I	[30]
IL-6	I	I	[30]	IL-13	N	I	[30]
IL-8	I	I	[22]	IL-17	I	I	[12]
IL-12	I	N	[30]	IL-21	I	N	[20]
IL-18	I	N	[13]	IL-22	I	I	[5]
IL-23	I	N	[34]	IFN-γ	I	I	[30]
IL-27	I	N	[34]	LIGHT	I	I	[8]
TNF-α	I	I	[30]	TL1a	I	I	[3, 28]
TL1a	I	I	[18]				

cells. It is involved in many inflammatory diseases including rheumatoid arthritis, autoimmune encephalomyelitis, asthma, and IBD [6, 11, 17, 18, 26, 38]. In fact, accumulating evidence shows that TL1A exerts its effect most profoundly in the gut. In this chapter, we will first discuss the genetic evidence showing the association of TL1A to IBD. We will then discuss both the upstream regulators and downstream targets of the TL1A-DR3 signaling pathway. Lastly, the role of the TL1A-DR3 signaling pathway in autoimmunity and inflammatory disease will be reviewed.

TL1A (*TNFSF15*) Is an IBD Severity Associated Gene

As a result of recent advances in genetic statistical theory, the availability of affordable high-throughput genotyping and the development of the HapMap, researchers are now able to perform genetic association studies on a scale that until recently was thought to be impossible. There has been a plethora of such genome-wide association studies (GWASs) published within the last few years in an increasing number of genetically complex diseases with IBD leading the way.

The first GWAS of IBD provided evidence that variation in *TNFSF15*, the *TL1A* gene, is linked to CD in Japanese and both CD and UC in the British population [14, 42]. Moreover, additional GWAS have revealed a significant association of variants of the *TL1A* (*TNFSF15*) gene with CD in several European cohorts [41, 42], in US Jewish patients [27], in pediatric IBD patients [16], and in combined data from the NIDDK IBD Genetics Consortium, the Belgian-French IBD Consortium, and the Wellcome Trust Case Control Consortium [4]. *TL1A* is the only gene that has been associated with both Asian and Caucasian IBD. These GWAS show that association between *TL1A* polymorphisms and CD has been found in every ethnic (both Asian and Caucasian IBD) and age groups (both adult and pediatric IBD) that were studied.

Haplotypes composed of 5 *TNFSF15* SNPs were observed to confer significant CD risk (*haplotype A*) and protection (*haplotype B*), using both case/control and family-based study designs [4, 14, 42]. Protective association between CD and UC with the same *haplotype B* in a Los Angeles based cohort was also observed [27]. Interestingly, there are ethnic differences in IBD susceptibility of TL1A. In contrast to the protective association seen in non-Jews, the opposite trend towards a risk association with haplotype B was observed in Ashkenazi Jews [27]. Furthermore, haplotype B is correlated with a more severe CD disease course since there is an association between *TNFSF15 haplotype B* and small bowel surgery in Jewish patients only ($p = 0.006$, OR: 2.2, 95% CI: 1.25, 3.75.) [27].

To determine if the statistical association of *TL1A* polymorphisms reflects an effect on protein function and/or expression, a detailed molecular phenotypic analysis of patients with particular TL1A haplotype was performed. There is an earlier onset of TL1A secretion and a higher amount of secreted TL1A in Jewish CD patients with *haplotype B* compared to *haplotype A* upon stimulation of FcγR with immune complexes (IC) [18]. The association of *TL1A haplotype* B and elevated TL1A expression was further accentuated in Jewish patients with antibody titers to *E. coli* outer membrane porin C ($OmpC^+$), a group characterized by more severe CD course [2, 18, 21]. Jewish CD patients who are $OmpC^+$ and carry *haplotype B* have increased secretion of soluble and membrane TL1A in response to IC, as well as increased baseline membrane TL1A expression, when compared to *haplotype A* [18]. In non-Jewish $OmpC^+$ patients with *haplotype B*, we only observed an increased secretion of soluble TL1A in response to IC, but with a distinct and delayed kinetics compared to Jewish $OmpC^+$ patients of the same haplotype [18]. The higher baseline and faster kinetics of TL1A expression on antigen presenting cells in Jewish but not non-Jewish $OmpC^+$ CD patients carrying *haplotype B* suggests a higher capacity of TL1A-dependent T cell activation [28, 29] leading to a more severe CD disease course as measured by clinical parameters including fibrostenosis, internal perforation, perianal penetration, and small bowel surgeries. Taken together, these studies implicate *TL1A* as an IBD severity gene. Defining CD patients by ethnicity, serotyping, and genotyping may identify a subset of IBD patients who would best respond to therapeutic blockade of TL1A function.

Upstream Regulators of the TL1A-DR3 Signaling Pathway

Although TL1A is found in many tissues including plancenta, lung, kidney, endothelium, skeletal muscle, pancreas, spleen, prostate, small intestine, and colon [19, 31], an important feature of TL1A is that its expression is inducible and not ubiquitously expressed by all cells of the tissue. For example, the expression of TL1A is increased in inflamed tissue of colon and small bowel of CD patients with positive staining of TL1A specifically in T cells and macrophages in tissue sections from small bowel and colonic lamina propria [3, 28]. In particular, lamina propria, but also peripheral $CD4^+CCR9^+$ T cells, constitutively express membrane TL1A

and are especially sensitive to TL1A stimulation [23, 25]. The findings that TL1A expression is increased following immune activation implicate a central role of TL1A in modulating inflammation. Therefore, understanding the upstream regulators of TL1A expression is likely to provide insight into this cytokine's role in inflammatory diseases.

Using umbilical vein endothelial cells (HUVEC), phorbol myristate acetate (PMA), TNFα, and IL-1α can induce TL1A expression whereas IFN-γ has a negative effect on TL1A expression [19]. Because inflammatory bowel diseases reflect an aberrant immune reaction to microbial flora and highlight the critical role of the host/microbial interface, antigen recognition is hypothesized to be an important inducer of TL1A expression in APC. Indeed, activation of FcγR signaling pathway by IC induces an up-regulation of TL1A mRNA and both membrane bound and secreted protein in monocytes and dendritic cells (DC) (Fig. 29.1) [29]. Signal transduction by activating FcγR involves downstream mediators including Signal Transducers and Activators of Transcription (STAT)-mediated signaling and increase in intracellular calcium levels and may be required for effective TL1A induction. However, the exact FcγR signaling components that mediate TL1A expression in APC remain to be determined.

The findings that FcγR signaling can trigger TL1A expression in APC implicate a potential role of opsonized pathogens, microbes, and Toll-like receptor (TLR) signaling pathways as potential upstream activators of TL1A expression in APC. Recently, it was found that several types of microbial organisms including gram negative organisms (*E. coli, E. coli* Nissle1917, and *S. typhimurium*), gram positive organisms (*L. monocytogenes* and *S. epidermidis*), a microaerophilic organism (*C. jejuni*), and obligate anaerobes (*Bifidobacterium breve, B. thetaiotaomicron, Clostridium A4*) can induce TL1A expression in primary human monocytes and monocyte-derived DC [35]. Microbial-activated TL1A was in part mediated by TLR 1, 2, 4, and 9 signaling pathways and dependent on downstream p38 MAP kinase and NF-κB activation (Fig. 29.1) [28, 35]. The role of TLR 4 signaling and the involvement of NF-κB pathway in activating TL1A expression is independently confirmed using the human monocyte cell line U937 [10]. Interestingly, bacterial induction of TL1A expression was significantly decreased when microbes were killed either by heat or sonication [35]. The more efficient induction of TL1A by live bacteria over dead bacteria may be due to differences in efficiency in the uptake of bacteria by APCs, bacteria processing, or in the generation of reactive oxygen species, and these hypotheses are being investigated.

Negative regulators of the TL1A signaling pathway in APC also exist and may serve to maintain gut immune homeostasis. Our group showed that TLR8 or TLR7/8 ligand (R848) can inhibit TL1A production in a dose-dependent manner (Fig. 29.1) [32]. In support of this finding, TLR8 is found to be an X-linked IBD susceptibility gene with both common predisposing and protective haplotypes [33]. These associations emphasize the importance of TLR8 signaling in IBD and may represent a novel therapeutic target by inhibiting TL1A expression.

In T cells, TL1A expression is also inducible. TL1A mRNA and protein levels are up-regulated in $CD4^+/CD8^+$ lymphocytes in inflamed intestine of IBD patients

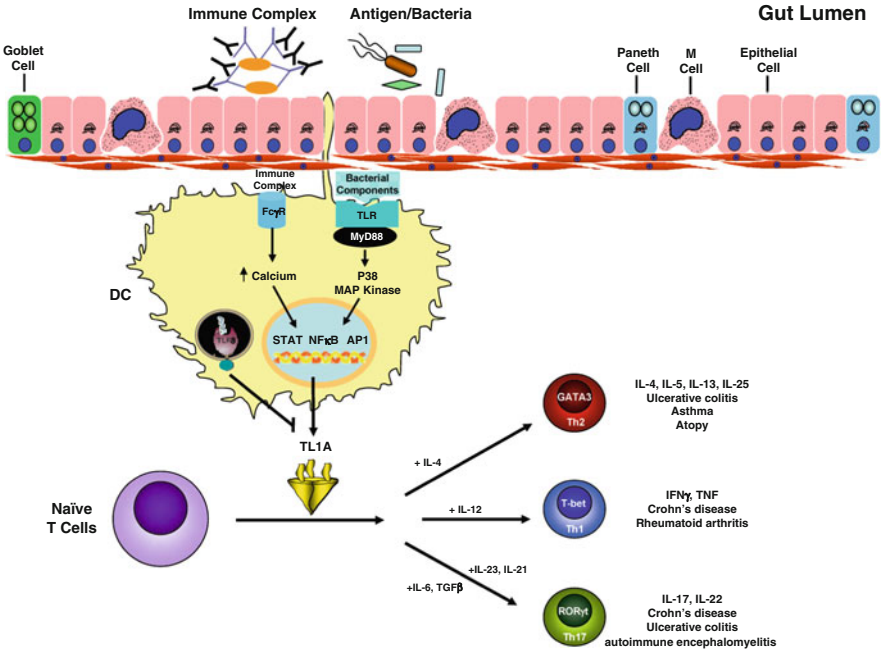

Fig. 29.1 Working hypothesis of TL1A in inflammatory diseases. Intestinal immune system is in close apposition to luminal antigens/bacteria, which are separated by a layer of epithelial cells. Goblet cells contribute to the formation of the protective mucus layer, M cell and dendritic cell (DC) sample intestinal luminal contents. Overresponse to antigens, either through the Toll-like receptors (TLR) or immune complex via FcγR, results in stimulated dendritic cells (DC) and other APC, which lead to an up-regulation of TL1A. TLR8 is a negative regulator of TL1A expression. TL1A synergistically enhances the potential of IL-4, IL-12, or IL-23 in the generation and effector function of Th1, Th2, and Th17 cells. For each T helper cell differentiation program, specific transcription factors and cytokine milieu are required and indicated in the figure. Terminally differentiated T helper cells are characterized by a specific combination of effector cytokines (indicated in the figure) that orchestrate effector function of the adaptive immune system, and the characteristic cytokin profile in CD and UC as in Table 29.1

[3, 28], suggesting that TL1A expression can also be induced in activated T cells. We found that TCR activation by phytohaemagglutinin (PHA) can induce TL1A mRNA and membrane-bound protein on a fraction of T cells [28]. $CCR9^+$ T cells represent a small subset of peripheral blood lymphocytes that have an activated phenotype, enriched in the lamina propria and intraepithelial lymphoid compartment of the small intestine, and express several TNF-like molecules [23]. Interestingly, TL1A is almost exclusively expressed in $CCR9^+CD4^+$ T cells (25%) compared to $CCR9^-CD4^+$ T cells (2.5%) which is consistent with the induction of TL1A on activated T cells that are enriched in the gut [25]. A recent study also showed that activation of peripheral blood T cells and Jurkat cells by PMA/ionomycin also induced TL1A and that the degree of activation results from the *-358T/C* promoter SNP [15]. The promoter activity of risk *-358C* allele is higher than that of non-risk

-358T allele in activated lymphocytes [15], implicating the higher transcriptional activity of risk *-358C* promoter allele confers susceptibility to IBD. Electrophoretic mobility shift assay showed that the *-358T/C* promoter SNP influences nuclear protein binding only when nuclear extracts from lymphocytes were used, suggesting that this is a T cell specific regulatory SNP [15]. However, the specific nuclear factors that bind to the *-358T/C* promoter SNP and cause the difference in promoter activity between *TNFSF15* risk haplotype in T cells need to be identified.

Downstream Effects of the TL1A-DR3 Signaling Pathway

TL1A plays important role in modulating adaptive immune response (Fig. 29.1). In the Th1 effector arm, activation of the DR3 receptor by TL1A or agonistic anti-DR3 monoclonal antibody enhances IFN-γ production from peripheral and mucosal T cells as well as NK cells in a dose-dependent manner [24, 28]. This occurred independently of, but also in synergy with IL-12/IL-18 [24, 28]. $CD4^+CCR9^+$ T cells represent approximately 2–4% of peripheral blood $CD4^+$ T cells and contain differentiated Th1 cells that are specifically enriched in the intestinal immune compartment [23]. We observed that $CD4^+CCR9^+$ T cells not only constitutively express TL1A, but also are extremely sensitive to TL1A stimulation since even 100 pg of TL1A enhance IFN-γ production by up to 10-fold [25]. Since most of the activated T cells express DR3, TL1A may induce IFN-γ through T cell–T cell interaction. Recent studies have shown that TL1A can also be induced in APC by FcγR signaling [29] and microbial organisms [35] suggesting that augmentation of Th1 immune response by TL1A may also occur through APC-T cell interaction. Indeed, in autologous APC-T-cell co-cultures, TL1A production by monocytes or DC-potentiated IFN-γ production by $CD4^+$ T cells [29, 35]. In a murine model of chronic mucosal inflammation, TL1A enhanced Th1 effector function by up-regulating IFN-γ production in gut-associated lymphoid tissue (GALT) $CD4^+$ cells under conditions that stimulated Th1 fate, indicating that TL1A is an important modulator in the development of gut mucosal inflammation [38]. In addition to enhancing Th1 effector function, TL1A may also be involved in differentiation of Th1 cells [26]. The physiological relevance of TL1A in promoting Th1 differentiation is illustrated by the fact that the percentage of IFN-γ^+ $CD4^+$ T cells is greatly reduced in TL1A deficient mice [26].

Recently TL1A was also found to be important in promoting Th17 effector cell function. The expression of DR3, the receptor for TL1A, was selectively elevated in Th17 cells, and TL1A could promote the proliferation of effector Th17 cells [26]. The dendritic cells isolated from TL1A-deficient mice exhibited a reduced capacity in supporting Th17 differentiation and proliferation [26]. TL1A may also be involved in differentiation of Th17 cells as the percentage of IL-17^+ $CD4^+$ T cells is greatly reduced in TL1A-deficient mice [26]. In line with these findings, we have demonstrated that during chronic colitis TL1A up-regulated IL-17 production from Th17 cells in GALT $CD4^+$ T cells and synergized with IL-23 to induce IL-17 by

these cells [38]. Confirming the findings in the mouse, TL1A also promoted the differentiation of human T_H17 cells from naive $CD4^+$ T cells leading to enhanced IL-17 and IL-22 secretion. Additionally, TL1A stimulation of $CD4^+CD45RO^+$ memory T cells also enhanced IL-17 production and induces a unique cell population of IL-17/IFN-γ producing T_H17 cells. Furthermore, TL1A also enhanced the IL-17 and IL-22 production by committed $CD45RO^+CCR6^+$ Th17 cells suggesting that TL1A is able to induce Th17 differentiation and enhanced IL-17 secretion from committed Th177 cells (Michelsen et al. unpublished data).

These findings indicate that TL1A is required for the optimal differentiation as well as effector function of Th17 cells. Interestingly, a conflicting report using DR3 deficient mice showed that TL1A/DR3 signaling is not essential for polarization of naïve $CD4^+$ T cells into Th1, Th2, or Th17 effector cell subtypes [17]. Instead, DR3 expression is required on T cells for immunopathology, local T cell accumulation, and cytokine production [17], suggesting that TL1A/DR3 signaling is important to co-stimulate antigen-induced expansion of primed T cells in the target organ of T cell-mediated autoimmune and inflammatory diseases.

In addition to mediating Th1 and Th17 responses, the role of TL1A in Th2-mediated functions is demonstrated in a mouse model of allergic lung inflammation [11]. Fang et al. showed that TL1A-DR3 signaling pathway is required to exert Th2 effector function in Th2-polarized CD4 cells and co-stimulated IL-13 production, a Th2 cytokine, by glycosphingolipid-activated NKT cells [11]. TL1A-DR3 signals also increased the number of IL-13 producing NKT cells, by inducing IL-13 producing cells [11]. The importance of TL1A in Th2 effector function is further illustrated by the fact that in vivo blockade of TL1A-DR3 signaling diminished production of Th2 cytokines such as IL-4, -5, and -13 [11]. Together, this study implicates TL1A in augmenting Th2 effector function.

Role of TL1A in Inflammatory Diseases

The interaction between TNF and its respective receptors is believed to be important in the pathology of inflammatory diseases as neutralizing TNF–TNFR interaction can result in the reduction of inflammatory disease severity. The TL1A-DR3 signaling pathway is also likely a central modulator of severity of inflammatory diseases (Fig. 29.1). Using either TL1A- or DR3-deficient mice or neutralizing antibodies to TL1A, reduction in inflammatory disease symptoms has been found in murine models of experimental autoimmune encephalomyelitis (EAE) [17, 26], colitis [38], asthma and atopy [11, 17], and arthritis [6].

In humans, evidence is accumulating that demonstrates the importance of TL1A-DR3 pathway in inflammatory diseases such as IBD and RA. In addition to GWAS association of TL1A to IBD, functional data demonstrating that TL1A is a central immune modulator of innate immune response and in the differentiation of Th1 and Th17 CD4+ T cells implicate a central role of TL1A in the severity of gut mucosal inflammation. In RA, TL1A expression is elevated in monocytes in

synovial fluids/tissue of rheumatoid factor (RF)-seropositive RA patients but not in RF-seronegative subjects [7]. These studies in mice and humans indicate that blockage of TL1A may have therapeutic benefit in inflammatory diseases including IBD and RA.

Conclusion

TL1A/DR3 signaling appears to have pleiotropic effects that include: amplifying the innate immune response, modulating adaptive immunity by augmenting Th1, Th2, and Th17 effector cell function, and T cell accumulation and immunopathology of inflamed tissue (Fig. 29.1 and Table 29.1). Given its immune modulatory effects, blocking TL1A-DR3 signaling is a promising therapeutic strategy in a variety of T cell-dependent inflammatory diseases.

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Chapter 30
The Role of TNFRSF25:TNFSF15 in Disease. . . and Health?

Taylor H. Schreiber, Dietlinde Wolf, and Eckhard R. Podack

Abbreviations

TNF	tumor necrosis factor
TNFSF	tumor necrosis factor superfamily
TNFRSF	tumor necrosis factor superfamily receptor
Teff	effector T cell
Treg	regulatory T cell
BSA	bovine serum albumin
IFN	interferon
RF	rheumatoid factor
DSS	dextran sodium sulfate
kDA	kilo Dalton
aa	amino acid
PBL	peripheral blood lymphocytes
ECD	extra-cellular domain
NK	natural killer
NKT	natural killer T cell
TLR	toll like receptor
TRADD	TNF receptor associated death domain
FADD	Fas associated death domain
RIP	receptor interacting protein
NFkβ	nuclear factor kappa beta
IL	interleukin
TCR	T cell receptor

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Introduction

The tumor necrosis factor superfamily (TNFSF) consists of at least 19 ligands and 30 receptors (TNFRSF) that serve distinct roles in the maintenance, activation, and termination of immune responses. As a whole, little is understood about where and when these receptor:ligand pairs are expressed, what signaling events are precipitated by their triggering, how these signaling pathways modulate innate and adaptive immunity, and whether crosstalk exists between various TNFRSF members. Here we discuss the history of one of the recently described TNFSF members, TNFSF15 (TL1A) and its receptor, TNFRSF25 (TNFR25), data presented at the 12th international TNF conference as well as recent data from our laboratory.

Structure, Expression and Signaling

TNFRSF25 was first described 13 years ago (1996) by different groups and under the names Death Receptor 3 (DR3) [9], WSL-1 [14], Apo-3 [17], lymphocyte-associated receptor of death (LARD) [30], TNF receptor-related apoptosis-mediated protein (TRAMP) [5], and TR3 [34]. Today, the most broadly used common name for TNFRSF25 is DR3; however, because of the growing number of non-apoptosis-inducing functions of DR3, we refer to it simply as TNFR25.

TNFR25 is a type I membrane protein of 417 amino acids (aa) and has a calculated molecular weight of approximately 45 kDa. An N-terminal signal sequence (aa 1–24) is followed by an extracellular domain (ECD; aa 25–198), a transmembrane domain (aa 199–224), and an intracellular domain (ICD; aa 225–417). The ECD contains four cysteine-rich pseudo-repeats and two potential N-linked glycosylation sites. The ICD contains a death domain (DD) which is essential for signaling. TNFR25 is closely related to TNFR1 (29% overall, 28% in the ECD, 32% in the ICD, and 48% in the death domain). It was mapped in humans to the short arm of chromosome 1, at position 1p36.3 and in mice to chromosome 4, region E1 [5, 9, 15, 17, 30, 36] . At the protein level mouse and human TNFR25 are 63% homologous (94% in the DD and 52% in the ECD) [36]. Although not definitively tested it is likely that TNFR25 is a homotrimer like other members of this family [2, 10, 21].

TNFR25 expression in mice and humans is restricted predominantly to lymphocyte-rich tissues like spleen, thymus, and peripheral blood lymphocytes (PBL) and is less abundant in small intestine and colon [9, 15, 17, 30, 34, 36]. At least 13 human [30, 38] and three mouse [36] splice variants have been reported so far. We have identified 10 splice forms of TNFR25 in C57BL/6 mice (Fig. 30.1).

Germ line deletion of TNFR25 results in impaired negative selection of thymocytes; however, no other phenotypic changes were observed in peripheral lymphocytes as compared to wild-type lymphocytes [37]. Similarly, a dominant negative transgene of TNFR25 (DN-TNFR25) expressed under the CD2 promoter and enhancer which blocks signaling of endogenous TNFR25 had no overt phenotypic effect. On the other hand the full length TNFR transgene and the Δ5,6-TNFR25

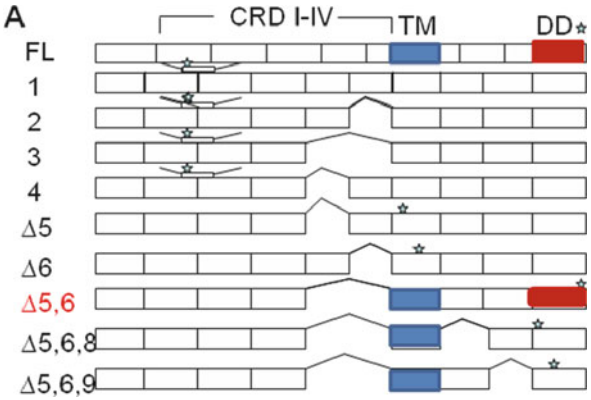

Fig. 30.1 Splice forms of mTNFR25. RT-PCR products from murine cell lines and murine tissues were cloned and confirmed as splice forms of mTNFR25 by sequencing. CDR, cysteine rich domain; TM, transmembrane domain; DD, death domain; asterisks, stop codons

transgene caused spontaneous Th2 polarization of CD4+ T cells which was associated with increased susceptibility to allergic lung inflammation [11].

At a cellular level TNFR25 is expressed at low levels on naïve CD4+ and at even lower levels on naive CD8+ T cells but is up-regulated after activation on both [30, 34]. Also a subpopulation of CD11c+ and NK cells express it. NKT cells express high levels whereas it is not found on B cells [11]. Interestingly, TNFR25 is highly and constitutively expressed by CD4+FoxP3+ T regulatory cells (Treg) ([26] and TH Schreiber unpublished data).

The only known ligand for TNFR25 is TL1A (TNFSF15), and to date, aside from a decoy receptor in humans (DcR3/TR6) [20], this remains a monogamous receptor:ligand pair, although the existence of a second receptor for TL1A has been proposed [1]. TL1A was first described in 2002 by Migone et al. [20] as a member of the TNF superfamily. It is a type II membrane-bound protein but can also be processed into a soluble form. Mouse TL1A consists of 252 aa and shares a 63.7% sequence homology with its human counterpart, mapped to chromosome 9q32. TL1A is constitutively expressed by endothelial cells, especially vascular endothelial cells in the kidney and prostate, and can also be induced by IL-1β or TNFα [1, 20]. Similar to TNFR25$^{-/-}$ mice, germ line deletion of TL1A does not result in any gross developmental or phenotypic abnormalities in lymphoid or non-lymphoid tissues in mice ([26] and TH Schreiber unpublished data). Robust expression of TL1A by macrophages and dendritic cells occurs rapidly after triggering of TLR4, TLR11, or FcγR but not by TLR2 or TLR5 ligands and not in B cells [11, 18, 28]. A consensus on the role of TLR ligands on TL1A induction remains to be reached however, and some reports suggest that TLR-induced expression of TL1A is FcγR-dependent, which can be inhibited by other TLRs such as TLR8 [28, 29]. TL1A is not expressed by resting CD4+ or CD8+ T cells, but can be induced upon activation by TCR stimulation or exposure to LPS [11, 27]. The crystal structure for human TL1A was recently solved and reported to exist as a homotrimer with the longest cytoplasmic domain loop of any of the TNFSF members for which the structure has been solved [12].

Two distinct signaling cascades downstream of TNFR25 have been described that can lead to either NFκB activation or apoptosis. After binding of TL1A to TNFR25 the intracellular signaling molecule TNFR-associated death domain (TRADD) is recruited via the death domain (DD) of TNFR25. As an adaptor molecule, TRADD then recruits TNFR-associated factor 2 (TRAF2) and receptor interacting protein 1(RIP1) to form the so-called complex 1 [3, 19, 40]. This complex 1, containing TRADD, RIP, TRAF2, initiates a downstream signal transduction pathway leading to the activation of NFκB and MAPK (ERK1/2, JNK and p38) [31, 40]. One of the TL1A-induced downstream targets of NFκB was recently described as the anti-apoptotic factor cIAP2 [40]. Alternatively, if complex 1 dissociates from the receptor, TRADD associates with Fas-associated death domain (FADD) and caspase 8. This initiator caspase then induces the activation of effector caspases, such as caspase 3 which ultimately leads to cell death [19]. It remains unclear under what conditions the TRADD- or FADD-mediated signaling pathways prevail following TNFR25 triggering.

TL1A:TNFR25 in T Cell Function

TNFR25 is mostly expressed on T-lymphocytes and is up-regulated rapidly by splicing involving protein kinase C (Fang, unpublished data) following their activation by T cell receptor (TCR) stimulation. The initial function described for TNFR25 on T-cell enriched human lymphocytes was to enhance T cell responsiveness to IL-2 [20]. In these early studies, Migone et al. determined that TNFR25 signaling subsequent to activation with anti-CD3 and anti-CD28 led to enhanced T cell proliferation via increased expression of CD25 and CD122, as well as enhanced expression of IFNγ and GM-CSF but not IL-2, IL-4, IL-10, or TNF. These authors also determined that freshly isolated T cells are not susceptible to TNFR25-induced apoptosis by TL1A treatment, suggesting that the default signaling pathway for TNFR25 may be via complex 1 and not FADD. Shortly thereafter, TL1A-induced IFNγ expression was also reported in preparations of mononuclear cells isolated from the lamina propria, the magnitude of which was mildly increased in patients with active Crohn's disease [4]. In the first of a continuing series of contributions to this field, Stephan Targan's group next demonstrated that in the absence of TCR signals, TL1A leads to a dose- and IL-12/IL-18-dependent increase in IFNγ production by CD4+, CD8+, NK, and NKT cells [23]. These studies were then further defined when it was demonstrated that although either TCR signaling or IL-12/IL-18 are required for TL1A to enhance IFNγ production by T cells, IL-12/IL-18 are not required in addition to TCR signaling for TL1A to enhance IFNγ production [27]. The enhancement of IFNγ production by TL1A was most notable in T cells that were sub-optimally activated by TCR stimulation. These studies also observed that TL1A enhanced IFNγ production in only a subset of CD4+ T cells, which was subsequently found to contain gut-homing CCR9+CD4+ lymphocytes [25]. These early studies consisted entirely of in vitro or ex vivo analysis of lymphocytes exposed to

TL1A in combination with various cytokine or costimulatory molecules and established TNFR25 as a receptor that functioned to enhance Th1, but not Th2, cytokine secretion by activated T, NK, and NKT cells, potentially by lowering the threshold for TCR-induced activation.

TL1A:TNFR25 in Auto-aggressive Disease

These early functional data were complemented by human genetic studies implicating TNFR25:TL1A in autoimmune disease, particularly rheumatoid arthritis, inflammatory bowel disease, and type 1 diabetes [6, 13, 22, 23, 32, 41]. Because each of these diseases are variably characterized by a combination of type-1 inflammation, dependence upon immune complex formation or reactivity to antigens which should normally not cross the threshold for TCR activation, there was urgent need to begin examining the function of TL1A:TNFR25 in disease model systems in vivo. In the first of these studies, Cassatella et al. [8] demonstrated that immune complexes were potent inducers of soluble, but not membrane bound, TL1A in monocytes via binding to FcγRII/III. TL1A was subsequently found to be highly expressed by mononuclear phagocytes in synovial tissue samples from patients that were rheumatoid factor (RF) positive, but only weakly expressed in RF patients.

Our understanding of TL1A:TNFR25 in auto-aggressive disease models was greatly expanded in 2008 first with a pair of reports from our group and Linda Burkly/Chen Dong, which were followed with reports from Richard Siegel's, Stephen Targan's, and Eddie Chung Yern Wang's groups; each of these reports were first submitted between July–November of 2007 [7, 11, 18, 26, 33]. Fang et al. utilized a TNFR25-dominant negative (DN) transgenic mouse model together with a pair of TL1A-blocking or TNFR25-agonistic antibodies to study the effect of TL1A:TNFR25 in the development and severity of allergic lung inflammation. These studies provided first-evidence for a role of TL1A in the development of Th2-mediated inflammation associated with the production of IL-4, IL-5, and IL-13 that was dependent upon TNFR25 and NKT cells [11]. During primary antigen stimulation, TNFR25 was not required for but enhanced the production of IFNγ, IL-4, IL-10, and IL-13 by CD4+ lymph node cells. In contrast, during secondary antigen stimulation TNFR25 was required for the efficient production of IL-4, IL-5, IL-10, and IL-13, but not IFNγ, by CD4+ lymph node cells stimulated under ThN conditions [11]. Antibody-mediated blockade of TL1A was sufficient to prevent allergic lung inflammation in this model. In the same issue of the *Journal of Experimental Medicine*, Pappu et al. reported the generation of $TL1A^{-/-}$ mice [26]. In this report, the recently identified Th17 lineage of CD4+ T cells [39] were found to highly express TNFR25. TL1A signals as well as wild type, but not $TL1A^{-/-}$, dendritic cells enhanced the differentiation and proliferation of Th17 cells but were not required for the differentiation or proliferation of Th1-polarized CD4+ T cells. In accordance with these findings, $TL1A^{-/-}$ mice were found to have reduced disease severity and inflammatory cytokine production experimental allergic encephalomyelitis (EAE) [26], a Th17-dependent inflammatory disease

model [16]. The role of TL1A:TNFR25 in both allergic asthma and EAE were independently confirmed by Meylan et al., a report which also demonstrated that TNFR25 signals enhanced effector cell proliferation within the target organ, but not systemically. Further, these authors provided data confirming early reports that implicated TNFR25 signaling in CD4+ T cells in the production of IL-2, which subsequently enhanced CD4+ T cell proliferation and IFNγ production in the context of TCR stimulation [18]. The only disagreement between these three reports is whether or not TL1A enhances the differentiation of Th17 cells [18, 26].

Despite a number of early reports implicating TL1A expression in the development of inflammatory bowel disease, Takedatsu et al. [33] provided the first study to examine TL1A in an experimental model of chronic colitis in 2008. Using a model of dextran sodium sulfate induced chronic colitis, these studies demonstrated that expression of TL1A was increased in the mesenteric lymph nodes (mLN) and colon as well as by dendritic cells isolated from mLN of mice with chronic colitis. Accordingly, CD4+ T cells isolated from gut-associated lymphoid tissue demonstrated enhanced IFNγ, IL-6, and IL-17 secretion following ex vivo stimulation with TL1A either alone or in combination with IL-12 or IL-23. When administered either concurrent with the induction or during the recovery phase of DSS-induced chronic colitis, TL1A blocking antibodies significantly enhanced weight-gain during the recovery phase. Administration of anti-TL1A at the time of disease onset slightly reduced weight loss, which however failed to reach statistical significance. Treatment with TL1A-blocking antibodies reduced the expression of IFNγ, IL-6, and IL-17 by gut-associated lymphoid cells [33]. Parallel observations were also made by Bull et al. [7] in studying inflammatory arthritis in wild-type and TNFR25$^{-/-}$ mice. Similar to DSS-induced chronic colitis, the induction of articular inflammation by injection of methylated BSA was not significantly altered in TNFR25$^{-/-}$ mice, whereas the early resolution of inflammation was impaired in the presence of TNFR25 and correlated with decreased disease severity and bone destruction [7]. These results were recently confirmed using a similar arthritis model by Zhang et al., [42] who also demonstrated that TL1A can contribute to humoral responses including the production of Th2-polarizing cytokines such as IL-4 . It is intriguing that in both of these disease models, the contribution of TL1A toward pathological inflammation is most noticeable during the resolution, not the induction, of disease.

At the 12th international TNF conference, recent data on the role of TL1A:TNFR25 in disease were presented by Richard Siegel, Stephan Targan, and Eckhard Podack [35]. As mentioned above, TNFR25 is expressed by FoxP3+CD4+ regulatory cells (Treg). Podack presented data to show that an agonistic antibody to TNFR25, 4C12, inhibited the suppressive activity of Treg. Moreover, TNFR25 signaling inhibits the induction of FoxP3 on antigen specific CD4+ T cells by CD103+ dendritic cells. These data indicate that TL1A expression can disable the suppressive activity of preexisting Treg and prevent the de novo generation of Treg. The TL1A function on disabling Treg therefore supports a proinflammatory environment and explains why inhibition of TL1A is an effective strategy to block inflammatory disease.

TL1A:TNFR25 in Health?

Perhaps what is most conspicuous regarding the state of knowledge of TL1A:TNFR25 is that while this receptor ligand pair is clearly implicated in autoaggressive immunopathology, no obvious susceptibilities have yet been observed in mouse strains with germ line deficiencies in either TL1A or TNFR25; a state that clearly indicates the beneficial traits associated with genetic conservation of TL1A:TNFR25 expression have yet to be discovered. The roles that TNFR25 signaling plays in lowering the threshold for T-cell activation, enhancing proliferation of TCR-stimulated effector cells, and subsequent production of various effector cytokines by those cells could have obvious benefits in the protection from and clearance of various pathogens, particularly in the mucosal compartment. The relative efficiency of TL1A as a vaccine adjuvant in comparison to other TNFSF members underscores this specialization [14, 35]. To date, however, a contribution of TNFR25 signaling toward the protection or clearance of any pathogens has yet to be described.

As was reported during the 12th international TNF conference, our group has studied the role of TL1A:TNFR25 signaling on CD4+FoxP3+ regulatory T cells (Treg). In agreement with early observations by Pappu et al. [26], we have recently confirmed that TNFR25 is highly and constitutively expressed by Treg but is only expressed at a low level on non-activated CD4+FoxP3-conventional T cells (Tcon) (TH Schreiber unpublished data). By utilizing either the TNFR25-agonistic antibody, 4C12, together with various combinations of Tcon and/or Treg expressing DN-TNFR25 we have determined that TNFR25 signaling on Treg inhibits their ability to suppress the proliferation of Tcon in in vitro proliferation assays to a similar degree as GITR-agonistic antibodies (TH Schreiber unpublished data). Given the diverse activities of TNFR25 on Teff polarization, proliferation, and effector function, it is possible that a similar spectrum of Treg functions may be controlled by TNFR25 signaling events. Although only scattered reports exist, the suggestion that TNFR25 is important for the deletion of self-reactive thymocytes [37] indicates that under certain conditions TL1A can protect from inflammation [1], and the relatively unexplored role for constitutive expression of TNFR25 on Treg may implicate TNFR25 expression on Treg as a factor in the evolutionary conservation of this TNFSF receptor:ligand pair.

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Chapter 31
Identification of New Candidates as Mucosal Vaccine Adjuvant in TNF Family Cytokines

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Mucosal Immunity and Vaccine

Mucosal immunity forms the first line of defense against various infectious diseases. The majority of emerging and re-emerging pathogens, including *Vibrio cholerae*, pathogenic *Escherichia coli*, HIV (human immunodeficiency virus), and influenza virus, invade and infect via the mucosal surfaces of the host gastrointestinal, respiratory, and/or genitourinary tracts [3]. An important aspect of the immune response at mucosal surfaces is the production of polymeric immunoglobulin (Ig) A antibodies (Abs), as well as their transport across the epithelium and release as secretory IgA [2]. Because this IgA response represents the major mechanism of defense against viral and bacterial infections, recent efforts have been focused on the development of vaccines that are capable of efficiently inducing IgA production, as well as cytotoxic T-cell activation, in mucosal tissues.

Mucosal vaccines administered either orally or nasally are effective in inducing antigen-specific immune responses in both the systemic and the mucosal compartment [10]. Because of this two-layered protective immunity, the use of mucosal vaccines is likely to be an ideal strategy for combating both emerging and re-emerging infectious diseases (Fig. 31.1). However, the mucosal antigen-specific immune response is weak, because most protein antigens, such as non-living macromolecules or protein-subunit antigens, can evoke only a weak or undetectable adaptive immune response when they are applied mucosally [1]. Therefore, one strategy to overcome the weakness of the immune response is the co-administration of a mucosal adjuvant with the vaccine antigen [6]. Unfortunately, the development of a safe and effective mucosal adjuvant has proved to be challenging. As a potent mucosal vaccine adjuvant, cholera toxin (CT) or heat-labile toxin has been used

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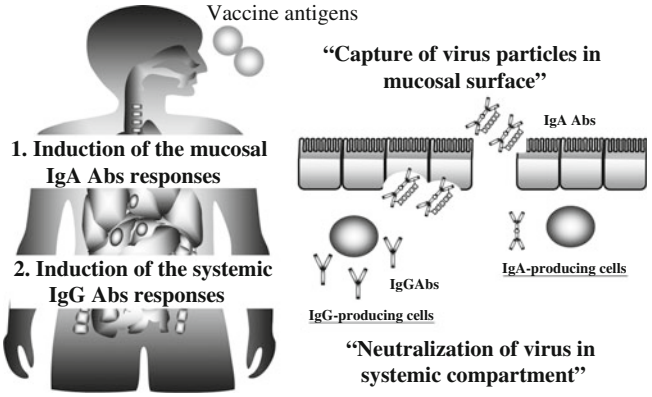

Fig. 31.1 Mucosal vaccine strategy

in experimental studies. However, the watery diarrhea induced by the administration of these toxins precludes their use as oral adjuvants in humans [4]. In addition, a recent report showed that administration of a human vaccine containing inactivated influenza virus, with heat-labile toxin as a mucosal adjuvant, resulted in a very high incidence of Bell's palsy [9]. Therefore, development of novel mucosal vaccine adjuvants with high efficacy and safety is urgently required for clinical applications.

Previously, we tested the potential for tumor necrosis factor (TNF)-α to function as a mucosal vaccine adjuvant. We showed that TNF-α could substantially enhance antigen-specific Ab responses at both the systemic and the mucosal level in mice [7, 8]. Furthermore, we have utilized a phage display system to create a functionalized TNF-α, and we have developed a mutant form of TNF-α, namely mTNF-K90R, which possesses six times the in vitro bioactivity and 13 times the in vivo bioactivity as wild-type TNF-α [11]. In addition, we previously demonstrated that intranasal administration of a model antigenic protein (ovalbumin; OVA) together with mTNF-K90R effectively induced the production of both antigen-specific IgG in the serum and IgA at mucosal sites (Fig. 31.2). Importantly, although mTNF-K90R had a potent adjuvant effect on mucosal immunity, it did not elicit signs of excessive inflammation, such as edema or fibrosis [7, 8]. These findings indicate that our technique of creating bioactive mutant cytokines might be attractive for designing novel mucosal adjuvants that are effective and safe.

Identification of New Candidate Mucosal Vaccine Adjuvants Among TNF Superfamily Cytokines

Previously, we reported that use of TNF-α as a mucosal vaccine adjuvant augmented both the antigen-specific systemic IgG Ab response and the mucosal IgA Ab response in mice [7, 8]. However, it is not yet known which of the TNF superfamily cytokines is the most potent mucosal adjuvant for augmenting antigen-specific

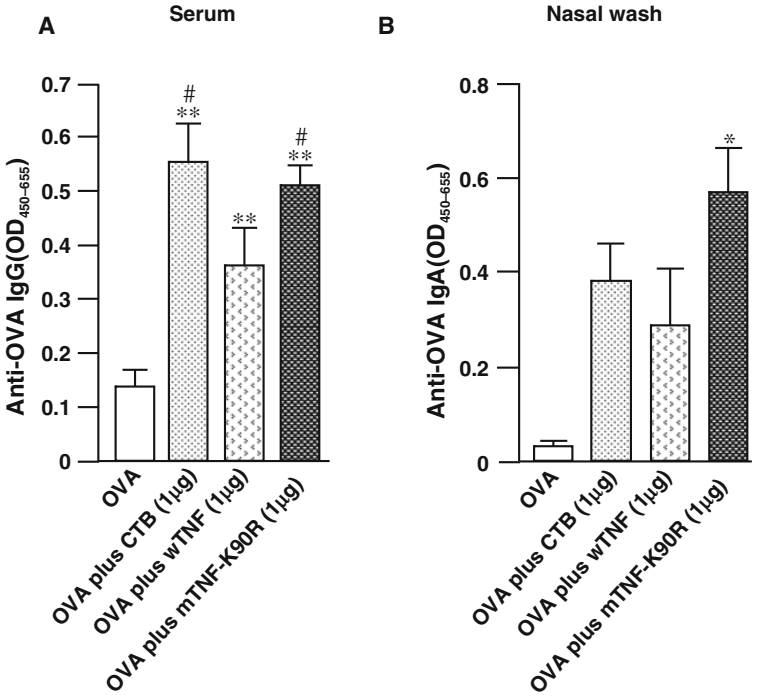

Fig. 31.2 Ovalbumin (OVA)-specific Ig antibody response after nasal immunization with OVA plus adjuvant. BALB/c mice were intranasally immunized once a week for three consecutive weeks with OVA alone, OVA plus 1 μg cholera toxin B subunit (CTB), OVA plus 1 μg wild-type tumor necrosis factor (wTNF)-α, or OVA plus 1 μg mutant (m)TNF-K90R. Serum and nasal wash was prepared 1 week after the last immunization and analyzed by ELISA (enzyme-linked immunosorbent assay) for OVA-specific IgG (**a**) at a 1:100 dilution of serum and OVA-specific IgA (**b**) at a 1:8 dilution of nasal wash. Data represent the means of absorbance a wavelength of 450 nm (reference wavelength, 655 nm). ND, not detected. Data are presented as means ± SEM ($n = 7$; $^{*}P < 0.05$, $^{**}P < 0.01$ versus value for group treated with OVA alone, by ANOVA; $^{\#}P < 0.05$ versus value for group treated with OVA plus wTNF-α, by ANOVA)

Ig Ab responses in both the mucosal and the systemic immune compartment. We attempted to identify the powerful mucosal adjuvants among 16 different TNF superfamily cytokines.

Currently, more than 40 members of the TNF/TNF receptor (TNFR) superfamily have been identified, and the majority are expressed by immune cells. The biological functions of this system encompass beneficial and protective effects in inflammation and host defense, as well as a crucial role in immune organogenesis [5]. Some TNF superfamily cytokines already being used as vaccine adjuvants[0] to enhance primary and memory immune responses against cancer and infectious diseases are among the candidates for selection of a powerful but safe adjuvant. For example, 4-1BBL, CD27L, CD30L, GITRL, LIGHT, OX40L, and TNF-α,

which have costimulatory functions for the survival, expansion, and effector function of T cells, enhance systemic immunity to co-administered antigens [12]. However, there have been no comparative studies of the potential of TNF superfamily cytokines as mucosal vaccine adjuvants. To compare the mucosal adjuvant activities of TNF superfamily cytokines, we intranasally immunized mice with 100 μg OVA plus each of 16 different TNF superfamily cytokines (APRIL, BAFF, 4-1BBL, CD27L, CD30L, CD40L, EDA, GITRL, LIGHT, LT-α, OX40L, TL1A, TNF-α, TRAIL, TRANCE, and TWEAK; 1 μg/mouse) three times at weekly intervals. Intranasal immunization with OVA plus TL1A gave significantly higher OVA-specific IgG responses in the serum than did immunization with OVA alone (Fig. 31.3a). The OVA-specific IgG level in TL1A immunized mice was of similar magnitude to that induced by cholera toxin B subunit (CTB). In addition, intranasal immunization with OVA plus APRIL or TNF-α gave a strong OVA-specific IgG response in the serum. The highest OVA-specific IgG Ab responses were seen in mice immunized with OVA plus TL1A as a mucosal adjuvant, from

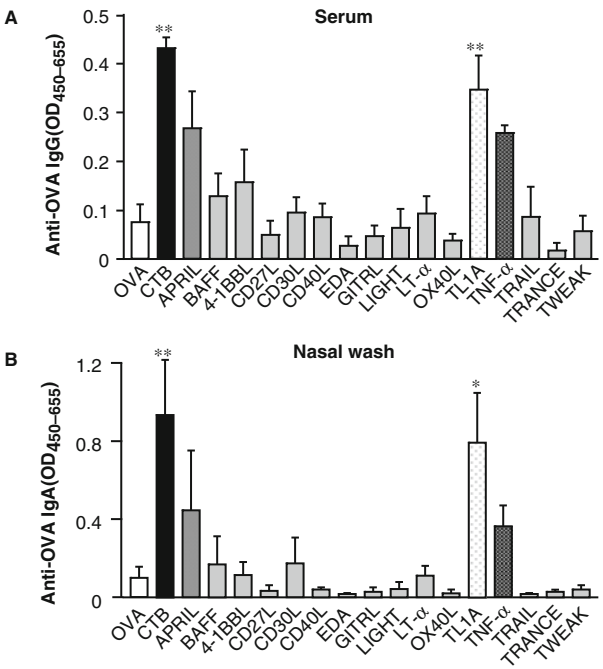

Fig. 31.3 OVA-specific Ig responses to intranasal immunization with ovalbumin (OVA) plus tumor necrosis factor (TNF) superfamily cytokines. BALB/c mice were intranasally immunized three times at weekly intervals with OVA alone, OVA plus cholera toxin B subunit (CTB), or OVA plus each TNF superfamily cytokine. Serum and nasal wash were collected 7 days after the last immunization and analyzed by ELISA for OVA-specific (**a**) IgG at a 500-fold dilution and OVA-specific (**b**) IgA at a 50-fold dilution. Data are presented as means ± SEM ($n = 5$; * $P < 0.05$, ** $P < 0.01$ versus value for group treated with OVA alone, by ANOVA)

among 16 different TNF superfamily cytokines (Fig. 31.3a). Next, to identify the characteristics of TNF superfamily cytokines as mucosal adjuvants, we examined OVA-specific IgA responses in nasal washes. Mice immunized with OVA plus APRIL or TNF-α tended to show strong OVA-specific IgA responses. Importantly, the OVA-specific IgA level was significantly higher in TL1A-immunized mice than in mice immunized with OVA alone; the level was similar to that induced by CTB (Fig. 31.3b). Collectively, these results demonstrated that TL1A was the most potent mucosal adjuvant among the TNF superfamily cytokines for inducing OVA-specific systemic IgG and mucosal IgA Ab responses; TL1A might therefore be candidate for a mucosal vaccine adjuvant to replace toxin-based adjuvants.

Next, to clarify the mechanisms of the immune response to the addition of each TNF superfamily cytokine, we analyzed the release profiles of cytokines from splenocytes of immunized mice. Culture supernatants from OVA-stimulated splenocytes collected from immunized mice were assessed for the Th2-type cytokines IL-4 and IL-5 and the Th1-type cytokines IFN-γ and TNF-α, using a multiplexed immunobead-based assay. Splenocytes from mice immunized with OVA plus APRIL, TNF-α, or TL1A exhibited higher levels of Th2-type cytokines (IL-4 and IL-5) than those immunized with OVA alone. In contrast, there was little difference in Th1-type cytokine (IFN-γ and TNF-α) secretion among all types of immunized mice. These results suggested that the addition of APRIL, TNF-α, or TL1A as a mucosal vaccine adjuvant induced a strongly polarized Th2-type immune response. Collectively, our results indicated that TL1A induced the strongest mucosal immunity among the TNF superfamily cytokines. This is the first report to demonstrate the activity of TL1A as a mucosal adjuvant inducing antigen-specific systemic and mucosal immune responses in mice.

Concluding Remarks

To our knowledge, this study is the first to clearly demonstrate the potential of TNF superfamily cytokines as mucosal adjuvants. In summary, among the TNF superfamily cytokines, APRIL, TL1A, and TNF-α induced two layers of protective immunity when administered intranasally with a vaccine antigen. TL1A was more effective than other TNF superfamily cytokines as a mucosal vaccine adjuvant. In the future, application of our technology to the creation of mutant TL1A could lead to the generation of a promising mucosal adjuvant that is both effective and safe.

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Chapter 32
The TWEAK/Fn14 Pathway in Tissue Remodeling: For Better or for Worse

Linda C. Burkly and Taeko Dohi

Introduction

Tissues subjected to stress, for example due to mechanical injury, environmental exposures, or endogenous signals, possess a remarkable ability to respond, adapting to limit stress, protect the tissue from further injury, and promote normal tissue recovery. This involves the coordinated activation and regulation of multiple processes, including infiltration by inflammatory cells to remove damaged cells and debris, angiogenesis, extracellular matrix turnover, and progenitor cell expansion and differentiation to regenerate normal tissue. However, in some circumstances inappropriate tissue responses occur, for example acute or prolonged overreactions, wherein the underlying processes are exaggerated and dysregulated, resulting in maladaptive tissue remodeling. A prominent feature of such pathological tissue responses is robust inflammation, as occurs in chronic autoimmune and inflammatory diseases, leading to cell death, progressive damage, and pathological remodeling of the disease target tissue.

Physiological and pathological tissue remodeling are mediated by a complex network of cell types and molecular players which are still being unraveled and identified. In the past decade, the cytokine tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) and its receptor FGF-inducible molecule-14 (Fn14) have emerged as a ligand/receptor pair of the TNF superfamily that is prominently featured in normal and pathological remodeling of tissues. TWEAK expressed primarily as a soluble cytokine by infiltrating leukocytes mediates multiple activities through Fn14 upregulated locally in injured and diseased target tissues, including proinflammatory responses, angiogenesis, and cell growth and death. This paradigm applies broadly across a variety of organ systems (Fig. 32.1). A growing body of evidence supports the thesis that these TWEAK/Fn14-induced activities contribute to normal tissue regeneration and repair when appropriately orchestrated

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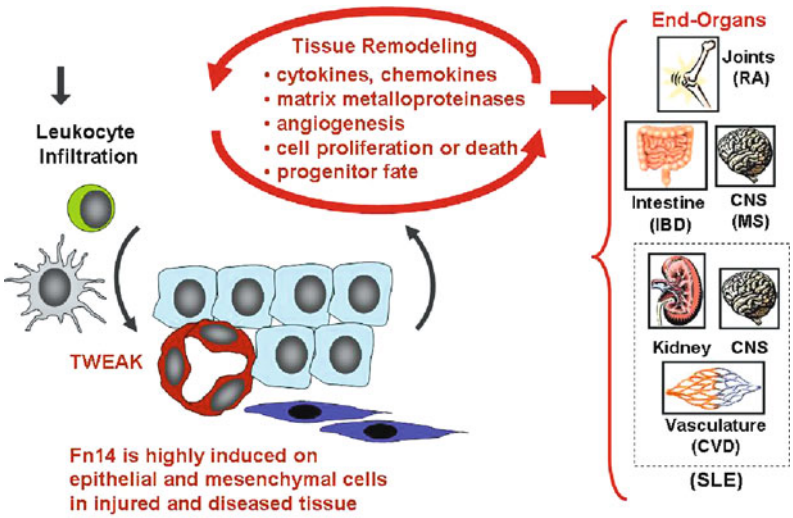

Fig. 32.1 TWEAK/Fn14 pathway coordinates local tissue remodeling. TWEAK expressed primarily as a soluble cytokine by infiltrating leukocytes mediates multiple activities through Fn14 upregulated locally on epithelial and mesenchymal cell types in injured and diseased target tissues, including proinflammatory responses, angiogenesis, cell growth and death, and progenitor responses. TWEAK/Fn14-induced tissue remodeling is a mechanism that is broadly applicable across a variety of organs/disease target tissues. When appropriately orchestrated after acute tissue injury, these activities contribute for better, to normal tissue regeneration and repair, but when exaggerated and dysregulated, these activities contribute for worse, to progressive local tissue damage and maladaptive remodeling. RA, rheumatoid arthritis; IBD, inflammatory bowel disease; MS, multiple sclerosis; CVD, cardiovascular disease; SLE, Systemic Lupus Erythematosis

after acute tissue injury, as supported in acute models of liver and skeletal muscle injury; in contrast, when exaggerated and dysregulated, these TWEAK/Fn14-induced activities contribute to progressive local tissue damage and maladaptive remodeling, as supported in models of inflammatory disease. Given the role of the TWEAK/Fn14 pathway in regulating cell survival, growth, migration, and angiogenesis, implications for the role of this pathway in tumor biology have also been discussed [1, 2].

Complementing two comprehensive review articles on the TWEAK/Fn14 pathway [1, 2] and a synthesis of evidence supporting the above-stated thesis [1, 3], this proceeding focuses on the effects of the TWEAK/Fn14 pathway on tissue remodeling, primarily effects contributing to worsening outcomes, through review of our expanding knowledge of pathological roles of TWEAK/Fn14 in models of disease. This proceeding also highlights our novel observations of the effects of the TWEAK/Fn14 pathway on tissue remodeling in contexts of intestinal injury [4], where (1) TWEAK signaling through Fn14 upregulated on intestinal epithelial cells regulates epithelial cell turnover, which may be beneficial or

detrimental to tissue repair, depending on the context, and (2) TWEAK/Fn14-mediated induction of innate immunity promotes local intestinal inflammation and tissue degrading enzymes, thereby contributing to tissue destruction.

TWEAK

TWEAK is synthesized as a Type II transmembrane protein in the endoplasmic reticulum and considered to be primarily a soluble cytokine due to efficient cleavage by members of the furin protease family located within the trans Golgi network [5]. TWEAK is constitutively expressed by leukocytes, including monocytes/macrophages, dendritic cells, neutrophils, natural killer cells, and T cells, and by cell types that reside in the tissues, including endothelial cells, although there is relatively little known about the regulation of TWEAK expression [1].

The presence of membrane bound TWEAK has also been observed [6–8], although these reports need to be interpreted cautiously as we have not reproduced this finding with erythrocytes, peripheral blood T cells, and IFNγ-stimulated monocytes from healthy subjects (LC Burkly, unpublished). However, we and others have found cell surface TWEAK on the monocytes from some patients, particularly those with a clinical isolated syndrome suggestive of multiple sclerosis (CISSMS) that later converted to definitive MS [9]. Although larger studies are needed to confirm these intriguing findings, they suggest the possibility that surface TWEAK expression by monocytes may be a marker of monocyte activation which is known to correlate with MS and other autoimmune disease activity.

Given that leukocytes are a major source of TWEAK, it is not surprising that TWEAK expression can be increased locally in the target tissues in animal models of disease with inflammatory cell infiltrates. Notably, TWEAK levels are also elevated in the tissues and fluids of humans with inflammatory diseases, including the synovial tissues, synovial fluid, and sera of patients with rheumatoid arthritis (RA) [10], in the sera of patients with multiple sclerosis (MS) (LC Burkly, unpublished) as well as in MS brain tissue where it was associated with chronic active lesions but not inactive lesions [11], and in the sera of patients after ischemic stroke [12]. In addition, in patients with systemic lupus erythematosus (SLE), urinary TWEAK levels are elevated in patients with nephritis and correlate with renal disease activity scores [13]. The elevated TWEAK levels in human autoimmune and inflammatory diseases and associations with disease activity support its potential role in disease pathogenesis.

Fn14

Fn14, the widely recognized TWEAK receptor, is the smallest TNFR superfamily member with only one cysteine-rich domain (CRD) [14]. Originally cloned as a bFGF-inducible molecule [15], Fn14 is also highly induced in response to other

growth factors, including PDGF and VEGF, on the surface of epithelial cells, endothelial, and other mesenchymal cell types. Importantly, Fn14 is normally expressed at low levels in healthy tissues, and highly induced in contexts of tissue injury and disease, likely in response to one or more of these growth factors [3].

In addition to growth factors, a variety of other Fn14-inducing stimuli have been more recently identified, providing further clues as to where, when, and how the TWEAK/Fn14 pathway may function. For example, pertinent to the topic of this proceeding, our studies showed that Fn14 expression was induced on intestinal epithelial cells through Toll-like receptor (TLR) signaling by an oligodeoxynucleotide containing CpG motifs which mimics bacterial DNA [4]. This observation suggests that in the context of intestinal injury, epithelial cells responding to microbial components may upregulate Fn14, which then engages constitutively available TWEAK and induces an innate inflammatory response. Consistent with TWEAK/Fn14 pathway regulation of innate immunity, with little if any effect on systemic adaptive immunity in this study [4] and other studies [16, 17], Fn14 is not expressed by resting or activated T or B cells.

The highly inducible nature of Fn14 expression has also been clearly established in vivo in numerous experimental contexts of injury and disease, including the liver after partial hepatectomy and chemical injury [6, 18] and (J. Tirnitz-Parker, personal communication) heart after myocardial infarct [19] and (R. Liao, personal communication), vasculature after balloon catheter injury [14] and in atherosclerotic plaques [20], skeletal muscle after chemical injury [21], kidney after acute kidney injury (AKI) [22], intestinal epithelium after 2,4,6-Trinitrobenzene sulfonic acid (TNBS)-induced injury [4] and γ-irradiation injury (T. Dohi, personal communication), and spinal cord of mice with experimental autoimmune encephalomyelitis (EAE) [23] and in models of stroke [24, 25]. Importantly, the regulation of Fn14 expression appears to be similar between mouse and humans [3], with relatively low Fn14 levels in normal human tissues and strong Fn14 expression in human inflammatory disease tissues, including RA synovium [10], MS brains [11] and inflammatory liver diseases [26]. Particularly pertinent to this article, Fn14 has also been shown to be upregulated in colonic biopsies from patients with human inflammatory bowel disease (IBD) as compared to healthy subjects, and particularly in those with ulcerative colitis (UC) (Fig. 32.2).

Fn14 Signaling

Once upregulated, Fn14 signals through a 28 amino acid cytoplasmic tail that associates with TNF receptor associated factors (TRAFs) to activate NFκB. Interestingly, TWEAK not TNF is capable of inducing prolonged NFκb activation through biphasic induction of the canonical and noncanonical pathways [27]. The prolonged NFκB activation by TWEAK may have implications for its unique contribution to the pathogenesis of chronic inflammatory diseases. Through this activity, TWEAK/Fn14 signaling may promote inflammation and tissue damage, driving

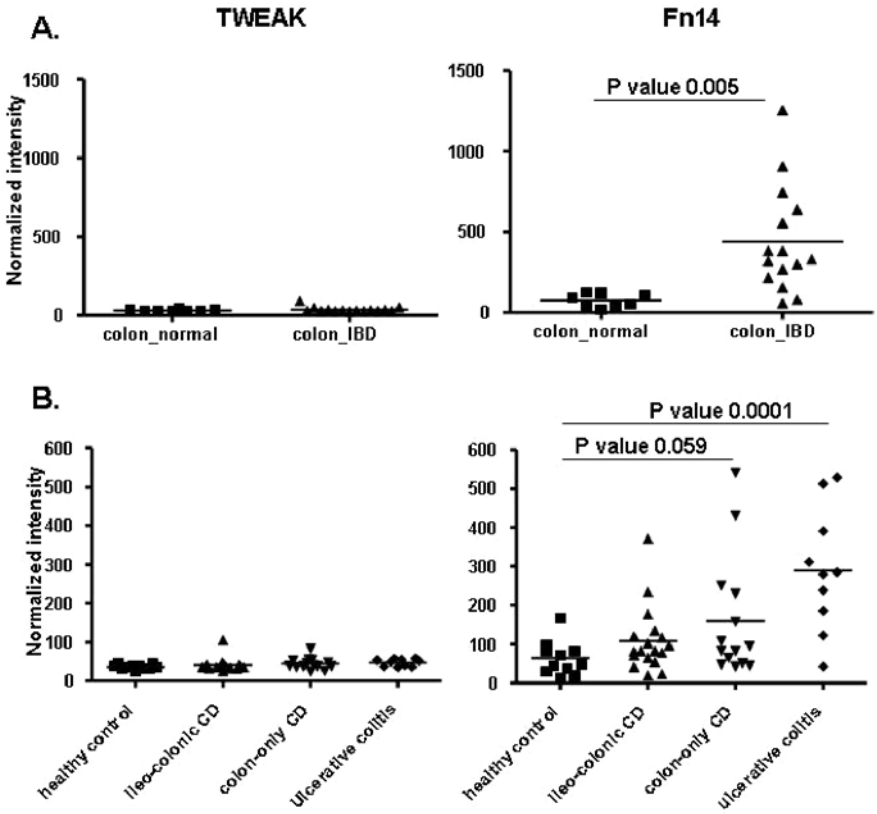

Fig. 32.2 TWEAK and Fn14 mRNA expression in human colon biopsies from patients with IBD and healthy control subjects. (**a**) Raw unfiltered dataset of [48, 49], made publicly available through the National Center for Biotechnology Information (NCBI) GEO Datasets, accession number GSE4183, are plotted as normalized intensity, with $n = 8$ normal controls and $n = 15$ with IBD. (**b**) Raw unfiltered dataset of [50], made publicly available through the NCBI GEO Datasets, accession number GSE10616, are plotted as normalized intensity. Colon biopsies were from healthy controls $n = 11$; the most proximal affected segment of the colon of Crohn's disease patients with ileo-colonic $n = 18$, and colon-only $n = 14$; $n = 10$ with UC. In both (**a**) and (**b**), the normalized intensity values are shown in linear scale (converted from raw data provided as normalized intensity log base 2), with horizontal bars indicating mean values. Both datasets show that TWEAK mRNA is constitutively expressed at low levels and is not different between normal and IBD subjects, whereas Fn14 is present at relatively low levels in healthy controls and increased in IBD patients

end-organ pathology through upregulation of adhesion molecules on endothelial cells, tissue production of cytokines, chemokines and degradative enzymes, promotion of angiogenesis, vascular permeability, and pathological hyperplasia [3].

TWEAK-induced death of Fn14-expressing cells may also be an important mechanism contributing to tissue remodeling in vivo. The ability of TWEAK to signal through Fn14 to mediate cell death was initially described using tumor

cell lines, and may occur through multiple pathways, including caspase-dependent and caspase-independent, with features of both apoptosis and cathepsin-B dependent necrosis [5, 28]. Since Fn14 lacks a death domain, the signaling mechanism is not understood, although two recent studies elucidated a mechanism involving TRAF2/cIAP depletion in TWEAK-primed tumor cell lines that resulted TNFα/TNFR1 mediated tumor cell death [29, 30]. The cell killing mechanism remains to be determined in other tumor cell lines and cell types where TWEAK-induced death is TNF-independent [28]. Thus far, there have been a few reports where TWEAK was shown to induce cell death in normal, primary cell types in vitro, including human peripheral blood monocytes [7] and NK cells [31]. It is intriguing to speculate that this activity is beneficial, as a means for TWEAK to contribute to the resolution of ongoing inflammation. TWEAK has also been shown to induce death of murine cortical neurons under conditions of hypoxia in vitro and in vivo [24], possibly also reflecting a beneficial function, a means to promote the removal of injured cells. The latter is also suggested by our recent observation that TWEAK promotes apoptosis of intestinal epithelial cells after γ-irradiation injury [4]. In other contexts such as in inflammatory diseases, TWEAK-induced cell death may contribute to progressive tissue destruction and impede repair, as suggested in TNBS-induced colitis, where TWEAK exacerbates the severity of epithelial ulcers [4]. It is currently not known in these intestinal models whether TWEAK act alone or indirectly involves TNF. However, in a model of acute folic acid-induced kidney tubular cell injury, TWEAK may act in conjunction with TNF to promote tubular cell death [22].

Role of TWEAK/Fn14 Pathway in Disease

In recent years, evidence has greatly expanded for pathological tissue remodeling roles of the TWEAK/Fn14 pathway, spanning a wide range of organ systems (Table 32.1.)

Direct evidence for the disease-driving capacity of TWEAK/Fn14 signaling, including tissue degeneration and maladaptive remodeling, comes from studies of TWEAK overexpression in mice. Perhaps the most dramatic example of this is the profound dilated cardiomyopathy exhibited by TWEAK transgenic (Tg) mice [32]. In another striking example, TWEAK overexpression in skeletal muscle or by exogenous administration has been reported to induce skeletal muscle wasting in young mice [33]. TWEAK overexpression also promotes features of neuroinflammatory and neurodegenerative disorders, including exacerbated MOG-induced EAE in TWEAK Tg mice [34], and breakdown of the blood–brain barrier induced by intracerebral TWEAK injection in normal mice [35]. Furthermore, exogenous TWEAK administration in normal mice induced many features of inflammatory kidney disease, including the production of chemokines, other proinflammatory cytokines, mild kidney inflammation, and glomerular and tubular proliferation [36, 37, 38]. Still more recently, it has been shown that

Table 32.1 TWEAK/Fn14 pathway-mediated pathological tissue remodeling: evidence from TWEAK overexpression and pathway inhibition in disease models

Disease target tissue (human disease)	Potential Fn14-expressing cell types in the diseased tissue	Effect of TWEAK overexpression	Animal Model	Effect of TWEAK/Fn14 pathway inhibition	References
Heart (heart failure)	Cardiomyocytes, fibroblasts, and vascular cells	Dilated cardiomyopathy and heart failure in TWEAK Tg mice; similar phenotype in normal mice injected with a TWEAK-expressing adenoviral vector			[32]
Intestine (Inflammatory bowel disease)	Intestinal epithelial cells, fibroblasts, smooth muscle cells, and vascular cells		TNBS-induced colitis in Balb/c mice	TWEAK KO mice exhibit reduced clinical disease severity, ulcers, and inflammatory cell infiltration; similar effect in wild-type mice treated with a TWEAK-blocking mAb	[4]
			TNBS-induced colitis in C57Bl/6 mice	TWEAK or Fn14 KO mice exhibit reduced ulcers and inflammation score	[4]
Kidney (inflammatory kidney diseases)	Kidney glomerular mesangial cells and podocytes, tubular epithelium, vascular cells, and macrophages	Exogenous TWEAK administration induces chemokines, macrophage infiltration, and proliferation of glomerular and tubular cells			[36, 37, 38]

Table 32.1 (continued)

Disease target tissue (human disease)	Potential Fn14-expressing cell types in the diseased tissue	Effect of TWEAK overexpression	Animal Model	Effect of TWEAK/Fn14 pathway inhibition	References
Kidney (lupus nephritis)			Chronic GVHD-induced lupus nephritis by H-2^{bm12} splenocyte transfer into H-2^{b} mice	Fn14 KO hosts have reduced proteinuria, kidney cell proliferation, Ig deposition, proinflammatory cytokines, chemokines, and macrophage infiltrates; similar findings in normal hosts treated with a TWEAK-blocking mAb	[17]
Kidney (acute renal failure)			Folic acid-induced acute kidney injury in mice	TWEAK KO exhibits improved renal function and decreased tubular cell death; similar effects in wild-type mice treated with TWEAK-blocking mAb as well as reduced chemokines and tubulointerstitial inflammation	[38, 42]
Liver (inflammatory liver diseases)	Liver progenitor cells (oval cells), biliary duct epithelial cells, stellate cells, hepatocytes, and vascular cells	Proliferation of liver progenitor cells in TWEAK Tg mice, and in mice injected with a TWEAK-expressing adenoviral vector			[26]

Table 32.1 (continued)

Disease target tissue (human disease)	Potential Fn14-expressing cell types in the diseased tissue	Effect of TWEAK overexpression	Animal Model	Effect of TWEAK/Fn14 pathway inhibition	References
Joint (rheumatoid arthritis)	Fibroblast-like synoviocytes, chondrocytes, osteoblasts, mesenchymal lineage progenitors, and vascular cells		Pristane-primed, collagen-induced arthritis in mice	TWEAK-blocking mAb reduced clinical severity, joint inflammation, cartilage and bone loss, and synovial angiogenesis	[16]
			Collagen-induced arthritis in mice	TWEAK-blocking mAb reduced clinical severity, joint inflammation, and synovial angiogenesis	[39]
Nervous tissue (neuroinflammatory/neurodegenerative diseases)	Astrocytes, microglia, neurons, and vascular cells	Intracerebral injection of TWEAK in normal mice disrupts the neurovascular unit resulting in increased blood–brain barrier permeability with cerebral edema			[35]
Nervous tissue (stroke)			Permanent MCAO	Fn14 KO mice exhibit reduced infarct volume and cerebral edema, and increased motor activity	[44]

Table 32.1 (continued)

Disease target tissue (human disease)	Potential Fn14-expressing cell types in the diseased tissue	Effect of TWEAK overexpression	Animal Model	Effect of TWEAK/Fn14 pathway inhibition	References
				TWEAK-blocking mAb reduced infarct volume	[24]
				Fn14-Fc fusion protein reduced infarct volume, microglial activation and neuronal cell death, and cerebral edema, and improved motor function	[25, 35, 44]
Nervous tissue (multiple sclerosis)		TWEAK Tg mice exhibit increased EAE clinical severity	MOG_{35-55} and pertussis toxin-induced EAE in mice	TWEAK-blocking mAb reduced clinical severity and neuroinflammation	[34; 23]
			MOG_{1-125}-induced EAE in rats, and $PLP_{139-151}$ induced EAE in SJL/J mice	Antibodies to TWEAK or Fn14 raised by DNA vaccination reduced EAE clinical severity	[40]
			Cuprizone-induced demyelination	TWEAK KO mice exhibit delayed demyelination and microglial/macrophage accumulation	[43]
Skeletal muscle (muscle wasting diseases)	Skeletal muscle myofibers, and myoblasts	TWEAK Tg mice or normal mice treated with exogenous TWEAK have reduced body weight and skeletal muscle atrophy			[33]

Table 32.1 (continued)

Disease target tissue (human disease)	Potential Fn14-expressing cell types in the diseased tissue	Effect of TWEAK overexpression	Animal Model	Effect of TWEAK/Fn14 pathway inhibition	References
Vascular (atherosclerosis)	Vascular cells, macrophages, and foam cells	Exogenous TWEAK exacerbates vascular and renal lesions, promotes chemokine expression and macrophage infiltration in atherosclerotic plaques and kidney	$APOE^{-/-}$ mice fed a hyperlipidemic diet	TWEAK-blocking mAb attenuates vascular and renal lesions, chemokine expression, and macrophage infiltration in atherosclerotic plaques and kidney	[20]
			$APOE^{-/-}$ mice fed a normal diet	Fn14-Fc fusion protein treatment did not prevent lesion initiation but inhibited some features of plaque progression, and induced a unique, advanced plaque phenotype with increased macrophage content and smaller macrophage size	[41]

in ApoE–/– mice fed a hyperlipidemic diet, exogenous TWEAK injection exacerbates the severity of atherosclerotic plaques and renal lesions [20]. These observations demonstrate that overly activated TWEAK/Fn14 signaling can induce tissue pathologies and imply an important contribution of endogenous TWEAK/Fn14 pathway signaling in inflammatory diseases.

The disease-driving activities of the endogenous TWEAK/Fn14 pathway have indeed been validated in multiple, independent animal models of human autoimmune and inflammatory disease by treatment with TWEAK neutralizing monoclonal antibodies (mAbs) and in TWEAK or Fn14 deficient mice (Table 32.1). Disease severity was reduced by anti-TWEAK-blocking antibodies in collagen-induced arthritis [16, 39], a model of human RA, and in peptide-induced EAE [23, 40]. In chronic graft versus host disease-induced lupus-nephritis, a model of human SLE nephritis, either anti-TWEAK mAb treatment of wild-type mice or Fn14 deficiency reduced disease severity [17]. Recently, disease-modifying effects were also observed in a model of human atherosclerosis, by Fn14-Fc fusion protein treatment of ApoE–/– mice [41], as well as a beneficial effect of anti-TWEAK mAb treatment in ApoE–/– mice fed a hyperlipidemic diet [20].

In addition, anti-TWEAK mAbs or TWEAK or Fn14 deficiency has proven beneficial in animal models of acute injury, wherein the underlying processes induced and pathological outcomes are akin to those in human inflammatory diseases. For example, in folic-acid induced AKI, a model featuring interstitial inflammation and tubular cell death that are likewise observed in human inflammatory kidney diseases, TWEAK inhibition resulted in reduced renal inflammation and improved renal function [38, 42]. In TNBS-induced colitis, a model with some features of IBD including inflammation, epithelial ulcers and crypt deformity, anti-TWEAK mAb treatment of wild-type mice, TWEAK or Fn14 deficiency resulted in reduced clinical severity, inflammation, and colon epithelial damage and crypt abnormality [4]. In the cuprizone-induced model of demyelination, a model which shares some features of MS, TWEAK-deficient mice exhibited significant delays in both microglial/macrophage accumulation at the site of CNS injury and demyelination [43]. In the setting of permanent distal middle cerebral artery occlusion (MCAO), a model of human stroke, blocking TWEAK with anti-TWEAK mAb or an Fn14-Fc fusion protein, or Fn14 deficiency reduced the infarct volume and ischemia-associated inflammatory response [24, 25, 35, 44]. Thus the pathological contribution of the TWEAK/Fn14 pathway has been demonstrated in disease models across a variety of organ systems, supporting that TWEAK/Fn14 pathway activation is a universal mechanism contributing to pathological tissue damage and maladaptive remodeling associated with autoimmune and inflammatory disorders.

Role of TWEAK/Fn14 in Intestinal Inflammation and Injury

In recent years, epithelial cells have been increasingly recognized as critical players in the innate immune system of the intestine, with roles in both health and inflammatory disease [45–47]. It is now appreciated that mucosal health is maintained by

normal epithelial turnover, maintenance of the epithelial barrier integrity, and antimicrobial responses, all of which involve an underlying innate response by mucosal epithelial cells to commensal flora. Although not fully understood, it is thought that in addition to the usual players, namely various types of mucosal leukocytes, the epithelia can contribute to inappropriate responses to commensal or pathogenic microbes, diet or stress, thereby contributing to inflammatory bowel diseases, characterized by chronic inflammation, intestinal tissue destruction, and insufficient or abnormal repair reflected by crypt branching in case of ulcerative colitis and the development of strictures, which characterizes the lesion of Crohn's disease.

We investigated the role of the TWEAK/Fn14 in the gastrointestinal tract since inducible expression of Fn14 is well documented in epithelial cell types and TWEAK/Fn14 signaling regulates innate immune responses in epithelial cells. Based on prior demonstrations of the role of the TWEAK/Fn14 pathway in pathological tissue remodeling in models of inflammatory disease (see above), we hypothesized that this pathway might promote an inappropriate innate response on the part of epithelial cells expressing Fn14 in the injured gut, thereby contributing to tissue damage and impeding tissue repair. The results of these studies have recently been published [4].

Using a model of acute intestinal damage induced by TNBS in Balb/c mice, we found that Fn14 was upregulated early (day 3) and later (day 10), and TWEAK was constitutively present, suggesting that this pathway may play a role in TNBS-induced colitis. To study the role of the TWEAK/Fn14 pathway in this context, we used TWEAK-deficient mice. These mice were healthy and normal with no detectable difference from wild-type mice, including no difference in intestinal morphology or gene expression profiles from wild-type mice. In addition, analysis of immune compartments revealed no apparent differences in immune cell subsets.

TWEAK deficient and wild-type Balb/c mice were given TNBS/EtOH intrarectally, causing colon epithelial injury, resulting in acute inflammation and further tissue damage. Early clinical responses, including weight loss, diarrhea, bloody stool and mortality, resulting from the initial tissue injury and innate inflammatory responses to that injury, were analyzed on day 3. A second dose of TNBS was administered on day 7, resulting in a later response involving innate as well as adaptive immune components by day 10. We found that TWEAK deficiency reduced clinical severity at both the early and later time points as compared to that in wild-type mice. Consistent with this, histology scores reflecting the extent of epithelial ulcers and colon inflammation, and corresponding crypt deformity, were reduced in TWEAK deficient mice. Further analysis showed reductions in both neutrophil and macrophage infiltrates in the TWEAK deficient mice. Likewise, reductions in clinical severity and histological score were obtained with anti-TWEAK mAb treatment of wild-type mice as compared to control Ig treated mice, with either prophylactic or therapeutic dosing regimens. Notably, consistent with the lack of Fn14 expression on lymphocytes, TWEAK pathway deficiency did not significantly impact the systemic adaptive immune response to TNP, as measured by sacral lymph node TNP-specific T cell proliferation and circulating anti-TNP antibody titers. Thus these studies reveal a novel aspect of local innate immune regulation by

the TWEAK/Fn14 pathway that contributes to pathology severity in the context of intestinal injury and inflammation.

In order to elucidate the mechanism for protection from TNBS-induced colitis in TWEAK-deficient mice, gene expression profiling was conducted from colon tissue of TWEAK deficient and wild-type mice. Strikingly, we found that a wide array of chemokines and MMPs commonly induced by NFκB signaling were greatly increased after TNBS-induction in wild-type mice, but only modestly induced, if at all, after TNBS treatment of TWEAK deficient mice. In addition, cytokines well known to play a role in intestinal inflammation, such as IL-1 and IL-6, were highly upregulated in TNBS-induced wild-type but not TWEAK deficient mice. Thus it appears that by eliminating TWEAK/Fn14 signaling and its downstream NFκB activation, the pathogenic induction of proinflammatory genes and tissue degrading enzymes was reduced and pathological colon remodeling thereby markedly attenuated.

To further elucidate the pathogenic mechanism of TWEAK in TNBS-induced colitis, we identified the cells expressing TWEAK and its receptor Fn14. TWEAK protein was expressed at low levels by mucosal epithelial cells and did not increase after TNBS injury. TWEAK may also be expressed by infiltrating leukocytes; however, our tissue analysis was equivocal, warranting further investigation. As expected based on the highly inducible nature of Fn14 expression, Fn14 was expressed at minimal levels before disease induction and dramatically upregulated in the colonic mucosal epithelial cells after TNBS injury. The stimulus for Fn14 upregulation in vivo is currently not known, but may result from responses to microbial components or other cytokines after injury, as we found that Fn14 was induced by CpG (mimicking bacterial DNA) or TNF in colon explants. Furthermore, we directly demonstrated that TWEAK can induce innate inflammatory responses through Fn14 expressed by colon epithelial cells using in vitro cultures, as evidenced by significantly induced MMP-9, MCP-1, and KC production. Thus, our studies support that TWEAK signaling through Fn14 upregulated after TNBS injury on colonic mucosal epithelial cells, likely by exposure to microbial products or cytokines, promotes innate inflammatory responses including chemokines, cytokines, and MMPs that contribute to TNBS-induced colitis.

In addition to inducing colon epithelial damage through the induction of proinflammatory mediators, we wished to determine whether TWEAK might induce death of colon epithelial cells directly. In order to address this question, we employed a commonly used model of intestinal cell damage, namely γ-irradiation-induced injury. The rapidly dividing epithelial cells of the intestinal mucosa are particularly sensitive to γ-irradiation-induced DNA damage, resulting in their apoptotic death or cell cycle arrest to allow DNA repair. This model has a less prominent inflammatory component as compared to TNBS-induced colitis, due to the radiation-sensitivity of the hematopoietic cells as well as the intestinal epithelial cells. As expected, we found Fn14 expression highly upregulated in the intestinal tissue after γ-irradiation (T. Dohi, unpublished). Notably, we also found that crypt cell apoptosis was significantly reduced in TWEAK or Fn14 deficient mice as compared to wild-type mice. These findings indicate that TWEAK regulates

intestinal epithelial turnover, suggesting that the TWEAK/Fn14 pathway directly promotes the apoptotic death of injured Fn14-expressing epithelial cells. We speculate that, in the context of γ-irradiation injury, this reflects a physiological role of the TWEAK/Fn14 pathway in promoting removal of DNA-damaged cells. However, if this activity is exerted to excess, it may also promote death of cells that would otherwise undergo cell cycle arrest and DNA repair, thereby impeding tissue repair. In addition, in the context of TNBS-induced colitis, this death-promoting activity of the TWEAK/Fn14 pathway may play a pathological role, acting in conjunction with the TWEAK/Fn14-induced innate inflammatory activities (described above) to promote epithelial ulcers and interfere with epithelial repair (Fig. 32.3).

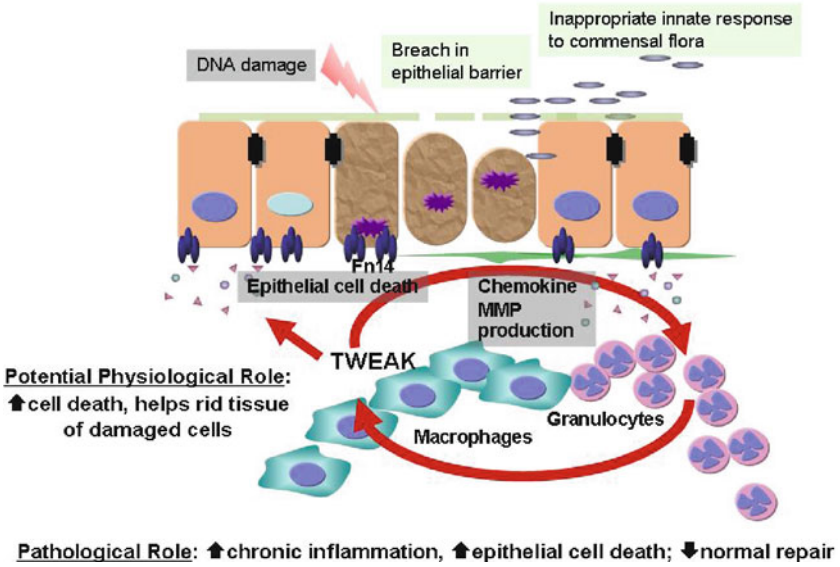

Fig. 32.3 TWEAK Signaling through Fn14 upregulated on intestinal epithelial cells: potential physiological and pathophysiological roles. TWEAK regulates epithelial cell turnover after γ-irradiation, promoting apoptosis of DNA-damaged cells. In the context of TNBS-induced colitis, this death-promoting activity of the TWEAK/Fn14 pathway may play a pathological role, acting in conjunction with the TWEAK/Fn14-induced innate inflammatory activities to promote epithelial ulcers and interfere with epithelial repair

Summary and Perspective

In the past decade, the TWEAK/Fn14 pathway has emerged as a prominent pathway in tissue remodeling, contributing to improved outcomes in health and to worsening outcomes in disease, through its induction of multiple activities, including inflammation, angiogenesis, regulation of cell survival and growth, and tissue progenitor cells. Advances in our understanding have derived largely from studies of

the TWEAK/Fn14 pathway in experimental models of acute injury and repair and in models of autoimmune and inflammatory diseases. Importantly, the highly evolutionarily conserved nature of this pathway argues the importance of its function and relevance of the findings in animal models to man. The pathological role of the TWEAK/Fn14 pathway has been validated in diverse organ systems, using a variety of animal models of autoimmune and inflammatory disease, and notably, TWEAK and Fn14 expression are elevated in many of the human disease counterparts.

We revealed a novel aspect of innate immune regulation by the TWEAK/Fn14 pathway through our studies in a model of TNBS-induced intestinal injury and colitis, a model with features of human IBD. Specifically, TWEAK signaling through Fn14 upregulated after TNBS injury on colonic mucosal epithelial cells, likely by their exposure to microbial products or cytokines, promotes their innate inflammatory response including chemokine, cytokine, and MMP production, thereby contributing to exarcerbate TNBS-induced colitis. We also employed the model of γ-irradiation injury as a tool to investigate the effect of TWEAK/Fn14 signaling on intestinal epithelial turnover. We discovered that TWEAK/Fn14 signaling promotes apoptotic death of injured cells. We speculate that this is a direct mechanism whereby TWEAK may contribute to epithelial destruction and impede repair in the inflammatory disease context. While further studies are warranted to investigate the role of the TWEAK/Fn14 pathway in other models of IBD and its expression in relation to disease in human IBD patients, these studies suggest that targeting the TWEAK/Fn14 pathway may be a promising approach to IBD treatment that will attenuate local tissue damaging activities and allow mucosal healing.

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Chapter 33
TWEAK and the Kidney: the Dual Role of a Multifunctional Cytokine

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Introduction

Acute kidney injury (AKI) is a syndrome characterized by an acute loss of renal function. The incidence of AKI is 208 per million population in Europe. There is no established therapy to accelerate the recovery and attempts at preventing AKI are not universally effective. The treatment of most forms of AKI is symptomatic and consists in substitution of renal function by dialysis if renal failure is severe. Despite the reversibility of the loss of renal function in most patients that survive, the mortality of AKI remains high (over 50%) [41].The tubular epithelium is a key cell in the renal injury. Indeed, tubular cell death plays an important role in the AKI and is the main histological correlation with the degree of renal failure [37, 51]. The tubular epithelium also contributes to renal failure by secreting pro-inflammatory cytokines and by the epithelium–mesenchymal differentiation originating fibroblasts and promoting tubular atrophy and interstitial fibrosis [40, 54]. These events are important contributors to progression of chronic kidney disease.

Cytokines of the TNF superfamily have a role in different physiopathological processes [22]. TWEAK is a recently identified cytokine of the TNF superfamiliy that activates the Fn14 receptor. TWEAK has multiple functions of potential physiological and pathological relevance. TWEAK regulates cell proliferation, cell death, cell migration, cell differentiation, neo angiogenesis, and inflammation [16, 24, 25, 32, 36, 48, 55]. TWEAK may have different functions dependent on the microenvironment and cell type, the basis of which is not currently known. The receptor, Fn14, was identified as an early response gene whose transcription was induced by FGF-1 (fibroblast growth factor-1) in murine fibroblasts [27] and its expression is induced during tissue injury, repair, and remodeling [10].

The multiple functions of TWEAK suggest that the TWEAK/Fn14 system plays a role in the pathogenesis of human diseases, and that this system may be a therapeutic target in multiple pathologies. However, the precise role of TWEAK in

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different diseases has to be defined, since it can be deleterious or beneficial depending on the context [3, 59]. Thus, it is necessary to understand the role of TWEAK in each pathology, and in each phase of the pathology, to maximize the benefit and minimize the potential adverse effects of a therapeutic intervention. A detailed understanding of the regulation of TWEAK functions and their modulation by the microenvironment in order to apply this knowledge to the clinic is a new frontier. In addition, the variety of TWEAK functions suggests the existence of key regulatory molecules, of therapeutic interest, that determine the response, depending on the environment or the cell type. Understanding these key molecules offers an opportunity for the identification of signal transduction pathways critical for tissue pathology and repair.

Current evidence suggest that TWEAK and Fn14 can play a role in different processes relevant in renal injury, such as regulation of the survival/proliferation of renal cells, tissue repair following injury, and the inflammatory response. Nevertheless, information on the role of TWEAK and Fn14 in specific pathologies is incomplete. TWEAK plays a role in inflammatory and ischemic injury in other organs such as in the central nervous system injury models of experimental autoimmune encephalomyelitis and ischemic/reperfusion brain injury [7, 31]. In this chapter, we aim to provide a comprehensive review of the role of TWEAK/Fn14 pathway in kidney injury based on published data. We describe the regulation of the expression of TWEAK and Fn14 in the kidney, the actions of TWEAK on renal cells in vitro and in vivo, and the effect of TWEAK blockade or absence in three different experimental models of human disease: acute kidney injury, uninephrectomy-induced renal hyperplasia, and experimental lupus nephritis.

TWEAK and Fn14 Expression in Renal Cells

TWEAK, in contrast to other TNF superfamily members, is a widely expressed cytokine, and can be found at high levels in pancreas, intestine, heart, brain, lung, ovary, vasculature, and skeletal muscle, and at lower levels in liver and kidney [5, 26]. The TWEAK receptor, Fn14, is expressed by many cell types [3]. One interesting aspect of Fn14 expression is that resting tissue levels of Fn14 are usually low, but are highly regulated in response to injury. The potential sources of TWEAK in the kidney include infiltrating monocytes and T lymphocytes, tubular epithelial cells, and mesangial cells [18, 20, 21, 35]. Resident kidney cells, such as tubular cells, mesangial cells, and podocytes, express Fn14 and respond to TWEAK stimulation [12, 18].

Renal murine tubular cells constitutively express TWEAK and Fn14. Basal Fn14 expression is low but it is quickly induced by pro-inflammatory cytokines as TNFα and INFγ [18]. The early peak of Fn14 expression is consistent with other reports, as Fn14 is an immediate-early response gene [27]. In addition, the growth factors present in serum also increased TWEAK and Fn14 expression in tubular cells in a more sustained manner [48]. Growth factors also upregulate Fn14 in endothelial cells [9].

TWEAK Biological Activity in the Kidney

Recent studies have revealed that the TWEAK-Fn14 pathway likely plays a role in a variety of cellular processes and in the pathogenesis of several human diseases [59]. Experimental studies have also shown a role for TWEAK/Fn14 in models of human kidney disease. Renal disease may result from injury to the glomeruli, the tubules, or the renal vessels. A series of reports have highlighted the role of TWEAK/Fn14 in tubular and glomerular cell injury.

Pro-inflammatory Effect of TWEAK in the Kidney

Tubular cells compose most of the mass of the functioning kidneys and behave as a central cell type in renal inflammation [6]. Tubular cells release cytokines that promote infiltration by inflammatory cells [56]. The transcription factor NFκB plays a key role in the inflammatory response of tubular cells. In resting cells, inactive NFκB proteins are present in the cytoplasm bound to inhibitor subunits (IκBs). When IκBs are phosphorylated and degraded, active NFκB migrates to the nuclei where it activates transcription. TWEAK activates NFκB in murine tubular epithelial cells, via Fn14, through this classical pathway [47]. As a result, TWEAK induces the expression and secretion of MCP-1, IL-6, and RANTES [47]. Different patterns of NFκB DNA-binding complexes were obtained in cells that were treated with TWEAK or TNFα, suggesting differential NFκB activation by these closely related cytokines [47]. The delayed induction by TWEAK of NFκB DNA-binding complexes, not induced by TNF, is consistent with the reported ability of TWEAK to activate NFκB through the non-canonical pathway [46] and suggest that TWEAK and TNFα are not redundant cytokines in renal injury. TWEAK also induced the expression of inflammatory mediators in glomerular mesangial cells and podocytes through NFκB activation [4, 12].

Cell culture results may be reproduced in vivo. Systemic TWEAK administration in vivo induces sustained renal NFκB activation and tubular expression of chemokines [12, 47]. Moreover, injection of TWEAK led to interstitial kidney infiltration by macrophages and T cells [12, 47]. However, in healthy kidneys, TWEAK does not induce glomerular inflammation, but TWEAK contributes to glomerular injury when Fn14 has been previously upregulated locally as in lupus nephritis [61].

TWEAK and Renal Cell Death

Apoptosis contributes to renal cell loss [49]. Indeed, several members of the TNF ligand superfamily, such as FasL, TNFα, and TRAIL, induce apoptosis in stressed renal cells [23, 39, 43, 44]. The name TWEAK derives from its weak capacity to induce apoptosis. TWEAK frequently requires co-stimuli, such as INFγ, to induce apoptosis [35]. Nevertheless, TWEAK has been shown to promote apoptosis, by

itself, in neurons, monocytes, certain tumor cell lines, and mesangial cells [4, 20, 33, 45]. TWEAK does not induce apoptosis in non-stimulated tubular epithelial cells. However, the lethal effect becomes apparent in the presence of several (not one) inflammatory mediators released during AKI, such as TNFα and INFγ [14, 18, 42]. This requirement for both INFγ and TNFα is novel. TNFα and INFγ increased Fn14 expression in tubular cells. In this context, upregulation of Fn14 may underlie the sensitization to apoptosis. In fact, Fn14 transfectants become sensitive to TWEAK-induced cell death [34]. However, the level of Fn14 expression is not the only mechanism involved, since INFγ or TNFα alone and serum also increase Fn14 expression but do not sensitize to cell death. The signaling pathway leading from Fn14 to cell death remains poorly understood. Fn14 lacks the death domain (DD) and this suggests that direct recruitment of DD-containing adapter proteins are not the primary mode of inducing cell death. Recently, it has been reported that Fn14 activation recruits TRAF2 and induces lysosomal degradation of the cellular inhibitor of apoptosis 1 (cIAP1). TWEAK-induced cIAP1 degradation sensitizes immortalized tumor cells to TNFα-induced apoptosis, thus providing an additional molecular mechanism for the TNF/TWEAK interaction [57]. TWEAK/TNFα/INFγ-induced tubular apoptosis is associated with caspase-8 activation and recruitment of the mitochondrial pathway [18]. TNFα alone may induce a delayed apoptosis in tubular cells, but the time course differs from that of TWEAK/TNFα/INFγ [18]. In addition, the intracellular molecular mechanisms differ between death induced by TNFα and by cytokine combination. The pancaspase inhibitor zVAD prevented TWEAK/TNFα/INFγ-induced apoptosis but led to a dramatic increase in necrosis, and this necrotic cell death is not observed with TNF alone and zVAD [18].

By contrast, TWEAK or INFγ alone induce mild apoptosis in mesangial cells under basal conditions and have a synergistic effect when combined [4, 12].

TWEAK and Renal Cell Proliferation

Kidney tissue cell number is carefully regulated through the balance between mitosis and cell death. An imbalance between these processes can result in disorders of cell number characterized by an excessive, such as proliferative or collapsing glomerulonephritis or insufficient cell number, such as AKI or chronic kidney disease [40]. Several TNF family members can either induce apoptosis or proliferation depending upon experimental conditions [1, 29]. TWEAK induces proliferation in quiescent tubular cells [48]. The proliferation induced by TWEAK is enhanced in the presence of the mitotic factors from serum. These factors increased both TWEAK and Fn14 expression and this may facilitate an enhanced proliferative response [48]. These results suggest that TWEAK actions over renal cells are modulated by the cell microenvironment as in the presence of pro-inflammatory cytokines that also increase Fn14 expression, and TWEAK potentiates cell death [18]. Since Fn14 receptor is the mediator of both the proliferative and the lethal potential of

TWEAK [18, 48] we have hypothesized that serum and inflammatory cytokines may have opposite effects on other cell cycle regulatory proteins. In this regard, they have different effects over cell survival proteins. Thus, TNF increases pro-apoptotic proteins and down-regulates anti-apoptotic proteins, while serum has opposite effects [17, 42].

TWEAK-induced proliferation is cell type-specific [8, 9, 16, 24]. However, there is little information about the molecular pathways that mediate TWEAK-induced proliferation. TWEAK can activate cell proliferation-related pathways, such as NFκB and MAPKs in endothelial cells [9], and PI3K/Akt in osteoblastic cells [2], but the role of these pathways in TWEAK-induced proliferation was not explored. In this regard, our group found that in tubular cells TWEAK activates ERK1/2, p38 MAPK, PI3K/Akt, and NFκB and that these pathways cooperate in TWEAK-induced proliferation in a manner that disruption of one of them prevents the proliferation [48]. The proliferative effect of TWEAK was also observed in vivo in healthy kidneys from mice injected with exogenous TWEAK [48].

Glomerular cell proliferation is an important feature of the histopathology of renal lupus. Gao et al. [12] recently found that TWEAK also induces proliferation in mesangial cells and in podocytes.

TWEAK and Fn14 in Experimental Kidney Disease

The studies summarized above show that TWEAK has different biological actions over resident renal cells: inflammation, proliferation and apoptosis, and that these effects are microenvironment dependent (Table 33.1). These data suggest that the TWEAK/Fn14 pathway may play a key role in kidney diseases.

Acute Kidney Injury

AKI is characterized by tubular cell apoptosis, compensatory tubular cell proliferation leading to regeneration, inflammatory cell infiltration, and mild fibrosis in a chronic phase. An overdose of folic acid induces AKI in humans [28] and mice.

Table 33.1 TWEAK actions on renal cells

Cell type	Action	Reference
Proximal Tubular	Proliferation through NFκB, MAPKs and PI3K/Akt activation	[12, 18, 47, 48]
	Inflammation by NFκB activation	
	Apoptosis in inflammatory environment	
Podocytes	Inflammation	[12]
	Proliferation	
Mesangial cells	Inflammation by NFκB activation	[4]
	Proliferation	[12]
	Apoptosis in the presence of INFγ	

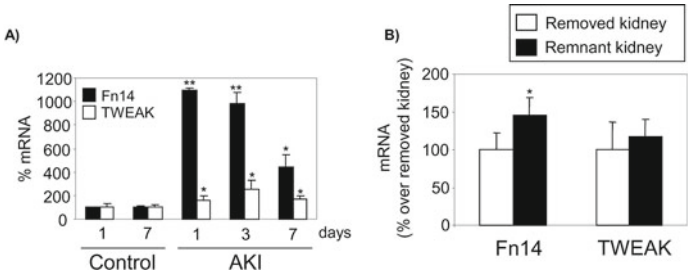

Fig. 33.1 Expression of TWEAK and Fn14 during acute kidney injury (AKI) and in compensatory renal growth following uninephrectomy. The renal expression of TWEAK and Fn14 mRNA increases in (**a**) a model of AKI induced by a single injection of folic acids. Mean (±SEM), $^{*}p<0.05$ vs. control, $^{**}p<0.01$ vs. control, and in (**b**) remnant kidney following uninephrectomy when compared with the removed kidney. Mean (±SEM). $^{*}p<0.05$ vs. removed kidney

Experimental folic acid-AKI shares with human AKI the cellular processes cited above [38, 42]. In this model, expression of both TWEAK and Fn14 increased at the mRNA and protein level [18]. Fn14 was localized to injured, dilated tubules [18]. Fn14 expression was already very high at 24 h, while TWEAK increased more progressively, peaking at 72 h [47] (Fig. 33.1a). Moreover, during AKI multiple cytokines are released, including TNFα and INFγ [42, 47]. Cell culture data suggest that TWEAK may be deleterious in inflamed kidneys, since in this environment it promotes cell death. However, potential proliferative actions of TWEAK on tubular cells or progenitor cells or the recruitment of macrophages that participate in tissue repair might confer a protective role for TWEAK. The inhibition of TWEAK in vivo allowed studying the end result of its interactions with different molecules in the renal microenvironment. A neutralizing anti-TWEAK antibody decreased peak serum creatinine and resulted in significantly milder histological injury in folic acid-induced AKI [47]. Moreover, renal function was better in TWEAK KO mice and there was no impact of TWEAK targeting over functional recovery [47].

Several of the cell culture actions of TWEAK on tubular cells may contribute to the beneficial effect observed in vivo. In AKI an initial wave of cell death is followed by tubular cell proliferation that repopulates the tubules and promotes the recovery. TWEAK KO mice showed a decreased peak of tubular apoptosis followed by a decreased peak of proliferation [48]. Based on the ability of TWEAK to induce tubular cell death in culture in an inflammatory environment, in the presence of inflammation during AKI and in the temporal relationship between cell death and proliferation in AKI and the beneficial effect of TWEAK targeting on AKI outcome, we speculate that TWEAK targeting decreased the early cell death peak, thus leading to a lesser need of repair proliferation during recovery. However, these results do not rule out a proliferative role of TWEAK during the recovery phase in AKI.

During AKI infiltration of leukocytes depends on the local expression of inflammatory cytokines. Endothelial and tubular cells release cytokines and chemokines in response to various immune and non-immune factors [6, 56]. When TWEAK is neutralized, the earliest increased in injured kidneys' chemokine expression is not

modified, but by 72 h the tubular expression of chemokines (MCP-1, RANTES) and interstitial inflammation by macrophages are decreased. However, kidney IL-6 in the course of AKI is TWEAK independent [47]. Thus, although TWEAK modulates IL-6 expression in cultured tubular cells, in the AKI model there are other, more potent, stimuli that regulate the IL-6 expression.

In summary, preemptive TWEAK targeting during AKI improves renal function and decreases tubular cell death and compensatory tubular cell proliferation as well as interstitial inflammation. There is currently no information on the therapeutic effect of TWEAK targeting once renal injury has been established.

Non-inflammatory Renal Regeneration Model: Uninephrectomy

Compensatory renal hypertrophy and hyperplasia are observed following an acute loss of renal mass such as that induced by unilateral nephrectomy [52]. This occurs clinically following nephrectomy for benign or malignant conditions, in living kidney donors and in transplant recipients. Growth factors and cytokines, such as growth hormone and insulin-like growth factor, have been implicated in compensatory renal growth after uninephrectomy [11]. TNF superfamily cytokines participate both in parenchymal cell injury as well as in proliferation following loss of tissue mass. As an example, TNFα promotes hepatocyte apoptosis, but type 1 TNF receptor is also required for compensatory proliferation following partial hepatectomy [58, 60].

TWEAK induces cell proliferation in various cell types and may contribute to pathological or tissue repair compensatory hyperplasia after acute injury. Early reports disclosed Fn14 expression is upregulated during liver regeneration and in hepatocellular carcinoma [10]. Moreover, Fn14 contributes to nerve regeneration [53], TWEAK induces proliferation in liver oval cells [16], in astrocytes, and in synoviocytes in vitro [9, 19], and TWEAK/Fn14 pathway regulates skeletal muscle precursor cells [13].

Exogenous TWEAK has a proliferative action in the kidney in vivo, promoting tubular cell proliferation. In addition, endogenous TWEAK contributes to renal cell proliferation in compensatory renal growth following uninephrectomy [48]. This is a situation characterized by increased tubular cell Fn14 expression (Fig. 33.1b), and tubular cell proliferation in the absence of tubular injury or increased expression of inflammatory cytokines [52]. In fact, kidney TNFα and INFγ, factors associated with TWEAK-induced tubular cell death, are not increased in this model [15, 48]. The decreased tubular cell proliferation observed in uninephrectomized TWEAK KO mice and the increased proliferation observed in mice treated with exogenous TWEAK [48] suggest that Fn14 facilitates tubular cell proliferation in compensatory renal growth following nephrectomy by sensitizing the cells to TWEAK and cooperating with other mitogens as observed in culture. This information may be used to promote kidney growth in clinical situations characterized by an acute non-inflammatory loss of kidney mass and its relevance should be explored in tissue engineering.

Autoimmune Kidney Disease: Lupus Nephritis

TWEAK has a pro-inflammatory effect in mesangial cells and podocytes [4, 12], suggesting a possible role of the TWEAK/Fn14 pathway in the pathogenesis of glomerular injury. This role has been explored in experimental lupus nephritis as a model of immune-mediated glomerular disease. Initial evidence came from the finding that urinary levels of TWEAK in human patients with lupus nephritis correlate with renal disease inflammatory activity [50]. In an experimental model of lupus nephritis genetic deletion of Fn14 or neutralizing anti-TWEAK antibodies improved the outcome of glomerulonephritis [61]. Deficiency of Fn14 decreased proteinuria, glomerular IgG deposition, kidney cytokine expression (such as MCP-1, IL-6, RANTES, and IP-10), and macrophage infiltration without changes in autoantibody production [61]. These studies provide evidence that therapeutic modulation of TWEAK may reduce renal damage in lupus nephritis. Further studies addressed the relative contribution of Fn14 expression by resident kidney cells or by myeloid cells (macrophages) that infiltrate the kidney in lupus nephritis. Two groups of chimeric mice were studied; one chimera expressed Fn14 in kidney resident cells, while bone marrow-derived cells lacked Fn14. In the other chimera Fn14 expression is restricted to bone marrow-derived cells [30]. Fn14 expression on kidney resident cells was sufficient to promote an early inflammatory response and a rapid increase in albuminuria as a marker of glomerular injury, while Fn14 signaling from bone marrow-derived cells plays a role in albuminuria and inflammation at a later stage of the disease. These results are consistent with previous reports, since TWEAK is able to induce chemokine secretion in kidney cells leading to macrophage chemotaxis [4, 12, 47]. The observation is consistent with the current model of kidney inflammation in which resident kidney cells contribute to initiation of nephritis, in this case through the TWEAK/Fn14 pathway, while infiltrating macrophages play a later role, when they have migrated to the kidney.

Future Lines of Research

In conclusion, TWEAK activates Fn14 and it is a new mediator of glomerular and tubulointerstitial renal injury. The expression of the cytokine TWEAK and its receptor Fn14 are increased during inflammatory renal injury and Fn14 is increased in non-inflammatory renal regeneration but the functional significance of the increased Fn14 is different (Fig. 33.2). TWEAK actions depend on the cell microenvironment: TWEAK causes renal injury in an inflammatory environment but promotes renal regeneration in the absence of inflammation. Since, in human tubulointerstitial inflammatory nephropathies Fn14 expression is also increased, these results are relevant for a possible therapeutic application of anti-TWEAK antibodies, that are in phase I trials in rheumatoid arthritis patients.

TWEAK can also contribute to renal regeneration in a non-inflammatory microenvironment. This observation deserves more detailed studies aimed at trying to separate the deleterious and beneficial pathways activated by TWEAK with a view to therapeutic manipulation. The contribution of TWEAK/Fn14 to tubular

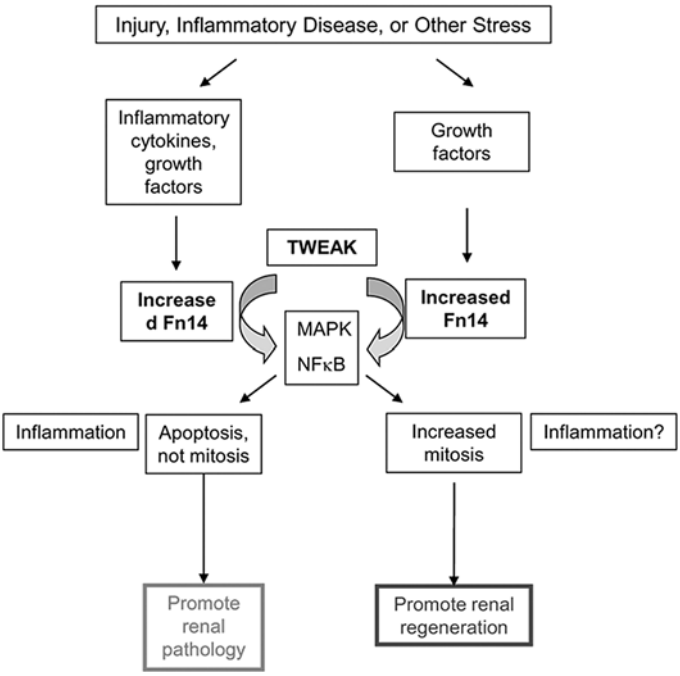

Fig. 33.2 TWEAK may contribute to renal injury in an inflammatory environment and to regeneration in the absence of inflammation. TWEAK activates the Fn14 receptor in renal tubular cells and activates MAP kinases (MAPK) and the canonical and non-canonical NFκB pathway. The activation of NFκB and MAPK contributes to the proliferative effect of TWEAK over tubular epithelium, such as during kidney growth following reduction in kidney mass which occurs in the absence of inflammation. In contrast, in an inflammatory environment, such as acute kidney injury, increased Fn14 expression enhanced the cell susceptibility to death induced by TWEAK. The activation of NFκB also increases the secretion of various chemokines, promoting macrophage recruitment. Functional studies have shown the relevance of this inflammatory response for promoting kidney pathology, but their potential participation in tissue repair in a microenvironment-dependent fashion has not been addressed

cell proliferation should be explored in the context of tissue engineering and renal regeneration.

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Chapter 34
TWEAK and TNF Regulation of Sclerostin: A Novel Pathway for the Regulation of Bone Remodelling

David M. Findlay and Gerald J. Atkins

Introduction

Bone remodelling is the critical process, by which skeletal mass and integrity are controlled. This process is disrupted in a number of bone pathologies, such as osteoporosis, and the focal bone loss that occurs in rheumatoid arthritis, around artificial joint prostheses or that induced by osteolytic tumours. A common feature of conditions that result in a net loss of bone is the presence of elevated levels of pro-inflammatory mediators and evidence has accumulated to show that a chronic inflammatory environment is both catabolic for bone and may also suppress bone formation. Here, we focus on the possible anti-anabolic roles in bone of TNFα and TNF-like weak inducer of apoptosis (TWEAK).

TWEAK

TWEAK, a member of the TNF ligand superfamily designated TNFSF12 [1], has pleiotropic effects, which include induction of pro-inflammatory mediators in a number of target cell types, including fibroblasts and synoviocytes obtained from RA and advanced OA patient tissues [2]. The receptor for TWEAK, fibroblast growth factor-inducible gene-14 (Fn14/TNFRSF12/TWEAK R), is widely expressed [3] and is up-regulated in the context of tissue injury and inflammatory responses [4–7]. Ligation of TWEAK to Fn14 has been shown to activate a limited set of signalling pathways involved in cell proliferation and differentiation [8], including NFκB activation, which is associated with its pro-inflammatory effects [3, 9]. TWEAK has also been shown to activate the mitogen-activated protein kinases (MAPK), JNK [10], ERK [10, 11] and p38 MAPK [5].

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A Role for TNF Family Members in Bone Remodeling

The TNF family members and their cognate receptors, RANKL/RANK/OPG and TNF/TNFR1, have central and well-established actions in bone remodelling [12]. Physiologically, RANKL expression by osteoblastic stromal cells mediates osteoclast formation by binding to RANK expressed by osteoclast precursors. Evidence suggests that RANKL and TNF are together involved in inflammatory bone remodelling [13–15]. Although the mechanisms remain to be fully elucidated, TNF can both enhance RANKL expression and sensitize preosteoclasts to RANKL [12]. TNF expression is markedly up-regulated in peri-prosthetic osteolysis [16], in association with polyethylene (PE) particles produced by wear of the prosthesis [17]. We [18] and others [19] have reported a significant role for TWEAK in the inflammatory bone remodelling seen in the mouse CIA model of rheumatoid arthritis. Serum TWEAK was elevated in CIA mice [18] and a neutralising TWEAK antibody significantly reduced the disease severity. Together, the data suggest that TWEAK may play roles in both joint inflammation and tissue damage in the context of RA. The extent to which TWEAK and TNF, also a known mediator of joint pathology in inflammation-driven bone remodelling, each contribute to the pathogenesis of CIA remains an open question. We have reported that human osteoblasts express Fn14 and that TWEAK exposure inhibited their expression of the key osteoblast gene, osteocalcin [18, 20], implying a role for TWEAK in osteogenesis. Ando and colleagues [11] reported that an initial effect of TWEAK on the mouse osteoblast cell line, MC3T3-E1, is to induce RANTES production and RANKL expression. The work described below suggests that TWEAK may contribute to physiologic human osteoblast function and/or their differentiation and so may be a key regulator of bone remodelling.

Human Osteoblasts and Osteoblast-Like Cell Lines Express Fn14 and TWEAK

We have found that all human primary osteoblast [20] and osteoblast-like cell lines (Fig. 34.1a–c) tested express high basal levels of cell surface Fn14. Human osteoblasts also express intracellular TWEAK, suggesting that TWEAK could have an autocrine role in osteoblast activity [20]. In addition, TWEAK mRNA levels may be regulated in an inflammatory milieu, since exposure of human osteoblasts to TNF and IL-1β decreased the expression of TWEAK mRNA in this cell type (Fig. 34.1d).

TWEAK Inhibits In Vitro Mineralisation and Antagonizes the Osteogenic Effect of TNF

The ability to form a mineralised matrix over time is a key functional indicator of osteoblast activity and osteogenesis. We have shown that TWEAK inhibits in vitro mineralisation by human osteoblasts and that this inhibitory effect could be reversed by concomitant incubation with a TWEAK neutralizing antibody. Conversely, TNF

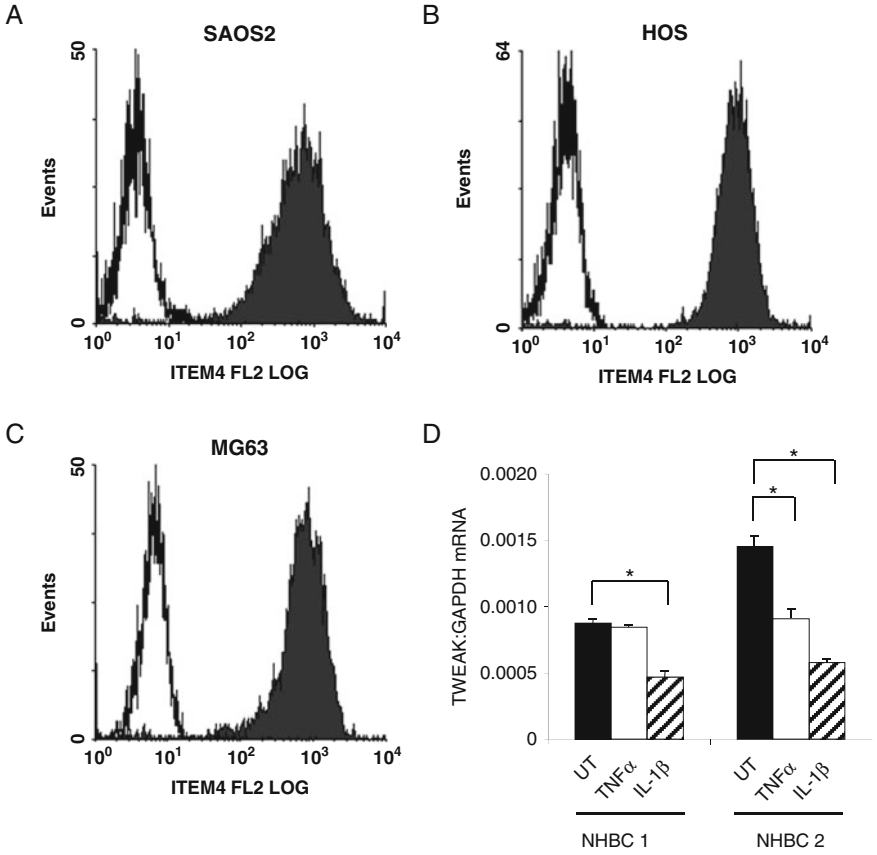

Fig. 34.1 Human osteoblast expression of Fn14 and TWEAK. All human osteoblastic-like cell lines examined expressed high levels of Fn14, including (a) SAOS2, (b) HOS and (c) MG63 cells. Immunofluorescence and flow cytometry were performed essentially as described previously [20]. (d) TWEAK expression in NHBC was regulated by a 48-h incubation with either recombinant human TNFα or IL-1β (R&D Systems). Real-time RT-PCR was performed as described previously [20]. Oligonucleotide primers designed in-house to amplify human TWEAK were forward primer: 5′-ATCGCTGTCCGCCCAGGAGC-3′ and reverse primer 5′-CTGTCTGGGGATTCAGTTCCG-3′, which amplify a 86-bp product. Data shown are means of triplicate reactions ± SD, and are representative of two independent experiments. Asterisks denote significant difference compared to untreated (UT) ($p < 0.01$)

promoted in vitro mineralisation by human osteoblasts. Interestingly, TWEAK antagonized this effect in a dose-dependent fashion [20]. These results suggest that TWEAK is a negative regulator of osteoblast differentiation and osteogenesis.

Effect of TWEAK and TNFα on Osteoblast Proliferation

Osteoblast proliferation is another important determinant of the osteogenic effect and is inversely related to osteoblast maturation. We have described the use of

carboxyfluorescein diacetate succinimidyl ester (CFSE) to measure proliferation of human osteoblasts [21, 22]. CFSE-labelled human osteoblasts exhibited enhanced proliferation in response to TWEAK, showing reduced numbers of cells in the parental population and increased numbers of cells having three or more cell divisions. TNFα is also mitogenic for NHBC and this effect is enhanced in the presence of TWEAK, to the extent seen with TWEAK alone [20].

Effect of TWEAK and TNF on Osteoblast Osteogenic Gene Transcription

We have also investigated the effects of TNF and TWEAK on the expression of a number of osteoblast-associated genes under two sets of conditions, viz. basal conditions for 3 days and conditions permissive of mineralization (the latter containing supplemented phosphate levels) over 3 weeks. Under basal conditions, the osteoblast transcription factors, RUNX2 and osterix, together govern osteoblast lineage commitment and their subsequent differentiation [23]. We found that TWEAK significantly inhibits RUNX2 mRNA expression, in the co-presence or absence of TNF, although TNF alone had no discernible effect on RUNX2 levels [20]. TWEAK dose responsively increased osterix mRNA levels by up to ninefold, consistent with it holding osteoblasts at an immature stage of differentiation. In contrast, TNF alone had no apparent effect on osterix expression, the combined effect of TWEAK/TNF on osterix being similar to the effect of TWEAK alone [20]. These data implicate TWEAK as an important controller of osteoblast differentiation. The effect of TWEAK exposure on RUNX2 is consistent with the emerging negative role of RUNX2 in controlling osteoblast proliferation [24, 25]. Also consistent with the effect of TWEAK on osteoblast proliferation is the stimulatory effect on osterix expression, which has been shown itself to promote cell proliferation and decrease expression of osteoblast genes such as osteocalcin and alkaline phosphatase [26]. TWEAK dose-dependently down-regulated the expression of genes associated with osteoblast differentiation and mineralisation, including osteocalcin, BSP1 [20], alkaline phosphatase and osteopontin (Atkins, unpublished data). TNF had qualitatively similar effects on these genes, although quantitatively it varied from considerably more potent than TWEAK (e.g. on the osteocalcin gene) to approximately equipotent with respect to other genes (e.g. BSP1) [20].

Under conditions permissive of osteoblast differentiation and in vitro mineralization, we found that, while osteogenic genes such as OCN, BSP1 and OPN were more potently down-regulated by TNF in short-term dose–response studies compared with TWEAK, a different pattern of gene responsiveness emerged during a 3-week differentiation period. Early strong suppression of OCN transcription by TNF, for example, was followed by partial release from this suppression, consistent with our finding of an overall positive effect of TNFα on mineral apposition by

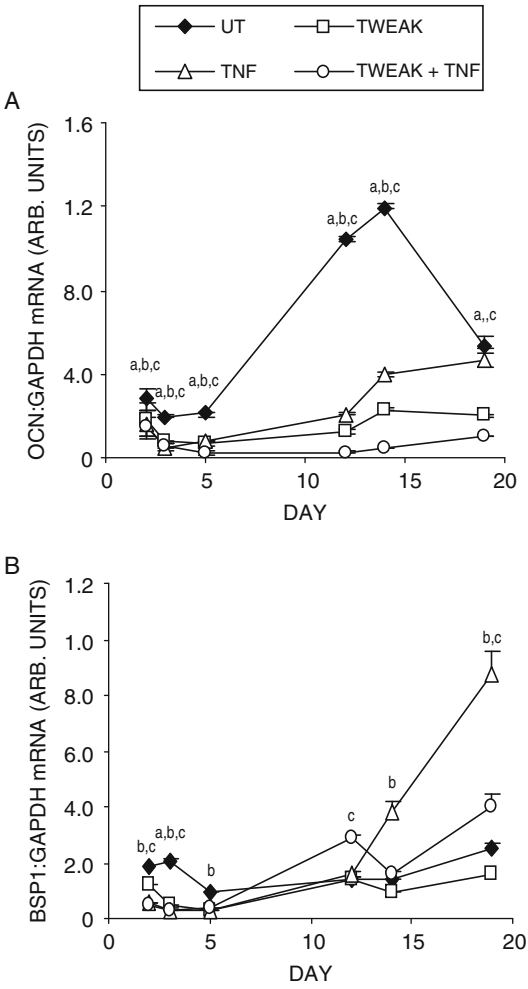

Fig. 34.2 Effect on osteogenic gene expression as a function of time of recombinant human (rh) TWEAK (50 ng/ml) [18], rhTNFα (1 ng/ml; R&D Systems, MN, USA) or a combination of both cytokines. Human osteoblasts were cultured for up to 3 weeks under mineralising conditions and analysed at the times indicated by real-time RT-PCR for (**a**) OCN and (**b**) BSP1 using a published protocol [20]. Significant differences to control (untreated) levels are indicated by *a* for TWEAK, *b* for TNF and *c* for the combination treatment ($p < 0.05$). Data shown are normalised to GAPDH mRNA levels and are means ± SD of triplicate reactions. Similar results were obtained in two independent experiments

human osteoblasts (Fig. 34.2a). TWEAK, on the other hand, suppressed OCN transcription throughout the culture period (Fig. 34.2a), in fact antagonizing the TNF effect on OCN transcription. Similarly, both TWEAK and TNF suppressed BSP1 mRNA expression, a gene associated with mineral deposition, in the early phase of the mineralising cultures. However, expression was later induced by TNF to a level above that seen in control cultures, again consistent with an osteogenic effect of TNF, but this was not seen in cultures exposed to either TWEAK or TWEAK/TNF (Fig. 34.2b). Thus, while the short-term effects of TWEAK resemble those of TNF, these cytokines have divergent effects on osteoblast differentiation, with TWEAK exerting the more dominant inhibitory effect.

Interaction Between TWEAK and TNF

TNF ligand family members are capable of modulating each others' activities. For example, TNF synergizes with RANKL in the induction of osteoclast formation [16, 27]. Given that the microenvironment encountered by cells during physiologic or pathologic bone remodelling, for example in RA, consists of a complex cytokine milieu, such interactions are likely highly relevant and important. For example, we recently reported that TWEAK is a mediator of joint erosion in the mouse CIA model, a model in which TNF is also expressed and is implicated in the mechanism [18]. Our data indicate that TWEAK and TNF modulate each other's activities with respect to human osteoblast behaviour, and the functional outcome likely depends on the relative expression of each cytokine and their respective receptors. It is also likely that there are species-specific effects of TWEAK, as neither TWEAK nor TNF caused detectable phosphorylation of p38 MAPK, or activation of the Akt survival pathway, in human osteoblasts in contrast to effects of TWEAK in mouse MC3T3-E1 osteoblast-like cells [11].

The Wnt Signalling Pathway and Inhibitors: Sclerostin

In investigating the mechanisms for the inhibitory effects of TWEAK on osteoblast differentiation and function, it is important to consider the possibility of interaction with a central regulator of this process, the Wnt signalling pathways. The canonical Wnt-signalling pathway is the best described of these [28, 29]. In this pathway, Wnt ligands bind to frizzled (Fzd) and LRP5/6 co-receptors on target cells, preventing the proteosomal degradation of β-catenin and promoting the formation of transcription complexes with TCF/LEF transcription factors, resulting in the downstream transcription of osteogenesis-related genes. Several inhibitors of the Wnt pathway have been identified, including Dikkopf 1 (DKK1), secreted Frizzled-related protein (sFRP) and sclerostin [28]. It is noteworthy that impaired osteoblast function on bone surfaces adjacent to sites of inflammation, in a murine model of rheumatoid arthritis, was associated with up-regulated expression of a number of Wnt inhibitors, including DKK1 and sFRP1 [30]. Sclerostin is the product of the *SOST* gene, mutations in which cause conditions with high bone mass, and is a key negative regulator of bone formation [29, 31, 28]. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength [32]. A major activity of sclerostin appears to be the inhibition of Wnt/β-catenin signalling [33].

TWEAK Alone and in Combination with TNF Induce Sclerostin Expression

We have reported that TWEAK can induce the expression, at both the mRNA and protein levels, of sclerostin in a dose-dependent fashion [20]. Sclerostin mRNA levels remained elevated relative to control conditions throughout the mineralisation

period, increasing further towards the end of the 3-week differentiation period. TNF by itself had a mild effect but combined with TWEAK to further increase sclerostin expression. The induction of sclerostin transcription by TWEAK and TWEAK/TNF was highly dependent on JNK and ERK1/2 phosphorylation [20]. Sclerostin expression in normal bone is virtually restricted to mature osteocytes embedded in mineralised bone, with some expression by osteoblasts [31]. Our unpublished observations concur with this, that sclerostin expression normally coincides with in vitro mineralisation by NHBC (Atkins et al., unpublished), and in some cases where mineralisation is increased, for example by the anabolic drug, strontium ranelate [34], sclerostin levels also increase both as a marker for osteocytes arising in the cultures and in an apparent bid to regulate the anabolic effect. However, the expression pattern of sclerostin in vivo has not been tested under inflammatory conditions. It is possible that less mature osteoblasts may be induced to express sclerostin under inflammatory conditions. Notably, while TWEAK induced proliferating cells to express sclerostin, as judged by their expression of the nuclear proliferation antigen Ki67 [20], there was an inverse relationship between intracellular sclerostin expression and the proliferative potential of primary osteoblasts following TWEAK treatment (Fig. 34.3). It is possible that by inducing sclerostin expression TWEAK may cause premature differentiation into a mature osteocyte-like phenotype, thereby bypassing the mineral apposition phase of osteoblast differentiation [35] and explaining the observed inhibitory effect on mineralisation [20]. This possibility is the subject of ongoing research.

Our preliminary data indicated that the exposure of human osteoblasts to recombinant human sclerostin has remarkably similar effects to treatment with TWEAK and TWEAK/TNF, with respect to the suppression of osteocalcin and RUNX2 expression [20]. We are currently exploring in greater detail the involvement of sclerostin in TWEAK-mediated effects on osteoblasts, using human cell line models. Notably, blockage of sclerostin activity is currently being explored as a novel treatment option for osteoporosis [36]. A recent report [37] identified TNF-mediated induction of DKK1 expression in a mouse model of inflammatory arthritis and in human RA.

Concluding Remarks

Taken together, these data provide a number of potential mechanisms for the anti-anabolic effects of an inflammatory environment in bone. The induction of expression of inhibitors of the Wnt and/or BMP signalling pathways is a novel potential mechanism, by which TWEAK alone, or in concert with TNF, might regulate physiologic osteoblast differentiation and mineralisation, and suppress these processes in chronic inflammatory disease states. In an inflammatory setting, TWEAK or TWEAK/TNF may act to regulate osteoblast proliferation, inhibit the osteogenic activity of osteoblasts and perhaps also promote the formation and activity of osteoclasts, as depicted in (Fig. 34.4). TNF has been shown to both directly and indirectly activate osteoclast activity [12, 27]. A single report has implicated

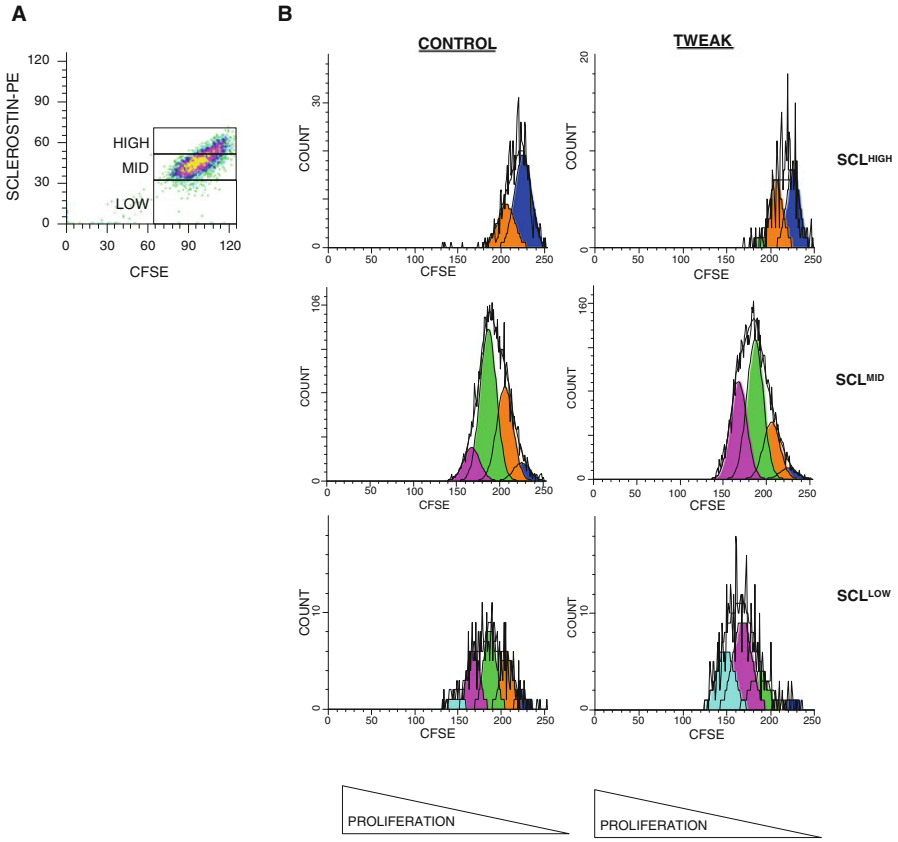

Fig. 34.3 Sclerostin (SCL) expression is inversely related to cell proliferation. NHBC were labelled with CFSE and cultured for 3 days in the presence or absence of recombinant human TWEAK (50 ng/ml), as described [20]. Cells were analysed by flow cytometry for intracellular expression of sclerostin by a published method [20], using a commercially available monoclonal antibody (MAb) against human sclerostin (R&D Systems) or an isotype-matched negative control MAb. Data were analysed using Modfit^{TM} software (Verity, NJ, USA). (**a**) Cells were gated on low, mid and high levels of expression of sclerostin, the 'low' gate completely overlapping the level of staining generated by the negative control MAb. (**b**) Cell divisions were calculated as described [20], the proportion of cells having undergone successive divisions represented by coloured peaks, with the *dark blue* peak representing undivided cells and the *light blue* peak the cells that had undergone four divisions. Even though TWEAK induces sclerostin expression [20], cells expressing high levels (SCL^{HIGH}) proliferated the least while the cells expressing low levels of intracellular sclerostin (SCL^{LOW}) displayed the highest proliferative potential

TWEAK as a direct regulator of osteoclast activity in the absence of evidence for Fn14 expression [38]. This has yet to be confirmed by others, and evidence from our laboratories instead implies that the effect is indirect, via effects on osteoblastic stromal cells (Atkins, unpublished data). Together, these observations give new insight

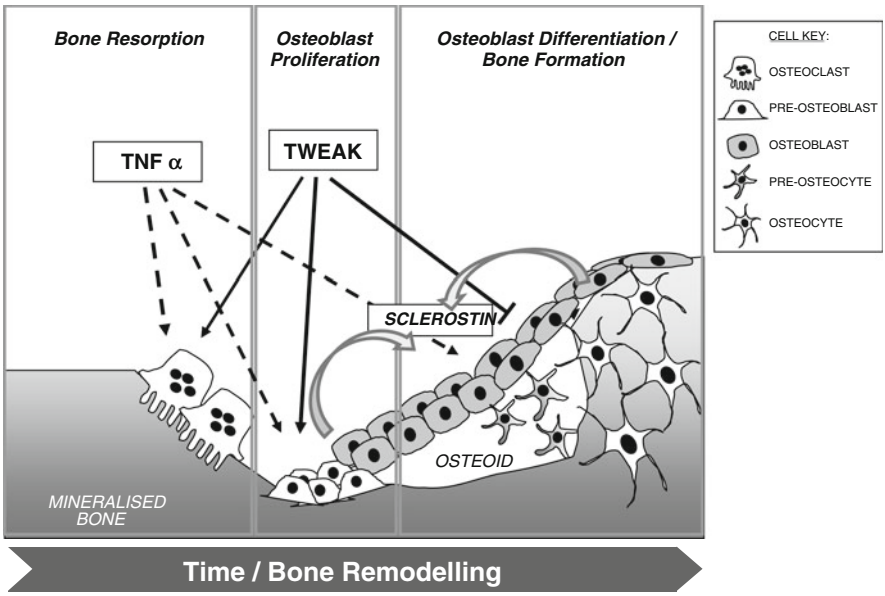

Fig. 34.4 Cartoon depicting the anti-anabolic actions of TWEAK in bone remodelling, here divided into the phases of bone resorption, osteoblast proliferation and osteoblast differentiation accompanying bone formation, as inferred from the work described in this manuscript and that reported by others and discussed in the text. In an inflammatory environment, TWEAK may act (*solid arrows*) on cells of the osteoblast lineage to stimulate osteoblast proliferation and also produce inhibitors of bone formation, including sclerostin (*curved arrows*), which acts back on bone forming osteoblasts to limit both osteoblast differentiation and the production of new bone. TNFα also has mitogenic effects on osteoblasts and induces the production of sclerostin (*dashed arrows*) but the overall effect is positive on bone formation perhaps because of the transient nature of these effects. TNFα together with TWEAK modulate each other's activity and the net effect would depend on their relative abundance. TWEAK may also activate bone resorption by promoting the formation and activity of osteoclasts, as has been reported for TNFα

into the mechanism of physiologic bone remodelling and the bone loss and lack of repair that are observed in a number of clinically important bone loss pathologies, including osteoporosis and RA.

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Part VIII
Control of Lymphocyte Function and Repertoire by the TNF Family

Chapter 35
Workshop Summary: Control of Lymphocyte Function and Repertoire by the TNF Superfamily

Carl F. Ware and Jeffrey L. Browning

The diversification of the TNF superfamily in regulating *lymphocyte function* is astounding. This diversity of function is reflected in genetic expansion and linkage of several TNFR within conserved immune response loci. Several TNFR, including TNFR2, HVEM, OX40, 41BB, and DR3 reside on Chromosome 1p36, and their ligands retained as functional paralogs of the MHC gene complex on Chromosome 6. These TNF superfamily genes encode the molecules that control intercellular communication pathways involved primarily in regulating cellular immunity. In this session, the science presented underscored the diversity of roles played by the TNF superfamily in regulating lymphocyte function.

New results from the investigation of the herpesvirus entry mediator (HVEM; TNFRSF14) revealed that HVEM can be activated by five known functional ligands (*T. Cheung and C. Ware*). Two canonical TNF superfamily ligands, LIGHT (TNFSF14) and LTα, and three unconventional ligands that belong to the immunoglobulin superfamily BTLA (B and T lymphocyte attenuator), CD160, and the viral envelop glycoprotein D (gD) of Herpes Simplex virus can activate HVEM signaling of the transcriptional factor, NFκB RelA, which controls genes involved in cell survival. These new insights into the biophysics of the ligand–receptor interactions in the HVEM pathway suggest unanticipated functional consequences of this cosignaling network.

The studies presented by *E. Gallagher* take a close look at mitogen-activated protein kinase signaling pathway in T- and B-cell interactions utilizing a Mekk1 kinase domain-deleted mouse model. The results demonstrated that this MAP3K plays a key role in thymus-dependent responses, and B- and T-cell signaling. Careful

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analysis revealed defects intrinsic to the B cell in signaling via CD40 and BAFFR involved in activating JNK and p38.

Dendritic cells (DC) are specialized antigen-presenting cells that efficiently present antigen and cosignals that aid in T-cell activation and proliferation. These activated T cells also concurrently deliver signals back to the Ag-bearing DC in a process that has been termed DC licensing. The licensed DC can then cross-prime a CD8+ T-cell responses so that infected cells can be eliminated from the tissue. *L. Summers deLuca and J. L. Gommerman* define a new role for the lymphotoxin (LTαβ)-LTβ receptor) in the licensing of DC.

Lymphotoxin β receptor pathway is well known for mediating signals that are essential for formation of secondary lymphoid organs, including lymph nodes and intestinal Peyer's patches. *R. Fava, J. Browning and colleagues* investigate the role of LTβR in forming tertiary lymphoid structures at sites of chronic inflammation. In contrast to the pro-inflammatory pathways controlled by TNF, the Fava group has determined whether blockade of the "homeostatic" pathways in secondary and "ectopic" or tertiary lymphoid tissues is under the control of LTβR. This intriguing hypothesis is examined in a mouse model of Sjogren's syndrome.

Inflammatory and homeostatic processes can go on simultaneously through signaling by the CD137 (4-1BB) pathway. *D. Jiang and group* demonstrate that CD137 is expressed by cells in the bone marrow and CD137 ligand is expressed on hematopoietic progenitor cells setting the stage for this pathway's role in myelopoiesis. A dichotomy revealed in the expression of CD137 in activated immune cells promotes macrophage colony formation, but during homeostasis this pathway appears to limit myelopoiesis.

These reports clearly demonstrate the diversity of function roles the TNF superfamily plays in lymphocyte biology and predicts the possibility of manipulating these pathways to control pathogenic processes.

Chapter 36
The Canonical and Unconventional Ligands of the Herpesvirus Entry Mediator

Timothy C. Cheung and Carl F. Ware

Introduction

The maintenance of immune homeostasis requires a balance between stimulatory and inhibitory pathways. The herpesvirus entry mediator (HVEM; TNFRSF14) [1] serves as one of two key entry routes used by herpes simplex virus-1 and herpes simplex virus-2 to infect cells [2]. The selection of HVEM as a route of infection is intimately linked with the capacity of HSV to modulate immunity. As a cellular signaling receptor, HVEM functions as a molecular switch for pathways that can stimulate or inhibit hematopoietic cell activation. The HVEM-regulated pathways impact T and B cell activation [3, 4], dendritic cell proliferation [5], and protection of mucosal epithelia from damage during inflammation [6]. HVEM has five known functional ligands: two canonical TNF superfamily ligands (LIGHT: TNFSF14 and LTα: lymphotoxin-a) [7] and three unconventional ligands that belong to the Ig superfamily [B and T lymphocyte attenuator (BTLA), CD160, and the viral envelop protein of herpes simplex virus envelope glycoprotein D (HSV gD)] (Fig. 36.1a) [8]. The shared receptor usage of HVEM's ligands, LIGHT and LTα, by the LTβ receptor and the two receptors for TNF suggests these molecules are part of a larger signaling network whose ramifications have not been fully elucidated [9]. Recent insights into the biophysics of the ligand–receptor interactions in the HVEM pathway suggest unanticipated functional consequences of this cosignaling network.

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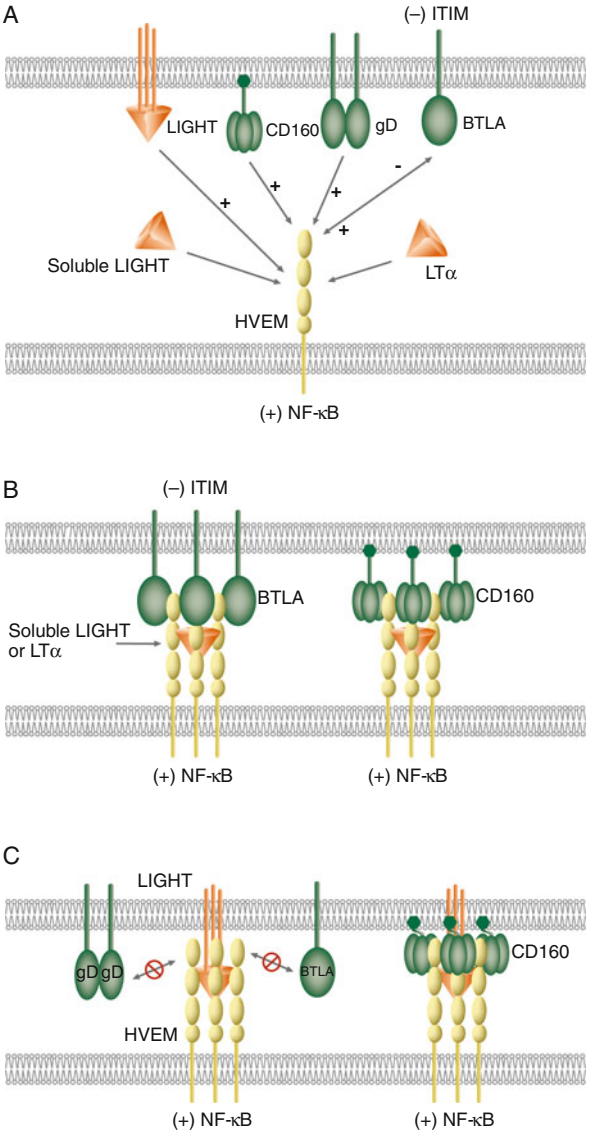

Fig. 36.1 Schematic illustrations of molecular interactions between HVEM and its ligands. (**a**) Canonical and unconventional ligands of HVEM. LIGHT and LTα are the canonical ligands as well as positive activators of HVEM. Ligation of LIGHT or LTα activates HVEM-dependent NF-κB signaling. BTLA, CD160, and HSV gD serve as unconventional ligands for HVEM. Ligation of CD160 or gD to HVEM induces NF-κB activation. Bidirectional signaling occurs between HVEM and BTLA. HVEM-mediated BTLA signaling induces tyrosine phosphorylation of the ITIM in BTLA, providing an inhibitory signal to T cells. BTLA, CD160, and gD bind HVEM at the CRD1, but LIGHT and LTα interact with HVEM in the CRD2 and 3. The binding of gD to HVEM blocks HVEM binding to LIGHT or BTLA (not shown in diagram). (**b**) Cooperative binding between soluble LIGHT, LTα, HVEM, BTLA, and CD160. *Left complex* shows formation of

Canonical Ligands: LIGHT and LTα

Lymphotoxin-α and LIGHT are members of the tumor necrosis factor superfamily (TNFSF) having a common structural motif that forms TNFR binding site. LIGHT was initially identified as cellular ligand for HVEM through the characterization of a distinct 30 kDa HVEM-binding protein on the surface of an activated human CD4+ T cell hybridoma (II-23) [7]. LTα is one of the original tumor necrosis factors [10]. LTα contains a classic signal cleavage site and is secreted as a homotrimer, while LIGHT is a type 2 transmembrane glycoprotein. The extracellular domain of LIGHT may be cleaved from the surface and released in a functional soluble form [11].

The LIGHT gene is located on human Chr 19p13 and a genetic paralog of LTβ, FasL, and TL1A [12]. LIGHT shares significant amino acid sequence homology with the C-terminal receptor-binding domains of LTβ (34% identity), and it shares binding to the LTβR, which engages the heterotrimer, LTα1β2. LIGHT, like all TNFSF members, forms a trimeric complex [13, 14] that allows multivalent binding with cell surface receptors. Receptor clustering is the key initiating step in the activation of TNF receptor signaling [15].

Both LIGHT and LTα bind to a similar region of HVEM. The binding site of LIGHT and LTα on HVEM were mapped on cysteine-rich domain-2 (CRD2) and CRD3 using HVEM mutants. Although the binding sites of LIGHT and LTα on HVEM are distinct, it is likely that their binding sites are topographically overlapping as the molecules are cross competitive [7]. It has been shown that HVEM has a stronger binding avidity to LIGHT than LTα [7], and LIGHT-induced HVEM signaling results in the recruitment of the TNF receptor-associated factor 2 (TRAF2) to the cytoplasmic tail of HVEM. The activation of a TRAF-dependent NF-κB pathway provides positive costimulation and prosurvival signal to T cells [13]. Although there have been many studies on the binding, structure and function of LTα in particular, in relation to TNFR1 and TNFR2, the distinct role of LTα on the HVEM signaling network remains unclear. However, LTα enhanced binding interactions between HVEM and BTLA [16, 17] presumably through oligomerization of HVEM. Additional studies are needed to further define the impact of LTα in the LIGHT–HVEM–BTLA/CD160 signaling system.

The expression of LIGHT is regulated at the transcriptional level [7, 14]. LIGHT is inducible but transiently expressed on the surface of activated T lymphocytes

Fig. 36.1 (continued) a LIGHT–HVEM–BTLA trimolecular complex with three molecules of soluble LIGHT, HVEM, and BTLA. *Right complex* shows the assembling of LIGHT–HVEM–CD160 trimolecular complex with three molecules of soluble LIGHT and HVEM, and nine molecules of CD160. (**c**) Model of membrane LIGHT, HVEM, BTLA, and CD160 interaction. Membrane LIGHT competes with BTLA for the binding with HVEM. The binding of membrane LIGHT to HVEM prevents BTLA or gD from binding to HVEM due to membrane restriction (*left complex*). The flexibility of the GPI-link in CD160 may accommodate the membrane restraint for the formation of membrane LIGHT–HVEM–CD160 trimolecular complex (*right complex*)

[7, 14]. Although both LIGHT and LTα are expressed in activated T cells, the transcriptional regulation of their genes appears to be mediated via different signaling pathways [7]. LIGHT expression was detected in MCF10A breast epithelial line [18] and melanoma cells [19]. Thus, LIGHT appears to have a broader range of expression compared to LTα, which is limited to activated T cells, B cells, NK cells, and LTi cells.

Unconventional Ligands: BTLA and CD160

BTLA and CD160 were originally identified as receptors for HVEM [20, 21] involved in activating inhibitory signaling. However, recent studies demonstrated that both BTLA and CD160 serve as activating ligands for HVEM [8]. BTLA or CD160 binding to HVEM induced HVEM-dependent NF-κB activation, demonstrating bidirectional signaling between HVEM and BTLA (Fig. 36.1a) in cells interacting in *trans* [8]. BTLA appears to form dimers as a membrane protein, providing a basis for oligomerizing HVEM that leads to TRAF2 recruitment and activation of NF-κB RelA. These results highlight the complexity of LIGHT–HVEM–BTLA/CD160 cosignaling networks.

In contrast to the TNFSF ligands, BTLA is a type 1 transmembrane protein with a single intermediate (I) type Ig domain. Three conserved tyrosine-based signaling motifs, two ITIM, and a Grb-2 recognition consensus are present in the cytoplasmic domain of BTLA in both mouse and human [22]. The utilization of the ITIM and recruitment of the protein tyrosine phosphatases, Src homology domain (SHP)-1 and SHP-2, into the cytoplasmic tail of BTLA appear to be HVEM dependent [23, 24]. However, the distinct role of the ITIM and the contributions of SHP-1 and SHP-2 in BTLA intracellular signaling have not been precisely defined. BTLA is expressed in a broad range of hematopoietic cells, including mature lymphocytes, splenic macrophages, dendritic cells, as well as T and B cells in the developing thymus [25, 26], and thus the effects of BTLA signaling may modulate many aspects of innate and adaptive immunity.

CD160 was originally identified as a binding partner of MHC class I molecules with weak binding affinity [27]. Recent studies by Cai et al. [21] demonstrated that CD160 exhibited specific binding to HVEM and that signal transduction mediated through HVEM and CD160 was shown to be inhibitory to T cells. CD160 is also a member of the Ig family with a single Ig V-like domain and a predominant glycosylphosphatidylinositol (GPI) motif, which enables anchorage to the cell surface. It has been shown that the GPI anchored CD160 on activated T cells was cleaved by a metalloprotease [28]. Furthermore, additional isoforms of CD160 have also been reported. Giustiniani et al. [29] have recently indentified three isoforms of CD160 (CD160ΔIg-GPI, CD160-TM, and CD160ΔIg-TM) that were generated by alternative splicing. Both CD160ΔIg-GPI and CD160ΔIg-TM have a deletion of the Ig domain, which is likely to abolish their binding capability to HVEM. Additionally, CD160-TM and CD160ΔIg-TM also contain transmembrane

and intracellular domains. Although the distinct role of these new isoforms in LIGHT–HVEM–BTLA/CD160 cosignaling system remains to be determined, it is anticipated that the newly found isoforms, in particularly the CD160-TM, would allow intracellular signaling. Unlike BTLA, CD160 assemble as a trimer with interchain disulfide bridges [30]. The trimeric form of CD160 along with its high binding avidity to HVEM enables it to function as a highly effective ligand for HVEM. CD160-expressing EL4 cells specially activated HVEM-dependent NF-κB reporter in transfected 293T cells and induced nuclear translocation of RelA in HT29 colon cell line, which naturally express HVEM [8]. The expression of CD160 is restricted to the hematopoietic compartment. CD160 is expressed in NK and NKT cells, γδT cells, intestinal intraepithelial T cells, $CD8^{+}CD28^{-}$ T cells, and a subset of CD4 T cells, but not in B cells [30]. CD160 mRNA was detected in spleen, peripheral blood, and lymphocytes in the small intestine [30].

Distinct Ligand Binding Sites on HVEM

HVEM is a typical TNFRSF member with four cysteine-rich domains (CRD). The 11 disulfide bonds in the ectodomain create an elongated structure. The presence of at least two distinct ligand-binding sites in topographically separate regions of HVEM allows this receptor to simultaneously interact with multiple ligands. LIGHT and BTLA bind on opposite sides of HVEM. BTLA and CD160 engage residues in the N-terminal CRD1 of HVEM, whereas the contacts of LIGHT and LTα are located in CRD2 and CRD3. The binding of soluble LIGHT to HVEM does not inhibit either BTLA or CD160 binding to HVEM [8, 16, 31], substantiating the conclusion that LIGHT and BTLA have distinct binding sites. Interestingly, soluble LIGHT and BTLA bind cooperatively to HVEM. Soluble LIGHT or LTα enhanced the binding of BTLA to HVEM [16, 17]. This result is consistent with a view that LIGHT clusters HVEM, which in turn, increases the avidity for BTLA (Fig. 36.1b). This interpretation suggests that soluble LIGHT, HVEM, and BTLA form a trimolecular complex.

We suggested a model in which the LIGHT–HVEM–BTLA trimolecular complex is likely to contain a trimer of soluble LIGHT with three molecules of HVEM and BTLA. However, the trimolecular complex of LIGHT–HVEM–CD160 would contain a trimer of LIGHT and three HVEM, and three trimers of CD160 (Fig. 36.1b). The formation of these higher ordered complexes not only enhances binding between HVEM, LIGHT, LTα, BTLA, and CD160, but also enhances HVEM clustering and, importantly, HVEM signaling.

Viral Ligands of HVEM and BTLA: HSV gD and UL144

HSV-1 gD is a type 1 transmembrane glycoprotein. The N-terminal ectodomain contains three N-glycosylation sites and six cysteine residues for the formation of three disulfide bridges. Sequence structure analysis revealed an IgV-like domain at

the N- and C-terminal extensions [32, 33]. Although there is no significant sequence homology between gD and other cellular ligands of HVEM, gD shows direct binding with HVEM. The binding site of HSV gD was mapped in the CRD1 of HVEM [34]. The gD site is topographically close to the BTLA site on HVEM, but their binding sites are not identical [16]. The unique location of the gD site on HVEM enables gD to perform two distinctive functions. Firstly, the binding of gD-Fc on HVEM prevents HVEM from binding to both LIGHT and BTLA. The ectodomain of gD serves as a multi-function inhibitor, which not only blocks binding of HVEM to LIGHT, but also to BTLA. Secondly, gD forms a stable dimer with a disulfide link between two subunits [35, 36]. The dimeric nature of gD allows it to oligomerize HVEM, serving as a functional ligand. The gD–HVEM complex is likely to contain two molecules of gD and two HVEM. Direct evidence for supporting this hypothesis came from the observation that the binding of gD-Fc to HVEM activated NF-κB [8].

UL144 is a herpes virus ortholog of HVEM [37] that binds BTLA [16], but not to the canonical TNF ligands, LIGHT and LTα. UL144 is encoded within the ULb' region of human cytomegalovirus, a β-herpesvirus. The protein contains two CRD homologous to CRD1 and 2 of HVEM explaining the lack of binding of the TNF-related ligands. The protein is highly variable in the ectodomain [38] but contains a conserved short intracellular domain. The role of UL144 in the pathogenesis of CMV is unknown, but the engagement of BTLA suggests a possible role in immune evasion.

The *Trans* and *Cis* of HVEM Signaling

In general, HVEM is viewed as a positive immune regulator since it activates NF-κB transcriptional programs that are involved in cell survival and proliferative responses. In addition, ligation of BTLA or CD160 to HVEM also activated NF-κB in a TRAF2-dependent pathway, providing a prosurvival signal for T cells [8]. In this setting, the ligands and receptors function in *trans* between interacting cells. However, our recent studies indicate that HVEM and BTLA can interact in *cis*, laterally within the same membrane [8]. Flow cytometric analysis demonstrated that *cis*-interaction between HVEM and BTLA is the predominant complex expressed on the surface of naïve human and mouse T cells, and the formation of HVEM–BTLA *cis*-complex inhibited HVEM-dependent NF-κB activation. The heterodimeric complex of HVEM and BTLA uses the same site in CRD1 as for *trans*-interaction, with only the ectodomain of BTLA required to form the *cis*-complex. The HVEM–BTLA *cis*-complex competitively inhibits *trans*-signaling by all its cellular ligands, providing a mechanism for maintaining T cells in a resting state. As the binding sites of BTLA and CD160 on the CRD1 of HVEM are topographically close to each other, the receptor binding domains of BTLA and CD160 also act as a competitive inhibitors blocking BTLA or CD160 from interacting with HVEM in *trans* and inducing NF-κB activation.

Interestingly, herpes simplex virus envelope glycoprotein gD also forms a *cis*-complex with HVEM. *Cis*-association between gD and HVEM blocks *trans*-interaction of LIGHT, BTLA, or gD to HVEM in the *cis*-complex. This is consistent with the observation that gD expressing cells were resistant to HSV infection, indicating that gD might interfere with its endogenous receptors [39]. We propose that the formation of *cis*-complexes between HVEM and BTLA, CD160 or gD competitively (against BTLA, CD160 and gD) or non-competitively (against LIGHT and LTα) inhibits HVEM activation by ligands expressed in the surrounding microenvironment, allowing T cell to remain in the naïve state.

Although LIGHT interacts with HVEM in a topographically distinct site, which differs from the BTLA binding site, membrane LIGHT inhibits HVEM–BTLA *trans*-interaction [16]. These results suggested that the proximity of the membrane may sterically exclude HVEM from binding BTLA when membrane LIGHT occupies its binding site in the CRD2 and 3 regions. Promoted by high affinity binding, the LIGHT–HVEM *trans*-complex, may in turn, sterically compete with membrane BTLA from binding HVEM, thus acting in a noncompetitive fashion to disrupt inhibitory signaling by BTLA (Fig. 36.1c, left complex). We propose that the proximity of TNF homology domain of LIGHT to the membrane surface could prevent BTLA from engaging HVEM when HVEM engages LIGHT in *trans*. Similarly, the close proximity of the base of the LIGHT trimer to the membrane surface would also prevent access of gD to CRD1 of HVEM (Fig. 36.1c, left complex). However, the inherent flexibility of the GPI linkage in CD160 might allow CD160 to accommodate the steric requirement for the formation of the membrane LIGHT–HVEM–CD160 trimolecular complex (Fig. 36.1c, right complex).

The LIGHT–HVEM–BTLA/CD160 cosignaling system has the potential to simultaneously deliver stimulatory and inhibitory signals between interacting cells. Clear evidence indicates that HVEM–BTLA play a counter-regulatory role with the LTβR system in controlling dendritic cell proliferation within lymphoid tissues [5]. *Cis*-association between HVEM and its unconventional ligands, as well as the formation of the higher ordered trimolecular complexes with soluble LIGHT and LTα add additional levels of regulatory complexity in the LIGHT–HVEM–BTLA/CD160 system. The outcome of the signal transduction process mediated via HVEM not only depends on the timing of expression of individual signaling molecules, but also the distinctive combinations of molecules within the *cis*- and *trans*-complexes.

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Chapter 37
The Lymphotoxin Pathway as a Novel Regulator of Dendritic Cell Function

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Dendritic cells (DC) are critically required for the host response to antigen (Ag) [1, 2]. Upon exposure to Ag, DC take up Ag within the peripheral tissues and subsequently migrate in response to chemokine gradients into the lymphatics [3]. During this initial exposure to Ag, DC become activated by the sensing of microbe-associated molecular patterns (MAMPs) and consequently upregulate co-stimulatory molecules such as B7.1 and B7.2 so that they may optimally prime Ag-specific $CD4^+$ T cells [4–6]. DC leave the tissue and enter the lymphatics where they journey to the inflamed draining lymph node (LN) [7, 8]. Upon entry into the LN via the subcapsular sinus, DC "find" rare Ag-specific CD4+ T cells by taking advantage of the intricate organization of the LN tissue itself [9]. Once DC encounter Ag-specific T cells, the T cells become activated to proliferate. These activated T cells also concurrently provide signals back to the Ag-bearing DC in a process that has been termed DC licensing. Licensed DC can then cross-prime a $CD8^+$ T-cell response so that pathogen may be cleared. The nature of these helper T cell-derived signals and their impact on the Ag-bearing DC remain poorly elucidated.

It is increasingly clear that additional signals beyond the core co-stimulation molecules B7.1 and B7.2 are required to shape the nature of the adaptive T-cell response. For instance, tumor necrosis factor (TNF) family members including CD40 ligand (CD40L), RANK ligand, Lymphotoxin-αβ (LTαβ), and LIGHT are rapidly upregulated on Ag activated $CD4^+$ T helper cells [10, 11]. During the immune response, DC:T-cell interactions result in the ligation of CD40 and RANK on DC, and this has been shown to promote the cross-priming capacity of DC and DC survival [12–16].

Signaling via the lymphotoxin-β receptor (LTβR) has been implicated in a number of settings that can influence the activation of Ag-specific T cells. For instance, signaling of the LTβR is required for the optimal secretion of lymphocyte-attracting

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chemokines [17]. Moreover, LTβR signaling on high endothelial venule (HEV) cells is critically required for the appropriate sulfation and luminal display of peripheral lymph node addressing (PNAd) [18, 19]. Thus, inhibition of LTβR signaling in the context of T-cell priming within the draining lymph node could cause a number of problems including altered T-cell migration to LN and impaired positioning within the LN parenchyma. Furthermore, LTβR signaling in other lymphoid tissues, and in some non-lymphoid tissues, is important for various aspects of tissue homeostasis (Table 37.1).

Interestingly, LTβR signaling has been implicated in the etiology of a number of autoimmune diseases in rodent models. These include diabetes, experimental autoimmune encephalomyelitis (EAE), uveitis, inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and transplant tolerance, all of which involve T-cell activation (Table 37.2). The question of the mechanism of action of LT inhibition in the context of these settings is a daunting one. How does one separate the pleiotropic effects of LTβR signaling on the immune system in order to understand the relative contribution of this pathway in the context of autoimmunity? In particular, as all of these autoimmune models require the inappropriate activation of auto-specific T cells, how does the LT pathway facilitate this process? These questions will guide our thinking on how to best use LT pathway inhibitors in the clinic.

To begin to answer this question, one must start with the earliest events of T-cell priming and query the role of the LT pathway in this context. LTβR is widely expressed on a number of cell types including stromal cells, HEV, follicular dendritic cells, and DC. In contrast, the expression of the LTαβ ligand is limited to the hematopoietic compartment [20]. A small amount of LTαβ is expressed on resting B cells; however, the level of LTαβ on activated germinal center B cells is significantly higher [21, 22]. Likewise, LTαβ is highly expressed on Ag-activated T cells in vivo and in vitro. However, the expression of LTαβ is tightly regulated as it is typically not observed on naïve T cells and is rapidly downregulated on the surface of activated T cells following exposure to Ag. Indeed, the expression of LTαβ on activated T cells seems to follow similar kinetics as the expression of CD69, peaking during the first 36 h of the immune response [11]. Since it is during this period that T cell:DC encounters are most frequent, and because the LTβR is expressed on DC [23], an important question is whether LTβR signaling in DC has any functional relevance on the adaptive immune response.

Our lab first assessed this question by using a competitive inhibitor of the LT pathway, LTβR-Ig. This agent acts as a soluble decoy, inhibiting all LTβR-mediated signaling via either LTαβ ligand or the alternative ligand LIGHT. Furthermore, this agent will prevent LIGHT binding to HVEM. In order to ascertain a role for LTβR signaling in DC function during an in vivo immune response, we immunized mice with ovalbumin (OVA) protein Ag due to the availability of T-cell receptor (TCR) transgenic mice specific for OVA peptides presented in the context of MHC class I (OTI mice) and MHC class II (OTII mice). Specifically, mice were given an adoptive transfer of $CD4^+$ OTII OVA-specific T cells followed by a subcutaneous injection of OVA plus LPS adjuvant (Fig. 37.1). By pre-injecting OVA-specific $CD4^+$ T cells, the dynamics of DC:T cell interactions were synchronized to occur within the first

Table 37.1 LTβR signaling in tissue homeostasis

Lymphoid organogenesis	LTβR signaling is required for development of LN and Peyer's patches, with discrepant requirements for LTα and LTαβ for some LN	[26–28]
Lymphoid microenvironments	**Spleen** Constitutive LTβR signaling is required to retain FDC network, germinal center formation, marginal zone, and B cell–T cell boundaries	[17, 29–32]
	LN Constitutive LTβR maintains chemokine gradient-driven B-T zones, germinal center formation, FDC networks LTβR signaling enables HEV-specific gene expression (MAdCAM, GlyCAM, PNAd, and the sulfotransferases that mediate PNAd glycosylation) and function and thereby regulates lymphocyte entry into LN	[11, 18, 33, 34]
	Thymus LTβR signaling regulates expression of chemokines (CCL19, CCL21) by thymic medullary epithelial cells (mTEC) that allow proper migration of developing thymocytes. Absence of LTβR expression results in reduction of the size and number of mTEC	[35–37]
	GALT Constitutive LTβR signaling is required for the formation of isolated lymphoid follicles and colonic patches and for recruitment of B cells to and IgA production within the lamina propria	[38–41]
Dendritic cell homeostasis	DC-intrinsic LTβR signaling is required for homeostatic maintenance of splenic DC. HVEM-BTLA signaling counterregulates LTβR-driven homeostatic expansion of DC	[23, 42–44]
Liver function	LTβR signaling via T-cell-derived LIGHT regulates hepatic expression of enzymes involved in lipid metabolism. LTβR signaling by T-cell-expressed LTαβ regulates liver regeneration and LTα and LTβ have been implicated in the induction of hepatocellular carcinoma	[45–48]
NK, NK-T cells	LTβR signaling is required for NK and some populations of NK-T cell development	[49, 50]
Tertiary lymphoid structures (TLS)	LTβR signaling is required for chemokine and adhesion molecule expression in TLS formation	[51–53]

Table 37.2 Involvement of the lymphotoxin pathway in autoimmune disease

Type I diabetes (TID)	Prevention of TID with therapeutic LTβR-Ig in NOD mice, reversal of insulitis with therapeutic inhibition	[54] [55]
	Prevention of TID dependent on continued LTβR signaling inhibition by ectopically expressed LTβR-Fc	[56] [57]
	Absence of LN following embryonic inhibition of LTβR protects from TID in adult NOD mice	[58]
	Insulitis facilitated by LTβR signaling-dependent TLS	[59]
	Human TNF/LTα polymorphisms associated with TID with protective and susceptible haplotypes identified	
Rheumatoid arthritis (RA) (mouse model: collagen-induced arthritis, CIA)	Prophylactic LTβR-Ig inhibits CIA and therapeutic LTβR-Ig-alleviated CIA	[60]
	Inhibition of LT pathway in $LT\alpha^{-/-}$ mice or by therapeutic LTβR-Ig increased CIA severity	[61]
	Preventative and therapeutic depletion of LTα-expressing Th1 and Th17 cells relieves CIA	[62]
	LTβR stimulation of RA synoviocytes induces production of inflammatory mediators	[63]
	LTβR, LIGHT, and LTβ are expressed in RA synovium	[62, 64, 65]
Multiple sclerosis (mouse model: EAE)	Inhibition of monophasic and relapsing-remitting EAE with prophylactic LTβR-Ig treatment	[66]
	Prophylactic and therapeutic LTβR-Ig reduces EAE disease severity	[67]
	Prophylactic depletion of LTα-expressing T cells reduces EAE severity	[62]
Sjögren's syndrome	LTβ expression is elevated in salivary glands of Sjogren's patients	[68]
	LTβR-Ig reduced salivary gland T- and B-cell infiltration, ablated FDC, and restored saliva flow	[69]
Inflammatory bowel disease	LTβR signaling inhibition attenuates colitis in two transfer models of colitis	[31]
	Therapeutic LTβR-Ig treatment of mice with chronic DSS-colitis attenuates disease	[70]
	Th2-type colitis is sensitive to LTβR inhibition via dissolution of colonic patches	[41]
	LTβR-Ig treatment ameliorates TNBS-colitis	[71]
	Opposing roles for T cell and B cell-expressed LTαβ in the regulation of DSS-colitis	[72]
Uveitis	LTβR signaling inhibition protects from actively induced but not adoptively transferred uveitis	[73]
Transplantation/GVHD	Combined LTβR-Ig and α-CD40L treatment completely prevents GVHD	[25]
	LTβR-Ig completely inhibits GVH skin disease and reduces cutaneous ICAM-1 expression	[74]
	Either LTβR-Ig or LTβ blockade inhibits intestinal allograft rejection by CTL	[24]
	LIGHT-HVEM blockade ameliorates GVHD	[75]
	LTβR-Ig and CTLA4-Ig combined treatment induces long-term islet allograft survival	[76]
	Donor-derived LTα contributes to GVHD and is required for GV leukemia	[77]

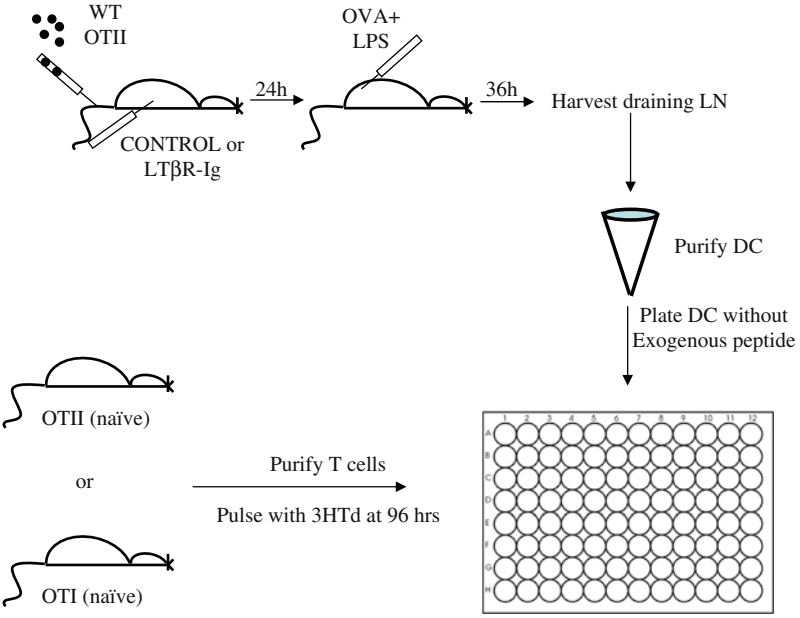

Fig. 37.1 Experimental setup for measuring DC function. This setup is an "in vivo/ex vivo" assay of DC function. The experiment examines the role of LTβR signaling in the host for optimal DC function. The experiment does not distinguish effects of LTβR signaling on the stroma versus DC-intrinsic LTβR signaling

36 h of the immune response, and if OTII T cells were not co-injected, we found that DC-specific readouts ex vivo were weak. Accordingly, 36 h post-immunization, we removed the draining LN, digested these tissues, and purified DC. Purified LN DC from control or LTβR-Ig-treated mice were then plated at equal numbers with naïve OVA-specific $CD4^+$ T cells (OTII) or naïve OVA-specific $CD8^+$ T cells. Using the proliferation of these naïve T cells as a readout, we were able to ascertain the functionality of DC ex vivo. Furthermore, given that we do not add exogenous peptide Ag to these DC:T cell co-cultures, we are assaying only the maturation/Ag presentation events that occur in vivo.

With this assay in place, we made the discovery that DC from LTβR-Ig-treated mice clearly exhibited functional defects when assayed ex vivo [11]. However, it is important to note that the effects of LTβR-Ig in vivo are multiple (see Table 37.1) and our experiment was not able to pinpoint the exact effect of LTβR-Ig on the DC themselves. Given the important role of LTβR signaling on lymphoid tissue stromal cell homeostasis, there was the possibility that DC were dysfunctional in LTβR-Ig-treated mice due to poor migration to the draining LN or disorganized positioning within the LN paracortex. To eliminate the possibility of a positional defect within the draining LN, we used fluorochrome-associated OVA protein that provides

a fluorescent readout when taken up and digested within subcellular compartments of antigen presenting cells (DQ-OVA). By flow cytometry and fluorescence microscopy, we established that DC were the only cell type that was processing OVA in the draining LN, and that DC from LTβR-Ig-treated mice exhibited no obvious defect in their ability to home to the LN, process OVA, or position within the paracortex [11].

However, it still remained possible that the LT pathway was having an effect on the LN stroma that resulted in modified DC function. Moreover, since LTβR-Ig will neutralize LIGHT, the role for LIGHT:LTβR or LIGHT:HVEM signaling in our system was unresolved. To answer these questions, we turned our attention to the Ag-specific T cells that condition or "license" DC during the immune response. We reasoned that if there is truly a role for LTαβ:LTβR signaling during $CD4^+$ T cell:DC communication, then DC that are conditioned by LTαβ-deficient $CD4^+$ T cells should exhibit a functional defect. We took this reasoning a step further and hypothesized that the Ag-specific $CD4^+$ T cells which rapidly upregulate LTαβ upon Ag exposure were the relevant LTαβ-expressing cell for delivery of DC "licensing"

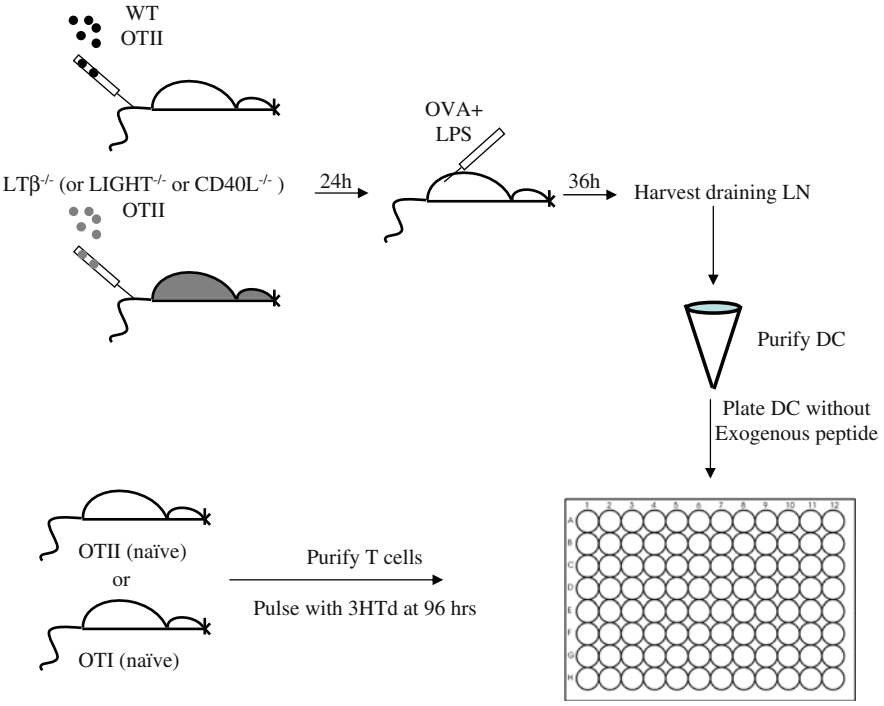

Fig. 37.2 Experimental setup for assaying DC:T-cell cross-talk. Using the "in vivo/ex vivo" assay of DC function described in Fig. 37.1, the genotype of the OVA-specific $CD4^+$ T cell (OTII T cell) is manipulated. In this manner, the relevance of the expression of TNF superfamily member ligands (LTβα, LIGHT, and CD40L) on Ag-specific T cells only is assessed in the context of DC conditioning. LTβR signaling in the host remains intact and the lymph node architecture is preserved

signals. To test this, we exploited our OTII-dependent system (Fig. 37.1) and modified the genotype of the OTII T cells (Fig. 37.2). We performed an adoptive transfer of WT versus LTβ$^{-/-}$ OTII T cells into WT mice. In this context, the only cells incapable of expressing LTαβ are the Ag-specific T cells. Using this approach, we found that the DC defect we observed with LTβR-Ig was fully recapitulated. Thus, our experiment showed that LTαβ expression on Ag-specific T cells was critical for DC function. We also exploited this system further to evaluate the role of LIGHT during T cell:DC cross-talk. We performed an adoptive transfer of LIGHT$^{-/-}$ OTII T cells and extracted the DC post-immunization. In contrast to the mice that received LTβ$^{-/-}$ OTII T cells, DC extracted from mice receiving LIGHT$^{-/-}$ OTII T cells did not exhibit a defect in their ability to prime naïve T cell ex vivo. Therefore, at least in the context of protein Ag, expression of LIGHT by Ag-specific T cells is not required for DC function.

The expression of LTαβ and its counter-receptor LTβR during T cell:DC cross-talk is reminiscent of the expression of CD40L on activated Ag-specific CD4^{+} T cells. Indeed, stimulation via CD40 is considered the classical "DC licensing" signal. Yet our results showed that the expression of LTαβ on Ag-specific T cells was essential for DC function and could not be replaced by CD40L. In our system, we also found the converse to be true: if we performed an adoptive transfer of CD40L$^{-/-}$ OTII T cells and immunized recipient mice, the extracted DC were likewise unable to prime T cells ex vivo. These results beg the question of how the LTβR and CD40 signaling pathways are working together to provoke full DC functionality. We found that if we administer a large amount of an anti-LTβR agonist antibody to mice that had received CD40L$^{-/-}$ OTII T cells that DC functionality was restored. Likewise, anti-CD40 agonist antibodies could rescue DC function in mice that had received LTβ$^{-/-}$ OTII T cells. While these experiments argue in favor of redundancy between LTβR and CD40 signaling, the agonist Ab experiments certainly do not mimic what occurs in vivo when the ligands for the LTβR and CD40 are expressed fleetingly and in small amounts. Future experiments addressing the unique downstream effects of activation of LTβR versus CD40 in DC during the immune response, where CD40L and LTαβ are expressed in a physiologically regulated context, will reveal how different TNF family members contribute to CD8^{+} T-cell cross-priming.

Our experiments have revealed a novel role for the LTαβ/LTβR pathway in DC function. Much remains to be learned on the downstream signals provided by LTβR stimulation in DC. However, modulation of DC function could be at the heart of the mechanism of action of LTβR-Ig in a plethora of autoimmune models, and dissection of the contribution of LTβR on DC in these models is required. In the context of allograft tolerance, inhibition of LTβR had an impact on the CD8 response [24], suggesting that LTβR signaling may play an important role in DC-mediating CD8^{+} T-cell cross-priming. This would have important implications for scenarios where one wishes to reduce immunity to protein Ag, as in autoimmunity, or boost responses to tumor antigen. Interestingly, the most profound repression of graft versus host disease was achieved by dual administration of CD40 and LTβR pathway blockers [25]. Our data suggest that these pathways are not redundant and are

providing unique signals to DC. Perhaps it's time to think about co-therapies to multiple TNF family members for treating aggressive autoimmune disease?

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Chapter 38
Involvement of the Cytokine Receptor CD137 in Murine Hematopoiesis

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Introduction

CD137 (TNFRSF9, 4-1BB, induced by lymphocyte activation, ILA) is a member of the tumor necrosis factor (TNF) receptor family [11, 24]. Expression of CD137 is strictly activation dependent and transient on immune cells [25]. High levels of CD137 can be found on activated T cells and cross-linking of CD137 delivers potent costimulatory signals to T cells. Recombinant CD137 ligand or agonistic anti-CD137 antibodies enhance T-cell activity and immune responses leading to the elimination of even established tumors in mice [4, 20, 28, 30]. Activated natural killer (NK) cells express CD137 too, and NK cells are essential for the anti-tumor effects of CD137 agonists [20]. Humanized anti-CD137 antibodies are currently undergoing phase I clinical trials for cancer immunotherapy [7, 29].

CD137 ligand is expressed by antigen presenting cells (APC), and APC use CD137 ligand to costimulate CD137-expressing, activated T cells. The CD137 receptor/ligand system is capable of bidirectional signaling, a property it shares with several other members of the TNF receptor/ligand families [6]. The molecular basis of bidirectional signaling is that CD137 ligand, just as CD137, is expressed as a transmembrane protein on the cell surface and can transmit a signal into the cell it is expressed on, a process referred to as reverse signaling [26].

Treatment of peripheral human monocytes with CD137 ligand agonists enhances cellular activation as evidenced by expression of activation markers (TNF, IL-6, IL-8, ICAM) and inhibition of the anti-inflammatory cytokine IL-10 [14]. The CD137 ligand signal synergizes with LPS in inducing cytokine release in human monocytes [17], and an association of the cytoplasmic domain of CD137 ligand with that of Toll-like Receptor (TLR)-4 has been demonstrated in murine macrophages [9]. CD137 is also expressed by cells of the vascular wall at sites of inflammation [1] and CD137 is involved in the recruitment of monocytes into the inflammatory tissue [5, 22].

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The CD137 ligand signal also induces release of the potent monocyte growth factor macrophage colony-stimulating factor (M-CSF) and prolongs survival of monocytes [15]. CD137 ligand cross-linking induces even growth of monocytes. Specifically, it induces DNA replication, synthesis of cell material, and an increase in cell size and nuclear division. However, an increase in cell number was not noticed, rather multinucleated giant cells appeared [16]. Therefore endomitosis, rather than proliferation (characterized by an increase in cell numbers), was induced by CD137 ligand signaling in peripheral human monocytes.

Since peripheral monocytes are assumed to be mature and terminally differentiated cells they were regarded not to be able to proliferate anymore. Therefore, it was surprising to observe induction of monocyte growth by CD137. This prompted the hypothesis that CD137 may exert similar growth-promoting effects on the precursors of monocytes, the hematopoietic stem and progenitor cells.

Effects of CD137 on Murine Hematopoietic Progenitor Cells

CD137 and Its Ligand Are Expressed in the Bone Marrow

In order for the CD137 receptor/ligand system to play a role in hematopoiesis the CD137 and its ligand would need to be expressed in the bone marrow. Indeed, CD137 was found to be expressed on few but distinctive cells among murine bone marrow cells, including myeloid progenitor cells as detected by immunostaining and flow cytometry [8, 18]. Whether CD137 can also be expressed by bone marrow stromal cells is not known. CD137 ligand is expressed on hematopoietic stem cells, common myeloid progenitors, and granulocyte–macrophage progenitors by flow cytometry [8, 18]. The expression of CD137 and CD137 ligand in the bone marrow cells was also detectable at the mRNA level by reverse transcription-PCR. These data showed that the CD137 receptor/ligand system could have a function in hematopoiesis.

CD137 Induces Proliferation and Colony Formation of Hematopoietic Progenitor Cells

In order to investigate a potential function of the CD137 receptor/ligand system in hematopoiesis bone marrow cells were isolated from the femur bones of BALB/c mice. For cross-linking of CD137 ligand the cells were cultured in tissue culture plates that were coated with a recombinant fusion protein consisting of the extracellular domain of human CD137 and the constant domain (Fc) of human IgG1 (CD137-Fc) or an Fc control protein.

CD137-Fc protein induced attachment and spreading of most bone marrow cells, whereas the cells in the control wells remained round and did not attach. Attachment

and spreading in response to CD137 was accompanied by morphological changes of the cells, which became apparent on days 2–3 and were most prominent on day 7. During the first 3 days, CD137-Fc-treated cells grew a tail-like extension on one end and lamellipodia on the opposite end. By day 7, the tails had grown longer, sometimes reaching five times the length of the cell body, whereas the lamellipodia were changing to filopodia. Some cells had several branches and displayed star-like shapes. From days 10–17 filopodia disappeared and tails shortened. Some cells adopted round and flat ovoid shapes with big nuclei [8].

CD137-Fc treatment increased the cell numbers in bone marrow cultures after day 3 and cell numbers peaked at day 10, whereas in the Fc control condition, most cells were dead after day 7. The ability of CD137 to maintain cell survival was even more potent than that of granulocyte colony-stimulating factor (G-CSF). The CD137-induced increase in cell number was dose dependent. On day 7, the difference in cell number between the CD137-Fc and the Fc control treatment was significant starting from a coating concentration of 1.2 μg/ml and reaching saturation at 20 μg/ml. CD137 treatment also induced cell proliferation and cell division as detected by measuring DNA synthesis, using ^{3}H-thymidine incorporation and carboxyfluorescein diacetate succinimidyl ester labeling, respectively [8].

CD137-Fc needed to be immobilized onto the tissue culture plates in order to induce proliferation and survival of bone marrow cells. When CD137-Fc was added to the cells as a soluble protein and its immobilization to the tissue culture plates was prevented by precoating the plates with bovine serum albumin (BSA), no increase in cell numbers and cell proliferation was observed, proving that CD137-Fc in its soluble form was not active.

The subset of bone marrow cells that are considered to be most enriched for hematopoietic progenitor cells is negative for lineage-specific markers (lin^-) and positive for CD117 (c-kit), the receptor for stem cell factor. Lin^-, $CD117^+$ cells constitute only 0.5–1% of bone marrow cells. CD137-Fc induced similar morphological changes in lin^-, $CD117^+$ cells as in total bone marrow cells. CD137-Fc also increased proliferation of the lin^-, $CD117^+$ cells to the same extent (about 5-fold) as that of total bone marrow cells. But total cell proliferation was about 10-fold higher in lin^-, $CD117^+$ cells than in unfractionated bone marrow cells.

Proliferation induced by CD137-Fc was confirmed by colony forming assays. Bone marrow cells were cultured in methylcellulose medium containing 10% bovine calf serum on tissue culture plates with immobilized CD137-Fc or Fc protein. There were in average only 3±1 colonies in the Fc-coated dishes compared to 40±10 in the CD137-Fc-coated dishes. In addition, colonies on Fc protein contained fewer than 100 cells whereas colonies on CD137-Fc protein were composed of up to 5,000 cells. The lin^-, $CD117^+$ cells had formed colonies at a much higher rate than bone marrow cells. CD137 induced similar looking colonies in total bone marrow cells and lin^-, $CD117^+$ cells, implying that it were the lin^-, $CD117^+$ cells among the bone marrow cells that responded to CD137. Most colonies (80.8 ± 1.9%) were colorless with dark, dense cores or consisted of granular or foamy cells. Based on these morphological characteristics they were identified as CFU

granulocyte–macrophage (CFU-GM) [3, 31]. CFU macrophage (CFU-M), recognizable by their larger and individually identifiable cells, were also induced by CD137 and constituted 16.9 ± 1.4% of the colonies. Very few (2.3 ± 0.5%) CFU granulocyte (CFU-G) (smaller and more tightly packed cells) could be identified. No additional growth factors besides CD137 were present in this assay to support colony formation [8].

The cell proliferation and colony formation induced by CD137 was independent of granulocyte–macrophage colony-stimulating factor (GM-CSF), since a neutralizing anti-GM-CSF antibody had no influence on the morphology, proliferation rate, or the colony formation of lin^-, $CD117^+$ cells. These data clearly demonstrate that CD137 and its ligand can amplify hematopoietic progenitor cells.

CD137 Induces Cell Differentiation Toward Macrophages

Generally, growth factors that induce proliferation of hematopoietic progenitor cells also induce cell differentiation to a specific lineage. The induction of colony formation, especially of the CFU-GM type, suggested that CD137 induces differentiation of bone marrow cells as well as lin^-, $CD117^+$ cells to the myeloid lineage. This was confirmed by analyzing cell surface marker expression. Lin^-, $CD117^+$ cells cultured for 7 days in the presence of immobilized CD137-Fc protein contained around 30–40% of $CD11b^+$, $CD14^+$, $F4/80^+$, or $CD11c^+$ cells, while expression of these markers in the Fc controls was negligible. The absolute numbers of live cells expressing these myeloid markers were also enhanced accordingly. Total bone marrow cells cultured on immobilized CD137-Fc protein had increases in $CD11b^+$, $CD14^+$, $F4/80^+$, or $CD11c^+$ cells, although mature myeloid cells, such as monocytes and macrophages, among total bone marrow cells that are known to respond to CD137 ligand signals with survival and proliferation may have contributed to the numbers of live cells in that case.

The comparable numbers of cells expressing macrophage markers (CD14, F4/80) and the dendritic cell (DC) marker (CD11c) which CD137 ligand signal had induced in lin^-, $CD117^+$ cells were compatible with macrophage and DC differentiation. In order to clarify the nature of cells that had been derived from hematopoietic progenitor cells after differentiation with CD137 protein, their biological activities were assessed by functional assays. Lin^-, $CD117^+$ cells that were exposed for 7 days to CD137-Fc exhibited a substantially enhanced phagocytic ability compared to Fc-treated cells. In an allogeneic mixed lymphocyte reaction where CD137-Fc-treated cells were used as stimulator cells, they were unable to induce T-cell proliferation. CD137-Fc-treated cells secreted high amounts of IL-10 but little IL-12 upon LPS stimulation. These data indicated that CD137 induced differentiation to macrophages but not to DCs.

CD137 ligand cross-linking has been reported previously to support DC maturation. But in these studies DCs were derived from human umbilical cord blood $CD34^+$ cells or peripheral monocytes via culture with cytokine cocktails for

1–7 days before exposure to CD137 protein [10, 13, 19]. The above-described findings are not contradictory to these reports; rather they demonstrate wider biological activities of CD137 in myelopoiesis. While CD137 enhances maturation of immature DCs derived from hematopoietic progenitor cells, it will induce macrophage differentiation in hematopoietic progenitor cells when given as a first and sole signal [8].

The CD137 receptor/ligand system is involved in a number of different immune activities [4, 28, 30] , and reverse signaling by CD137 ligand has been documented in monocytes, DCs, B cells, and T cells where it has been shown to regulate activation, survival, proliferation, and apoptosis [26]. The data by Jiang et al. (2008) [8] added murine hematopoietic progenitor cells to this list and demonstrated that CD137 ligand signals also regulate myelopoiesis as depicted in Fig. 38.1.

Fig. 38.1 Schematic diagram of CD137-induced myelopoiesis

Other Activities of CD137 in Hematopoiesis

Aside from the above-described study by our laboratory several other reports demonstrated activities of the CD137 receptor/ligand system in hematopoiesis. Recently, Lee et al. (2008) [18] found CD137 ligand expression on hematopoietic stem cells, differentiating common myeloid progenitor cells and granulocyte–macrophage progenitor cells, and that CD137 expression was inducible on activated myeloid progenitors. These authors concluded that CD137–CD137 ligand interactions limited myelopoiesis and the development of DCs since they found that steady-state numbers of granulocyte–macrophage progenitor cells, myeloid lineage cells, and mature DCs were higher in CD137- and CD137 ligand-deficient mice than in wild-type mice. Also, the absence of interaction between CD137 and CD137 ligand led to an enhanced differentiation of progenitor cells to DCs [18].

The discrepancies between the study by Lee et al. (2008) [18] and our study may reflect the different function of the CD137 receptor/ligand system in the regulation of hematopoiesis, particularly myelopoiesis and DC development during different physiological states. Lee et al. (2008) [18] investigated the effects of CD137 on hematopoiesis in a steady-state condition while our study reflects more the situation during an infection, when high levels of CD137 are expressed. After all expression of CD137 is strictly activation dependent [26]. In order to allow clearance of the pathogen, it would be beneficial if CD137 ligand signaling would induce myelopoiesis in an inflammatory condition. That would not exclude that CD137 may inhibit myelopoiesis at steady-state conditions in order to maintain homeostasis.

Our data are partly in agreement with earlier studies which demonstrated that CD137 protein in combination with M-CSF increases proliferation of bone marrow-derived osteoclast progenitors but inhibits M-CSF + receptor activator of nuclear factor κB ligand (RANKL)-induced osteoclast differentiation [23, 27]. Some of the osteoclast progenitor cells that proliferated in response to CD137 + M-CSF in the study by Saito et al. (2004) [23] may also have been contained within the lin^{-}, $CD117^{+}$ cell population of our study. What is surprising, however, is that CD137 protein inhibited osteoclast differentiation while it promoted macrophage differentiation, since both cell types are closely related if not identical. This difference could be due to the different starting cell populations or the presence of M-CSF.

A positive effect of CD137 on myelopoiesis is supported by a CD137-mediated enhancement of DC survival and functions during an activated immune state. Choi et al. [2] reported that during LPS-induced inflammation and *Propionibacterium acnes* infection CD137 functioned as a survival factor for DCs by maintaining high levels of the anti-apoptotic proteins Bcl-2 and Bcl-XL.

A role of the CD137 receptor/ligand system in hematopoiesis is also evident from mice that were treated with anti-CD137 antibodies. Mice injected with anti-CD137 antibodies had a 10-fold increased number of bone marrow cells that had a hematopoietic stem cell phenotype [21]. Also, CD137-deficient mice have an increased number of myeloid precursor cells in bone marrow, spleen, and blood [12]. It is tempting to speculate that this increase in precursor cells is caused by a missing differentiation signal due to the absence of CD137 or its blocking by the antibody. Interestingly, transgenic mice overexpressing CD137 ligand on APC have a threefold elevated number of macrophages [32]. Anti-CD137-treated mice display additional hematopoietic defects such as thrombocytopenia, anemia, and lymphopenia [21]. The underlying mechanisms for these effects are currently unknown.

The analysis of CD137- and CD137 ligand-deficient mice has provided additional insight into the involvement of these molecules in hematopoiesis. CD137-deficient mice have normal development of lymphoid organs and normal numbers of T and B lymphocytes, suggesting that CD137 is not required for normal lymphocyte development in steady-state conditions.

Conclusion

The CD137 receptor/ligand system is best known for regulating the activities of mature immune cells. Several recent reports demonstrate that the CD137 and its ligand are expressed in the bone marrow and also regulate hematopoiesis, in particular myelopoiesis. Depending on whether an immune activation state or a steady-state condition exists, CD137 and CD137 ligand may enhance or inhibit myelopoiesis, respectively.

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Chapter 39
LTBR-Pathway in Sjogren's Syndrome: CXCL13 Levels and B-cell-Enriched Ectopic Lymphoid Aggregates in NOD Mouse Lacrimal Glands Are Dependent on LTBR

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The TNF Family and Inflammatory Versus Homeostatic Pathways

The fact that TNF receptor family members are involved in the control of diverse gene products that effect both pro-inflammatory and homeostatic functions related to immune protection offers multiple targets for clinical intervention in a range of disease contexts. The stunning success of anti-TNF-alpha therapy in the treatment of the inflammatory disease rheumatoid arthritis perhaps best illustrates the vast potential of antagonism of TNF family members in clinical medicine [1]. The involvement of other family members, such as CD40, in many other immune regulated diseases will also no doubt lead to similar success stories. In contrast to the pro-inflammatory pathways controlled by TNF-alpha, our lab has begun to determine whether antagonism of the "homeostatic" pathways in secondary and "ectopic" or tertiary lymphoid tissues that are under the control of the TNF receptor family member lymphotoxin-beta receptor (LTBR) might represent a useful target in the treatment of certain diseases such as Sjogren's syndrome where frank inflammation is not the primary pathogenic impetus.

Ectopic Lymphoid Tissue Development and Homeostasis in Disease

Currently there is a keen interest in understanding the underlying biology and the overall importance of the development of "ectopic lymphoid tissue," also referred to as "tertiary lymphoid organs or TLO," in the wide variety of organs and disease settings in which they occur. Ectopic lymphoid tissues often display features that are virtually identical to the micro-architecture found in bona fide secondary

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lymphoid follicles [2] including high endothelial venules expressing peripheral node addressins (PNAd), clearly segregated regions of T-cell and B-cell aggregation, FDC networks, and, as very recently reported in the landmark paper from Grabner et al., even the collagen-fibril conduits which transport chemokines and small antigens into follicular centers exactly like the conduits found in lymph nodes [3, 4]. It is especially interesting that ectopic follicles can sometimes support germinal center reactions [5–7]. Yet much remains to be learned about the uniformity of ectopic lymphoid tissue in the context of different organs and the underlying supportive biology in different organs. One central theme, however, is the universal involvement of the lymphotoxin-beta receptor pathway in the development and homeostatic maintenance of the ectopic follicles that have been described to date.

What Is the Role of Ectopic Follicles in Disease

As scientists, we tend to first think in "black and white" terms, and thus there presently are two "camps" advancing the opposing views that ectopic follicles must be either *detrimental* factors in disease that support pathogenesis or that they are simply *harmless* bystanders that have no role in the local pathogenesis. Another view, however, is that just like secondary lymphoid organs (lymph nodes), the role of ectopic follicles may be very much context dependent. That is, ectopic follicles may have the capacity to support all manner of immune responses, some which may enhance autoimmunity (local initiation of naïve T cells, propagation of auto-reactive cells, expansion of antibody producing cells) and some that may be antagonistic (generation of T-regs, Th2 polarization). To date, there is only a small amount of direct experimental evidence in support of either possibility. A prominent example is the elegant set of experiments by Nasr et al. that have shown that skin explants taken from RIP-LT transgenic mice that contain ectopic lymphoid tissue comprise all the elements required to support skin-explant rejection by naïve recipient mice that lack secondary lymphoid organs; in contrast, grafts of normal skin lacking ectopic lymphoid tissue are not rejected by such recipient mice [8].

Clinical Utility of Dispersing Ectopic Follicles in Sjogren's Syndrome

The same transgenic mice and powerful chimeric antagonists, such as the chimeric antagonist LTBR-Ig, that were developed for use in defining the biology of ectopic follicles, can now be used in animal models of human disease. The goal of such studies, of course, is to determine for which specific disease/organ contexts there are "clinical" benefits from interfering with putative functions of ectopic follicles. Our lab, in collaboration with investigators at the University of Bergen, Norway, has chosen to use NOD mice and the well-known antagonist LTBR-Ig to evaluate the importance of ectopic follicles to the Sjogren's syndrome-like changes

that spontaneously arise in this mouse strain [9–11]. We have begun carefully to characterize and compare the exact nature of ectopic follicles that develop in both the salivary and the lacrimal glands of NOD mice as they spontaneously develop the hallmark features of Sjogren's syndrome (decreased saliva and tear-fluid secretion). It is important to note that the type of exocrine gland that is primarily affected in the NOD mouse model of Sjogren's syndrome (submandibular versus lacrimal) is determined by gender, with submandibular glands primarily affected in female mice while lacrimal glands are primarily affected in male NOD mice. The basis of this gender-determined pathogenesis is not understood. It is important to acknowledge that wide variation in the severity of disease symptoms has been reported in different NOD mouse colonies in different animal facilities around the world [12]; however, we found that during the 3 years of our investigation, the NOD mice kept at our two facilities in Bergen, Norway, and the VA Medical Research Facility in Vermont have developed aspects of Sjogren's-like disease to very similar extent, including the incidence of disease, the presence of ectopic follicles and HEVs in exocrine glands, the appearance of FDC networks in submandibular glands, and even the degree of loss of salivary secretion in female mice with disease progression (Fava, unpublished results). Concerning the role of LTBR in the development of ectopic follicles in submandibular glands of female NOD mice, the reader is referred to published results from this portion of the investigation in which systemic blockade of the LTBR axis in female NOD mice was shown to prevent formation of ectopic follicles and reduce the extent of loss of salivary secretion rates [13].

Role of LTBR in Lymphocytic Infiltrates in Lacrimal Glands

Compared to the salivary gland aspect of Sjogren's syndrome, much less work has been done to detail the pathology of the lacrimal gland and the relationship of these changes to reduction of tear-flow rates in NOD mice [9]. Recently, a landmark paper was published in which the progression of lacrimal disease of males of the NOD strain was carefully documented and demonstrated to constitute a useful model for the "dry eye" (keratoconjunctivitis sicca) aspect of Sjogren's syndrome [11]. It was shown therein, by using one of several possible approaches to measure tear-fluid secretion rates, that as the mice age and lymphocytic infiltrates accumulate in lacrimal glands, a progressive loss of tear-fluid secretion rate occurs and significant alterations in the nature of the tear-fluid proteome also occurs, [11]. In that study the secretion rate of tear fluid was measured after a maximal stimulation by injection of pilocarpine, a parasympathetic nervous system agonist, in the manner routinely used to measure saliva secretion in female NOD mice. In confirming and complementary studies, our lab has recently measured the unstimulated, basal tear-fluid secretion rates in male NOD mice as well, and while the volume of tear-fluid secreted per unit time is considerably smaller than after pilocarpine stimulation, we too found a significant reduction in the secretion rates as the male NOD mice increase in age (8–12 weeks) and lacrimal gland disease progresses (Fava, unpublished results).

Involvement of the LTBR Axis in Lacrimal Gland Pathology

It was well established in early studies that male NOD mice develop aggregates of leukocytes in lacrimal glands as they age beyond 5 weeks, and that high endothelial venules develop near the infiltrating cells and contribute to lymphocyte accumulation in lacrimal glands [14]. Using this as our starting point, we examined in more detail the exact nature of these leukocyte infiltrates, the involvement of activation of the lymphotoxin-beta receptor pathway in their formation, and the overall gene expression patterns of lacrimal glands in male NOD mice. Replicate histologic analyses of lacrimal glands taken from male NOD mice at selected points from age 8 to 16 weeks, together with analyses of glands from several mice at 1 year of age (data not shown), confirmed the early development and long-term presence in lacrimal glands of numerous, MECA-79-positive PNAd-expressing high endothelial venules (HEV), as was previously reported [14]. MECA-79 is a monoclonal antibody that recognizes most forms of PNAd expressed on functional HEV. The presence of the MECA-79-positive PNAd-expressing HEV in lacrimal glands was virtually obliterated when male NOD mice were given the systemic LTBR antagonist, LTBR-Ig, from 8 to 16 weeks of age (data not shown), similar to the effects of LTBR-Ig upon peripheral lymph nodes [15]. In conjunction with the disappearance of HEV in lacrimal glands of LTBR-Ig-treated mice, a large reduction (approximately fivefold) occurred in the absolute numbers of B lymphocytes present in the glands (data not shown). We determined the relative numbers of T cells, and of B cells, which predominated in the lacrimal infiltrates by approximately fourfold over T cells at 16 weeks of age. Using FACS analysis of isolated cells from glands, we found that the vast majority of B cells in the massive aggregations present in lacrimal glands were simple follicular B cells (data not shown).

Affymetrix Chip Analysis Implication of LTBR Pathway and Chemokine CXCL13

An analysis was performed of mRNA isolated from the lacrimal glands of groups ($n = 4$ mice) of untreated, LTBR-Ig-treated, and control MOPC-21 monoclonal antibody-treated mice to gain an overall picture of the gene expression pattern in each case and assess the impact of LTBR antagonism on the expression of mRNA for key factors of known importance to ectopic follicles that develop in other organs and disease contexts.

Four mice were used for each time point examined (8 and 16 weeks) and for each treatment (none, MOPC-21, LTBR-Ig) to obtain four independent total mRNA samples for calculation of a mean gene expression level for comparisons. The approximate fold change of differential expression was determined for the 39,000 genes represented on the Affymetrix chips. The “pie chart” shown in Fig. 39.1 summarizes the number of pathway-associated, differentially expressed genes that were

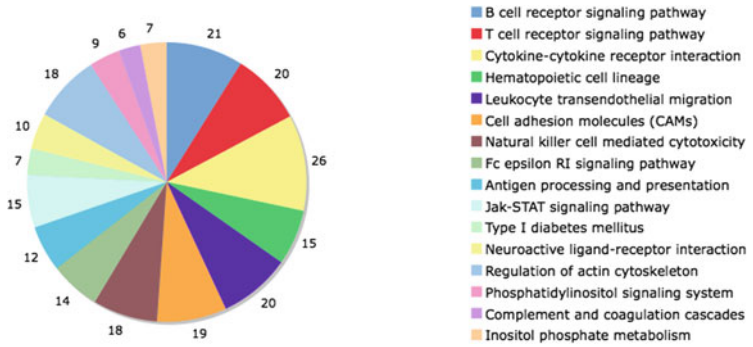

Fig. 39.1 KEGG pathways overrepresented by genes regulated by LTBR treatment

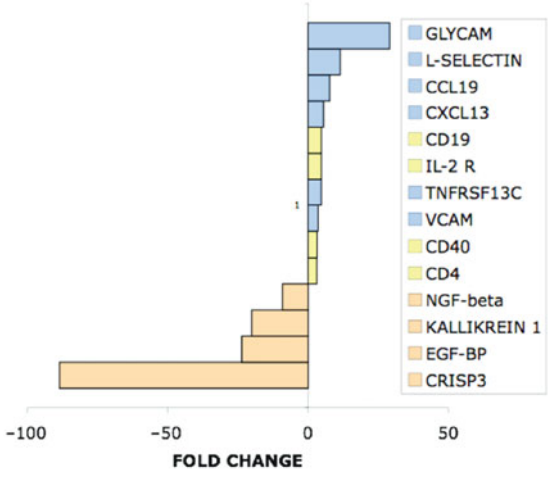

Fig. 39.2 Expression level in MOPC divided by LTBR-IgFc-treated mice

detected by comparing glands from LTBR-Ig- and MOPC-21-treated mice (treated from 8 to 16 weeks of age).

The bar graph shown in Fig. 39.2 highlights some of the larger changes among the 1200 genes that were differentially expressed. There was good reproducibility observed between replicate mice, and the final ~ fold changes shown in Fig. 39.2 are means of values from four mice. In general, the gene expression changes mirrored the histologic findings very well. It was interesting to find fairly large changes in key chemokines (CXCL13, CCL19) and in HEV-related genes glycam-1 and L-selectin, as well as many other molecules associated with B-cell and T-cell trafficking or that reflected signaling activity and antibody development (heavy chain genes). Some very large changes for genes for "functional" proteins present in lacrimal gland secretions such as EGF-binding protein, kallikreins, CRISP3, and others (data not shown) were also observed.

CXCL13 Production in Lacrimal Glands Lacking FDC Networks

The indication by Affymetrix Chip analyses of the involvement of CXCL13 in the recruitment of lymphocytes into lacrimal glands and the significant reduction of this chemokine mRNA by LTBR-Ig antagonism in male NOD mouse lacrimal glands were confirmed by real-time PCR and then at the protein level by CXCL13 ELISA of homogenates of replicate lacrimal glands from MOPC-21 control and LTBR-Ig-treated mice (data not shown). Using ELISA analyses, it was found that the CXCL13 protein content of male NOD lacrimal glands increased in parallel with the massive, predominately B-cell lymphocytic infiltration of the glands. By 16 weeks of age the CXCL13 protein content of diseased lacrimal glands approached approximately 1/3 of the CXCL13 content of peripheral lymph nodes collected from the same mice (data not shown).

Identification of the source(s) of CXCL13 in the lacrimal glands has been complicated by the unexpected absence of FDC networks in the ectopic lymphoid aggregates that formed in lacrimal glands. FDC networks are generally considered the most likely source of CXCL13 in lymphoid tissue [16], although additional sources such as monocytic cells [17], stromal cells [18], follicular B helper T cells [19, 20], follicular organizer cells [21, 22], and even smooth muscle cells in the context of aorta-associated ectopic follicles in atherosclerosis [4] have been described as sources of CXCL13 in recent years. The lack of FDC networks in diseased lacrimal glands (Fava, unpublished data not shown) is in marked contrast to the ectopic follicles that form in the salivary glands of female NOD mice, in which FDC networks are frequently observed [13]. Work is currently underway in our lab to identify more precisely the cellular sources of the large amounts of CXCL13 present in lacrimal glands of mice with Sjogren's-like disease. To date we have seen no evidence that the cells we can easily identify by immunofluorescent-CXCL13 staining of lacrimal gland infiltrates co-express CD45, CD14, CD11c, B220, or FDC markers (data not shown). In contrast, CXCL13 co-localization with FDC networks is consistently observed in immunofluorescent stains of submandibular gland ectopic follicles from diseased female NOD mice and of course in lymph nodes serving as positive controls for our investigation.

One hypothetical explanation for this difference between the two types of affected secretory glands in female and male NOD mice, although not yet supported with experimental evidence, may lie in the fact that lacrimal glands are part of a highly specialized and possibly unique "open" immune surveillance system that "washes" antigens from the ocular surface. The tear-fluid and antigens then drains "downstream" into a *bona fide* lymphoid tissue called "tear-duct-associated lymphoid tissue" (TALT) that initiates immune responses to protect the ocular surface [23]. An elegant paper was just published characterizing in great detail the ontology of TALT tissue development in mice which occurs independently of CXCL13, CCL21, CCL19, and surprisingly the transcription factors Id2 and ROR(gamma), factors that are generally required for "lymphoid tissue inducer cell" function during embryonic development of secondary lymphoid organs [24, 25]. The demonstration

of TALT as the ultimate site of initiation of immune responses to antigens encountered by the ocular surface immune responses in this "open" immune surveillance system allows the speculation that lacrimal glands may be unusual or even unique in lacking some currently unknown feature(s) that is intrinsic to other organs and that is required for the full "maturation" of ectopic follicles to the functional state (with highly segregated T- and B-cell areas, balanced T-/B-cell ratios, FDC networks). A possible candidate is lymphatic vasculature that that develops along with ectopic-follicles in other organs, but oddly is absent from lacrimal gland ectopic-lymphoid aggregates (Fava, data not shown). Thus the high levels of CXCL13 and predominately B-cell infiltrates in lacrimal glands of male NOD mice may reflect an aborted state of development of the typically more highly organized ectopic follicles found in most other organs, such as the submandibular glands in female NOD mice. Nonetheless, the powerful ameliorative effects exerted by LTBR antagonism in our experiments on both the CXCL13 content of glands and the associated B-cell aggregates, hold promise that the LTBR axis may be a useful target for treatment of Sjogren's syndrome, which we are continuing to investigate.

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Chapter 40
MEK Kinase 1: Kinase Domain Deficiency in Mice Reveals a Role in Orchestrating the Thymus-Dependent Immunity and TNFR Family Signaling

Ewen Gallagher

Introduction

MEKK1 was cloned as the second mammalian MAP3K after RAF1 [1] and was found to activate MAPK signaling by the phosphorylation of the activation loop of its upstream MAP2Ks (MKKs or JNKKs) [2]. Initially, MEKK1 was described as being a strong activator of JNK and p38 MAPKs in kinase assays [1] and overexpression was sufficient to render this MAP3K constitutively active in many cell line systems [2]. The constitutive activation of the overexpressed kinase proved problematic in the analysis of the biological role of this MAP3K until the later generation of *Mekk1* transgenics [3]. Motif analysis of the MEKK1 1493 amino acid coding sequence reveals a Really Interesting New Gene (RING), a Ubiquitin (Ub) Interaction Motif (UIM), and a kinase domain [3]. Like many MAP3Ks the MEKK1 kinase domain shares significant homology to the Ste11p kinase domain [1]. MEKK1 can form a signaling complex with its upstream MAP2Ks and MAPKs [2]. One of the consequences of MEKK1 overexpression in cell lines is elevated cellular proliferation and apoptosis [2], though the precise mechanisms underpinning these phenotypes are obscured by the gross activation of stress kinase signaling pathways, it was found that caspases could fragment the full-length MEKK1 protein [4].

To grain a greater physiological understanding of MEKK1 the *Mekk1* locus on chromosome 13 was disrupted to generate "knockouts" in embryonic stem (ES) cells by two different groups [5, 6], one deleting amino acids 1–132 within the amino-terminal regulatory domain of MEKK1 [6], disrupting the initial AUG start codon, and the other by substitution of the kinase domain with an in-frame *LacZ* gene to generate a fusion protein that is expressed at similar levels to the WT gene [5]. The subsequent analysis of *Mekk1* null ES cells identified roles for MEKK1 in

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MAPK signaling in response to the following agonists: sorbitol, nocadazole, taxol, lysophosphatidic acid (LPA), TNF-α, interleukin (IL)-1, cold shock, and serum. For none of these stimuli was MEKK1 found to be a critical factor for NF-κB activation [3, 5, 6]. Both the LPA and the serum signaling defects presented by *Mekk1*$^{-/-}$ ES cells have also been demonstrated to be important in growth factor-induced migration in Boyden chamber assays [5, 6].

Mekk1$^{-/-}$ mice display an eye-open at birth (EOB) phenotype, due to improper fusion of the murine eyelid during embryogenesis [3, 6, 7]. This null phenotype has its basis in defective cell migration and is observed in several differentiated cell types and this has been detailed in several recent reviews [2, 8]. In addition to improper eyelid fusion during embryogenesis at E13.5, *Mekk1*$^{-/-}$ embryos are anemic due to defects in erythropoiesis in the fetal liver and production of fetal liver-derived macrophages [9], a consequence is a greatly reduced frequency of homozygous *Mekk1* null postnatal embryos seen on the C57BL/6 genetic background. These early investigations into MEKK1-dependent embryogenic defects presented by *Mekk1* null mice were succeeded by subsequent postnatal investigations using *Mekk1 kinase-deficient* models (*Mekk1*$^{\Delta KD}$ or *Map3k1*$^{\Delta KD}$) [3, 10].

Activation of E3 Ub Ligase Itch by the T-Cell Receptor

Costimulation of T cells by cross-linking of the CD3 subunit of the T-cell receptor (TCR) and CD28 receptor induces rapid JNK activation and c-Jun phosphorylation [2, 10, 11]. Engagement of either receptor alone has only minor effects on JNK activity [2, 11]. This striking mode of TCR-dependent JNK activation in T cells was initially interpreted as inducing T-cell activation by up-regulating *Il2* expression [2, 11]. Costimulatory JNK activation was believed to activate T cells by enhanced AP-1-mediated transcriptional activity, since several AP-1 binding sites are located within the *Il2* promoter/enhancer [2, 11].

The later analysis of MAPK signaling in CD4^{+} T cells using *Mekk1*$^{\Delta KD}$ mice revealed that JNK1 kinase activity is important for TCR costimulation-dependent MAPK activation [10, 12]. Strikingly, *Mekk1*$^{\Delta KD}$ mice have defective TCR-dependent expression of T-helper (Th) cytokine (ILs-4, 5, 10, and 13) [10]. Careful genetic detective work and biochemical analysis identified activated JNK1 binding to the homologous to E6-AP carboxy-terminus (HECT) E3 Ub ligase Itch, a component of the intracellular ubiquitination machinery, by a δ domain that is proximal to the N-terminal HECT domain. Itch is then multiply Ser and Thr phosphorylated within its proline-rich region (PRR) by JNK1 at residues: Ser199, Thr222, and Ser232 [13]. PRR phosphorylation induces a conformational change within the E3 Ub ligase ablating an inhibitory interaction between the PRR and the WW region and the HECT domain [13]. This conformational change increases Itch HECT catalytic activity toward its substrates c-Jun and JunB [10, 13], which are then marked for proteasomal degradation following addition of Lys48-linked Ub [14]. The resulting elevated expression of *Jun* transcription factors then leads to enhanced AP-1-mediated transcription of Th2 cytokine genes, notably by enhanced binding to an AP-1 site in the *Il4* promoter [15, 16]. Analysis of *Mekk1*$^{\Delta KD}$ mice using an

$OVA_{323-329}$-alum allergy model identified also elevated IL-2 and IL-4 production in *Mekk1*$^{\Delta KD}$ T cells [17]. More recently, we have demonstrated that MEKK1 can scaffold Itch following costimulation and that this molecular interaction depends on MEKK1 kinase domain phosphorylation at residue Thr1381 within the kinase domain and an intact RING within the amino-terminal regulatory domain [12].

Thymus-Dependent Immune Responses and TNFR Signaling in B Cells

Further analysis of *Mekk1*$^{\Delta KD}$ mice revealed greatly reduced numbers of $B220^{+}GL7^{+}$ germinal center B cells following immunization with keyhole limpet hemocyanin (KLH) to stimulate a strong TD response (Fig. 40.1) [3, 18]. In addition, the $CD43^{-}$ B-cell population hypoproliferates in *Mekk1*$^{\Delta KD}$ mice, and there

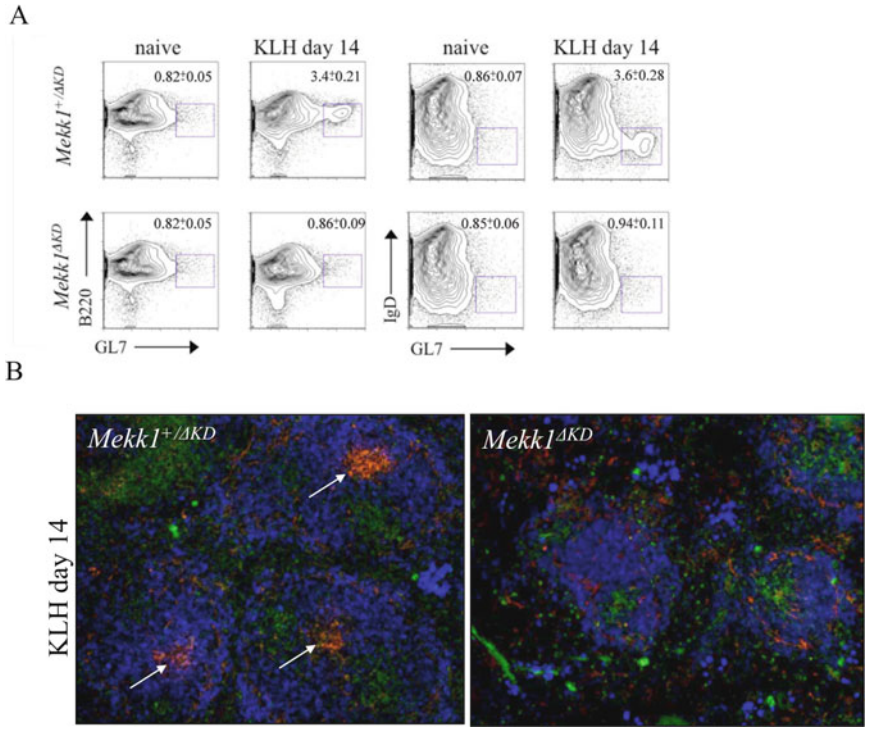

Fig. 40.1 Defective germinal center formation in *Mekk1*$^{+/\Delta KD}$ or *Mekk1*$^{\Delta KD}$ mice. (**a**) Flow cytometry of *Mekk1*$^{+/\Delta KD}$ and *Mekk1*$^{\Delta KD}$ $CD43^{-}$ splenocytes isolated 14 days after immunization with PBS or KLH. Numbers within the boxed areas indicate the percent B cells in outlined gates (mean s.e.m.; $n = 3$ mice per genotype; three separate experiments). (**b**). Confocal microscopy of WT and *Mekk1*$^{\Delta KD}$ spleen sections stained with anti-CD3 (*green*), anti-B220 (*blue*), and peanut agglutinin (*orange*); each image is one representative section ($n = 6$ spleens per genotype). *Arrows* indicate germinal center B cells. Numbers (*right margin*) indicate percent follicles containing germinal centers (mean s.e.m.). Original magnification, ×10

is reduced circulating immunoglobulin (Ig) M, IgG1, IgG2a, IgG2b, and IgG3 (Fig. 40.2) [18]. Long-term there is hyposplenism in *Mekk1*$^{\Delta KD}$ mice following TD responses [18]. In contrast to the strong deficits in TD response, there is no obvious deficit in immune responses following immunization with thymus-independent (TI)-I and TI-II antigens (Fig. 40.2) [18]. Adoptive transfer of WT and *Mekk1*$^{\Delta KD}$ bone marrow and reconstitution of B-cell-deficient μMT mice reveled that B cells are the principle cause of the TD phenotypes observed in *Mekk1*$^{\Delta KD}$ mice [18].

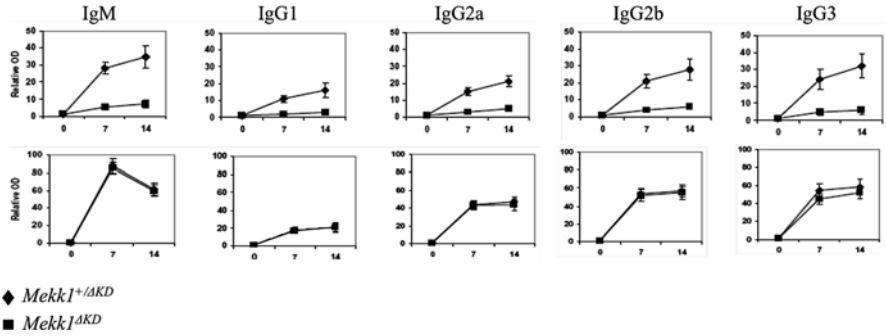

Fig. 40.2 Production of serum Ig following TD and TI immunization of WT or *Mekk1*$^{\Delta KD}$ mice. ELISA of circulating IgM, IgG1, IgG2a, IgG2b, and IgG3 anti-DNP (mean s.e.m) in WT (♦) or *Mekk1*$^{\Delta KD}$ (■) mice at the indicated time (days) after immunization with KLH (*upper panels*) or ficoll (*lower panels*) ($n = 3$ mice per genotype)

Careful examination of *Mekk1*$^{\Delta KD}$ B cells revealed defects in TNFR family member JNK and p38 MAPK signaling, notably for CD40 and BAFF receptors [18]. $CD40_L$-treated *Mekk1*$^{\Delta KD}$ B cells exhibit defective gene expression of several critical receptors: intercellular adhesion molecule-1 (ICAM-1), Fas (CD95), and CXC chemokine receptor (CXCR) 4 [18]. Analysis of *Mekk1*$^{\Delta KD}$ B-cell cytokine production revealed reduced production of the inflammatory cytokine IL-6 following stimulation by $CD40_L$ [18]. *Mekk1*$^{\Delta KD}$ B cells are also defective in *activation-induced cytidine deaminase* (*Aicda*) expression and exhibit significantly reduced Ig class switching to IgG1 or IgG2a-2b following CD40 cross-linking and IL-4 treatment [18]. The molecular explanation for these many defects, most likely, lies in greatly reduced Jun transcription factor phosphorylation and expression observed in *Mekk1*$^{\Delta KD}$ B cells, and the consequently reduced transcriptional activation mediated by AP-1 following CD40 engagement [3, 18].

Two-Stage Cytokine Receptor Signaling

The genetic demonstration of the role of MEKK1 in TNFR family signaling lead to the evaluation of the mechanism of MEKK1 activation in B cells by TNFR family member CD40. Following engagement of CD40 and ligand-induced receptor trimerization TNF receptor associated factors (TRAFs) 2, 3, and 6 are recruited with rapid

kinetics to TRAF-binding sites within the cytoplasmic tail of the receptor [3, 18, 19]. In some respects confirming early biochemical purifications of TRAFs, the E1, E2 enzyme hetereodimer Ub-conjugating enzyme E2 variant 1 isoform A (Uev1a) –Ub conjugating enzyme 13 (Ubc13) and E3 ligase TRAF2/6 associate then associate at the receptor [20], and these form a ubiquitination machine that potentiates the noncanonical Lys63-linked ubiquitination of TRAF2/6 [21].

Cellular inhibitor of apoptosis protein (c-IAP) and c-IAP2, initially identified as factors that can regulate TNFR1 apoptosis upon their overexpression in cell lines [3], directly associate with the TRAF–CD40 receptor complex and mediate canonical Lys48-linked Ub on TRAF3 [18, 19]. In many ways canonical and noncanonical ubiqutination act in this model as the key posttranslational modifications that prime the TRAF–CD40 complex for the subsequent recruitment of the MAPK signaling module to the receptor. Recruitment of MEKK1 depends upon both TRAFs being modified by noncanonical Lys63-linked chains and scaffolding mediated by inhibitor of κB kinase-γ (IKK-γ) [3, 19].

Ubiquitinated TRAF2 associates with IKK-γ and MEKK1, while ubiquitinated TRAF6 forms a complex with IKK-γ and the TGF-β activated kinase 1 (TAK1) complex, at the CD40 receptor along with the MAP2K (MKK or JNKK) [3]. It is of fundamental importance to note that at this stage of CD40 signaling the TRAF–MAPK module that is present at the CD40 receptor is largely non-phosphorylated [19]. In contrast to TRAF2 and TRAF6, TRAF3 marked with Lys48-linked Ub becomes rapidly degraded by the 26S proteasome [19]. Degradation of TRAF3 then releases the brake upon TRAF2/6 MAPK signaling complexes, and this releases them into the cytoplasm [19]. Here the MAP3Ks become active, presumably by the release of auto-inhibitory mechanisms present at the receptor, and activate JNK and p38 MAPK by phosphorylation of the MAP2Ks [19]. Similar signaling mechanisms may operate at TNFR1 and BAFF-R receptors (Fig. 40.3), indicating that two-stage cytokine receptor signaling is of fundamental importance for many, if not all, TNFR family members and may well be relevant to other non-TNFR family member receptors that activate stress MAPK signaling [3].

Perspectives

From the early isolation of MEKK1 by cDNA library screening the analysis of *Map3k1* has only been resolved after careful mouse genetic analysis. MEKK1 kinase activity has proven to be a key regulator of the TD immune response and TNFR family signaling in lymphocytes (Fig. 40.3) [3, 18, 19]. It still remains to be determined precisely which B-cell genes are critical for germinal center formation defect present in $Mekk1^{\Delta KD}$ mice following TD antigen immunization, but the likelihood is that it may prove to be complex and multi-factorial in nature. A remaining issue for the role of MEKK1 in the regulation of the immune response is the contribution of the RING within the amino-terminal regulatory domain to the regulation of lymphocytes and TNFR family signaling. Given that the RING

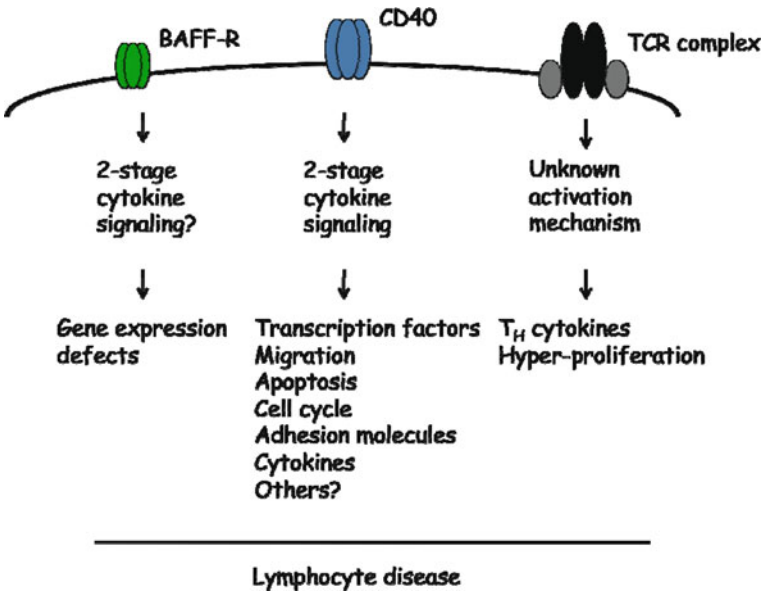

Fig. 40.3 Model summarizing MEKK1-dependent signaling events in B and T cells

has proven to be a mediator of Lys63-linked Ub it seems likely that this motif governs MEKK1 activation and would prove to have similar phenotypes to those observed in Mekk1$^{\Delta KD}$ mice [18]. After many years of detailed research MEKK1 now emerges as an attractive immune system target for drug discovery and therapeutic intervention. Indeed, early and somewhat nonspecific inhibitors of the MEKK1 kinase domain suggest that chemical inhibition may prove to be a valuable means of reducing the scale of immune responses involving T lymphocytes [22].

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Part IX
Advances in Applying Our Knowledge to Therapy: *Mechanisms of Pathological and Therapeutic Effects of the TNF Family*

Chapter 41
Introduction to the Session "Mechanisms of Pathological and Therapeutic Effects of the TNF Family"

Claude Libert

The prototype family member of the TNF family is TNF itself. Very early after its discovery and cloning, about 25 years ago, it became clear that TNF has very powerful and dramatic toxic activities when injected in experimental animals, such as mice and rats, and in humans. Also, in experimental animals, tumor regression was observed in many tumors, but the therapy was associated with very dangerous toxicity. The toxic response to TNF could best be compared with endotoxemia and septic shock, and hence TNF was considered as an essential mediator of these acute inflammatory conditions. TNF also proved to be a very strong inducer of gene expression programs through activation of signaling cascades, and because circulating TNF could be measured in several diseases, many research groups have embarked in studies to establish clearly in which inflammatory disease cytokines such as TNF should be considered a toxic mediator and hence a therapeutic target. A major breakthrough in this research were the studies which proved that TNF-neutralizing antibodies and soluble receptors protect arthritic patients. These data provided the community not only with very valuable research tools, but also with the belief that TNF is really to be considered as a drug target and that the market for anti-TNF strategies is huge indeed. In the mean time, more and more diseases are approached with anti-TNF therapies, but the search to increase the market is still ongoing, both in clinical trials and in fundamental research. Very instrumental in this search are the mouse models and tools which are available.

Besides the identification of the therapeutic niche of TNF inhibition, several other aspects of TNF and its family members are actively studied. First, there is a concern that anti-TNF therapies might have side-effects. There have been some reports about increased sensitivity for infectious diseases in some anti-TNF-treated patients. This is not surprising because TNF is an essential cytokine in the control of immune defense. Also, it is becoming clear that TNF-inhibiting strategies might cause problems in specific groups of patients and that it is instrumental that

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the research community gets a clear picture of the benefits and dangers of such TNF-blocking therapeutics. Second, the mechanisms by which TNF contributes to development of diseases are under intense investigation. For example, it remains to be studied what are the specific roles played by both the TNFRp55 and the TNFRp75 in certain pathologies, which cells are responding to TNF, which are the downstream mediators of TNF, and what is the impact of specific mutations on the response to TNF. Finally, the impact of TNF on basic physiological functions is still under intense research. It is known that TNF is essential in immune regulation, but that excess TNF has a whole spectrum of effects on the cardiovascular system, energy metabolism, and excretory functions. In order to understand the impact of TNF-inhibition and/or TNF application (in cancer therapy) it is essential to understand the overall impact of TNF on the different systems and physiological functions.

At the 12th International TNF Conference, many important new insights into these fundamental questions about the TNF family were reported.

Chapter 42
Mechanisms Regulating TNF-Driven Gut and Joint Inflammation

Dirk Elewaut, Peggy Jacques, Lode Melis, and Koen Venken

The Gut Joint Axis in Spondyloarthritis

Chronic inflammatory arthritis, a hallmark of a variety of inflammatory rheumatic diseases, is a lifelong condition, often with onset in early adulthood, with an important morbidity and even mortality in our society. It is estimated that approximately 2–3% suffers from chronic arthritis in our society. The coexistence of gut and joint inflammation is well established in spondyloarthritis (SpA), a cluster of interrelated rheumatologic diseases, characterized by a number of clinical and genetic features including peripheral arthritis (typical of lower limb joints) as well as inflammation of the axial skeleton (e.g., spine). Remarkably, other organs including skin (psoriasis) or the eye (anterior uveitis) may also be affected, indicating the systemic nature of these diseases. Various subtypes of SpA can be distinguished based upon clinical features, but an important overlap between them exists. The clinical subtypes include an kylosing spondylitis (AS, characterized by prominent inflammation of the axial skeleton – spine, sacroiliac joints, although other joints may also be affected), infection triggered reactive arthritis, some forms of juvenile chronic arthritis, arthritis in association with inflammatory bowel diseases (IBD), and some types of psoriatic arthritis.

Two decades ago, it was shown that a high frequency of inflammatory gut lesions could be observed in patients from various subtypes of SpA presenting with inflammatory joint symptoms but lacking any clinical signs of gastrointestinal symptoms [1–4]. This observation is particularly interesting because an anatomic linkage between these two organs is lacking. Inflammatory gut lesions are found in up to 90% of the patients, depending on the SpA subtype [5].

Furthermore, 6–13% of these patients eventually develop IBD, particularly Crohn's disease (CD) [6–10]. The occurrence of subclinical bowel inflammation was shown to appear in all known SpA subsets. Strikingly, a remarkable clinical relationship between gut and joint inflammation was found in prospective follow-up

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studies as bowel inflammation occurred more frequently in SpA patients suffering from peripheral arthritis compared to patients without this condition. Similarly, remission of joint inflammation was associated with disappearance of gut inflammation. Conversely, persistence of peripheral arthritis was usually accompanied with persistence of bowel inflammation [7–9].

In addition to shared clinical features, a number of common genetic predispositions were identified linking SpA and CD pathogenesis to immune-mediated inflammatory diseases. Hence, the strong genetic association of SpA with the human leukocyte antigen (HLA) B27 has extensively been documented [11–14] and very recently an association with IL-23 receptor polymorphisms in both CD and AS was reported [15–17].

Modeling Spondyloarthritis in Mice: Which Cells Are Targets of Pathogenic TNF?

Despite the frequent coexistence of gut and joint inflammation in SpA, very few models, however, exist in which gut and joint inflammation appear simultaneously. One such a model is HLA-B27 transgenic rats which develop an SpA-like phenotype with colitis and arthritic features (for review, see [18, 19]). However, in patients, the bowel inflammation is most typically confined to the terminal ileum rather than the colon. More recently, a mouse model with enhanced TNF mRNA stability has been described in which a strong relationship between gut and joint inflammation is found. By deletion of the AU-rich elements in the regulatory sequences of the murine TNF genome, a chronic and dysregulated TNF production occurred leading to an inflammatory disease characterized by the simultaneous occurrence of a Crohn-like inflammatory bowel disease and a joint disorder [20]. Recently it was shown that this articular disease involves both peripheral synovitis, enthesitis, and sacroiliitis, thereby covering many features of the SpA concept [21]. This model is particularly useful to examine mechanisms of action in TNF-driven gut and joint inflammation. Signaling through TNF receptor I (TNFRI) was previously shown to be essential for the development of both the bowel inflammation and the arthritis [20], but the cellular targets of TNF remained poorly defined (reviewed in [22]). Recently, however, Armaka et al. demonstrated using Cre/loxP-mediated TNFR1 expression in mesenchymal cells that TNFRI expression within the stromal compartment provided a sufficient target for TNF in the development of both gut and joint inflammation [21]. Therefore, in the presence of chronic TNF overexposure, signaling through TNFRI in synovial fibroblasts and intestinal myofibroblasts appears to be sufficient to develop combined gut and joint pathologies, a typical feature of SpA. This finding may also be of clinical relevance in SpA patients as recently other gene polymorphisms in SpA were unraveled, including a link to TNFR1 [23]. Further research is needed to explain the precise effector pathways by which mesenchymal cells can induce bowel inflammation and arthritic disease.

NKT Cells: Regulators or Effectors in Arthritic Disease?

Natural killer T cells constitute a subset of glycolipid reactive T lymphocytes that have important immunoregulatory properties. The most abundant and best characterized subset has an invariant TCR alpha chain with a typical Vα14-Jα18 rearrangement as well as a restricted TCR Vβ repertoire, which is referred to as invariant NKT (iNKT) cells [24]. Following antigen presentation of glycolipid antigens by the MHC class I-like molecule CD1d, iNKT cells quickly respond through the production of large amounts of Th1, Th2, and Th17 cytokines [25–27]. Through this capacity, these cells seem to play a pivotal role in various autoimmune diseases, although they can both enhance [28, 29] or ameliorate disease pathology [30–32]. Several glycolipids, capable of activating iNKT cells have been characterized. The first and best studied is α-galactosylceramide, derived from marine sponges, which is not expressed by mammalian cells. More recently, exogenous microbial [33–35] or endogenously derived CD1d ligands such as iGb3 [36, 37] have been reported.

In view of the ability of iNKT cells to produce large quantities of immunoregulatory cytokines, several investigations have been conducted to assess the capacity of iNKT cells to modulate autoimmune diseases. In the context of animal models for type 1 diabetes and multiple sclerosis [38], results obtained in these models have been rather confusing as opposing roles have been described. There are several potential explanations for these apparent discrepancies. First, there is evidence from some autoimmune disease models that iNKT cells naturally influence autoimmunity, while in other models, intentional activation of iNKT cells by α-GalCer is required to elicit a regulatory function. In addition, in some cases iNKT cells promote autoimmunity, even in the absence of α-GalCer administration. When iNKT cells are activated by exogenous glycolipids, disease modulation depends strongly on the type of ligand being administered. This has been exemplified by the clinical superior protection of OCH, a sphingosine-truncated analog of α-GalCer, in EAE [39]. Such findings appear to be relevant to the study of autoimmune rheumatic disorders including rheumatoid arthritis, SLE, and Lyme disease.

In rheumatoid arthritis, a dichotomous effect of iNKT cells on arthritis severity and incidence has been described. In collagen-induced arthritis (CIA), several studies aimed to modulate the Th1/Th2 balance toward the Th2 direction, using exogenous stimulation of iNKT cells. Therefore, the effect of repeated administration of OCH, which preferentially promotes Th2-type cytokine secretion, on disease severity was compared to α-GalCer [40]. It was reported that OCH showed more potency than α-GalCer to suppress disease. However, repeated administration of α-GalCer has previously been shown to cause a Th2-type immune deviation [40, 41]. Because a key difference between repeated administration of α-GalCer versus OCH appears to be secretion of IFN-γ upon the first administration of α-GalCer, the role of IFN-γ by iNKT cell activation was examined subsequently [30]. It was shown that the effect of IFN-γ, released upon exogenous iNKT cell activation, in disease pathogenesis is highly time dependent. While IFN-γ in the early phases of disease

contributes to arthritis severity, it protects against arthritis at later time points [30]. Thus, iNKT cell activation in CIA clearly has time- and ligand-dependent immune modulatory effects.

In contrast with the results of iNKT cell activation studies, CIA appeared to proceed milder in the absence of iNKT cells or CD1d, although the impact of disease severity seems to be rather limited. More profound are the results in antibody-mediated models of arthritis, by transfer of anti-collagen type II monoclonal antibodies or of K/BxN serum, which induce an inflammatory cascade by activation of complement and Fc-γ receptor pathways. Because inflammation occurs in the absence of a primary cellular immune response in either of these antibody-induced models of arthritis, they are considered useful in exploring the terminal effector mechanisms of RA. In these models, a considerable reduction in disease severity was observed in the absence of iNKT cells and, conversely, iNKT cell activation aggravated joint inflammation [30, 42]. In the K/BxN serum transfer model, it was shown that iNKT cells traffic to the synovium, a site where they are usually absent, and are accompanied with significant changes in the cytokine balance within the joint, by altering TGF-β1 levels [42]. It was proposed that iNKT cell activation in the serum transfer model occurred independently of TCR ligand recognition, but rather through Fc-γRIII engagement [43]. This in turn would lead to secretion of IL-4 and IFN-γ and subsequently to down-modulation of TGF-β1 levels. However, this finding still needs to be confirmed. Thus, also in other models of arthritic disease iNKT cells seem to play an important role but their effect may depend on a number of variables including timing, mode of activation, type of inflammation, and perhaps genetic background of the models used. It should be noted that all of the above-mentioned models are experimentally induced models by breaching tolerance or by transferring arthritogenic serum.

More recently, we evaluated the role of iNKT cells in the $TNF^{\Delta ARE}$ mouse model, a mouse model of SpA in which the disease spontaneously occurs, and found that iNKT cells become activated in the absence of exogenous stimulation. Activation was shown to occur through an indirect pathway that involves CD1d-mediated presentation of endogenous glycolipids by inflammatory dendritic cells. Chronic iNKT cell activation through this pathway was found to attenuate Crohn's like ileitis and joint inflammation. These findings may therefore represent a naturally occurring counter regulatory mechanism to dampen TNF-driven inflammation [44].

In summary, while the clinical efficacy of TNF blockers has well been established over the past years, the cellular mode of action of TNF in immune-mediated inflammatory diseases was less clear. The finding that a single cell type is sufficient to model the entire disease spectrum elicited by TNF production opens new avenues for even more targeted therapeutic approaches. Alternative modes of intervention may involve selective modulation of immune regulatory cells, of which iNKT cells are a prominent member.

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Chapter 43
Lessons from Anti-TNF Biologics: Infliximab Failure in a TRAPS Family with the T50M Mutation in *TNFRSF1A*

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Tumour necrosis factor (TNF) receptor-associated syndrome (TRAPS) is a chronic inherited autoinflammatory disorder. Typical features of TRAPS include recurrent fever, myalgia, rashes, and joint and abdominal pains. At the molecular level, TRAPS is associated with autosomal dominant mutations in the gene encoding the 55 kDa TNF receptor (*TNFRSF1A*). TRAPS affords a unique opportunity to study the biology of TNF in humans, as it is the only human disease currently known to be caused by mutations in the TNFR1 receptor. Although the inflammatory attacks of TRAPS generally fit with the notion of TNF as an inflammatory cytokine, there remain a number of questions to be answered. In particular, why do only certain patients present with cachexia, why do some patients develop systemic amyloidosis and not others, and why is erosive arthritis not seen in TRAPS although it is observed in TNF transgenic mice [20]? Perhaps some of these outcomes are related to the specific mutations seen in TRAPS, whereas others may be the result of still-undefined environmental or genetic factors. With the identification of new TRAPS mutations, it is likely that additional pathogenetic mechanisms will be identified.

We have undertaken a number of experimental strategies in an attempt to elucidate the basic biology underlying disease penetrance and pathogenesis in cells taken from patients with TRAPS [24, 25]. Alternatively, other groups have studied cell lines transfected with mutant receptor constructs [27, 28, 33, 36, 37, 38] or animal disease models [14]. The latter resource may be particularly helpful in delineating factors that precipitate episodes, in understanding the predilection of TRAPS for certain anatomic sites, and in screening the efficacy of new anti-inflammatory agents. Studies of new TRAPS mutations may also give insight into the factors that determine the penetrance of *TNFRSF1A* mutations and may provide evidence that TNF receptor mutations play a role in the pathogenesis of other inflammatory disorders.

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Assessment of the Effect of Anti-TNF Biologicals in an Irish Family with the T50M Variant of *TNFRSF1A*

TRAPS is characterized by spontaneously relapsing and remitting bouts of systemic inflammation, and treatment of TRAPS and related disorders involves targeting the immune cascade [4]. Successful therapeutic intervention is often achieved through introduction of anti-TNF agents. TNF is a molecule which exerts many of its biological actions through binding to TNF receptor 1 (TNFR1), and TNF receptors are central to cytokine inflammatory response. TNFR1 activation has one of two effects – either signalling apoptosis through death domain proteins and caspase cascade activation or triggering inflammatory response via activation of the transcription factor NF-κB. The anti-TNF agent, infliximab, neutralizes the biological activity of TNF by binding with high affinity to both the soluble and transmembrane forms of TNF and inhibits or prevents the effective binding of TNF with its receptors. Importantly, infliximab (Remicade®) has been shown to exert beneficial therapeutic action through induction of apoptosis in patients suffering from Crohn's disease, psoriasis, and HIV. Consistent results from clinical trials have also demonstrated the efficacy of TNF-neutralizing therapies in different autoimmune diseases. Based on these results the rationale for anti-TNF therapy, including TNF receptor fusion proteins (etanercept) and anti-TNF antibodies (infliximab), in autoinflammatory disorders appeared clear, especially in TRAPS related to mutations in the 55 kDa TNF receptor superfamily 1A (*TNFRSF1A*) gene.

As etanercept (Etan, Enbrel®) is a fusion protein comprised of the extracellular domain of TNFR2 and the hinge and Fc domains of IgG_1, it has been specifically advocated as a substitute for the low levels of (missing) soluble mutated receptor observed in many (but not all) patients with TRAPS. However, the variability in TRAPS attacks in the same patient renders treatment efficacy extremely difficult to ascertain. TRAPS patients can also develop amyloidosis in association with a persisting acute-phase response, giving the disease its life-threatening aspect. Treatment with etanercept has therapeutic benefits in some TRAPS patients and can control the clinical attacks [14, 17]. In addition, etanercept also results in improvement of renal function and reduction in amyloid deposition [9]. Infliximab had also been used to prevent attacks in TRAPS patients, but with limited success [7, 8, 15, 35, 39]. Worryingly, infliximab has been found to actually trigger acute inflammatory responses in some patients [4, 10, 15, 34]. Elucidation of the mechanisms underlying this paradoxical response is therefore important in order to efficiently target this anti-TNF therapy to the correct patients.

In a recent study [24], we investigated the effects of the anti-TNF agents, infliximab and etanercept, in a large family of TRAPS patients, all of whom carry the T50M variant of *TNFRSF1A* (Fig. 43.1). We demonstrated for the first time that these anti-TNF agents exert different effects on peripheral blood mononuclear cells (PBMCs) isolated from TRAPS patients. Importantly, infliximab failed to induce apoptosis in T50M cells. It has been reported that conformational changes in the extracellular portion of TNFR1 interfere with receptor internalization and subsequent activation of apoptotic pathways [32]. In addition to the failure to internalize

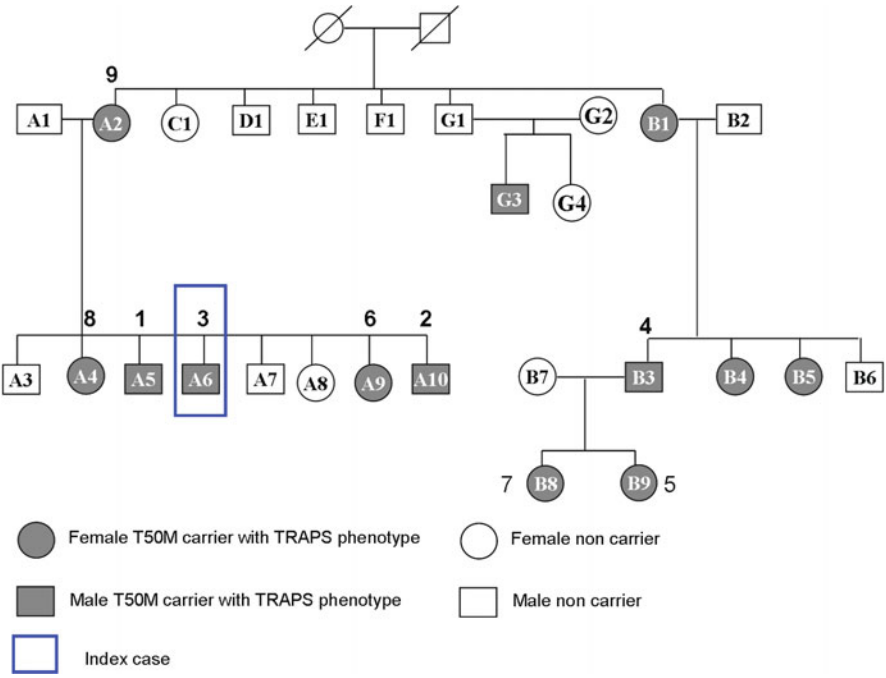

Fig. 43.1 Pedigree of the TNFRSF1A T50M variant family. Nine members from an Irish family in which several family members have TRAPS due to T50M mutation of the *TNFRSF1A* gene were recruited for this study. The index case represented in the table above as number 3 was referred to the Rheumatology Department, University Hospital Galway, West of Ireland, for investigation of arthritic symptoms. In grey are T50M variant carriers

TNFR1 receptors, the situation in T50M patients is likely to be compounded by the activation of anti-apoptotic c-Rel subunits. Upregulation of c-Rel has previously been shown to result in a concomitant upregulation of manganese superoxide dismutase, which in turn leads to mitochondrial degeneration and resistance to apoptotic induction [3]. Consistent with these findings, infliximab failed to induce apoptosis in any of the nine TRAPS patients we studied. This effect was specific to infliximab, as IgG_1 showed no effect, and etanercept induced a modest increase in apoptosis, in keeping with previous reports [23]. Furthermore, not only was there a failure of infliximab to induce apoptosis in T50M cells, but there was instead significant secretion of the proinflammatory cytokines IL-1β, IL-1R, IL-6, IL-8, and IL-12, [24]. While, to our knowledge, c-Rel has not been previously associated with TRAPS pathophysiology, our data are nevertheless consistent with previous reports of proinflammatory c-Rel-mediated IL-12 transcription in macrophages [30].

We hypothesize that binding of infliximab at the cell surface TNF/receptor complex not only results in a failure to induce apoptosis in certain TRAPS patients, but instead triggers a widespread inflammatory response, resulting from failure to shed infliximab-bound receptor from the cell surface leading to activation of anti-apoptotic c-Rel subunits. Importantly, in individuals who do not have TRAPS,

TNF-bound receptor is rapidly cleaved by metalloproteinase enzymes, yielding soluble TNFR1. However, certain *TNFRSF1A* mutations, including C52F and T50M [1, 21], and to a lesser extent H22Y, C33Y, and P46L [1], result in failed shedding of TNFR1 from the cell surface, leading to a hyperinflammatory response. In addition to elevated secretion of proinflammatory cytokines, we observed an inhibition in secretion of the anti-inflammatory cytokine IL-4 and inhibition of secretion

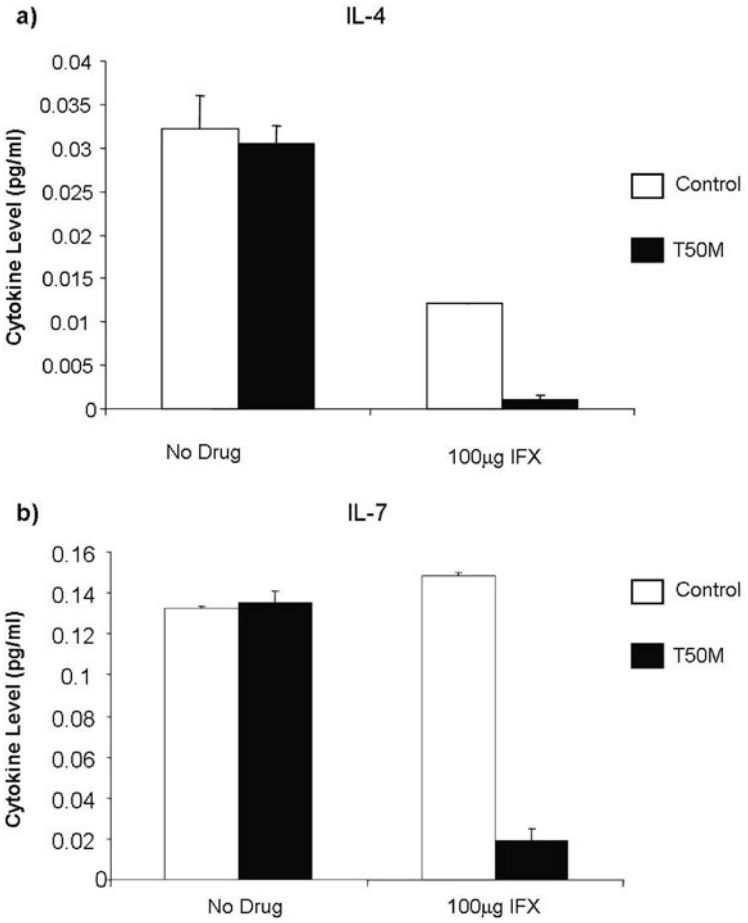

Fig. 43.2 Secretion level of (**a**) anti-inflammatory IL-4 and (**b**) anti-apoptotic IL-7 cytokines following infliximab treatment. Blood was obtained from nine TRAPS patients and nine healthy controls (approved by East London and City Health Authority Research Ethics Committee) and peripheral blood mononuclear cells (PBMCs) isolated using lymphoprep^{TM}fractionation protocol (Axis Shield, Dundee, UK). PBMCs were incubated with or without 100 µg/ml infliximab for 6 h at 37°C. Secreted cytokine concentrations were determined using a human multiplex bead immunoassay kit (Invitrogen, Paisley, UK) and analysed in duplicate, using a Luminex 100^{TM} instrument (Luminex, Riverside, CA, USA). Data analysis was performed using the Luminex 100 IS software version 2.3 and data displayed as mean ± SEM. All experimental data were gathered from a series of independent experiments ($n = 3$). Statistical analysis was by independent samples *t* test

of the anti-apoptotic cytokine IL-7, in PBMCs incubated with 100 μg infliximab from T50M TRAPS patients (Fig. 43.2). In the absence of a protective effect from IL-4 and IL-7, these individuals would be left particularly vulnerable to the harmful effects of proinflammatory cytokines.

The exact process allowing infliximab to induce a hyperinflammatory response is intriguing and warrants further study. It has been previously shown that the acute-phase inflammatory response is associated with production of pyrogenic cytokines, namely IL-1β, IL-6, and TNF, and acute-phase proteins, such as CRP and serum amyloid A [5]. We reported that an upregulation of pyrogenic cytokines is observed after infliximab stimulation in TRAPS patients [24]. Elevated IL-1β secretion induces the production of several cytokines, including itself, TNF, IL-6, IL-8, and IL-12. It also can be induced by acute-phase proteins, such as CRP [5]. In that light it is not surprising that IL-1 blocking drugs, such as anakinra, give encouraging results in patients with TRAPS. The cytokine activation cascade might also be activated by IL-6. We know that CRP is modulated by IL-6 and have observed increased IL-6 secretion in response to infliximab stimulation [24]. Monocytes and fibroblasts produce IL-8, a chemoattractant for neutrophils during inflammation, and this chemokine may have a role in the dermatological manifestations notable in TRAPS. As we have observed increased IL-8 secretion [24] in both TNF and infliximab-stimulated PBMCs isolated from TRAPS patients [24], our findings also suggest a possible role of neutrophils in TRAPS physiopathology.

Therapeutic Options in the Treatment of TRAPS: A Revised View

The genetic basis of TRAPS is now known. Not surprisingly, since there are multiple pathways that lead to fever and inflammation, there is also diversity in the mechanisms of this illness and its possible treatments, and established treatments may target either the underlying genetic defect or some downstream molecular or cellular event. Episodes of fever associated with TRAPS are usually characterized by a number of clinical manifestations (musculoskeletal and abdominal pain, rash, painful periorbital oedema) that greatly affect the patients' daily activities. In most patients, the symptomatic use of nonsteroidal anti-inflammatory drugs (NSAIDs) and oral steroids is able to control the clinical manifestations [19]. However, some patients have a high frequency of episodes of fever or develop a chronic, fluctuating disease course that causes severe limitations in normal social activities and requires prolonged therapy with steroids. These patients are at major risk of developing amyloidosis, the most severe long-term complication of TRAPS [18]. Corticosteroids, colchicine, and high doses of NSAIDs have all been used to treat TRAPS with varied success. Unlike familial Mediterranean fever (FMF), TRAPS patients generally respond poorly to colchicine [6]. TRAPS symptoms can, however, be relieved with high doses of prednisolone (>20 mg), although efficacy fades with time.

It has become increasingly evident that there are important differences in response to the various anti-TNF agents [15]. These differences could perhaps result

from the differential effects on apoptosis and proinflammatory signalling pathways that we have observed in cells incubated with etanercept and infliximab [24]. In assessing the physiological relevance of these observations, the ultimate question is whether there are differences in the capacities of infliximab and etanercept to block TNF-mediated disease processes. Both agents have been effective in providing symptomatic improvement and inhibition of structural damage progression in patients with rheumatoid arthritis [2, 16]. However, this is in marked contrast to results observed in treatment of TRAPS, where etanercept, but not infliximab, was demonstrated to be relatively effective in the attenuation of the symptoms. In the case of T50M TRAPS patients, we suspect that infliximab's affinity for the mutant receptor, which has a shedding defect, leads to constitutive activation. In addition some reports suggest a defect in internalization of the receptor and subsequent apoptosis machinery [32], leading to the survival of cells in an extremely high inflammatory environment. Importantly, the specificity of our results with infliximab is supported by the notion that infliximab is not known to bind to any antigen other than TNF, whereas etanercept binds equally well to both TNF and lymphotoxin α (LTα) (Fig. 43.3). Furthermore, each infliximab molecule is capable of binding to two TNF molecules, and up to three infliximab molecules can bind to each TNF homotrimer [31], thereby blocking all receptor binding sites on TNF (Fig. 43.3). In contrast, it is believed that the bivalent etanercept molecule forms a 1:1 complex with the TNF trimer in which two of the three receptor binding sites on TNF are occupied by etanercept, and the third receptor binding site is open. In addition, the p75 TNF receptor is known to have fast rates of association and dissociation with TNF [11], which suggest that etanercept might only transiently neutralize the activity of an individual TNF molecule. This was a relevant consideration given a distinct difference in clinical effectiveness of these two agents in patients with TRAPS [15]. Finally, while there are no published reports on the use of the fully humanized anti-TNF monoclonal antibody, adalimumab, in TRAPS, the above responses to infliximab caution against its use in TRAPS. This caution is also likely to be relevant to the newly developed certolizumab pegol, a pegylated antibody targeting TNF, which was recently approved for treatment of moderate to severe active rheumatoid arthritis (RA) in adult patients who have not responded to disease-modifying antirheumatic drugs (DMARDs). We suggest that the monoclonal anti-TNF antibodies, infliximab, adalimumab, and certolizumab, should be avoided in TRAPS.

What other treatment options are available? Recently, it was shown that treatment with anakinra can be effective in other autoinflammatory conditions, such as pyogenic sterile arthritis, pyoderma gangrenosum and acne (PAPA) syndrome, and familial Mediterranean fever, in which the mutated proteins have been shown to be functionally related to the inflammasome [22], as well as in patients with systemic-onset juvenile idiopathic arthritis [26]. Anakinra, a recombinant IL-1 receptor antagonist (IL-1-ra), has shown modest efficacy in the treatment of RA, but astonishing benefit in MWS, FCAS, and CINCA/NOMID, supporting a pivotal role for IL-1β in these hereditary autoinflammatory disorders. Interestingly, there are similarities in the response to anakinra treatment (anti-IL-ra therapy) observed in TRAPS patients

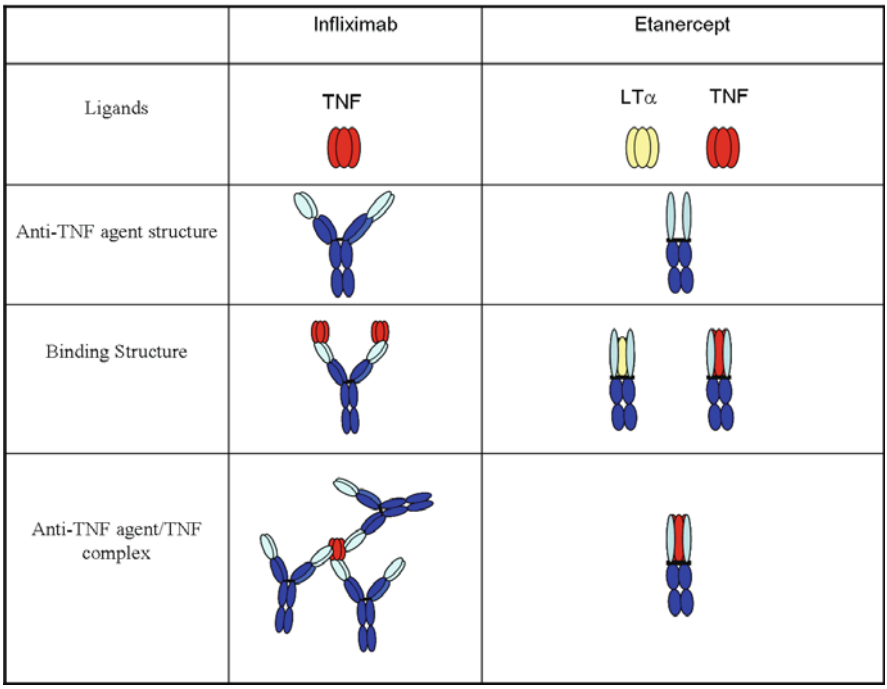

Fig. 43.3 Schematic illustrations summarizing similarities and differences between infliximab and etanercept. Infliximab neutralizes the biological activity of TNF by binding with high affinity to the soluble (free floating in the blood) and transmembrane (located on the outer membranes of T cells and other immune cells) forms of TNF and inhibits or prevents the effective binding of TNF with its receptors. Infliximab has mouse variable regions (*purple*) on both heavy and light chains linked to human IgG1 and κ constant regions (*light blue*). Etanercept contains the extracellular domain of human p75 TNF receptor (*dark blue*) and human IgG1 hinge and Fc domains (*light blue*). Infliximab binds TNF (*yellow*) but not LT (*green*). When present at a molar excess over TNF, three molecules of infliximab can bind to each TNF trimer. In contrast, etanercept binds TNF in a 1:1 stoichiometry, even when present in molar excess (Adapted from Scallon et al. [31])

and patients with other autoinflammatory conditions, including chronic infantile neurologic, cutaneous, articular (CINCA) syndrome/neonatal-onset multisystem inflammatory disease (NOMID), or Muckle–Wells syndrome [12] due to cryopyrin mutations. Cryopyrin is a key protein of a multiprotein cytoplasmic complex called the inflammasome. In the presence of a number of stimuli, cryopyrin oligomerizes and binds to other intracellular proteins. This association directly activates IL-1-converting enzyme/caspase 1, which in turn converts proIL-1β into the mature, active 17 KDa form.

Among reported patients with *TNFRSF1A* mutations, only a few displayed a particularly aggressive disease course that required almost continuous treatment with steroids. Recently, Gattorno et al. reported that five patients with aggressive disease course were treated with anakinra. All five of these patients showed a dramatic

response to the drug. Moreover, continuous treatment with anakinra completely prevented disease relapses during the follow-up period (4–20 months). This suggests that anakinra could be a valid alternative therapy for patients who require prolonged treatment with steroids or who experience frequent and long-lasting episodes of fever that led to severe limitations in their daily activities. These observations, together with the good response observed in several patients with TRAPS patients, raise the possibility that dysregulation of IL-1β production and secretion represents a possible final common pathway of different monogenic or multifactorial inflammatory disorders [22]. Consistent with this hypothesis, we observed an increase in IL-1β secretion in PBMCs isolated from TRAPS patients with the T50M mutation, when compared to healthy controls [24]. Is IL-1β contributing to the bulk of inflammatory responses responsible for the exacerbation of the phenotype observed in TRAPS patients and by extension contributing to the failure of treatment with infliximab as shown by Jacobelli et al.? If so, then IL-1β production and secretion is likely to play a major role in the pathogenesis of TRAPS.

Even if etanercept is still considered to be the first-line therapy for TRAPS, blocking IL-1β with anakinra is more effective than blocking TNF for some TRAPS patients, suggesting a role for both TNF-dependent and -independent pathways in the pathogenesis of TRAPS [35]. Therefore, anakinra combination therapy with another drug may be necessary to inhibit the exacerbated inflammatory response and secondary amyloidosis, which is the most threatening complication found in these patients. Although recent advances in treatment of hereditary autoinflammatory diseases have given reasons for optimism, the long-term risks and benefits of TNF and IL-1 inhibitors are not yet known [13, 29, 35]. All forms of TNF blockade are however associated with increased risk of infection and potential reactivation of tuberculosis, and possibly lymphoma – problems that have not come to light with anakinra. Indeed, it is worth bearing in mind that anakinra underwent early testing in extremely high doses among patients with severe sepsis without detriment [29]. IL-6 is also able to induce the cytokine activation. We know that CRP is modulated by IL-6, and IL-6 secretion is increased in T50M patients after infliximab stimulation [24]. One might therefore consider the role of IL-6 in TRAPS. Should this cytokine indeed play a central role in mediating the inflammatory response in TRAPS, then anti IL-6 therapy is also worthy of future consideration.

Conclusion

Therapy directed at blocking the effects of key cytokines has been remarkably effective in treating some cases of TRAPS. However, much work remains to be done to find effective therapies for the majority of these patients. It is also clear that there is still much to learn about the effect of TRAPS-causing mutations on TNF signalling pathways. Greater knowledge of the cellular pathways involved will however enable the development of more effective targeted medical intervention.

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Chapter 44
Modalities of Experimental TNF Blockade In Vivo: Mouse Models

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Introduction

In vivo, TNF is produced by many cell types, including lymphoid and stromal cells, and can exist in both membrane-bound and soluble forms. Systemic pharmacological or genetic TNF ablation in mice results in the impairment of host defense functions and of humoral immune responses as well as in multiple defects in lymphoid tissues (including disruption of primary B-cell follicles, absence of germinal centers (GC) and follicular dendritic cells (FDC) [1, 2]. These latter homeostatic functions of TNF were not known prior to knockout studies in mice. Moreover, it has been further demonstrated that TNF produced by B cells is one of important factors in the maintenance, at least in spleen [3].

In order to evaluate the effects of various blockers of human TNF on protective and homeostatic functions of TNF, we developed "humanized" mouse models which are free of any spontaneous disease.

Additionally, to uncover the role of TNF produced by specific cell types in development and maintenance of secondary lymphoid organs, we used mice with conditional inactivation of TNF gene restricted to either macrophages/neutrophils or B cells, or T cells, or to T cells + B cells. We also crossed these cell-type specific knockout mice to mice which produced only membrane-bound TNF [4, 5], thus allowing to evaluate the contribution of a unique cellular source of TNF in each of its two molecular forms (soluble versus cell-bound). Our results obtained using this experimental mouse panel unraveled several distinct contributions of TNF signals originating from B and T cells to the organogenesis and maintenance of distinct lymphoid tissues, such as spleen, LN, and PP, and to the efficiency of humoral immune responses to thymus-dependent antigens. The same panel of mice was also used to evaluate contribution of distinct sources of TNF into pathogenesis of several autoimmune diseases.

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Results and Discussion

Novel Humanized Mice for Studying TNF Inhibition In Vivo

Anti-TNF therapy is used for treatment of a growing number of autoimmune diseases, such as rheumatoid arthritis, Crohn's disease, ulcerative colitis, Behcet's disease, ankylosing spondylitis, psoriasis [6]. Currently, several drugs are available and multiple studies have shown that distinct anti-TNF agents may have different efficacy in patients, implying non-identical mechanisms of action, in particular, in Crohn's disease. However, the reasons for differential efficacy are still poorly understood and may include different kinetics, affinity, and dissociation of TNF–TNF blocker complexes between various TNF inhibitors, different half-life, and accessibility of the drugs and antibody-mediated killing of TNF-producing cells [6]. One of the obstacles to studying the effects of experimental TNF blockade in vivo with drugs used in clinics is the lack of appropriate animal model. Since the majority of anti-TNF agents (except Etanercept and XPro1595, see below) neutralize only human but not murine TNF, mice expressing human TNF are needed for evaluation of neutralizing effects. The first mice that expressed human TNF as pathogenic factor were generated by G. Kollias's lab almost 2 decades ago [7]. These mice bear multiple copies of the human TNF genomic fragment with disrupted 3'-UTR of the TNF gene which is necessary for correct posttranscriptional control of TNF biosynthesis. As a result, these mice express high amount of TNF produced by a variety of cellular sources and develop various pathologies, including polyarthritis. Such models proved to be extremely useful to investigate pathogenesis in TNF-driven arthritis, but obvious limitations are due to the fact that the disease is spontaneous, multiple organs are affected, and such mice usually die within several months unless they are treated with TNF blockers.

We decided to generate mice with a more "natural" control of human TNF expression and without spontaneous pathologies. In the first model, we integrated a 40 kb piece of human DNA, containing all three genes of the human TNF/LT locus under control of their natural promoters as a low-copy number transgene [8, 9]. Two founders were selected, one with a higher and the other with a lower level of TNF expression (as judged by induced production both in vivo and in vitro in response to LPS and other stimuli). The former, but not the latter, mice exhibited thymic atrophy, which was partly due to modest overexpression of human LT [9]. These transgenic mice have been backcrossed to mTNF/LT$^{-/-}$ background [10], resulting in generation of mice "humanized" for both TNF and LTa/LTb (Fig. 44.1a) and in such configuration none of the mice demonstrated thymic pathology. Naïve "humanized" mice did not produce detectable levels of hTNF, but LPS administration led to the spike of substantial levels of human TNF in sera (Fig. 44.1b). Moreover, lethal LPS/D-Gal liver toxicity mediated by pathogenic TNF could be prevented in these mice by administration of anti-TNF agents, such as Infliximab or drugs based on VHH from Camelids [11, 12] (Fig. 44.1c). Our preferred humanized mouse model

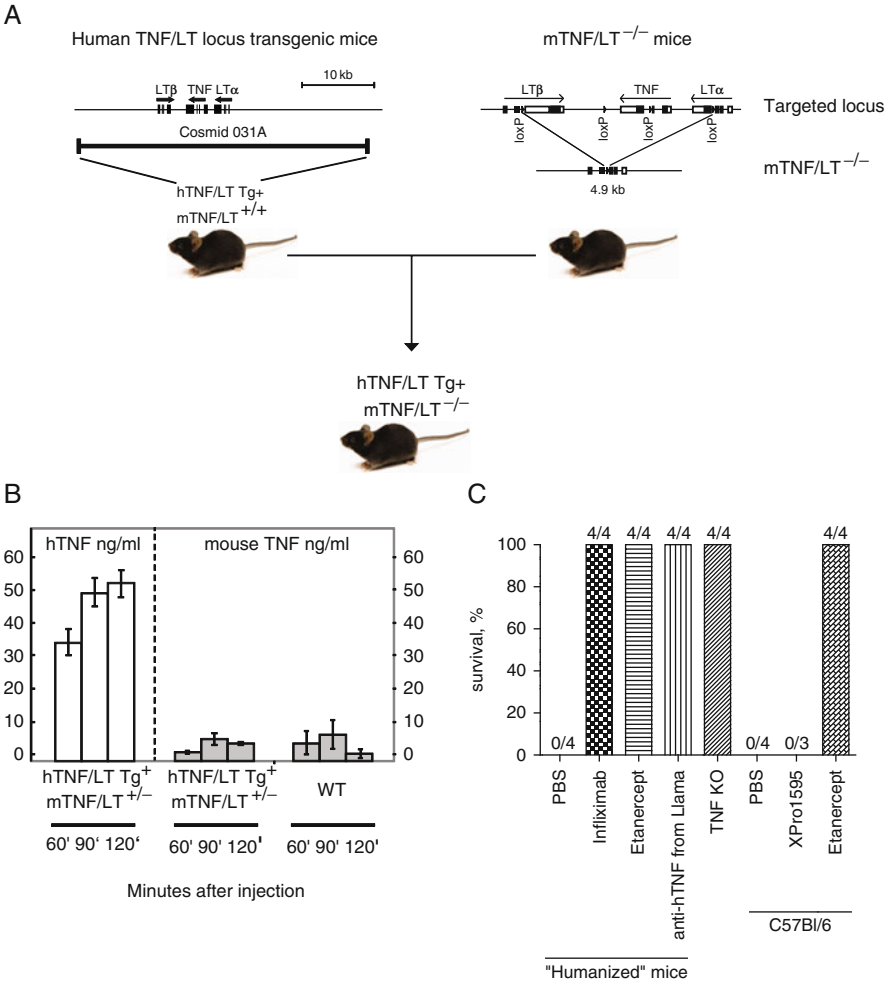

Fig. 44.1 Novel humanized mice for studying of TNF blockade in vivo. (**a**). Scheme of generation of hTNF/LT Tg+ mTNF/LT$^{-/-}$ mice. (**b**) Increased hTNF production in hTNF/LT Tg+ mTNF/LT$^{-/-}$ mice upon LPS administration. (**c**) Blockade of LPS/D-Gal septic shock in hTNF/LT Tg+ mTNF/LT$^{-/-}$ and C57Bl/6 mice by various TNF inhibitors. Blockers of human TNF, such as Etanercept (1 mg/mouse), Infliximab (1 mg/mouse), and VHH from llama (300 μg/mouse), can block pathogenic TNF in LPS/D-Gal toxicity model. XPro1595 (1 mg/mouse) does not rescue mice

is one which produces lower amounts of induced TNF and which contains exactly two copies of the human TNF gene [8] on TNF/LT triple-deficient background [10]. In our next humanization model the human TNF gene was knocked in into the exact position of the murine gene (Kruglov et al, in preparation) allowing to maintain both the "natural" control and the precise gene dosage.

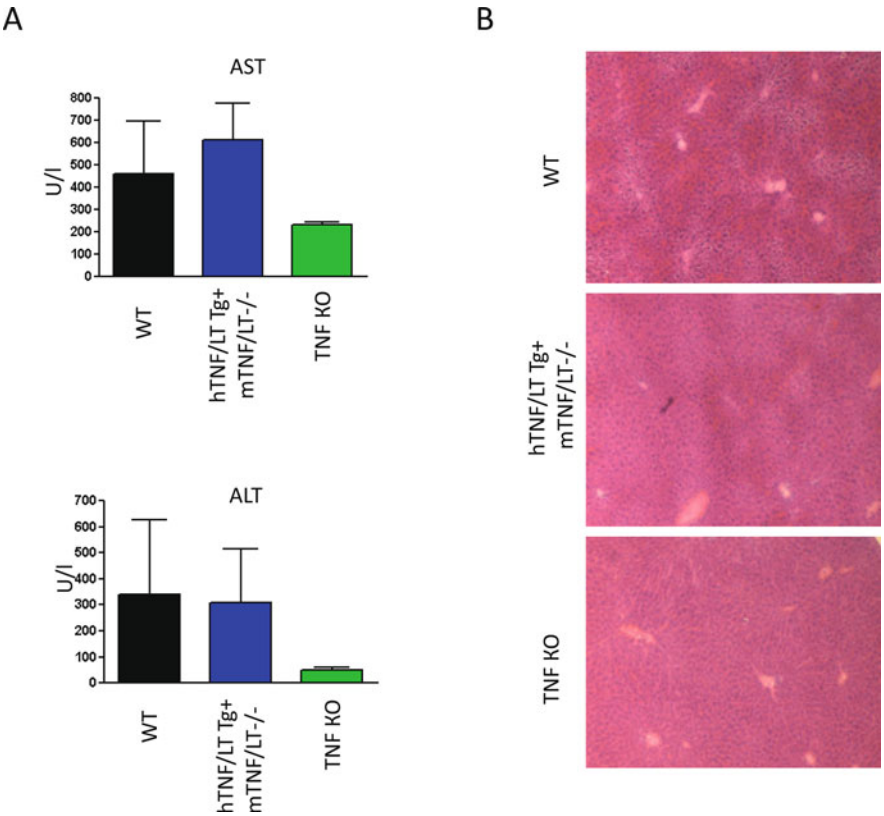

Fig. 44.2 Development of ConA-induced hepatitis in hTNF/LT-humanized mice. Mice were injected intravenously with 20 mg/kg of Concanavalin A. 8 h later, mice were bled, humanely sacrificed, and livers were assessed histologically. (**a**) Levels of ALT and AST in wild type (WT), hTNF/LT Tg+ mTNF/LT$^{-/-}$, and TNF KO mice. (**b**) Liver histology of WT, hTNF/LT Tg+ mTNF/LT $^{-/-}$, and TNF KO mice

It should be noted that faithful transmission of TNF-mediated signals in vivo requires "functionality" of both types of TNF receptors. It is well established that human TNF can bind and signal via murine TNFR1 (p55), while its ability to engage murine TNFRII (p75) remains controversial [13]. In our experiments with humanized mice autoimmune hepatitis induced by ConA was developed in all such mice and could be partly neutralized by anti-TNF treatment (Fig. 44.2 and data not shown). On the other hand, this disease model was reported to be dependent on both TNFR1 and TNFRII (p75) functions [14], suggesting at least partial functionality of the p75 signaling in our humanized mice. Nevertheless, the ultimate genetic mouse model for humanization of the TNF system may require additional humanization of the TNFR II gene.

Mouse Models to Evaluate Functions of TNF Produced by Distinct Cell Types or in Distinct Molecular Forms (or Both)

Previously, several mouse models have been engineered that allowed conditional ablation TNF production. $TNF^{flox/flox}$ mice have been generated [15] and crossed to B cell, T cell, and macrophage/neutrophil-specific Cre deleter mice (Tables 44.1 and 44.2). Additionally, $TNF^{flox/flox}$ mice were crossed to cell-specific Cre deleter mice allowing inactivation of the TNF gene in dendritic cells, mast cells, lymphoid tissue-inducing cells, and enterocytes (unpublished data).

Two groups have independently generated useful mice with mutation in the TNF gene which did not allow its gene product to be processed by specific protease and be secreted [4, 5]. As a result, these mice expressed only membrane-bound TNF on the surfaces of TNF-producing cells. Since these mice were generated using a knock-in approach, they can be crossed to cell type-restricted TNF-deficient mice to produce offspring, in which case one allele of TNF gene provides uncleavable TNF and the other allele can be inactivated by Cre-mediated deletion only in the selected cell types (ex. in B cells). As a result, all cell types, except the selected type, produce soluble secreted TNF from one of the alleles and the selected cell type (ex. B cells) produce only cell-bound TNF. Phenotypic features in such mice can be then tentatively linked to the function of soluble TNF produced by this selected cell type (Table 44.1).

These experiments allowed us to assign homeostatic functions of TNF in lymphoid organs to soluble TNF produced by B cells and also to membrane-bound TNF produced by T cells. Interestingly, spleen, lymph nodes, and Peyer's patches revealed distinct requirements with regard to the cellular source and molecular form of TNF (Table 44.1) (Tumanov et al. Blood, in press. [29]).

Distinct Functions of TNF Produced by Individual Types of Immunocytes in Disease

Mice with conditional TNF ablation were also evaluated in several pathophysiological models, including septic shock, ConA-induced hepatitis, and EAE.

TNF is pathogenic in several autoimmune diseases, such as rheumatoid arthritis, psoriasis, ankylosing spondylitis, Crohn's disease [6]. Anti-TNF treatment provides a successful therapy in many instances, although such therapy cannot be completely free of side effects in all individuals due to the spectrum of non-redundant beneficial functions of TNF. Of note, although TNF was thought to be involved in pathogenesis of multiple sclerosis, one clinical trial using anti-TNF therapy in this disease has failed [16]. However, in EAE, the murine model of MS, disease can be ameliorated by TNF blockade [17]. A study by Kollias's group has suggested that TNF plays a crucial role in the control of T cell immune response and that the loss of TNF may lead to prolonged persistence of autoreactive T cells, implying that they may be the cause of complications also in MS patients treated with TNF

Table 44.1 Genetic and pharmacological TNF ablation and its effects on lymphoid organs structure and susceptibility to various diseases

	Spleen			Lymph nodes		Peyer's patches		Disease models			
	B cell follicles	FDC	MZ	B cell follicles	FDC	B cell follicles	FDC	LPS/D-Gal toxicity	ConA-induced autoimmune hepatitis	Listeria infection	
C57Bl/6	+++	+++	+++	+++	+++	+++	+++	Susceptible	Susceptible	Resistant	
C57Bl/6 treated with Etanercept	++	–	+	+++	–	n.d	n.d.	Resistant	Resistant	Susceptible	[19, 23, 29]
C57Bl/6 treated with XPro1595	+++	+++	+++	+++	+++	n.d	n.d.	Susceptible	n.d.	Resistant	[20] and our unpublished data
TNFR1 KO	–	–	–	–	–	PP absent		Resistant	Resistant	Susceptible	[14, 24, 25, 26, 27]
TNFR2 KO	+++	+++	+++	+++	+++	+++	+++	Susceptible	Resistant	Resistant	[14, 25]
TNF KO	–	–	+	–	–	PP absent*		Resistant	Resistant	Susceptible	[1, 2, 28]
MN-TNF	+++	+++	+++	+++	+++	+++	+++	Resistant	Resistant	Susceptible	[15]
T-TNF	+++	+++	+++	++	++	+++	+++	Susceptible	Resistant	Partial susceptibility	[15, 29]
B-TNF	+	+	++	++	++	–	–	Susceptible	Susceptible	Resistant	[15, 29]

Table 44.1 (continued)

	Spleen			Lymph nodes		Peyer's patches		Disease models			
	B cell follicles	FDC	MZ	B cell follicles	FDC	B cell follicles	FDC	LPS/D-Gal toxicity	ConA-induced autoimmune hepatitis	Listeria infection	
C57Bl/6	+++	+++	+++	+++	+++	+++	+++	Susceptible	Susceptible	Resistant	
T,B-TNF	–	–	+	–	–	–	–	Susceptible	n.d.	n.d.	[15, 29]
Tm-TNF$^{\Delta 1\text{-}12}$	–	–	+	–	–	–	–	–	Susceptible	Partial susceptibility	[4]
Tm-TNF$^{\Delta 1\text{-}9,\ K11E}$	+	+	+	+	–	n.d	n.d.	Susceptible	Resistant	Partial susceptibility	[5]
Tm-T-TNF	+++	+++	+++	+++	+++	+++	+++	n.d.	n.d.	n.d.	[29]
Tm-B-TNF	+	+	+	++	++	–	–	n.d.	n.d.	n.d.	[29]

+++ – normal structure, ++ – reduced, + – strongly reduced or disorganized, – disrupted

*Different TNF KOs exhibit different phenotype from normal quantity of Peyer's Patches to complete absence

Table 44.2 Role of TNF produced by distinct cell types on EAE

Mice	Disease onset	Disease severity
TNF KO	Delayed	Increased*
T-TNF KO	As in wild-type mice	Reduced
MN-TNF KO	Delayed	As in wild-type mice
B-TNF KO	As in wild-type mice	As in wild-type mice

* – mice develop chronic, persistent EAE

antagonists [18]. In order to dissect the contribution of TNF produced by distinct cells in EAE model, we utilized mice with cell-specific TNF deletion in T cells (T-TNF KO), macrophages, and neutrophils (MN-TNF KO) and in B cells (B-TNF KO). Our analysis has uncovered the contribution of TNF from macrophages and neutrophils into disease onset, while TNF from T cells contributed to disease severity. TNF produced by B cells plays a critical homeostatic function in lymphoid tissues (see Table 44.1) However, it appeared not to have any effect in pathogenesis of EAE. In contrast to conventional TNF KO, prolonged persistence of autoreactive T cells has not been observed in T-TNF KO, MN-TNF KO, and B-TNF KO mice during the recovery stage. This suggests that cell type-restricted anti-TNF therapy may spare T cell homeostasis and could be potentially used for MS treatment, in the future.

In addition to EAE, other autoimmune disease models, in particular, experimental arthritis can also be dissected using the same mouse panels.

Effects of Non-conventional TNF Blockers on Acute Septic Shock and on the Structure of Secondary Lymphoid Organs

TNF is initially expressed as transmembrane trimeric molecule (tmTNF) that can be further cleaved resulting in the production of soluble TNF (sTNF). Studies in mice which expressed only uncleavable tmTNF implicated *soluble* TNF as the main driving factor for some of pathogenic functions of TNF [4, 5]. These findings also led to hypothesis that neutralization of only soluble TNF may block pathogenic functions of TNF without interfering with the beneficial effects. Remarkably, dominant negative TNF mutant (DN-TNF) has been reported to block selectively sTNF [19]. The follow-up studies using DN-TNF blocker (XPro1595) revealed that this agent could block development of arthritis without compromising protective TNF functions against infections mediated by tmTNF [20, 21]. It should be noted, however, that based on mouse data homeostatic functions of TNF in lymphoid tissues are largely provided by *soluble* TNF produced by B cells (Table 44.1).

To better evaluate the effects of DN-TNF antagonist in vivo, we set up the LPS/D-Gal septic shock model, where soluble TNF has a critical role in driving pathology. TNF blockade by Etanercept, soluble TNFR2 fusion protein, successfully protected mice from lethal toxicity, whereas XPro1595 administration at various doses and at

various time points showed very little or no protective effect (Fig. 44.1c and data not shown). This result may be explained by a too high concentration of pathogenic TNF in this very fast acute model, so that XPro1595 cannot neutralize it quickly enough.

Additionally, pharmacological or genetic TNF inhibition could result in disruption of the structure of secondary lymphoid organs in both humans and mice [22, 23]. Based on the results presented in Table 44.1 a significant component of homeostatic TNF-mediated signaling in lymphoid tissues is due to production of *soluble* TNF by B cells. To test the effects of XPro1595 in vivo on the structure of secondary lymphoid organs, we administered XPro1595 to mice twice in a 3-day interval. Similar schedule of treatment using Etanercept resulted in the loss of FDCs in mice ([23] and unpublished data). Somewhat surprisingly, immunohistochemical analysis of spleens from XPro1595 treated mice did not reveal appreciable defects in T/B cell zone segregation, FDC networks, and marginal zone integrity (Table 44.1). This unexpected result calls for additional evaluation of DN-TNF effects in vivo.

In conclusion, engineered mice described in this chapter have helped to better dissect TNF-mediated functions in health and disease and to link these functions to specific source of this cytokine. It is conceivable that more selective TNF blockers can be designed based on our mouse studies. In particular, it would be important to identify blockers that efficiently neutralize pathogenic TNF but only minimally disturb host defence TNF functions.

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Chapter 45
Tumor Necrosis Factor-α Signaling via TNFR1/p55 Is Deleterious Whereas TNFR2/p75 Signaling Is Protective in Adult Infarct Myocardium

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Background

Aging and Angiogenesis

Aging is associated with an increased risk for development of coronary and peripheral artery diseases [1]. The extent of ischemic damage and functional recovery in case of extensive impairment of vascularization due to arterial obliteration in either circulatory system largely depends on the development of new collateral blood vessels. Aging is also accompanied by a steady decline in immune functions that include but are not limited to defects in signaling pathways and altered cytokine (IFNγ, VEGF) expression [2–4]. Age-related impairment of angiogenesis has been documented in many previous studies [5–9]. Deficiencies in multiple components of ischemia-induced neovascularization including but not limited to inhibition of EC proliferation and function [7, 10–12], impaired expression of angiogenic growth factors, such as VEGF, bFGF, TGF-β, and PDGF [7, 13–15], were implied in delayed or failed angiogenesis in adult tissue. In addition, some recent studies have also demonstrated significant contribution of ischemia-induced inflammatory responses to delayed cutaneous wound healing with age [16, 17].

TNF, TNF Receptors, and Angiogenesis

Angiogenesis is accompanied by perivascular inflammation and monocyte/macrophage accumulation [18]. Tumor necrosis factor alpha (TNF-α), a

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macrophage/monocyte-derived pluripotent mediator, can function as an angiogenic factor in one system and as an anti-angiogenic factor in another system [19–22]. These mutually exclusive TNF-α effects were attributed to TNF-α concentration and duration of the exposure, that is, low concentrations and short exposure is angiogenic, whereas high concentrations and prolonged exposure is anti-angiogenic [23]. Interestingly, impaired TNF-α and other cytokine signaling in EC were correlated with enhanced apoptotic response in cutaneous microvasculature in adult tissue [24]. TNF-α has been reported to induce the expression of many immunologically relevant and angiogenesis-related genes [25, 26], through two different TNF-α receptors, TNF-αR1 (p55) and TNF-αR2 (p75) [27–29]. In ECs, TNF-α increases the expression of well-known angiogenic factors VEGF, bFGF, IL-8 [26]; however, the role of two distinct TNF-α receptors in mediating these responses is still unclear. Although the distribution of p55 is more widespread, p75 is present in greater amounts on endothelial and hematopoietic lineage cells [30]. Because p55 is largely known to mediate cytotoxic effects of TNF-α, whereas signaling through p75 is mostly implied in protective effects of TNF-α [31, 32], and because aging is associated with increased expression of p55 and decreased expression of p75 in lymphocytes from aged humans [33] we hypothesize that TNF signaling via its receptors may play significant and, perhaps, divergent effects in neovascularization, repair, and regeneration in adult tissue after acute myocardial infarction (AMI).

Aging, TNF Signaling, and Post-ischemic Recovery

Numerous reports suggest that VEGF and bFGF are critical growth factors in therapeutic angiogenesis [34–37]. Because treatment with TNF activates transcription factor NFκB and NFκB is known to regulate, both, VEGF and bFGF expression [26, 38], it is conceivable that impairment in TNF signaling may affect adversely post-ischemic recovery. Hence, it is possible that age-associated changes in TNF signaling may influence post-ischemic recovery processes in several ways. First, decreases with age expression of TNFR2/p75 coupled with post-ischemic increases in the systemic levels of TNF may favor apoptosis in adult cardiomyocytes and resident ECs [39] through impaired p75-mediated anti-apoptotic signaling via NFκB, subsequently leading to inhibition of angiogenesis. It is also conceivable that in adult tissue with decreased p75 receptor expression in general [40] unopposed signaling through p55 receptor increases predominantly pro-apoptotic cascade via increase in FADD, TRADD, and FASDD [33, 40]. Furthermore, it is also likely that previously reported decrease in VEGF expression in adult cells [2–4] may be a direct consequence of age-associated decrease in the expression/signaling via p75. In this case, not apoptosis, but rather direct inhibition of TNF-mediated angiogenic signaling (TNF→NFκB→VEGF, bFGF) could be responsible for failure of post-ischemic recovery and regeneration in adult tissue. Potential scenario could be as follows: in young tissue intact signaling through both p55 and p75 receptors assures proper NFκB activation, followed by transcriptional activation of VEGF and bFGF and

other TNF-induced NFκB-mediated pro-angiogenic genes (i.e., IL-8), whereas in cells from adult donors with decreased p75 receptor expression [33, 40] signaling through p55 receptor alone is not sufficient for proper NFκB activation [41] and regulation of transcription of pro-angiogenesis genes which, at least in part, may be responsible for impaired angiogenic response in adult tissue [5–9]. Finally, VEGF, which is an early angiogenic growth factor response to ischemia [42], has been shown to mobilize BM-derived EPCs in murine models and in humans [43, 44]. Hence, age-associated decrease in TNF-induced NFκB-mediated VEGF expression may be due to, in part, decreased p75 receptor expression, which may lead to subsequent decrease in mobilization and/or recruitment of BM-derived EPCs to the ischemic areas [41], thereby representing an additional mechanism of impaired repair, angiogenesis, and regeneration in adult coronary tissue.

Results

Constitutive Expression of p75 Is Decreased in Human PB EPCs from Donors of Increasing Age

We and others have previously reported that there is an inverse relationship between increased age and decreased angiogenesis [5, 7, 8]. Interestingly, similar inverse relationship between increased age and decreased expression of TNFR2/p75 has been reported, in contrast to increased levels of TNFR1/p55 in lymphocytes from adult donors [33]. To evaluate age-associated changes in TNFR1 and 2 levels in human ECs we measured p55 and p75 mRNA expression levels (using ribonuclease protection assay) in human PB EPCs isolated from donors of increasing age. There was no significant change in the levels of p55 expression in EPCs from elderly donors compared to more than 20 year younger adults (Fig. 45.1a). In contrast, there was more than 55% ($p<0.01$) decrease in p75 mRNA levels in EPCs from elderly donors vs. younger adults (Fig. 45.1b).

Post-AMI Survival and Functional Myocardial Recovery Is Impaired in Old p75KO Mice

It was reported previously that TNF receptor knockout (KO) mice reveal exacerbated damage and altered NFκB activation after ischemic injury [45]. We hypothesized that signaling through p75 may be essential in pro-survival signaling in adult tissue. To examine whether age-associated decreases in p75 expression may contribute to the failure of post-AMI recovery in adults we evaluated survival, cardiac function, angiogenesis, and recovery in a series of experiments in murine model of acute myocardial infarction (AMI) [ligation of the left anterior descending (LAD) coronary artery] in young and old p75KO and p55KO mice and age-matched wild-type (WT) controls. Overall surgical mortality death (within 24 h post-surgery) was

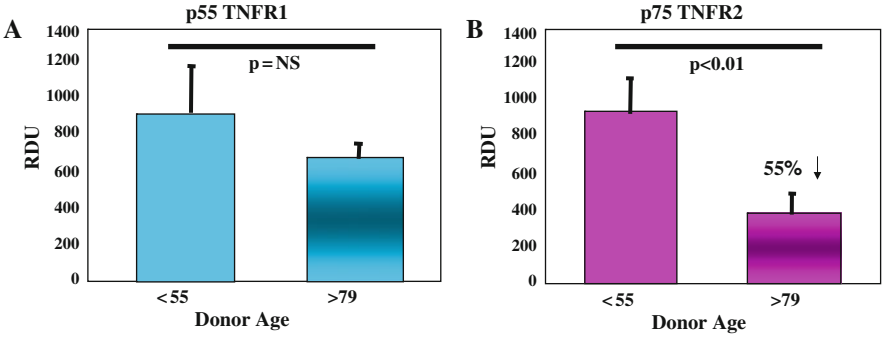

Fig. 45.1 p75 TNFR2 expression is decreased in peripheral blood EPCs from elderly donors. Human adult PB EPCs were collected and processed for evaluation of constitutive mRNA expression of TNF receptors p55 and p75 by ribonuclease protection assay. We divided (arbitrary) our donors into two groups: younger than 55 and older than 79 (n=5/age group). (**a**) Densitometric analysis of p55 TNFR1 mRNA expression in human adult PB EPCs. RDU –relative densitometric units. (**b**) Densitometric analysis of p75 TNFR2 mRNA expression in human adult PB EPCs

<4% and these animals were excluded from the study, because surgical and anesthesia errors could not be ruled out as the cause of death. The rest of all the deaths occurred within 1 week post-AMI, and although not all mice hearts that died within 7 days were examined, of those that were examined (~75%), most showed evidence of cardiac rapture in peri-infarct myocardium and some revealed pericarditis (data not shown). There was significant age-associated increase in post-AMI mortality in old WT mice (12% vs. 45% in young vs. old WTs) (Fig. 45.2a). Not surprisingly, post-AMI mortality in young p75KO mice was approaching the mortality in old WT mice (35% vs. 45% in young p75KO vs. old WTs) (Fig. 45.2a), suggesting development of an aging phenotype in young p75KO mice. Interestingly, more than half of the mice in old p75KO group (60%) died within 7 days post-AMI (Fig. 45.2a). In contrast, no post-AMI mortality was observed in young p55 mice and post-AMI mortality in old p55KO was only 10%, approximately the same as in young WTs (Fig. 45.2a). These latter data strongly suggest that lack of signaling through p55 appears to improve significantly post-AMI survival (better than in WT mice) in young p55KO, as well as in old p55KO mice (recovery in old p55KOs was as good as in young WTs).

For evaluation of infarct size and left ventricular (LV) dimensions transthoracic echocardiography was performed before surgery (LAD ligation), 7 and 28 days after surgery. We measured LV circumference in systole (LVs) and diastole (LVd) and these values were used to calculate change in fractional area using the following formula: [% LV FS=(LVd − LVs/LVd) × 100]. We also calculated infarct size in the same view by measuring infarct segment length and dividing it by diastolic circumference (×100). For both, change in fractional area and infarct size, we measured all parameters three times and mean of these values were used to make comparisons. Compared to young WT mice, between days 7 and 28 post-AMI functional recovery, as measured by percent decrease in left ventricular fractional shortening (%LV FS),

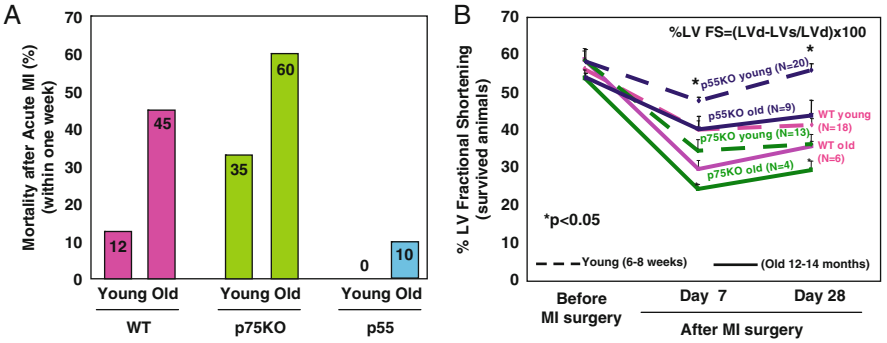

Fig. 45.2 (**a**) Post-AMI mortality is increased in WT old and TNFR2/p75 young and old mice and decreased in young and old TNFR1/p55 mice. Compared to WT young (6–8 weeks) mice within 7 days there was approximately threefold increase in post-MI mortality in old (12–14 months) WT and young p75KO mice. Post-AMI mortality in old p75KO mice was fivefold higher compared to young WT mice. No post-AMI mortality was observed in young p55 mice and post-AMI mortality in old p55KO was only 10%, approximately the same as in young WTs. (**b**) Impaired signaling through TNFR2/p75 worsens age-related decrease in LV FS in old p75KO mice, whereas signaling through TNFR1/p55 may be harmful for post-AMI recovery in murine model of AMI. No significant difference in LV FS was observed between mice of different genotypes before surgery. LV FS was significantly ($p<0.05$ day 7, and p=NS day 28) decreased in old WT and young p75KO mice. The worst recovery was observed in old p75KO mice. LV FS was significantly ($p<0.05$ day 7, and p=NS day 28) decreased in old p55KO vs. young p55KO on day 28 post-AMI, suggesting some age-associated impairment in post-MI recovery in old p55KO mice, although this degree of recovery was as good as in young WT mice

was considerably worse in mice of old WT and young p75KO mice (Fig. 45.2b). LV FS (%) was significantly worse on days 7 and 28 post-AMI in old p75KO mice compared to WT young (more than 20% decrease compared ($p<0.05$) and WT old and p75KO young (more than 10% decrease, $p<0.05$) (Fig. 45.2b). Our results strongly suggest that signaling through p75 may be a critical factor for the processes of post-ischemic recovery in adult tissue. Moreover, although lack of signaling through p55 reveals age-related decrease in LV FS in old vs. young p55KO mice (Fig. 45.2b), post-MI recovery of, both, young and old p55KO mice was significantly better statistically than in, both, young and old WT and p75KO age-matched counterparts, suggesting that signaling through p55 may be harmful for post-AMI recovery, as others have also shown previously [46, 47]. Please note that because of severe age-associated deficiency in old p75KO mice compared to very mild age-associated change in p55KOs in post-AMI recovery from here on all our data will give comparisons between young and old WT and p75KO mice only. *Ischemia-induced inflammatory infiltrate is increased and longer-lasting in p75KO and decreased in p55KO mice post-AMI.* To quantify post-AMI inflammatory infiltrate we evaluated the expression of myeloperoxidase-1 (MPO-1), a neutrophil marker, and CD-68, a glycoprotein normally expressed on macrophages, also known in mice as macrosialin. Compared to WT mice, quantification of MPO-1 (+) cells showed ~ 313% (p75KO) and 49% (p55KO) increases on day 7, and

$\sim$ 31% (p75KO) increase and 32% (p55KO) decrease on day 14 post-AMI ($p < 0.03$ for all, data not shown). Compared to WT mice, quantification of CD-68 (+) cells showed $\sim$ 5% (p75KO) and 24% (p55KO) decreases on day 7, and $\sim$ 85% (p75KO) increase and 39% (p55KO) decrease on day 14 post-AMI ($p < 0.05$ for all, data not shown), suggesting that in the absence of p75 signaling post-AMI inflammatory responses are increased and longer lasting, whereas in the absence on p55 signaling inflammatory responses decreased and are of short duration.

Cardiac Troponin I (cTnI) Expression Is Increased in Infarct Border Zone in p75KO Mice

To evaluate the magnitude of myocardial infarction and ongoing myocardial injury, we immunostained sections of infarct border-zone area with cardiac troponin I (cTnI), a marker that has proven to correlate well with infarct size and ongoing myocardial injury when measured in the serum [47] as well as in immunostained tissue [48]. Compared to normal non-infarct tissue (Fig. 45.3a, upper panel),

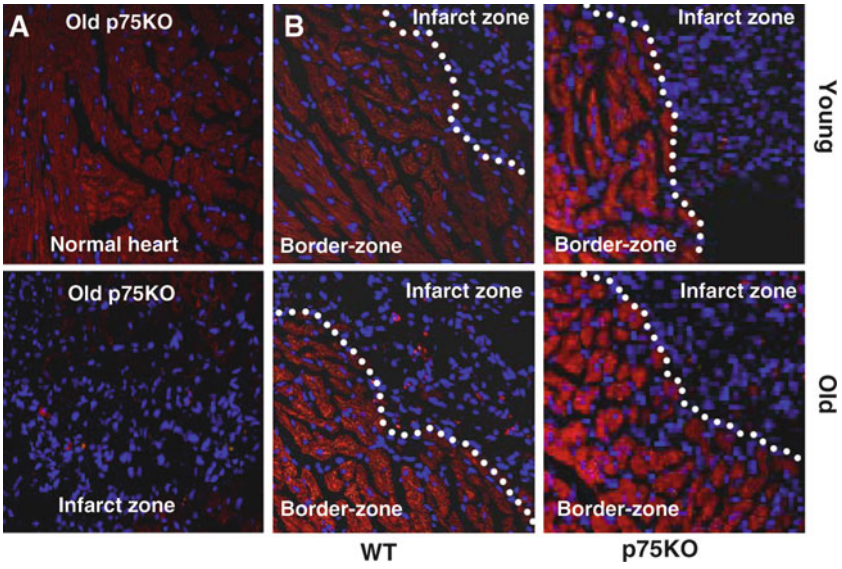

Fig. 45.3 Cardiac troponin I (cTnI) expression is increased in infarct border zone in p75KO mice. Representative confocal images of infarct border-zone myocardium of young and old WT and p75KO mice 7 days after AMI were stained with anti-cTnI antibodies (*red fluorescence*). cTnI expression was low in pre-surgery samples of all genotypes (p75KO old tissue is shown) and was consistently and substantially higher in the post-AMI myocardium of old WT and young and old p75KOs on day 7 post-AMI. Post-AMI hearts of at least three animals per genotype/time point were examined and similar cTnI expression pattern, as shown in these representative images, was observed in the tissues of young and old WT and p75KOs. *White dotted line* divides border zone from infarct area and *blue staining* is Topro-3 to identify nuclei

myocardial tissue expression level of cTnI on day 7 post-AMI was significantly increased in infarct border zone of young p75KO and old WT and p75KO mice (Fig. 45.3b, upper panel and lower left panel). cTnI expression levels were the highest in old p75KO mice (Fig. 45.3b, lower right panel), suggesting that extent of myocardial damage and ongoing myocardial injury is still in dynamics 7 days post-AMI in both young and old p75KO mice.

Capillary Density Is Decreased in Infarct and Infarct Border Zone in Old WT and Young and Old p75KO Mice

As a measure of collateral blood flow recovery after ischemia we evaluated capillary density in myocardium of young and old WT and p75KO mice 28 days after AMI surgery. Compared to young WT mice there was significant decrease in capillary density (first column – green staining) and the number of functional vessels (second column – red staining) in old WT mice in infarct and infarct border zone (Fig. 45.4a, b). Compared to both young and old WT mice post-AMI capillary density (green staining) and number of functional vessels (red staining) was further decreased in young as well as old p75KO mice (Fig. 45.4c, d). These data suggest age-associated decrease in post-AMI capillary density and patent/functional vessels in old WT mice and significantly greater deficiency in collateral vessel development and decrease in functional capillary network in young and old p75KOs.

Compared to Old WT Mice Post-AMI Functional Capillary Density Is Decreased in Old p75KOs in Infarct Border Zone

We quantified the capillary density and the number of functional vessels in old WT vs. old p75KO mice. To make sure that each and every endothelial cell is counted we used a Z-stack mode of confocal microscope and took at least 10 focal images each 0.5 μm thick (data not shown). We then used computer-assisted ImageJ (NIH software) to calculate the intensity of green (all ECs) and red (functional capillaries) in infarct myocardium 7 and 28 days post-AMI. When we compared capillary density in BS1-lectin-stained samples there was no significant difference between old WT and old p75KO mice (Fig. 45.5a). Between days 7 and 28 in old WT mice there was a significant increase in the functional capillary network (8.49±1.2 vs. 27.3±3.4, days 7 vs. day 28, respectively, $p<0.001$) (Fig. 45.5b), suggesting development of collateral vessels in old WT mice. Compared to old WT mice, the network of functional vessels in old p75KO mice was also not significantly different between old WT and old p75KO mice on day 7 post-AMI (8.49±1.2 vs. 11.2±3.4, old WT vs. old p75KO, respectively, p=NS) (Fig. 45.5b). Interestingly, by day 28 there was approximately threefold decrease in the functional capillary network in old p75KO mice compared to old WT mice (10.9±0.7 vs. 27.3±3.4, respectively, $p<0.001$) (Fig. 45.5b), strongly suggesting significant deficiency in development of collateral vessels in old p75KO mice post-AMI.

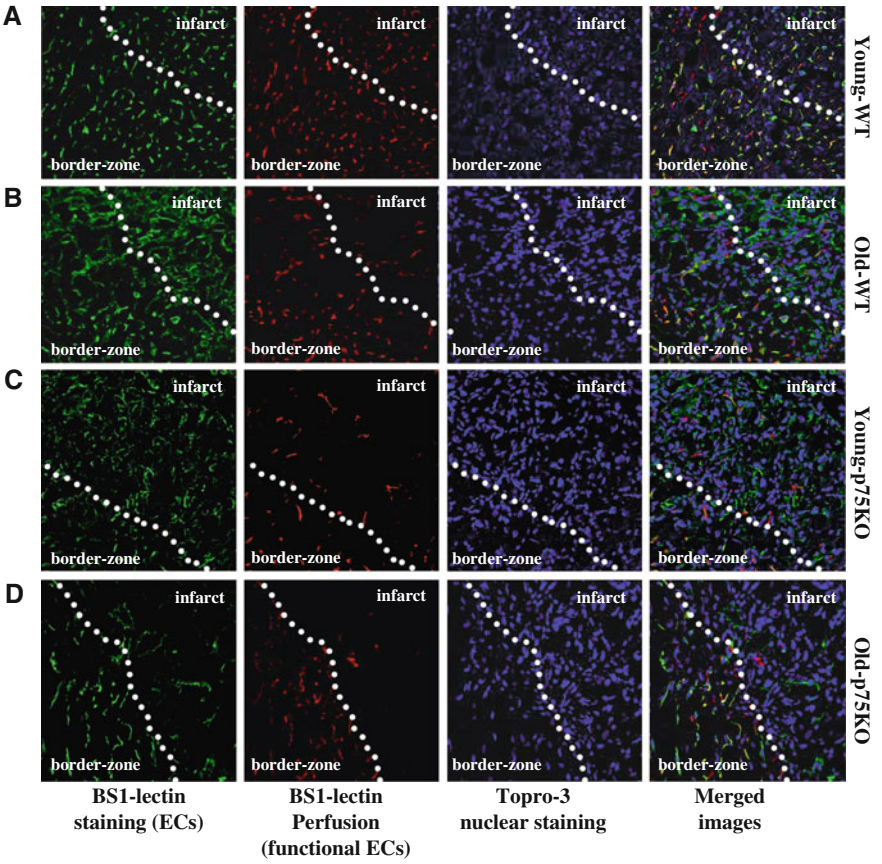

Fig. 45.4 Capillary density and the number of functional vessels are decreased in infarct and infarct border zone in old WT and mice of p75KO genotype. Five minutes before harvesting hearts mice were perfused with fluorescently labeled BS1-lectin (*red*) to identify functional/perfused vessels. After harvesting and fixation heart sections were again stained with fluorescently labeled BS1-lectin (*green*) to identify all vessels. (**a** and **b**) Representative confocal images of infarct border-zone myocardium of young and old WT mice. Compared to young WT mice capillary density and the number of functional vessels were consistently and substantially lower in the myocardium of old WT 28 days post-AMI. (**c** and **d**) Representative confocal images of infarct border-zone myocardium of young and old p75KO mice. Compared to young and old WT mice capillary density and the number of functional vessels were consistently and substantially lower in the myocardium of young and old p75KO mice 28 days post-AMI. *White dotted line* divides border zone from infarct area and *blue staining* is Topro-3 to identify nuclei. Post-AMI hearts of at least there animals per genotype/time point were examined and similar pattern, as shown in these representative images of randomly chosen areas of 0.09 mm^2 of myocardial tissue, was observed in the tissues of young and old WT and p75KO mice

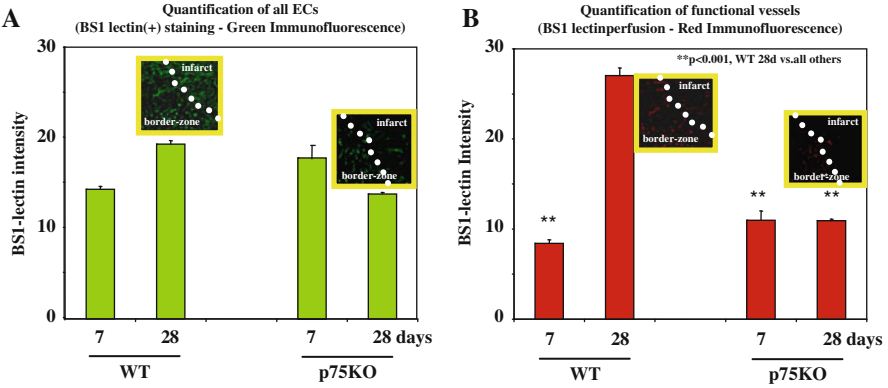

Fig. 45.5 The number of functional vessels is decreased in infarct border zone in old p75KO mice. (**a**) Quantification of all ECs. Insets are *green* BS1-lectin-positive cells; (**b**) quantification of functional capillary network. Insets are *red* BS1-lectin perfused, hence patent vessels. Compared to old WT mice capillary density and the number of functional vessels are significantly threefold lower in old p75KO mice 28 days post-AMI. Post-AMI hearts of at least three animals per genotype/time point were used to quantify the differences in capillary network between old WT and old p75KO mice

Ischemia-Induced Apoptosis Is Increased in the Myocardium of Old p75KO Mice 28 Days Post-AMI

To evaluate the apoptotic processes in infarct myocardium in young and old WT and p75KO mice we immunostained sections of infarct border-zone area for TUNEL, a marker of apoptosis, for BS1-lectin, a marker of ECs, and for TopRo3 to visualize nuclei. No apoptosis was detectable in hearts of WT or p75KO mice before AMI surgery (Fig. 45.6a, representative image of old p75KO heart). There were comparable increases in TUNEL (+) apoptotic cells (Fig. 45.6b, c, left column, green staining), as well as comparable decreases in BS1-lectin (+) cells (Fig. 45.6b, c, second column – red staining) on day 7 after AMI in ischemic myocardium of WT and p75KO old mice. By day 28, compared to old WT mice, p75KO old mice revealed extensive apoptosis in infarct area (Fig. 45.6d, e, left column – green staining) and significant decrease in BS1-lectin staining (Fig. 45.6d, e, second column – red staining), suggesting first that ischemia-induced myocardial apoptosis was augmented and long-lasting in old p75KO mice and second that there was a significant deficiency in functional collateral vessel development in old p75KO mice.

Expression of Several Angiogenic Growth and Stem Cell-Derived Factors and Their Receptors Is Decreased in Peripheral Blood EPCs from Adult Donors

Finally, to determine if, in addition to decrease in expression of p75 in adult EPCs (Fig. 45.1b), there may be alterations in the expression of other genes that are

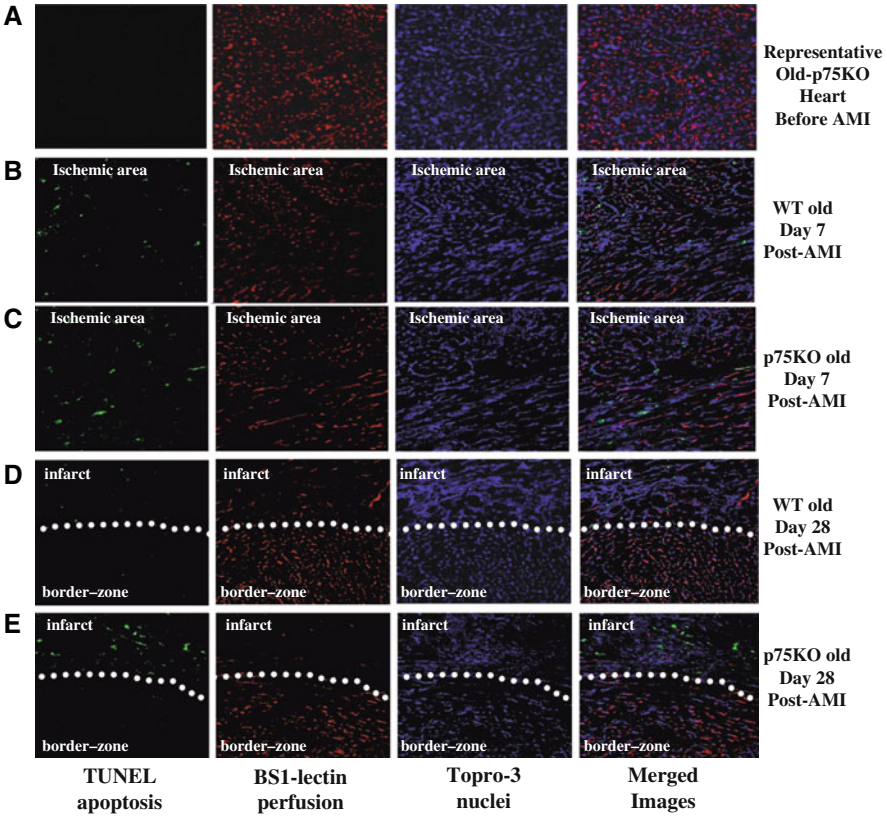

Fig. 45.6 Ischemia-induced apoptosis is increased in the myocardium of old p75KO mice after AMI. Representative confocal images of triple-stained heart muscle of old WT and old p75KO mice to visualize TUNEL (+) cells. TUNEL (*green*) identifies apoptotic cells, BS1-lectin (*red*) identifies functional endothelial cells, Topro3 (*blue*) visualizes nuclei. Representative images show significant increase in the number of TUNEL (+) cells in old p75KO mice in infarct area 28 days post-AMI. Post-AMI hearts of at least three animals per genotype/time point were examined and similar pattern, as shown in these representative images of randomly chosen areas of 0.09 mm^2 of myocardial tissue, was observed in the hearts of old WT and p75KO mice. *White dotted line* divides border zone from infarct area

involved in the processes of post-ischemic recovery, such as post-natal angiogenesis and regulation of stem and progenitor cell mobilization and recruitment to ischemic areas, we performed a series of qRT-PCR studies. We collected peripheral blood (PB) from healthy young donors (age 32±14) and from older patients (age 67±7) of our Catheterization Laboratory that came for a procedure. Please note that we did not need IRB approval for these studies because no identification of patients was required (except the age) and blood would otherwise be discarded. PB MNCs were isolated, then EPCs were grown on selective medium [41] and after being 5 days in the culture PB EPCs were harvested and processed for qRT-PCR. The expression of several potent angiogenic and pro-survival growth factors and cytokines such as VEGF-A, ANG-2, TGF-β, HIF-1α, ET-1 (Fig. 45.7a–e), as well

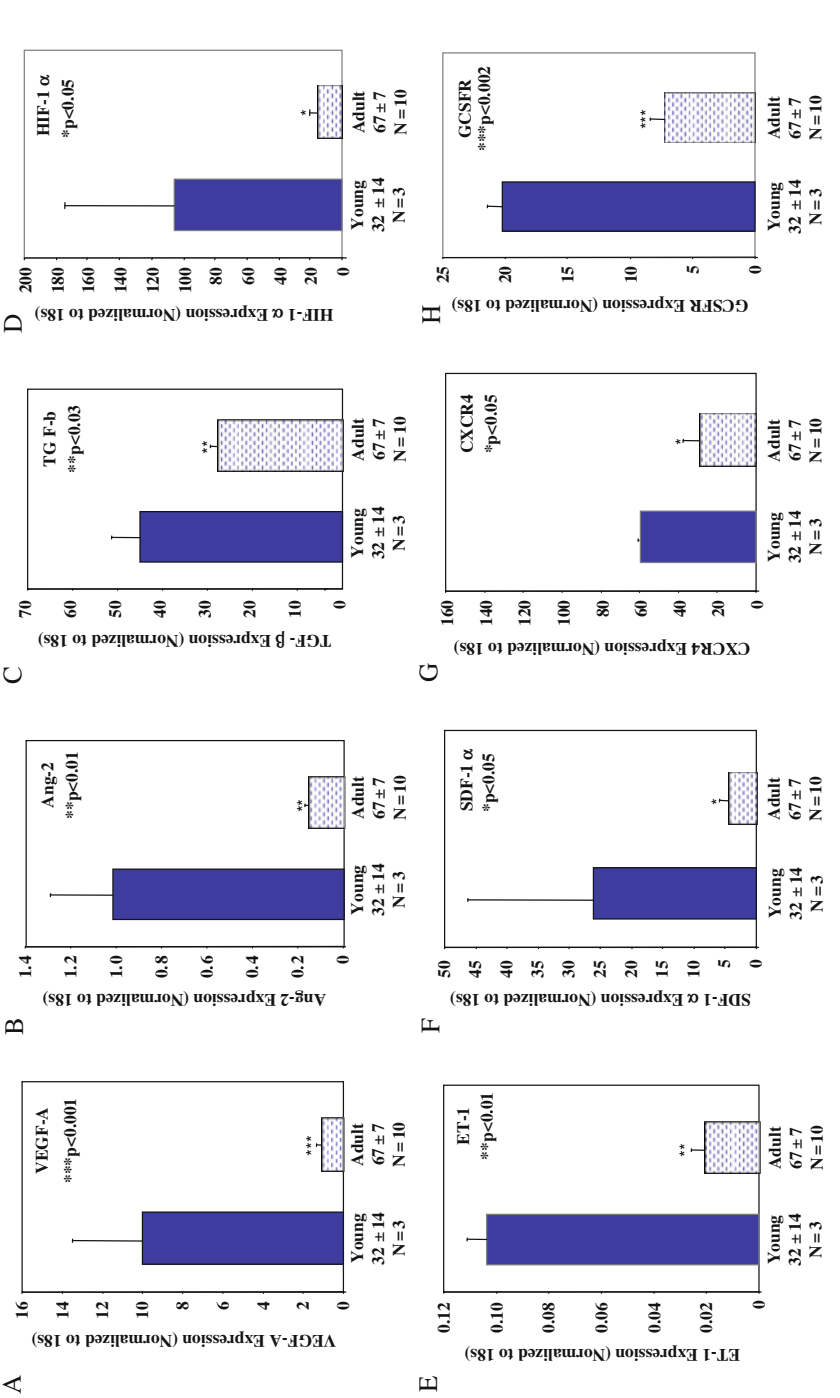

Fig. 45.7 Expression of several angiogenic growth and stem cell-derived factors and their receptors is decreased in peripheral blood EPCs from adult donors. qRT-PCR of PB EPCs from young normal volunteers vs. adult CAT lab patients. (**a–h**) Expression of several angiogenic growth and stromal cell factors and their receptors was decreased in PB EPCs obtained from adult patients. All experiments were conducted in triplicate. The differences were considered significant when $p<0.05$

as expression of -stromal cell-derived factors and their receptors, such as SDF-1α, CXCR4, and GCSFR (Fig. 45.7f–h) was significantly decreased (2–5-fold) in EPCs from adult patients when compared to the levels of these factors in the PB of normal volunteers. The significance of these finding may or may not be directly related or dependent on signaling through TNFR2/p75 pathway; we strongly believe that additional studies will be needed to elucidate the underlying molecular mechanisms through TNFR2/p75 that may affect expression of these genes.

Several major conclusions arise as a result of our studies: (1) expression of p75/TNFR2 is decreased in EPC from elderly donors; (2) loss of p75 expression impairs post-ischemic recovery in infarct myocardium and increases post-AMI mortality; (3) signaling via p55 TNFR1 is harmful for ischemia-induced repair and regeneration processes and absence of p55 receptor averts post-AMI mortality; (4) development of ischemia-induced functional collateral vessel network is mediated, at least in part, via p75 TNFR2; (5) with advanced age, signaling through p75 is required for collateral vessel development; (6) post-ischemic apoptotic responses in infarct myocardium are augmented in the absence of p75 TNFR2; (7) expression of several angiogenic growth and stromal cell-derived factors and their receptors is decreased in PB EPCs from adult donors.

Summary

Our data strongly suggest a critical role of TNF signaling via its receptors p55 and p75 in the processes of post-ischemic recovery in adult tissue after myocardial infarction, that is, signaling via TNFR1/p55 is *deleterious* and signaling via TNFR2/p75 is *protective* in repair and regeneration processes in adult infarct myocardium. As illustrated in Diagram 45.1a and b if occlusion of coronary artery is not resolved fast enough to prevent development of myocardial infarction a cascade of pathological events and morphological changes in the myocardium will develop beginning with necrosis, acute inflammation (heavy neutrophilic infiltrate), apoptosis, followed by macrophage and mononuclear infiltration to initiate fibrovascular response and granulation processes, which then may culminate in fibrosis and scar formation. As depicted in our hypothetical Diagram 45.1c we propose that therapeutic measures aimed toward either inhibition of signaling via TNFR1/p55 or overexpression of TNFR2/p75 in infarct myocardium may decrease ischemia-induced inflammatory responses, enhance neovascularization, and improve tissue regeneration, thereby minimizing myocardial fibrosis and improving the poor recovery and development of severe ischemia-induced damage in adult coronary diseases. Indeed, in support of our hypothesis several other research groups, independently of our group, had reported that myocardial injection of mesenchymal stem cells, genetically modified to overexpress TNFR2/p75, attenuates inflammation and cardiac dysfunction following myocardial infarction [49]. Another group had recently reported that signaling via both p55 and p75 is necessary to prevent reperfusion

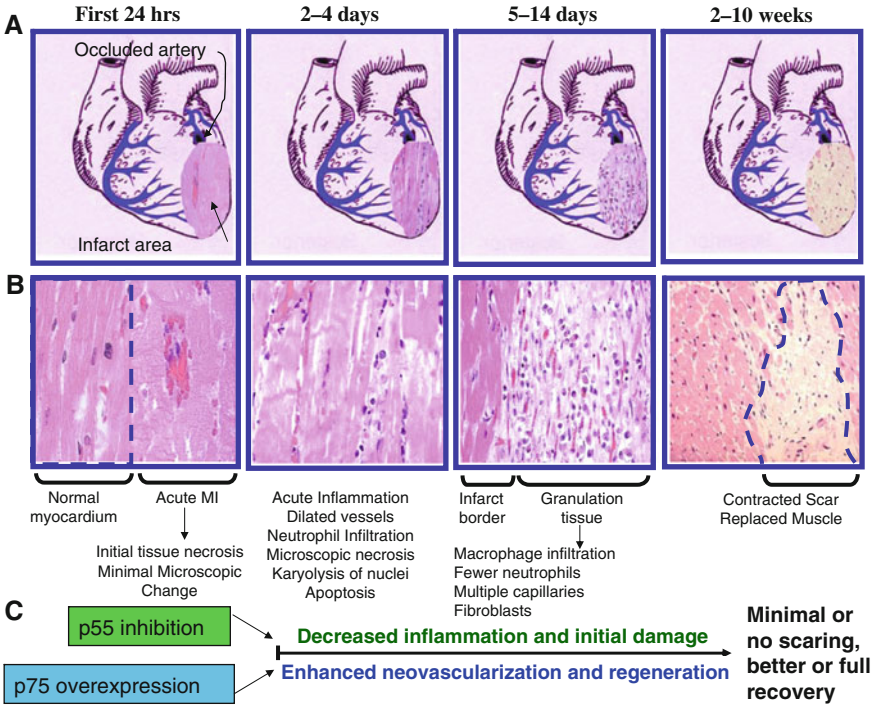

Diagram 45.1 (**a** and **b**) Microscopic morphologic changes that evolve over time after AMI. Evolution of microscopic morphologic changes after AMI: within first 24 h – myocardial fibers become wavy, edema, hemorrhage, coagulation necrosis, and early neutrophilic infiltrate develops; 2–4 days – loss of nuclei, microscopic necrosis, heavy neutrophilic infiltrate, and dilation of vessels develops; 5–14 days – macrophage and mononuclear infiltration and fibrovascular responses begin followed by prominent granulation; 2–10 days – fibrosis with scarring develops. (**c**) Hypothetical effect of modulation of TNF receptor expression on AMI recovery outcome. Inhibition of p55 receptor in first hours and days after AMI may decrease ischemia-induced acute inflammation and myocardial tissue damage, whereas p75 receptor overexpression within days and weeks after AMI may enhance collateral vessel development and myocardial regeneration

injury after AMI during late preconditioning; however, they found that only signaling through p75 is protective in non-preconditioned myocardium [50]. Because post-AMI recovery is an "evolutionary" process and inflammatory cytokine levels, specifically TNF, are significantly increased in infarct myocardium we strongly believe that further studies aimed toward modulation of TNF receptor p55 and/or p75 expression at different evolutionary stages of myocardial infarction may have important overarching implications for (1) prevention of AMI-mediated myocardial injury, (2) augmentation of myocardial repair and regeneration, and (3) improvement of therapeutic outcome of stem and progenitor cell therapy for cardiovascular diseases.

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Chapter 46
TNF-α Regulation of CD38 Expression in Human Airway Smooth Muscle: Role of MAP Kinases and NF-κB

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Introduction

CD38 is a type II transmembrane protein possessing multiple enzymatic activities. The receptor function of this 45 kDa glycoprotein has been studied extensively in hematopoietic cells. In non-hematopoietic cells, including airway smooth muscle (ASM) cells, CD38 has been investigated for its role as an enzyme involved in Ca^{2+} mobilization. The ADP-ribosyl cyclase activity of CD38 generates cyclic ADP-ribose (cADPR) from β-NAD [32]. The cADPR is known as a potent Ca^{2+} releasing agent in a variety of mammalian as well as invertebrate systems [13, 41, 50, 53]. In smooth muscle cells, a variety of cytokines induce the expression of CD38. The inflammatory cytokine TNF-α induces CD38 expression in myocytes from airways and myometrium [5, 12]. The mechanisms involved in the TNF-α regulation of CD38 expression are currently being investigated. Due to the multiple roles TNF-α plays in inflammatory disorders, it is seen as an attractive target for anti-inflammatory therapy. In this chapter, yet another signaling pathway downstream of TNF-α is described in the context of airway physiology.

CD38/cADPR Signaling Pathway

CD38 belongs to a superfamily of enzymes called ADP-ribosyl cyclases. CD38 and CD157 are the mammalian members of the enzyme superfamily (reviewed in [40, 51]). CD38 is expressed in a broad spectrum of mammalian tissues whereas the expression of CD157 is restricted to certain cells of hematopoietic origin (reviewed in [45]). There are three major enzymatic activities inherent in the members of ADP-ribosyl cyclase family. The ADP-ribosyl cyclase activity generates cADPR from NAD. The NAD-glycohydrolase activity generates ADPR from NAD, while

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cADPR hydrolase activity breaks down cADPR into ADPR [32, 41]. Although all the members of the superfamily possess these multiple enzymatic activities, the predominant enzymatic activity in each member varies. The ADP-ribosyl cyclase from the invertebrate *Aplysia californicum* (sea slug) generates predominantly cADPR from NAD, while the CD38 and CD157 generate mainly ADPR (~95%) with a marginal amount of cADPR (~5%) [32]. Recent investigations have revealed that this variability in predominant enzymatic activity is attributed to the critical amino acid residues found in the single catalytic pocket of the members [25, 26, 43]. Studies over the last several years have supported an important role for cADPR in Ca^{2+} release from endoplasmic reticulum (ER) in mammalian cells [30, 50, 53]. The cADPR-mediated Ca^{2+} release involves ryanodine receptor (RyR) channels in the ER, possibly involving the interaction of cADPR with FK506-binding protein (FKBP12.6) [53, 57]. Studies conducted in airway smooth muscle (ASM) cells isolated from various species confirmed that the CD38/cADPR signaling has a significant role in Ca^{2+} mobilization from the sarcoendoplasmic reticulum (SER), in response to contractile agonists [12, 36, 65]. There is evidence to support receptor subtype-specific recruitment of this signaling pathway in porcine ASM cells, although the nature of interaction between the agonist receptors and the CD38 is not clearly understood [65]. Recent evidence suggests a role for CD38 in store-operated calcium entry (SOCE) in primary cultures of HASM cells. The studies suggest that cADPR may be at least partially responsible for the CD38 regulation of SOCE in HASM cells [55]. As a result of the aforementioned studies, CD38/cADPR signaling pathway has emerged as a pivotal mechanism of cellular Ca^{2+} regulation in mammalian cells, including HASM cells. Since dynamic Ca^{2+} regulation in airway myocytes plays a crucial role in hypercontractility of ASM in airway disorders, the CD38/cADPR signaling is studied in the context of airway hyperresponsiveness (AHR). The pathologic significance of CD38 has already been reported in other mammalian systems. The most important pathological condition that brought CD38 to the limelight is chronic lymphocytic leukemia (CLL), in which elevated CD38 expression in lymphocytes is reported to be a negative prognostic marker [19]. However, the pathological role of CD38 as a molecule involved in Ca^{2+} regulation is in the non-insulin-dependent diabetes mellitus (NIDDM), where the deficiency of CD38 is thought to result in suppressed insulin secretion from the beta-cells [34, 46]. At least in one animal model, differential regulation of the CD38 enzymatic activities is known to play an important role in the pathogenesis of hypoxic pulmonary vasoconstriction (HPV) [17]. The potential role of CD38/cADPR pathway in the development of AHR has been studied in mouse models [14, 27, 28]. In cytokine-induced AHR models, CD38-deficient mice develop attenuated AHR compared to the wild-type mice [27, 28]. Tracheal rings isolated from CD38-deficient mice and exposed to TNF-α generate reduced isometric force in response to acetylcholine [27]. These findings suggest that suppressed AHR in CD38-deficient mice is largely attributable to the absence of CD38/cADPR signaling. The hypothesis that the CD38 has a potential role in the pathogenesis of airway inflammatory disorders, i.e., asthma, is also supported by in vitro studies conducted in primary HASM cells.

Regulation of CD38 Expression in ASM Cells

Cd38 Gene and Promoter

Located at chromosome 4, human *cd38* gene spans over 77 kb in size although a large part of the gene (~40 kb) is occupied by its first intron [47]. The first exon encodes the 5′untranslated region (5′UTR), N-terminal, and the transmembrane domain, while exons 2–8 encode the rest of the CD38 mRNA [47]. Alternate splicing leading to variant forms of CD38 mRNA has been reported in several tissues. The structure and function of *cd38* gene promoter have been studied by various investigators. Studies indicate that the classical promoter elements such as TATA box or CCAAT elements are absent in the *cd38* promoter region, indicating multiple transcription start sites in *cd38* gene [47, 56]. The *cis* regulatory elements of *cd38* gene are located both within and outside the 5′UTR. Analysis of a 3 kb putative fragment of *cd38* gene promoter revealed the presence of one NF-κB and six AP-1 response elements [61] (Fig. 46.1a). Electrophoretic mobility shift assays (EMSA) confirmed that nuclear proteins obtained from TNF-α-treated HASM cells bind directly to the putative NF-κB and AP-1 binding sites [61]. Mutagenesis studies showed that the NF-κB binding motif and some of the AP-1 binding sites are critical for the induction of *cd38* promoter by TNF-α [61] (Fig. 46.1b). The pivotal role of these two transcription factors in the pathogenesis of various inflammatory disorders has been well established [29, 48]. The finding that the binding motifs for NF-κB and AP-1 are critical for *cd38* promoter activity supports the pro-inflammatory role of CD38 in airway inflammatory disorders such as asthma. In addition to NF-κB and AP-1 binding motifs, other response elements have also been reported in the *cd38* gene promoter region. A *cis* element with particular importance to the pathophysiological role of CD38 in HASM cells is the glucocorticoid response element (GRE). Electrophoretic mobility shift experiments demonstrate that nuclear extracts from glucocorticoid-treated HASM cells show increased binding to some of the GREs, confirming the functional importance of these response elements [61]. Our earlier studies reported that TNF-α-induced CD38 expression is inhibited by the glucocorticoid dexamethasone in a concentration-dependent manner, possibly involving the GREs [38]. This is supported by the finding that promoter activation by TNF-α is ablated in cells treated with dexamethasone [61]. In addition to these classical response elements, binding motifs for additional *trans* elements, such as myo-D, GATA, and SP-1, have also been reported [56]. The non-coding first intron of the gene possesses regulatory elements with potential physiological significance. The retinoic acid response element (RARE) is located at the first intron of *cd38* gene [39]. Presence of a CpG island has been reported in a region spanning the first exon and first intron [23]. GC-rich CpG islands are potential targets of DNA methylation, which is a well-known epigenetic mechanism in the regulation of genes in response to environmental cues [52]. Presence of the CpG island in *cd38* gene along with several other response elements suggests that expression of this gene is regulated by a multitude of signals at various levels. The fact that some pro-asthmatic genes are

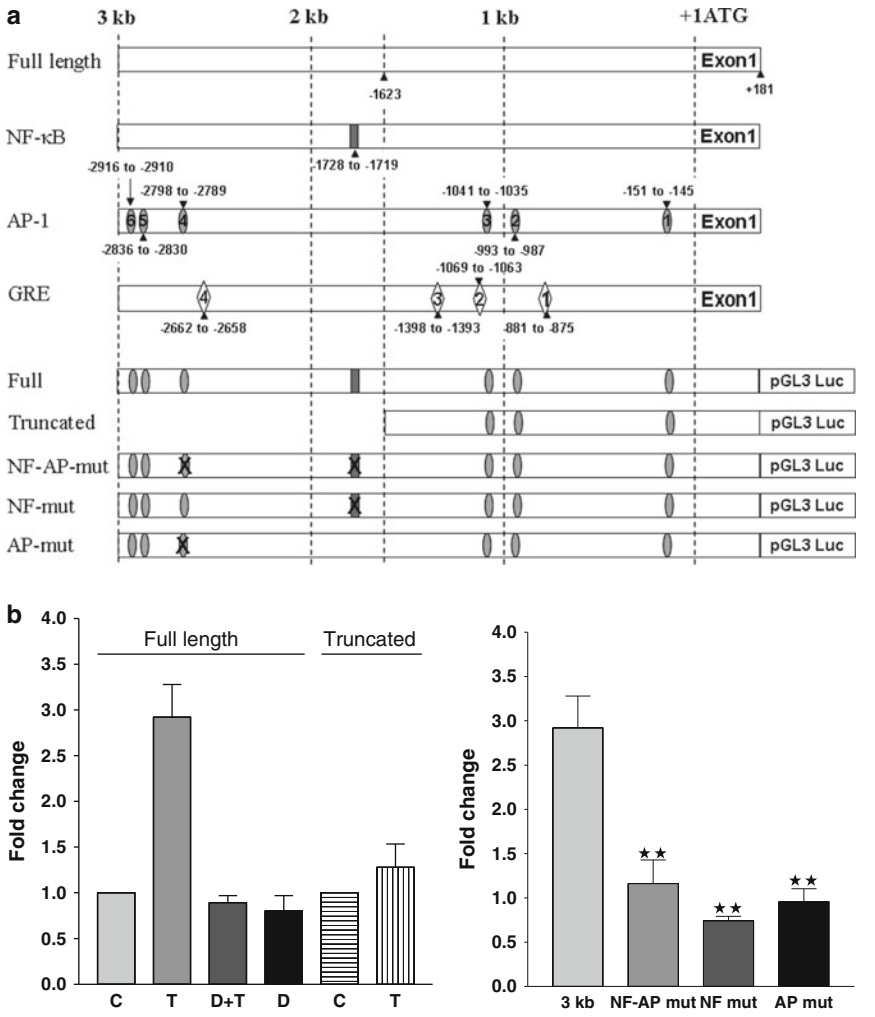

Fig. 46.1 Structure and function of *cd38* promoter. (**a**) The cloned 3-kb *cd38* promoter showing the location of the putative binding sites for NF-κB, AP-1, and GR (labeled NF-κB, AP-1, and GRE). Location of the putative binding sites for NF-κB, AP-1, and GRE on the full-length *cd38* promoter (full), the truncated 1.8 kb promoter (truncated), and the promoter constructs with mutations in the binding sites for NF-κB or AP-1-4 or both binding sites (NF-mut, AP-mut, and NF-AP-mut). The promoter was cloned in front of a luciferase reporter gene in the pGL3 plasmid and was used to transfect HASM cells. (**b**) Activation of the *cd38* promoter in the HASM cells. Luciferase activity was measured as an index of promoter activation with the Renilla luciferase activity (pRL-TK) to normalize for transfection efficiency. The normalized luciferase activity is expressed as the fold change compared to the control. *Left panel*: Activation of the full-length and truncated *cd38* promoters. TNF-α (T) causes activation of the 3-kb promoter as compared to control (C), which is inhibited in the presence of dexamethasone (D+T). There is no induction of truncated promoter by TNF-α. *Right panel*: TNF-α causes activation of the 3-kb promoter (3 kb), but not the constructs with mutations in NF-κB and/or AP-1 site 4 (NF-AP-mut, NF-mut, and AP-mut). Data are average of three independent experiments. Reprinted from Tirumurugaan et al. [60]

regulated through epigenetic mechanisms [1, 8, 31] indicates that CD38 also may be an important molecule with altered expression in airway inflammatory disorders like asthma.

Cytokine Regulation of CD38 Expression

The role of cytokines in CD38 expression in ASM cells has been investigated. In our previous studies involving HASM cells, we evaluated the effects of the Th2 cytokine IL-13 as well as the pro-inflammatory Th1 cytokines TNF-α, IL-1β, and IFN-γ on CD38 expression [11, 12]. HASM cells cultured under growth-arrested conditions exhibit very little constitutive expression of CD38. Following exposure to the cytokines, there is significant augmentation of CD38 expression in HASM cells [11, 12]. The induction of CD38 expression by cytokines is accompanied by elevated Ca^{2+} transients in response to contractile agonists [11, 12]. Antisense-mediated downregulation of CD38 expression in HASM cells attenuates the agonist-induced Ca^{2+} elevation, indicating that the cytokine-induced hypersensitivity in HASM cells is at least partially mediated through elevated CD38 expression [36]. The intracellular Ca^{2+} responses to contractile agonists following exposure to cytokines are also attenuated by 8-Br-cyclic ADP-ribose, a competitive inhibitor of cyclic ADP-ribose [11, 12]. The regulated expression of CD38 in HASM cells by inflammatory cytokines indicates a potential role for this gene in inflammatory diseases such as asthma. The potential role of CD38 in the development of AHR is further supported by studies in mouse models of asthma. Intranasal challenge of wild-type mice with TNF-α causes significant airway hyperresponsiveness with no evidence of significant airway inflammation [27]. However, in CD38 knockout mice, there is no evidence of airway hyperresponsiveness [27]. It is also of interest to note that intranasal IL-13 challenge causes airway inflammation and airway hyperresponsiveness in the wild-type, but not in the CD38 knockout, mice [28]. Both groups of mice develop significant airway inflammation following repeated intranasal IL-13 challenge [28]. These studies demonstrate a role for CD38 in the development of the asthmatic phenotype following cytokine challenge. Although TNF-α is traditionally thought to be a Th1-biased inflammatory cytokine, in contrast to asthma which is thought to arise from a Th2-biased inflammatory response, studies have provided support for the contention that TNF-α has a major role in asthma [22, 63, 65]. In this context, we have explored the mechanisms involved in the regulation of CD38 expression in HASM cells subjected to TNF-α exposure.

TNF-α and CD38 Expression

TNF-α

TNF-α is produced initially as a 25 kDa protein anchored to the cell membrane. The active 17 kDa TNF-α is generated by cleavage of the membrane-bound form by

the TNF-α-converting enzyme (TACE). The homotrimers of the biologically active TNF-α act on the TNF-α receptor subtypes 1 (TNFR1) and 2 (TNFR2) (reviewed in [54]). Among these two subtypes of receptors, TNFR1 is responsible for the pro-inflammatory functions of the cytokine [3, 24]. Secreted by macrophages, mast cells, and structural cells such as ASM cells, TNF-α acts on various cellular targets to induce the expression of an array of pro-inflammatory mediators [58]. Some important mediators induced by TNF-α are IL-8, eotaxin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) [58]. TNF-α mRNA and protein levels are elevated in the airways of patients with asthma [9, 65]. Administration of TNF-α to non-asthmatic individuals leads to airway hyperresponsiveness (AHR) and neutrophilia [59]. Genetic studies have reported polymorphisms in TNF-α gene that are associated with inflammatory diseases including asthma [2, 7, 42]. Human clinical trials involving TNF-α signaling modulators support the crucial role for TNF-α in the pathogenesis of severe asthma. Trials with anti-TNF-α molecules, etanercept (soluble TNFR) and infliximab (anti-TNF-α monoclonal antibody), have yielded favorable clinical outcomes in a subset of severe asthmatic patients [4, 6, 21, 22, 33].

TNF-α Signaling and MAP Kinases

TNF-α–mediated signaling in cells involves the activation of the MAP kinases and recruitment of several transcription factors including NF-κB and AP-1 [35, 62, 66]. NF-κB is known to regulate the expression of important pro-inflammatory genes and there have been suggestions that targeting NF-κB signaling may be useful in the management of asthma (reviewed in [20]). To this end, several small molecule inhibitors of NF-κB have been considered as potential therapeutic agents in asthma. In vitro studies in airway epithelial cell lines have demonstrated that small molecule inhibitors of I-κB kinase (IKK) inhibit the TNF-α-induced secretion of pro-inflammatory mediators by epithelial cells [49]. In experimental animal models of asthma, NF-κB and AP-1 oligonucleotides directly delivered into the lungs have been shown to inhibit airway hyperresponsiveness [15, 16]. In addition, inhibitors of the different MAP kinases have been shown to attenuate airway hyperresponsiveness in animal models. Ovalbumin-induced airway hyperresponsiveness is attenuated in guinea pigs following administration of a ERK MAP kinase inhibitor [10]. Similarly, aerosol-based administration of antisense oligos targeting p38α inhibits AHR and airway inflammation in an allergen-induced mouse model of asthma [18]. In humans, activation of ERK and p38 MAPKs is increased in asthmatic airways compared to non-asthmatic airways [44].

We have investigated the role of MAP kinases in the regulation of CD38 expression in response to TNF-α using the HASM cells as a model system. In ASM cells maintained under growth-arrested conditions, exposure to TNF-α causes transient activation of all three major MAP kinases [60]. Blocking the activation of the MAP kinases with selective inhibitors causes significant inhibition of TNF-α-induced CD38 expression (Fig. 46.2). This has been confirmed by recombinant expression

of dominant negative ERK and JNK MAP kinases as well [60]. Furthermore, inhibition of NF-κB through expression of dominant negative I-κBα also decreases TNF-α-induced CD38 expression in human ASM cells [38]. We have examined the mechanisms by which the MAP kinases regulate the expression of CD38 in human ASM cells. Our results support the conclusion that p38 and JNK MAP kinases have a large role in the transcriptional regulation of CD38 expression, while p38 and ERK MAP kinases regulate expression through transcript stability [60]. Presence of a regulatory mechanism involving the MAPKs and the transcription factors in CD38 expression is further confirmed by the glucocorticoid regulation of CD38 expression. Glucocorticoids are widely used as anti-inflammatory medication in asthmatics. Previous studies in our laboratory revealed that the TNF-α-induced CD38 expression in HASM cells is sensitive to glucocorticoid inhibition [38]. The glucocorticoid inhibition of CD38 expression in HASM cells is mediated through both transcriptional and post-transcriptional mechanisms [37, 38]. Some of the GREs found in the *cd38* promoter region are reported to be involved in the transcriptional regulation of CD38 expression [38]. RNA interference studies in HASM cells indicated a potential role for the dual specificity phosphatase-1 (DUSP-1) in mediating the glucocorticoid regulation of TNF-α-induced CD38 expression [37]. The DUSP-1-dependent mechanism attenuates the activation of MAPKs, thereby contributing to both transcriptional and post-transcriptional regulation of CD38 expression.

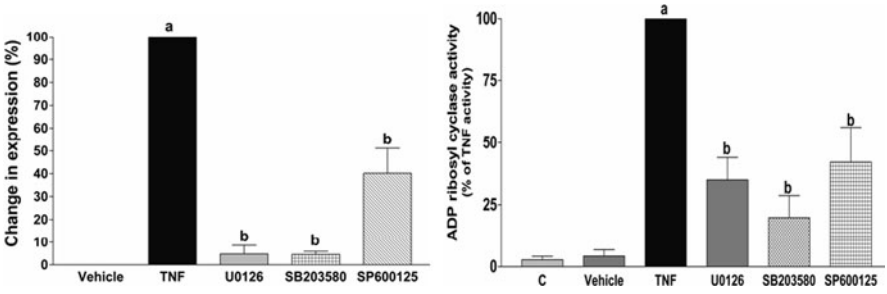

Fig. 46.2 Role of MAPK in CD38 expression in HASM cells. *Left panel*: TNF-α-induced CD38 mRNA expression in HASM cells exposed to pharmacological inhibitors of MAPK. CD38 mRNA expression is shown as the percentage of fold change of CD38 expression in cells exposed to TNF-α (TNF) for 24 h. Exposure to TNF-α in the presence of inhibitors of ERK (U0126), p38 (SB203580), or JNK (SP600125) MAPKs significantly attenuated the CD38 mRNA expression in HASM cells. Results are the average of six to eight independent experiments. *Right panel*: TNF-α-induced ADP-ribosyl cyclase activity in HASM cells exposed to the pharmacological inhibitors of MAPKs. ADP-ribosyl cyclase activity is shown as the percentage of cells treated with TNF-α (TNF) for 24 h. The TNF-α-induced ADP-ribosyl cyclase activity is inhibited in the presence of inhibitors of ERK, p38, and JNK MAPKs. Results are the average of four to seven independent experiments. (**a**) Significant increase ($p<0.05$) in expression compared to the control/vehicle-treated cells; (**b**) significant decrease ($p<0.05$) compared to the expression in TNF-α-treated cells. Reprinted with permission from Tirumurugaan et al. [60]

Concluding Remarks

CD38/cADPR signaling pathway has been implicated to have a potential role in the pathophysiology of many human disorders, including asthma. The inflammatory cytokine TNF-α augments the expression of CD38 in HASM cells through transcriptional and post-transcriptional regulatory mechanisms and this augmentation involves the activation of NF-κB and AP-1 and the major MAPKs. It is evident that CD38/cADPR signaling pathway is one of the downstream effectors through which TNF-α exerts its pro-inflammatory effects in ASM cells. Although the TNF-α receptors and the downstream mediators, such as transcription factors and protein kinases, are proposed as attractive therapeutic targets in airway inflammatory disorders, targeting these molecules currently presents many challenges. In this context, a downstream effector like CD38 may be considered a potential therapeutic target with minimal adverse effects on the parallel signaling pathways driven by TNF-α signaling. Although in vitro and animal studies have shown promise of CD38/cADPR pathway as a therapeutic target in AHR, the pathophysiological importance of these findings is yet to be established through studies in HASM cells isolated from asthmatic airways.

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Chapter 47
Functional Consequences of Disease-Associated Mutations in TNFR1 Elucidated by Transcriptome Analysis

Susana L. Rebelo, Paul M. Radford, Susan E. Bainbridge, Ian Todd, and Patrick J. Tighe

Introduction

Autosomal dominant mutations in the *TNFRSF1A* gene, which encodes the TNF receptor 1 (TNFR1), cause the autoinflammatory disorder tumour necrosis factor receptor-associated periodic syndrome (TRAPS; OMIM no. 142680) [1].

TRAPS is a key example of a group of hereditary periodic fever diseases and related autoinflammatory disorders characterised by chronic systemic inflammation without high titre autoantibodies or antigen-specific T cells that, as a whole, exhibit dysfunctions of innate immunity [2, 3].

Even though mutations in the ectodomain of TNFR1 have been recognised as the genetic defect underlying TRAPS since 1999 [1], the exact mechanism by which the periodic fevers are triggered is yet to be elucidated. Over the last decade several studies have tried to elucidate how mutant TNFR1 contributes to the inflammatory profile observed in TRAPS patients. This has proved both challenging and controversial. The inherent complexity of the TNFR1 signalling pathways and use of different cell types and assays in the various studies reported has contributed to some of the disparity in results.

This review looks at the current understanding of how mutant TNFR1 contributes to TRAPS pathogenesis and highlights some of our recent data investigating how mutant TNFR1 affects global gene expression in an epithelial and endothelial cell model.

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TNFR1 Mutations and TRAPS

More than 90 different variants of the *TNFRSF1A* gene have been reported (http://fmf.igh.cnrs.fr/infevers/) [4], around 60% of which are associated with TRAPS. Mutations affect the ectodomain of the mature TNFR1 protein (Fig. 47.1) and influence the structural integrity of the receptor resulting in protein misfolding [5–9]. Different mutations are associated with different phenotypes resulting in a highly heterogeneous clinical spectrum between patients [10, 11].

The majority of TRAPS-associated mutations are point mutations that result in single amino acid substitutions in cysteine-rich domain 1 (CRD1) and CRD2 of the ectodomain of the mature protein (Fig. 47.1). About half of these mutations involve

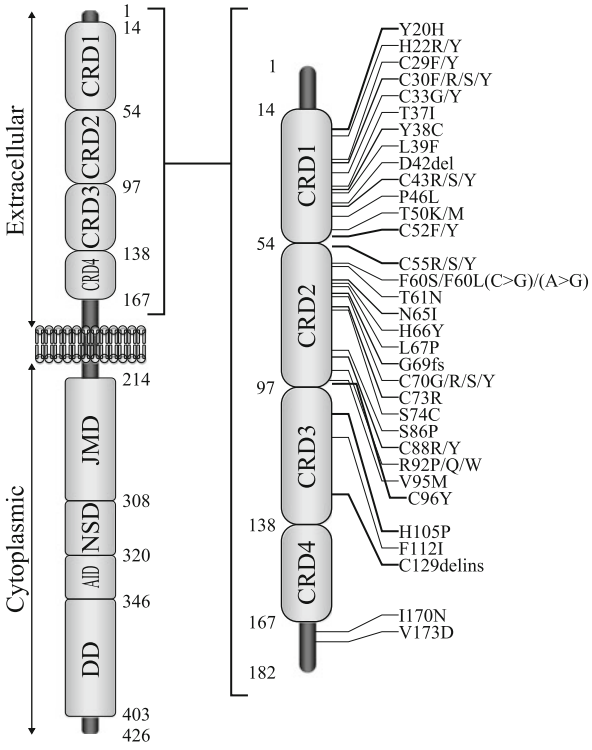

Fig. 47.1 Schematic representation of TNFR1 and ectodomain mutations associated with TRAPS. *Left*, model of TNFR1 domains highlighting the extracellular cysteine-rich domains (CRD)1–4 where TRAPS-associated mutations occur and the main cytoplasmic signalling domains of the receptor. The death domain (DD) is responsible for the recruitment of several interacting proteins and orchestrates the majority of signalling pathways engaged by TNFR1. The juxtamembrane domain (JMD), apoptosis signal-regulating kinase 1 interacting protein domain (AID) and the neutral sphingomyelinase domain (NSD) also contribute to signalling by serving as binding sites for several signalling regulators. *Right*, model of TNFR1 ectodomain highlighting the clustering of TRAPS-associated mutations in CRD1 and 2. List compiled from information contained in the INFEVERS database [4], available at http://fmf/igh/cnrs.fr/ISSAID/infevers/ (accessed 12.10.09)

substitutions of the cysteine residues that form disulphide bonds. These cysteine substitutions have a higher structural impact on the mature TNFR1 protein and are associated with a more severe phenotype and higher risk of developing amyloidosis [11]. In addition to the fully penetrant mutations, two polymorphic variants, R92Q and P46L, have been described as low-penetrance mutants that are associated with a milder and less characteristic phenotype [12].

Clinical Features

Diagnostic indicators of TRAPS include periodic bouts of inflammatory symptoms, often lasting longer than 5 days, that include fever, chest and abdominal pain, myalgia with migratory erythematous macular rashes, conjunctivitis and periorbital oedema, arthralgia or monoarticular synovitis and, in about 15% of patients, amyloidosis [11, 13]. The clinical spectrum observed is, however, much more heterogeneous. Cardiovascular [14–16], central nervous system [12, 17–19] and renal involvement [20] have all been described. Also, several inflammatory markers are raised in patients either during clinical attacks or constitutively. These markers include C-reactive protein, the erythrocyte sedimentation rate, interleukin-6 (IL-6) and IL-8 [21]. It is still not clear which inflammatory markers are raised as an immediate consequence of the effects of mutant TNFR1 and which are a secondary consequence of the inflammatory status of the patients [22].

Current therapeutic strategies target inflammation by blocking either TNF with etanercept [20, 21, 23–25] or IL-1 with anakinra [26, 27]. Disease response, however, varies widely between patients; adverse reactions and non-response to treatment have both been reported [28–31].

Dissecting the Molecular Pathogenesis of TRAPS

The original observations that patient cells showed increased cell surface expression of TNFR1 and exhibited a shedding defect upon stimulation led to the proposal of defective TNF neutralisation as a mechanism for TRAPS (the shedding hypothesis) [1]. Subsequent studies revealed that shedding is both mutation and cell-type specific [6, 10], thus implicating other mechanisms in disease pathogenesis.

Several studies have since identified additional pathogenic mechanisms in TRAPS. Our studies with transfected cells demonstrated that mutant forms of TNFR1 have reduced ability to bind TNF and are retained inside the cell (mainly in the endoplasmic reticulum) whilst maintaining signalling functions [5, 6, 8]. Others have since reported similar findings [9, 32].

Our molecular modelling of mutant TNFR1 suggested that mutant receptors are misfolded [8], which we have since confirmed in patient cells by demonstrating that mutants are antigenically altered [7]. Based on these findings, we and other investigators proposed that protein misfolding leads to ligand-independent receptor

aggregation and signalling as a fundamental pathological mechanism in TRAPS [5, 9, 33].

Alterations in cellular activation [34], apoptosis [33, 35] and the NF-κB pathway [36–38] in patient cells, with mutation-specific effects, have also been reported.

We propose that ligand-independent signalling by mutant TNFR1 contributes to the chronic inflammation observed in TRAPS patients by altering the balance of TNFR1 signalling pathways in the cell (Fig. 47.2).

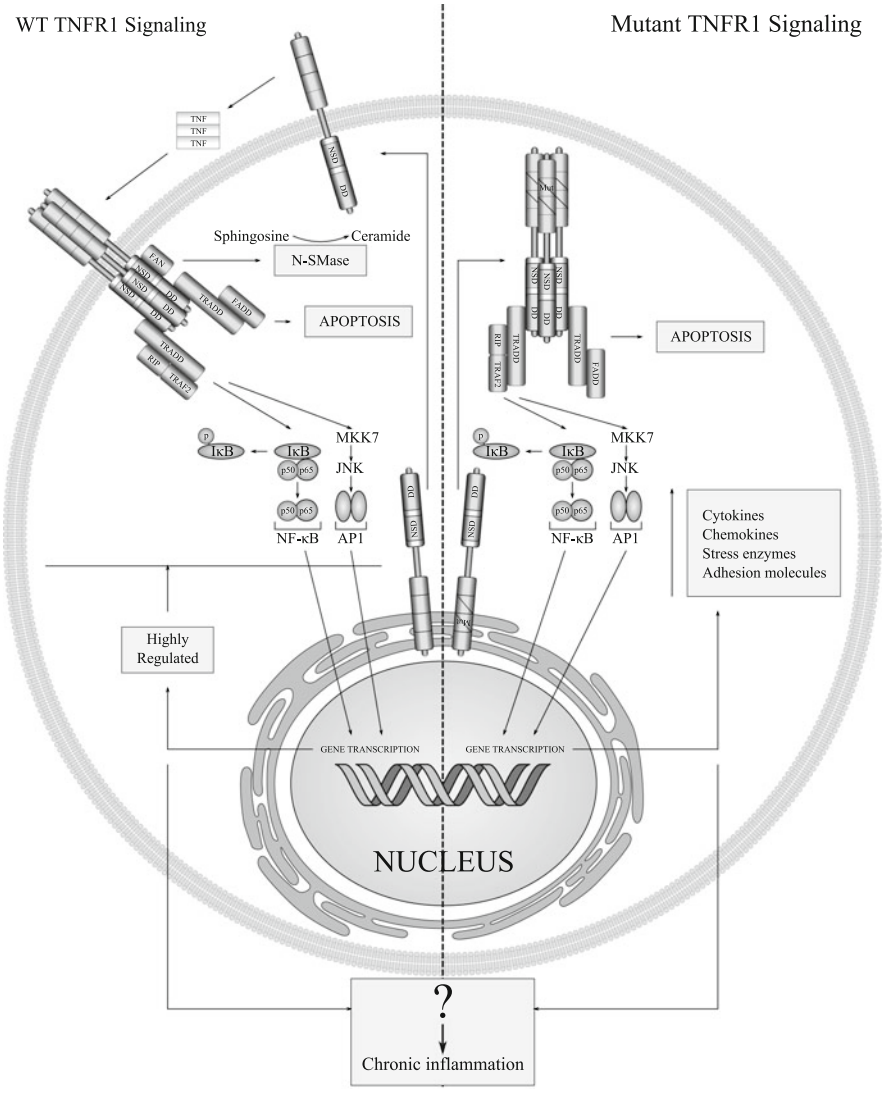

Fig. 47.2 (continued)

Altered Gene Expression Profile in Cells Expressing TNFR1 TRAPS Mutants

To investigate the extent of signalling pathways affected by mutant TNFR1 expression we investigated how TNFR1 influences global gene expression in SK-Hep-1 endothelial cells and HEK293 epithelial cells transfected with TRAPS-associated mutant or WT TNFR1. We also investigated whether the induced alterations differed between mutants and cell types and whether signalling by the TNFR1 death domain (DD) was involved.

Tables 47.1 and 47.2 summarise the genes that are most significantly differentially modulated by mutant TNFR1 expression in comparison to WT TNFR1 in transfected SK-Hep-1 and HEK293 cells, respectively.

Both cell type-specific and mutant-specific profiles were identified. In endothelial cells, an inflammatory gene expression profile associated with a deregulation in the cytokine network and related signalling pathways was identified (Table 47.1) [22], whilst in HEK293 cells a more general cellular stress response expression profile was revealed (Table 47.2) (S. Rebelo et al., manuscript in preparation).

The expression of most genes was induced by a death domain-dependent mechanism since they were not induced by expression of TNFR1 mutants with an inactivated death domain [22], thus supporting our hypothesis for ligand-independent activation of signalling by mutant TNFR1.

We have verified the expression of some of the novel markers identified in our gene expression profiling in patient cells [22]. Further validation work is currently underway. The various gene profiles identified highlight the variety of

Fig. 47.2 Model for altered balance in the TNF/TNFR1 regulated signalling pathways leading to the chronic inflammatory profile observed in TRAPS patients. WT TNFR1 signalling: basic schematic representation of signalling pathways (only main pathways are shown to reduce complexity). Upon TNFR1 engagement by TNF at the cell surface, the intracellular domain of TNFR1 through adaptor recruitment to specific subdomains is able to activate several downstream signalling pathways. These include neutral-sphingomyelinases (N-SMases), NF-κB, mitogen-activated protein kinases (MAPK) (e.g. JNK) and apoptotic pathways. The balance of signalling through these different pathways dictates cell fate, either apoptosis or cell survival, alterations in gene expression and pro-inflammatory consequences. In the absence of disease, these signalling pathways are tightly regulated by several cellular checkpoints and negative feedback loops that shut down constitutive signalling via the WT TNFR1 receptor. Mutant TNFR1 signalling: TRAPS-associated TNFR1 mutant misfolded proteins have impaired cellular trafficking and aggregate in the cytosol leading to ligand-independent pro-inflammatory signalling events. Constitutive upregulation of cytokines, chemokines, stress enzymes and adhesion molecules due to ligand-independent activation of TNFR1 signalling pathways by mutant receptor contributes to deregulated TNFR1 signalling in general. In patient cells, both WT and mutant receptor signalling pathways interact leading to an exaggerated inflammatory response lacking the normal negative feedback loops and resulting in chronic inflammation. FAN = neutral sphingomyelinase activation-associated factor; NSD = neutral sphingomyelinase domain, DD = death domain; RIP = receptor-interacting protein; TRAF2 = TNFR-associated factor 2; AP-1 = activator protein 1; Mut = mutant

Table 47. 1 Functional classification of selected genes modulated by mutant TNFR1 expression in transfected SK-Hep-1 cells. Values are the fold change of gene mRNA expression induced by each TNFR1 mutant in comparison with gene mRNA expression induced by WT TNFR1, identified by significance analysis of microarrays (SAM) with a false discovery rate (FDR) of zero

	TNFR1 mutant			
	C52F	C33Y	T50M	R92Q
Inflammatory molecules				
PTX3	10.2	7.2	7.9	5.2
CSF2/GM-CSF	8.9	7.1	9.9	8.6
CSF3/G-CSF	3.0	5.6	8.2	
CXCL1	4.5	5.3	5.7	
TNFSF7/(CD70)		2.5	2.9	
IL8	7.2	5		
CCL5/RANTES	4.1	4.3		
EBI3	2.1	2.1		
CCL2/(MCP1)				5.5
LTβ/(TNFSF3)				2.5
IL24		3.1		
CD58		2.3		
ICAM1		3.4		
Cellular receptors				
STAB2	2.5	2.1	3.4	1.8
SLC5A6	4.2	4.5	2.9	
INHBA	4.1	2.5		
CASH	3.5	2.4		
CD40			2.7	
Transcription factors and regulators				
NFKBIA	8.6	4.4	7.4	5.5
HSal2	2.4		2.3	2.0
NFKB1 p105	3.2	2.5	3.0	
NFKBIE		2.3	2.9	
NFKBIZ	3.3	3.2		
HHEX	2.6	2.8		
NRG1	2.7	2.0		
P38IP				2.0
ZNF267		2.6		
ELL2		2.3		
Apoptosis and cell cycle regulators				
BCL2A1	13	7.3	16.7	13.6
BID	1.7	1.6	2.5	1.9
SOD2	6.9	3.5	6.4	
G0S2	2.9	3.1	3.2	
MCTS1	2.1			1.9

Table 47.2 Functional classification of selected genes modulated by mutant TNFR1 expression in transfected HEK293 cells. Values are the fold change of gene mRNA expression induced by each TNFR1 mutant in comparison with gene mRNA expression induced by WT TNFR1, identified by SAM with a false discovery rate (FDR) of zero

	TNFR1 mutant			
	C52F	C33Y	T50M	R92Q
Stress enzymes				
SCCPDH (oxidoreductase)	6.4	6.4	7.8	7.2
SRD5A3 (oxidoreductase)	3.4	2.8	2.5	2.1
PA1 (phosphoprotein)	2.2	2.1	2.1	
Cytoskeleton and cell adhesion				
Keratin 19		15.8	17.2	17.0
Laminin, alpha 4	2.4	2.3	2.0	
Immune response				
DOM3Z	2.9	2.6	2.8	2.6
Beta-2-microglobulin	2.1	2.2	2.1	1.6
Transcription factors and regulators				
Zinc ribbon domain containing 1	1.9	2.0	2.2	2.2
Zinc finger protein 607	1.8	1.4	1.8	1.8
Nucleolar protein 66	1.5	1.9	2.0	
Methyl-CpG binding domain protein 2		0.3	0.3	0.2
Apoptosis and cell cycle regulators				
Catenin, beta like 1	1.5	1.7	1.9	1.8
PDGFA associated protein 1	2.7	4.5	3.1	2.4
Growth arrest-specific 2 like 3		2.0	1.9	1.8

signalling pathways that can be modulated by mutant TNFR1 receptor expression and emphasise the need for more physiologic models.

Also, since TNFR1 is ubiquitously expressed and patient cells have both a copy of WT and mutant TNFR, it is likely that the interplay between the signalling pathways engaged by WT and mutant receptors contributes to the autoinflammatory phenotype observed in patients [3] (Fig. 47.2).

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Chapter 48
Tumor Necrosis Factor-α and Kidney Function: Experimental Findings in Mice

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TNF-α is a pro-inflammatory cytokine that belongs to a family of both soluble and cell-bound cytokines which has a wide range of functions such as inflammation, lymphoid development, and apoptosis [10, 11, 29]. TNF-α is produced predominantly by white blood cells (monocytes and macrophages); however, it can be generated by endothelial cells; and, in the kidney, by mesangial and tubular epithelial cells, particularly upon inflammation and tissue injury [3, 7, 25]. Renal injury induced by angiotensin II (AngII) is mediated by a range of cytokines including TNF-α. AngII increases TNF-α production in mesangial, renal epithelial cells, and in mammalian heart [12, 17, 18]. Inhibition of angiotensin-converting enzyme (ACE) or AngII receptor type 1 (AT1) has been shown to attenuate tubulointerstitial injury and reduce expression of cytokines and matrix proteins [6, 25]. A relation between the renin–angiotensin–aldosterone system and the production of a pro-inflammatory cytokine, TNF-α, and its potential role in regulating cardiovascular function is increasingly evident from findings in many recent studies [8, 9, 13, 14].

Hypertension is considered as a low-grade inflammatory condition induced by the presence of various pro-inflammatory cytokines, including TNF-α. Recent studies have implicated an involvement of TNF-α in the development of AngII-dependent hypertension [16] as well as salt-sensitive hypertension induced by AngII [8, 9, 28]. It has been demonstrated that T cells are activated to increase the production of TNF-α in Ang II-induced hypertension [16]. AngII infusion failed to induce hypertensive responses in mice lacking the genes for TNF-α [28] or T-lymphocytes [16]. Renal vasoconstrictor response to acute AngII administration was also shown attenuated in TNF-α knockout mice [19]. TNF-α level in the plasma is also shown to increase in many other forms of hypertension including Dahl salt-sensitive hypertension [15], nitric oxide (NO)-deficient form of hypertension [4] as well as in DOCA salt-induced hypertension [8]. It is also reported that AngII-induced TNF-α production is associated with activation of NADPH oxidase and upregulation of NOS [21, 28, 30] and such inductions

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of oxidative and nitrosative stress are involved in the development of AngII-dependent hypertension [28]. Recent preliminary studies in our laboratory also showed that AngII induced increases in urinary excretion rate of nitrate/nitrite (NO metabolites) and 8-isoprostane (oxidative stress marker) is markedly attenuated in TNF-α knockout mice [19]. Collectively, all these studies suggest that TNF-α plays a key role in the pathogenesis of hypertension and cardiovascular disease, and targeting this pro-inflammatory cytokine may be a valuable strategy for lowering the incidence of hypertension and subsequent cardiovascular complications. However, the functional importance of TNF-α in regulating the hemodynamic and excretory function of kidney, an organ which is involved in the pathogenesis of essential hypertension, is not clearly defined until recently.

Direct Effects of TNF-α on Renal Hemodynamics and Excretory Function

In a recent experiments in anesthetized mice [27], we have examined the renal responses to incremental doses of TNF-α (0.1, 0.3, and 0.5 ng/g/min) infused intravenously for 75 min at each dose. Renal blood flow (RBF) and glomerular filtration rate (GFR) were determined by PAH and inulin clearance, respectively. The urine sample was collected from a cannula inserted into the bladder. Infusion of a low dose (0.1 ng/g/min), TNF-α did not cause any significant change in systemic arterial pressure (SAP) or renal vascular resistance (RVR). At this low dose, TNF-α did not also affect RBF, urine flow (V), or sodium excretion ($U_{Na}V$), but showed a decrease in GFR (−22%) as compared to basal values. However, TNF-α caused dose-dependent decreases in RBF (−11 and −23%) and in GFR (−30 and −41%) at higher doses (0.3 and 0.5 ng/g/min), respectively. There was no change in SAP or RVR during low-dose infusion, but high-dose infusion of TNF-α did cause significant reductions in SAP. Interestingly, there were significant increases in V (103 and 120%) and sodium excretion (142 and 204%) though there were reductions in RBF and GFR. These renal responses to TNF-α were abolished in mice pretreated with TNF-α blocker etanercept, confirming the specificity of these direct effects of TNF-α in the kidney. Etanercept (Immunex, CA), a TNF-α blocker, is a fusion protein which binds with circulating TNF-α and prevents its' action on the cellular TNF-α receptors [26]. Etanercept was administered (5 mg/kg, s.c.) in conscious mice 1 day prior to the experiment. A second dose was also given to the anesthetized mice 3 h prior to TNF-α infusion on the day of experiment [21, 22]. These findings demonstrate that TNF-α exerts vasoconstrictor action but causes diuresis and natriuresis in the kidney.

What Would Be the Mechanism (s) of These Renal Actions TNF-α?

It was reported that AngII treatment caused upregulation of the protein expressions of NAD(P)H oxidase enzyme subunit, $gp91^{phox}$ (Nox2), and its homologues Nox1

and Nox4 along with endothelial NO synthase (eNOS) enzymes in the myocardium [24, 28] as well as in aortic endothelial cells [30]. This upregulation of eNOS and NAD(P)H oxidases in response to AngII were absent in TNF-α knockout mice but reinstated when these knockout mice were treated a replacement therapy with human recombinant TNF-α [24, 28]. TNF-α knockout mouse also exhibited lower urinary nitrite/nitrate (NO metabolites) and 8-isoprostane excretion ($U_{Iso}V$; marker for endogenous superoxide activity) compared to that in corresponding wild-type strains though basal levels of renal hemodynamics and excretory parameters are same in both strains [19]. Collectively, these data suggest that TNF-α might produce its effects by regulating the activity of NADPH oxidase and NOS and thus influencing superoxide (O_2^-) and NO production. To determine the role of O_2^- production as well as its effects on NO bioavailability in the responses to TNF-α, we have recently conducted experiments in mice pretreated with a O_2^- scavenging agent, tempol, and with a NO synthase inhibitor, nitro-L-arginine methyl ester (L-NAME). Tempol was given at a rate of 2 μg/g/min starting 2 h prior to TNF-α infusion. L-NAME was given at a rate of 0.1 μg/g/min starting 2 h prior to TNF-α infusion. In these tempol and L-NAME pretreated mice, responses to a single dose of TNF-α (0.33 ng/g/min) were assessed on renal hemodynamics and excretory function. This single dose of TNF-α was selected from the findings with incremental doses of TNF-α [5, 24]. We considered this dose of TNF-α as physiologically relevant because this dose was lower than the dose that was used to induce cytokine-mediated cardiac dysfunction [5]. This dose did not alter systemic arterial pressure significantly and thus allowed us a reasonable assessment of the renal responses to TNF-α without any compounding arterial pressure-induced effects in the kidney. The responses to TNF were also assessed in mice pretreated with a TNF-α blocker, etanercept, as mentioned earlier.

Figure 48.1 illustrates the responses observed in these experiments in control and pretreated animals with tempol and L-NAME. TNF-α alone in control mice increased RVR and caused decreases in RBF and in GFR from the baseline value. However, these responses to TNF-α were completely abolished in etanercept-pretreated mice. In the animals treated with tempol, TNF-α decreased RVR and increased RBF from the baseline value. However, TNF-α also caused lesser degree of decreases (−21% vs −41%) in GFR in the animals treated with tempol compared to that in control animals. In L-NAME-treated animals, TNF-α infusion resulted in increases in RVR and decreases in RBF and GFR from the basal values.

Figure 48.2 illustrates the effects of TNF-α infusion on renal excretory function. TNF-α infusion alone in control mice caused marked increases in V, $U_{Na}V$ as well as fractional excretion of sodium (FE_{Na}) from their respective baseline values despite there were reductions in RBF and GFR. These effects of TNF-α were not observed when TNF-α was given to etanercept-pretreated mice. However, these diuretic and natriuretic responses to TNF-α persist also in mice pretreated with tempol or L-NAME. It was noted that the degree of TNF-α-induced increases in $U_{Na}V$ during tempol treatment was greater than that during TNF-α infusion alone. TNF-α infusion in control mice caused 38% increases in $U_{Iso}V$ from the baseline value, a response which was not observed when TNF-α was given to etanercept as well as tempol-pretreated mice. However, during L-NAME treatment, TNF-α caused

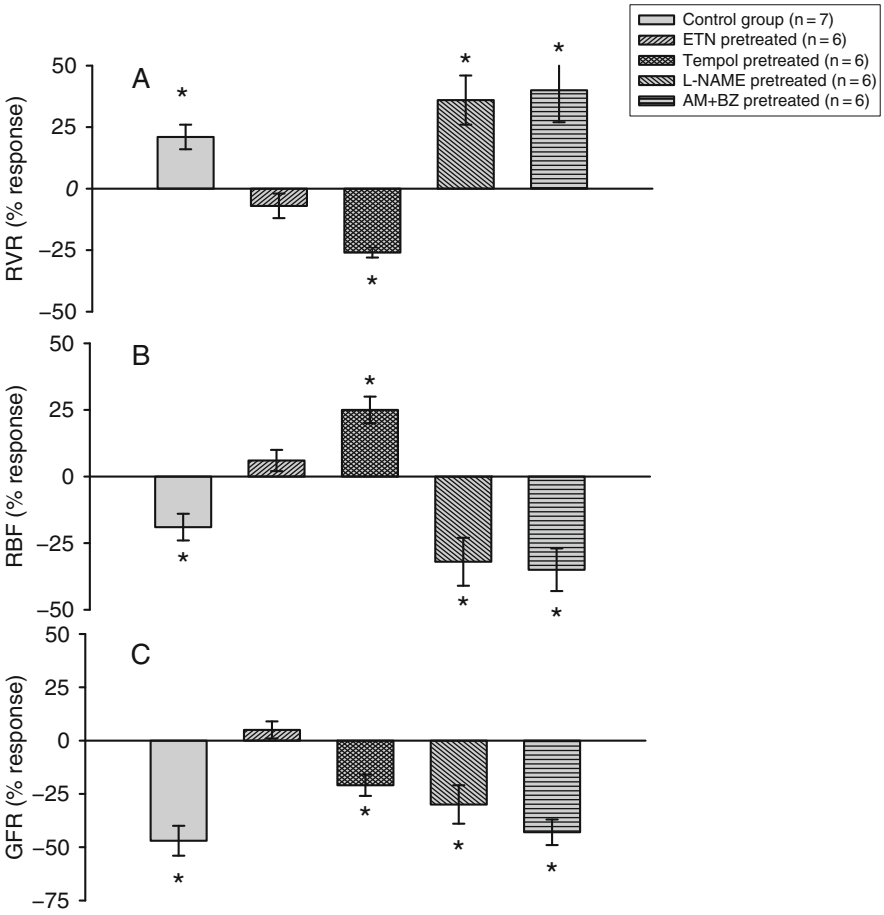

Fig. 48.1 Renal hemodynamic responses to TNF-α infusion. Percent (%) changes in renal vascular resistance (RVR; **a**), renal blood flow (RBF; **c**) and glomerular filtration rate (GFR; **c**) observed in different groups of mice are depicted in this figure. ETN, etanercept; L-NAME, nitro-L-arginine methyl ester. ∗, $P<0.05$ vs baseline values prior to TNF-α infusion

marked increases in $U_{Iso}V$ (53%) from the baseline value [27]. These data suggest that TNF-α induces renal vasoconstriction and hypofiltration via enhancing the activity of O_2^- and thus reducing the activity of NO. However, these findings could not explain the exact cause of these diuretic and natriuretic responses to TNF-α despite its vasoconstrictor and hypofiltration effects.

What Would Be the Mechanism of Natriuresis Induced by TNF-α?

From the findings of our experiments [27] described earlier, it seems clear that the diuretic and natriuretic responses to TNF-α were due to its direct influence on

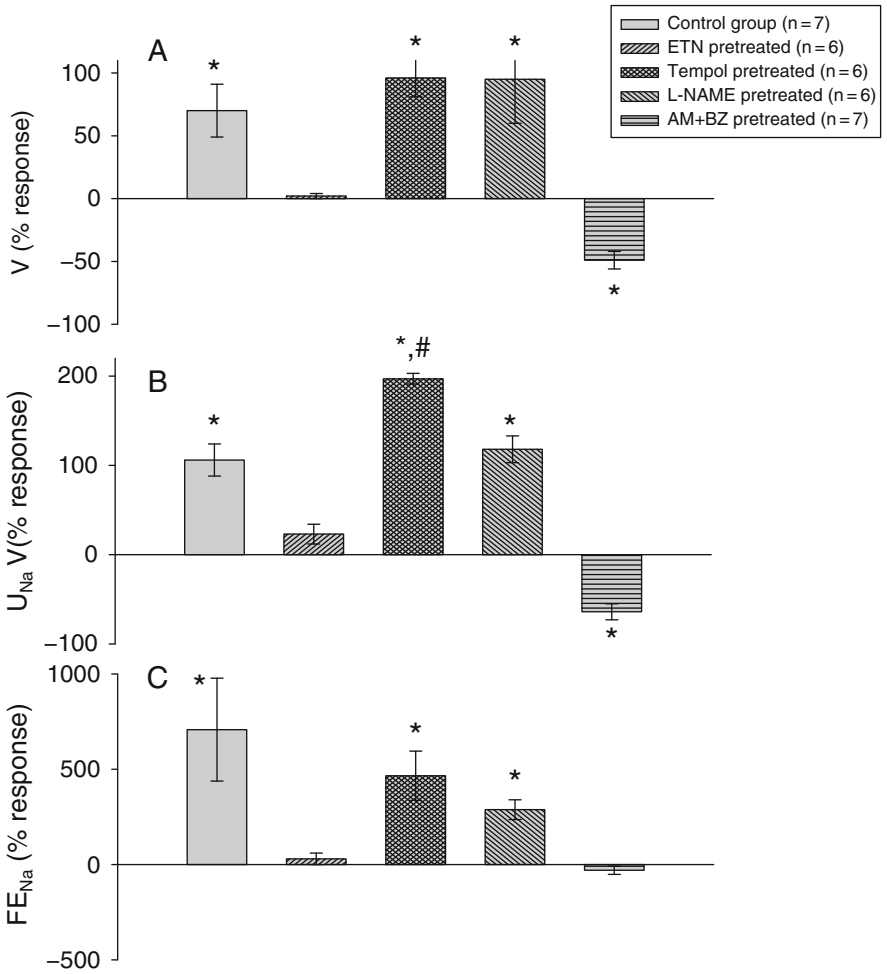

Fig. 48.2 Renal excretory responses to TNF-α infusion. Percent (%) changes in urine flow (V; **a**), sodium excretion ($U_{Na}V$; **b**) and fractional excretion of sodium (FE_{Na}; **c**) observed in different groups of mice are depicted in this figure. ETN, etanercept; L-NAME, nitro-L-arginine methyl ester. ∗, $P<0.05$ vs baseline values prior to TNF-α infusion. #, $P<0.5$ vs values obtained in control group

tubular sodium reabsorption since FE_{Na} increased during TNF-α infusion. It was reported in previous in vitro studies that TNF-α exerts direct inhibitory action on Na^+K^+ATPase, $Na^+K^+2Cl^-$ co-transporter [20], and the renal epithelial sodium channel (ENaC) activity which is mediated by ceramide and the PKC-dependent pathway [2]. Thus, it is conceivable that the natriuretic effect of TNF-α observed in in vivo preparations could be due to its direct inhibitory action on tubular sodium transporters. To examine the hypothesis that TNF-α-induced natriuresis is caused

by its direct inhibitory action on epithelial sodium transport, particularly in the distal tubular nephron segment, we further examined the renal responses to TNF-α infusion (0.33 ng/min/g; i.v.) in anesthetized mice pretreated with combined administration of amiloride (AM; 17 μg/kg/min; i.v.) and bendroflumethiazide (BZ; 2.8 μg/kg/min, i.v.) that would block distal tubular sodium transport [23]. These results are also illustrated in Figs. 48.1 and 48.2. TNF-α infusion in AM+BZ pretreated mice caused reductions of 35% in RBF and 43% in GFR which were similar to those observed in non-treated control mice in earlier investigation. However, contrary to the diuretic and natriuretic response observed in non-treated control mice, TNF-α in these AM+BZ pretreated mice resulted in a marked reductions in V (−49%) and $U_{Na}V$ (−64%) with no alteration in FE_{Na}. As FE_{Na} remained unchanged, the observed anti-natriuretic response to TNF-α in these AM+BZ pretreated mice was mainly related to hemodynamic changes but not due to its action on renal tubule. Thus, these data provide the evidence that TNF-α-induced natriuretic response results from its direct inhibitory action on distal tubular sodium transport.

Renal mRNA Expression of Oxidative and Nitrosative Enzymes in Response to TNF Infusion

As mentioned above that TNF-α produces its action by modulating the activity of NADPH oxidase and NOS [22, 30], we also measured mRNA levels of NADPH oxidase subunit gp91phox (Nox2) and its homolog Nox1 (oxidative enzymes) as well as different isoforms of NO synthase (NOS; nitrosative enzymes) in the kidney during infusion of TNF-α in different groups of animals pretreated with or without tempol or L-NAME. Figures 48.3 and 48.4 illustrate these findings for subunits of NADPH oxidase and NOS enzymes, respectively. We observed that TNF-α infusion (0.33 ng/g/min, iv for ~90 min) markedly increases the mRNA levels of Nox1 and Nox2 compared to untreated control animals (Fig. 48.3). This increase in Nox 1 but not Nox 2 expression in response to TNF-α infusion was attenuated in mice pretreated with TNF-α blocker, etanercept, as compared to the vehicle-treated animals. Nox 1 expression but not Nox 2 expression responses to TNF-α were also remained attenuated in the animals pretreated with tempol or L-NAME when compared to vehicle-treated animals.

TNF-α infusion also increases eNOS and iNOS mRNA expressions in the kidney (Fig. 48.4). The increased eNOS and iNOS mRNA expressions in response to TNF-α infusion were significantly attenuated in mice pretreated with TNF-α blocker etanercept. Interestingly, the mRNA levels of nNOS in the kidney were found to be reduced in response to TNF-α, in contrast to its effects on eNOS and iNOS mRNA levels. However, in etanercept-treated animals, TNF-α caused an increase nNOS mRNA expression indicating that TNF-α might exert a tonic suppression in the normal expression of this NOS isoform in the kidney. It was also reported earlier [1] that TNF-α-induced hypertension in pregnant rats was associated with a decreased renal nNOS activity, suggesting the opposing effect of nNOS against TNF-α-mediated

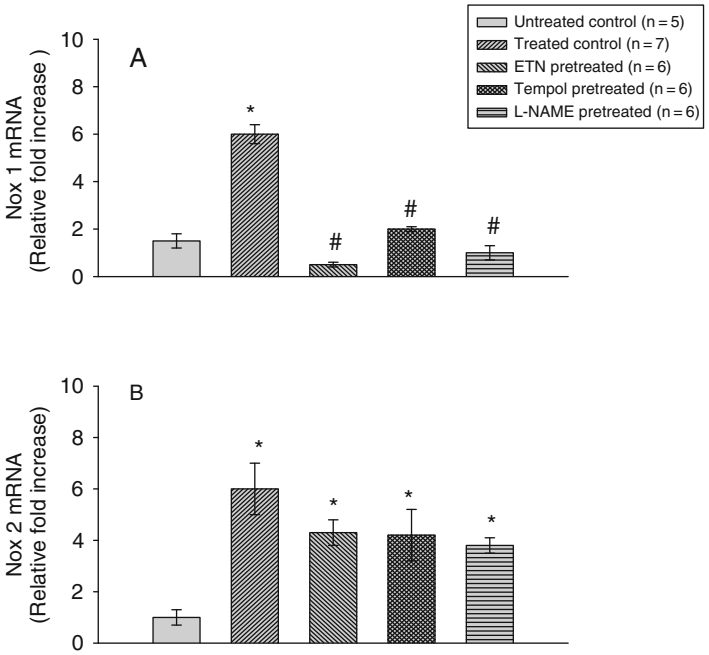

Fig. 48.3 Effects of TNF-α infusion on oxidative enzymes. Changes in mRNA expression of NADPH oxidase isoforms, Nox1 (**a**) and Nox2 (**b**), during TNF-α infusion in different groups of mice are depicted in this figure. ETN, etanercept; L-NAME, nitro-L-arginine methyl ester; AM, amiloride; BZ, bendroflumethiazide. ∗, $P<0.05$ vs values in untreated control group (not infused with TNF-α). #, $P<0.05$ vs values obtained in treated control group (infused with TNF-α)

action. TNF-α failed to increase these expressions in the animals treated with tempol or L-NAME.

Conclusion

The present data from our laboratory demonstrate that acute administration of TNF-α exerts renal vasoconstriction and hypofiltration but induces diuresis and natriuresis in the kidney. These findings suggest that an enhancement in O_2^- production and thus a reduction in NO bioavailability due to its interaction with O_2^-, mediate the renal vasoconstrictor action of TNF-α. The data also demonstrate the diuretic and natriuretic responses to TNF-α are due to its' direct inhibitory action on distal tubular epithelial sodium transport in the kidney.

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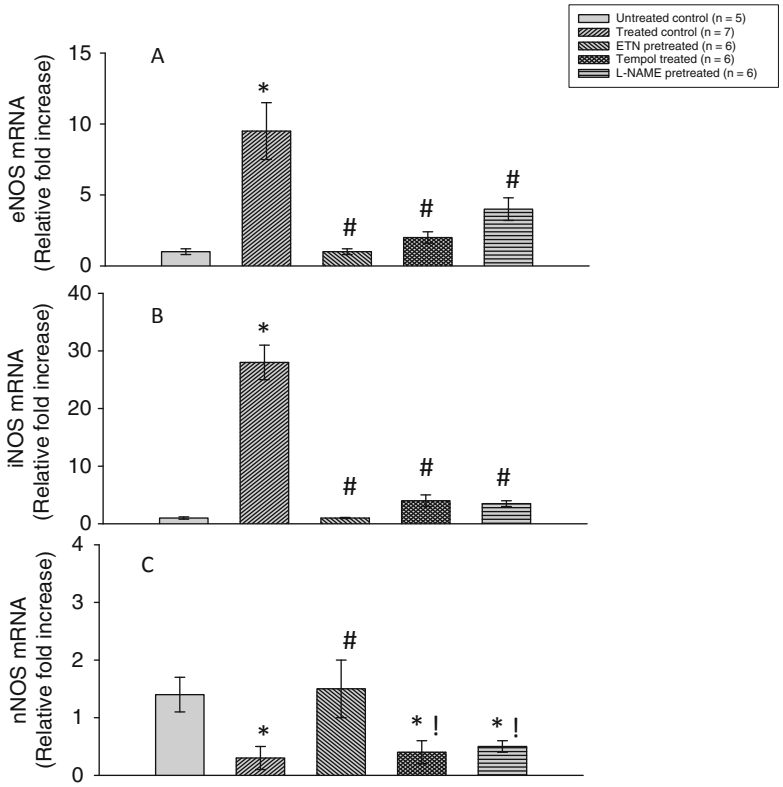

Fig. 48.4 Effects of TNF-α infusion on nitrosative enzymes. Changes in mRNA expression of NO synthase isoforms, eNOS (**a**), iNOS (**b**), and nNOS (**c**) during TNF-α infusion in different groups of mice are depicted in this figure. ∗, $P<0.05$ vs values in untreated control group (not infused with TNF-α). ETN, etanercept; L-NAME, nitro-L-arginine methyl ester; AM, amiloride; BZ, bendroflumethiazide. #, $P<0.05$ vs values obtained in treated control group (infused with TNF-α). !, $P<0.05$ vs values obtained in ETN-pretreated group

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Chapter 49
Strategies to Inhibit the Toxicity of Systemic TNF Treatment

Filip Van Hauwermeiren, Leen Puimège, Sofie Vandevyver, Tom Van Bogaert, Ineke Vanlaere, Liesbeth Huys, Lien Dejager, and Claude Libert

Introduction

Research in our unit has focused on several aspects of acute inflammatory shock induced by pro-inflammatory molecules, such as the cytokines TNF and IL-1, and the bacterial cell wall component, LPS. We have paid special attention to the study of TNF in mouse models for two reasons. First, TNF has a very potent anti-tumor effect, especially when combined with IFN-gamma (IFNγ), melphalan, or other chemotherapeutics. This anti-tumor activity is directed to the neovasculature of the tumor [1] and so it is applicable to all solid tumors regardless of their location, tissue type, or degree of progression. Second, TNF exerts very powerful pro-inflammatory effects. Indeed, administration of TNF to healthy volunteers, cancer patients, or experimental animals leads to systemic inflammation so serious that lethal shock can occur [2]. Chronically dysregulated TNF expression is also an essential step in the development of several diseases that impose a heavy social and economic burden, such as rheumatoid arthritis (RA), inflammatory bowel disease (IBD), and asthma [3]. The major aim of most of the unit's research has been the acquisition of novel insights into the endogenous mechanisms of protection against serious inflammation and their possible application to ameliorating the inflammation induced by TNF in disease and the potential development of TNF-based anti-cancer therapy. In this chapter we will discuss the three different approaches we have been using to discover relevant protective genes and molecules.

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Inhibition of Matrix Metalloproteinases

We have been studying the role of the family of matrix metalloproteinases (MMPs) in TNF-induced lethal shock. This family consists of almost 30 members. These proteases are centrally involved in several aspects of tumor progression ranging from tumorigenesis to invasion of tumors in the stroma and metastasis. However, it is now also clear that several MMPs play important roles in other important pathological and physiological processes, such as inflammation. Using a broad-spectrum MMP blocker and MMP-deficient mice, we found that inhibition of MMPs completely protected mice against TNF-induced toxicity and that the mediating molecules were in particular MMP2, MMP3, MMP8, and MMP9. We found that expression of these MMPs by macrophages and/or neutrophils caused these cells to move into the sinusoids of the liver and cause toxicity. Interestingly, the same broad-spectrum MMP blocker reduced the toxic effects of TNF/IFNg in anti-tumor studies without reducing their anti-tumor efficacy. This study has important implications in the MMP world as it was an elegant illustration that MMP inhibitors still have a future in cancer therapy, albeit as anti-inflammatory drugs rather than anti-cancer drugs [4, 5]. Follow-up studies have revealed that the natural MMP inhibitors, called TIMPs, may also inhibit the toxicity of TNF to some degree [6, 7].

Induction of HSP70

We have known for a long time that mice, which are sensitive to TNF, could be protected against its lethal effects by at least three different treatments: desensitization, tolerization, and administration of heavy metals. Desensitization, which is induced by a single injection of TNF, IL1, or LPS, provides a short period of protection against TNF lasting about 12 h [8]. Tolerance is better known in pharmacology and is induced by treatment of mice twice a day for 5 days with low doses of TNF. This treatment results in protection that lasts more than a week [9]. Finally, we showed that heavy metals can also protect against TNF. For example, treatment of mice for 1 week with zinc sulfate in the drinking water induces considerable protection [10]. By performing differential expression studies with microarrays and RNA isolated from livers of protected animals, we found that these animals expressed a typical set of genes, which we then studied in detail. One protective gene that was induced in all cases is the inducible *hsp70.1* gene. We found that whole-body heat shock of mice strongly induced HSP70 in different tissues and protected against TNF, but this was not the case in hsp70.1-knockout mice, which we generated in collaborative effort [11]. Induction of HSP70 in mice by heat shock (comparable with a sauna), by heavy metals (zinc sulfate in the food), or in HSP70 transgenic mice led to very significant protection against TNF/IFNg toxicities but without loss of the anti-cancer effect of TNF/IFNg in four different tumor models [12]. In conclusion, HSP70 has powerful effects in that its presence overcomes the effect of a lethal dose of TNF. So, HSP70 induction could enable safe anti-tumor therapy with TNF/IFNg.

Inhibition of Cytokines

Although we have been able to reduce the toxicity of TNF/IFNg in mouse models by using MMP inhibitors or HSP70 and at the same time maintain the anti-tumor activity of TNF/IFNg, we continued our search because neither MMP blockers nor HSP70 are suitable as therapeutic agents for humans. We believe that identifying cytokines that mediate the toxic effects of TNF is a top priority, because cytokines are usually produced in minute amounts and so they might be easier to target. As an example, we have been studying the role of IL-17 in the lethality of TNF. We clearly demonstrated that interleukin-17 and its receptor play an essential mediating role in TNF-induced lethal inflammatory shock. Antibodies against IL17 protect mice against TNF and IL17R-deficient mice are also protected. Most evident was protection against bowel necrosis. We also demonstrated that IL17 is induced by TNF locally in the Paneth cells of the small intestines. These cells, which are located in the crypts of the villi, share many characteristics with leukocytes, they are essential in innate host defense, and they specifically and strongly express IL17 after TNF induction. Expression of IL-17 by Paneth cells is a novel and very important finding that sheds light on the mechanism of innate immunity in the bowel and on the role of the mucosa in acute inflammation [13].

Conclusion

It has been known for a long time that TNF can display remarkable anti-tumor effects. Unfortunately the high doses of TNF needed to obtain this effect are accompanied by strong, shock-inducing inflammatory stimuli, impeding the clinical use of TNF. Research in our lab shows that the anti-cancer effect of TNF can be uncoupled from induction of inflammation, which means that we are indeed able to preserve the anti-tumor effect while inhibiting or preventing live threatening inflammation. This can be achieved by targeting different molecules, which shows that TNF toxicity depends on multiple mediators. Finding the key molecules that mediate TNF toxicity can be helpful in developing safer TNF-based anti-tumor therapies. As a possible windfall, such molecules might turn out to be important targets for therapy of TNF-driven pathologies.

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Part X
Advances in Applying our Knowledge to Therapy: *Rational Design of New Means for Therapeutic Modulation of Function of the TNF Family*

Chapter 50
Workshop Summary: Introduction to Rational Design of New Means for Therapeutic Modulation of Function of the TNF Family

Klaus Pfizenmaier and David E. Szymkowski

The conference session entitled "Rational Design of New Means for Therapeutic Modulation of Function of the TNF Family," as the title implies, explored multiple novel strategies to develop new drugs acting as agonists and antagonists of the TNF–TNFR superfamily for treatment of cancer and chronic or acute inflammatory diseases, respectively. The approved clinical use of agonistic superfamily ligands in cancer therapy is presently restricted to TNF itself, used in a few selected indications that allow locoregional application (e.g., isolated limb perfusion). However, other death ligands (in particular TRAIL) and TRAIL receptor-targeting reagents have received great attention because of their potential capacity to induce tumor-selective apoptosis. These pathways are under intensive clinical evaluation and new developments in this field were addressed by several presentations in this session. In contrast, the development and use of TNF antagonists is much more advanced and has proven over the past 10 years to be a great clinical and commercial success. As of this writing, there are now five anti-TNF biologics on the market (with two approved just in 2009), with annual class sales of over US$16 billion [1]. Remarkably, anti-TNFs will soon become the top-selling drug of any class (including small molecules) and have undoubtedly revolutionized the treatment of autoimmune diseases. However, the current anti-TNFs are first-generation drugs and their limitations have become apparent over years of clinical use. Thus, the development of new antagonists of TNF superfamily ligands was a major theme in the session.

This motivation to develop improved TNF antagonists has several reasons. For example, aside from being effective in only a fraction of patients, their limitations most notably include a universal "black box" warning regarding increased risk of tuberculosis and other infections, as well as warnings regarding increased susceptibility to demyelinating disorders and lymphomas. In their defense, the first generation of anti-TNF biologics was developed based on our limited

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understanding of TNF biology in the 1980s and early 1990s; for example, all approved anti-TNF biologics (including three full-length monoclonal antibodies, one Fab′ antibody fragment, and a TNFR2 receptor – IgG1 fusion) are essentially "me-too" drugs with respect to target specificity and selectivity. That is, these biologics bind to both membrane and soluble forms of TNF [2], though notably, etanercept (a TNFR2 receptor) also binds to LTα. Inevitably, as our knowledge of the importance of TNF in both health and disease has grown in the last 20 years, so too has our understanding of what constitutes an optimal therapeutic profile for a TNF inhibitor. Hence, a major theme of this session considered what attributes of "next-generation" drugs may help to improve the safety and efficacy of approved nonselective anti-TNFs. As a solution, several speakers highlighted the therapeutic potential of selective inhibition of specific aspects of TNF signaling. For example, Shin-ichi Tsunoda described specific inhibition of the pro-inflammatory TNFR1 receptor by R1antTNF, a mutated and pegylated form of human TNF [3]. In a similar vein, several talks in other sessions by Malú Tansey [4], Irène Garcia-Gabay [5], and one of us (Szymkowski) [6] highlighted development of a novel mutated human TNF that acts as a dominant-negative inhibitor of soluble TNF and spares transmembrane TNF signaling, possibly generating safety and efficacy advantages in neuroinflammatory and other diseases. In addition to the above-mentioned protein-based antagonists, a prototype of a small-molecule TNF antagonist based on macrocyclic chemistry has been developed and the first functional data were presented by Steven Hale [7]. This is the first example of a small molecule capable of effectively competing with TNF binding to its receptors. The attractiveness of a small-molecule inhibitor of protein ligand binding with the potential of oral applicability is obvious and progress will be followed eagerly by clinicians and patients alike. Finally, in the same vein, Marcos Milla described Roche's efforts to develop a small-molecule inhibitor of TNFα-converting enzyme (TACE/ADAM17) as a strategy to prevent transmembrane TNF release to the soluble form. TACE is indeed an old target in the TNF field, with inhibitors having been pursued at least since the early 1990s. Interest in this target remains high because a TACE inhibitor should skew TNF signaling from a soluble (putatively pro-inflammatory) form to a transmembrane (putatively immunoprotective) form. To provide context to such efforts, Anja Krippner-Heidenreich presented her lab's progress in understanding mechanistically why TNFR2 is preferentially activated by transmembrane TNF while TNFR1 is activated similarly by both TNF ligands. In addition, the growing understanding of signaling pathways downstream of TNF receptor activation has stimulated renewed interest in small-molecule inhibitors. As one example, Lih-Lin Ling described Wyeth's efforts to develop a small-molecule inhibitor of the Tpl2-MEK-ERK signaling pathway as a novel anti-inflammatory drug [8]. The promising results presented from several groups on small-molecule inhibitors provide a new boost to this research field and may lead to novel orally available therapies for autoimmune diseases, challenging the current dominance of anti-TNF biologics. Taken together, presentations in this session emphasized that TNF remains a promising drug target. More importantly, they demonstrate that both industrial and academic labs are exploiting advances in basic research to move

beyond "me-too" drugs, with the goal of improving the safety and efficacy profiles of next-generation anti-TNF biologics.

Beyond TNF itself, it is remarkable that almost every ligand–receptor pair in the TNF superfamily is currently being interrogated by one or more drugs in clinical trials [9]. Almost all of these candidates are biologics (frequently monoclonal antibodies or soluble receptor-Fc fusions), perhaps due to the historical difficulties encountered in developing selective small-molecule agonists or antagonists of protein–protein interactions. For example, the near future should see the first approval of biologics targeting two additional TNF superfamily pathways. The first is likely to be denosumab (to be marketed as Prolia), an anti-RANKL antibody which promises to become the first successful drug antagonizing the RANKL-RANK-OPG axis in bone remodeling [10]. Although the FDA has recently delayed its final decision, denosumab may be approved sometime by mid-2010. In addition, belimumab (anti-BAFF antibody, Benlysta) has proven efficacious in two pivotal Ph III trials and seems likely to be approved for lupus sometime in late 2010. Because signaling axes involving BAFF and the related ligand APRIL echo the complexity of signaling axes comprising soluble and transmembrane TNF and their signaling through TNFR1 and TNFR2 receptors, BAFF and APRIL represent another example where selective agonism or antagonism of key pathways may prove beneficial. For example, BAFF and APRIL signal differentially through their receptors TACI, BAFFR, and BCMA (BAFF through all three receptors, and APRIL only through TACI and BCMA). To add to this complexity, BAFF and APRIL may form heterotrimers with unknown biological significance. Although the therapeutic ramifications of selective inhibition of these five signaling axes are largely unexplored, it is likely that different inhibitors will have different pharmacological effects; hence, there is interest in developing selective biologics. This was exemplified at the meeting by the development of specific anti-APRIL antagonistic antibodies (as described by Jan Paul Medema) which are likely to have distinct effects from belimumab, and elsewhere by clinical development of atacicept, a TACI-Ig decoy receptor, that antagonizes both BAFF and APRIL.

Also in the pipeline are agonistic drugs primarily intended as cancer therapeutics, but also with potential in autoimmune inflammatory diseases. Reagents targeting TRAIL death receptors are presently the focus of several preclinical and clinical development programs. Given that the reported clinical data on TRAIL's application as a cancer drug indicate some, but not overwhelming, anti-tumoral activity, the generation of new, more effective TRAIL variants was a hot topic at this meeting. TRAIL ligand interacts with five known receptors with potentially opposing effects and several talks presented strategies to generate receptor-selective TRAIL variants. Improvement of the specific activity of recombinant-soluble TRAIL molecules to increase death receptor (DR4, DR5)-selective affinity and signaling capacity was reported by Wim Quax. Targeting TRAIL to the desired site of action through generation of fusion proteins comprising antibody or other specific targeting ligands is another approach to increasing its activity (Pfizenmaier, Razmara). This concept of targeted TRAIL molecules opens up the possibility of including additional specific functions via the fusion protein, e.g., inhibition of the targeted membrane molecule

coupled with TRAIL-mediated apoptotic signaling. A vivid example of the power of such a dual-function protein was presented by Marjaneh Razmara, who used an fn14(TWEAKR)-TRAIL fusion protein to effectively suppress inflammation in an autoimmune encephalomyelitis model in mice through simultaneous blocking of TWEAK inflammatory signals and induction of TRAIL-mediated apoptosis of T cells [11]. Similar concepts are presently being exploited for cancer therapy by one of us (Pfizenmaier) and others (as reviewed in [12] and [13]). Receptor-selective TRAIL variants as well as receptor-specific agonistic antibodies have the advantage of circumventing sequestration by TRAIL decoy receptors DcR1 and DcR2 that are frequently co-expressed on tumor cells, potentially interfering with activation of the death receptors, although the practical value of this strategy remains controversial. Regardless of this ongoing discussion, improvement of the intrinsic activity and selectivity of recombinant TRAILR-selective ligands may lead to the development of safer and more potent cancer therapies [7, 13, 14]. One such example already in clinical trials is Genentech's anti-TRAILR2/DR5 antibody apomab, which (as presented by Andreas Evdokiou) selectively stimulates DR5 death domain-mediated apoptosis in breast cancer models [15].

The function of a TNF superfamily member is sometimes irrelevant to its exploitation in the treatment of disease; for example, a receptor may simply serve as a convenient cell surface drug target. Such is the case for Genentech's program to develop a depleting anti-LTα antibody. As Jane Grogan described, this antibody binds cell surface LTα1β2 and thus depletes CD4+ Th1 and Th17 T helper cells; such activity is critically dependent on Fc-mediated killing by effector cells. The antibody is contemplated as a possible T cell-depleting therapy in autoimmune diseases [16].

In summary, the presentations in this session clearly demonstrated that the clinical development of anti-TNF biologics has been a major success in rational drug design and that this achievement is grounded firmly in the basic research of the 1980s and 1990s. At the same time, it was clear that drug designers must be conversant with today's basic research in their quest to improve next-generation anti-TNFs; for example, by reducing their significant side effects necessitating "black box" warnings. Finally, another important lesson learned from this session is that enormous potential exists to create new drugs targeting other members of the TNF superfamily. This is most obvious for biologics targeting the RANKL and BAFF pathways, which are on the cusp of market success, but also applies to the other ∼22 known receptor–ligand interactions of the TNF superfamily. Our growing understanding of the complexity of these signaling pathways is likely to keep both basic and applied researchers occupied for many years to come.

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Chapter 51
Anti-inflammatory Effects of a Novel TNFR1-Selective Antagonistic TNF Mutant on Established Murine Collagen-Induced Arthritis

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Anti-TNF Therapy for Autoimmune Diseases

Tumor necrosis factor (TNF) is a major inflammatory cytokine that, like the other members of the TNF superfamily of ligands, plays a central role in host defense and inflammation [1]. Elevated serum levels of TNF correlates with the severity and progression of the inflammatory diseases such as rheumatoid arthritis (RA), inflammatory bowel disease, septic shock, multiple sclerosis (MS), and hepatitis [6, 22]. Currently, TNF-neutralization therapies using Etanercept (Enbrel®), a soluble Fc-TNF receptor (TNFR) fusion protein, or Infliximab (Remicade®), a TNF-specific monoclonal antibody, have proven successful as a strategy for the treatment of RA [6, 31, 33]. Because TNF blockade not only improves symptoms but also suppresses joint destruction [19], it is viewed as a highly effective therapeutic approach. However, therapeutic efficacy may be accompanied by serious side effects, such as congestive heart failure [5], demyelinating disease [18], and lupus-like syndrome [26]. Most notably, the use of TNF blockade is associated with an increased risk of bacterial and viral infection [8, 17] as well as lymphoma development [2]. This is because under these conditions TNF-dependent host defense functions are also inhibited. On the other hand, administration of anti-TNF agent to patients with MS aggravated the disease [30, 32]. Thus, commercially available agents for TNF blockade, such as Infliximab and Etanercept, are contraindicated in demyelinating diseases. To overcome these problems, development of a new therapeutic strategy is required.

TNF exerts its biological functions by binding to one of two receptors, TNFR1 or TNFR2. It has been reported that the incidence and severity of arthritis were lower and milder in TNFR1-knockout mice than in wild-type mice [20]. Furthermore, previous studies demonstrated that transgenic mice with enforced expression of human

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TNF developed severe arthritis [3, 13]. Thus, the involvement of TNFR1 in arthritis pathogenesis is strongly implicated, because human TNF binds and activates only murine TNFR1 but not TNFR2. On the other hand, TNFR2 was shown to be crucial for the antigen-stimulated activation and proliferation of T cells [9, 14, 15], essential for cell-mediated immune responses to infection. Additionally, transmembrane TNF (tmTNF), the prime activating ligand of TNFR2 [10], was reported to be sufficient to control mycobacterium tuberculosis infection [23, 24], indicating the importance of TNF/TNFR2 function in this bacterial infection. Based on these studies, blocking TNF/TNFR1- but not TNF/TNFR2-interations is emerging as an effective and safe strategy for treating inflammatory diseases, which might overcome the risk of infections associated with the use of the currently available TNF blockades [16]. However, because TNFR1-selective antagonists had not yet been developed, the efficacy of this strategy has not been verified thus far. In the following section, we introduce our approach to creating TNFR1-selective mutants of TNF with antagonistic activity.

Creating Functional Mutants with Advanced Medical Applications

In order to generate proteins for therapeutic applications it is often desirable to alter the primary amino acid sequence to give artificial functional mutants with enhanced affinity for specific receptors. In recent years, phage display systems have been developed for constructing libraries of mutants displayed on a bacteriophage surface to facilitate rapid screening against a given target [4, 11, 25]. The main features of phage display procedure include the following points: (1) A selected foreign gene is first integrated into the phage genome or phage vector (phagemid vector) at the 5'-terminus of a gene (e.g., g3p) encoding an outer shell protein. The corresponding fusion protein is thus presented on the surface of the phage where it can interact with the target molecule. (2) The phage genotype (i.e., including the foreign gene) and phenotype (i.e., the foreign protein exposed on the surface of the phage) are correlated because only one phage can infect a single host bacterial cell. (3) A "library" of phage varieties numbering more than a billion in total can readily be made. (4) A desired phage clone from the library can be easily expanded by simply infecting a host bacterial culture. It is possible to create a library of random peptides, naive antibodies or cDNA-derived proteins, where the number of varieties can reach tens of millions to several billions. From such libraries desired phages (i.e., those with exposed proteins highly compatible with the target substance) can be selected, isolated and then expanded by application of a panning procedure (Fig. 51.1). Moreover, the relevant gene sequence is readily determined because the selected phage contains the corresponding gene that encodes the desired protein. The range of applications of the phage display method as a standard technology for quickly and efficiently screening molecules that bind to a particular target is constantly increasing.

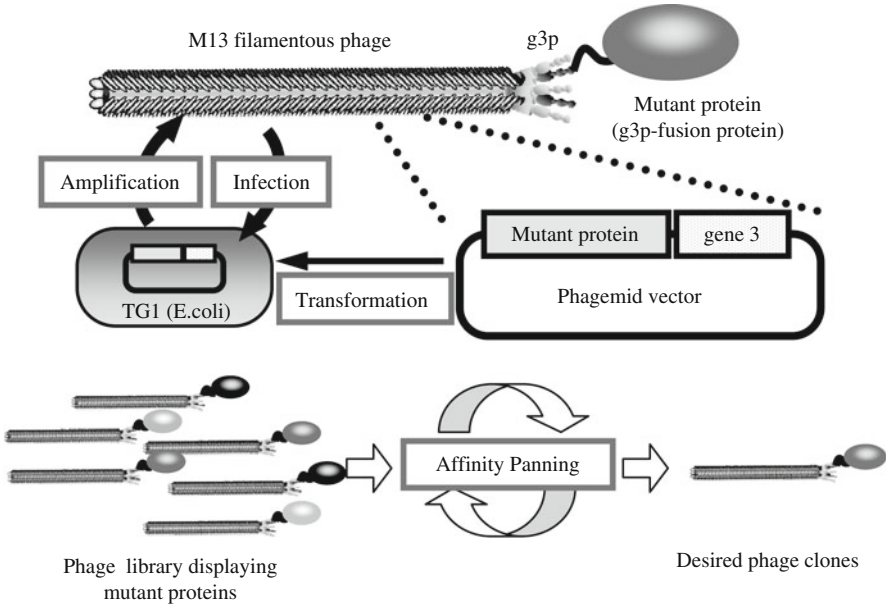

Fig. 51.1 Creation of functional mutants using phage display

In this respect, we previously constructed a phage library displaying structural mutant TNFs in which six amino acid residues (position 84–89) in the predicted receptor binding site were replaced with other amino acids. We then successfully identified the TNFR1-selective antagonistic mutant TNF (R1antTNF) from the library [29]. We showed that R1antTNF displays exclusive TNFR1 selective binding, thereby selectively inhibiting TNFR1-mediated biological activity in vitro (Fig. 51.2) without affecting TNFR2-mediated bioactivity. Additionally, it has been

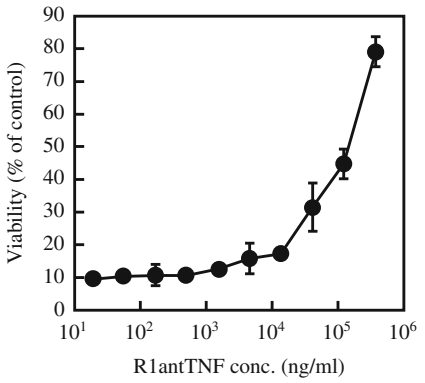

Fig. 51.2 Antagonistic activity of the R1antTNF. Serial dilutions of R1antTNF were mixed with human wtTNF-α (20 ng/ml) and then applied to HEp-2 cells. After 18 h, the inhibitory effects of R1antTNF on the cytotoxicity of wtTNF were assessed by using the methylene blue assay. The absorbance of cells without wtTNF was plotted as 100% viability

revealed that the therapeutic effects of R1antTNF in the acute lethal hepatitis models were as good as or better than those obtained using conventional anti-TNF antibody therapy [28]. By using these technologies, we were also able to create a number of functional agonistic mutant TNFs with high receptor selectability [21] and lysine-deficient mutants with full bioactivity [34]. Our system for applying the phage surface method to the construction of artificial functional mutants has not only led to the proposition of a biological DDS-based proteome drug development technology, but also redefines the concepts of protein engineering and the correlation between protein structure and activity in a way that far exceeds previous mutational technologies such as alanine screening.

Bioconjugation as a Polymeric DDS

The R1antTNF is also expected to have a therapeutic effect in chronic inflammatory disease models, such as collagen-induced arthritis model and experimental autoimmune encephalomyelitis model. However, as is the case for wild-type TNF (wtTNF), R1antTNF has a very short half-life (∼10 min) in the plasma of mice when administered intravenously. Thus, there is increasing need for a method to effectively extend the half-life of R1antTNF in plasma. It has been demonstrated that the attachment of water-soluble synthetic polymers, such as polyethylene glycol (PEG), to the surface of these proteins can significantly increase their half-life in vivo (Fig. 51.3). The covalent conjugation of proteins with PEG is specifically referred to as PEGylation. Based on these points, we have successfully developed a novel PEGylation system combined with an efficient method of creating functional mutants [35]. Our strategy

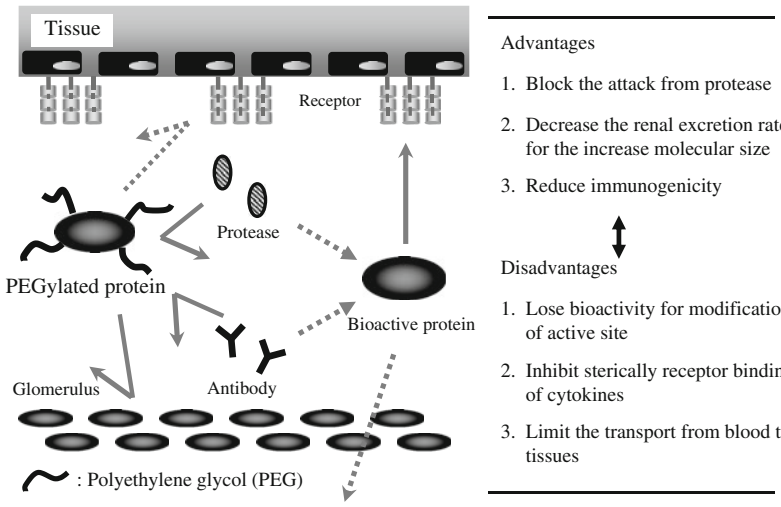

Fig. 51.3 Characteristics of PEGylation

first involves applying the phage display system to create a fully bioactive lysine-deficient mutant protein. Site-specific PEGylation to improve therapeutic potency is then performed using the lysine-deficient mutant. While conventional PEGylation of TNF caused a loss of bioactivity due to random introduction of PEG at the ε-amino groups of six lysine residues in monomer TNF, our site-specific PEGylation introduces PEG only at the NH_2 terminus via a lysine-deficient mutant TNF without loss of bioactivity. R1antTNF was generated using a phage library based on the lysine-deficient mutant of TNF [29]. Consequently, R1antTNF also lacked any lysine residues. Intriguingly, the N terminus of TNF is not indispensable for function because a deletion mutant of TNF lacking eight residues at the N terminus retains full bioactivity [7]. In such instances, site-specific PEGylated R1antTNF (PEG-R1antTNF) was uniform at the molecular level and had similar bioactivity (80%) to that of unmodified R1antTNF. Furthermore, introducing PEG only to the N-terminal amino group made it possible to produce molecularly stable bioconjugated proteins with nearly 100% yield. We have also demonstrated that PEGylation of R1antTNF greatly improves its ability to suppress arthritis (Fig. 51.4). This could be due to enhanced retention of R1antTNF in the circulatory system. In order to maximize the effectiveness of PEGylation, it is important to select the optimal molecular weight or type of PEG, by balancing favorable effects, adverse side effects, and dose schedule.

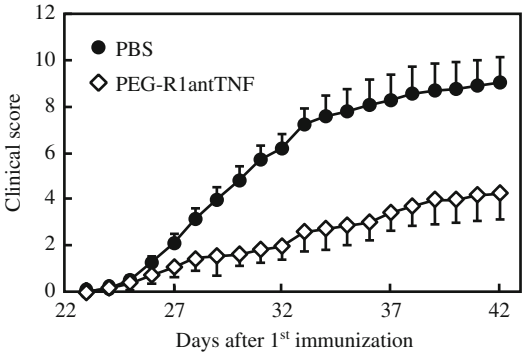

Fig. 51.4 Therapeutic effect of R1antTNF in CIA model. The severity of arthritis in CIA mice ($n = 10$) treated with PBS, PEG-R1antTNF (1 μg twice a day) for 3 weeks from 23rd day was assessed every day using an established macroscopic scoring system. Data of severity represent the mean ± SEM

A previous study using TNFR1- or TNFR2-deficient mice had indicated that TNF/TNFR2 interactions play an important role in antiviral-immune responses [12]. Therefore, we hypothesized that because it inhibits the bioactivity resulting from the interaction between TNF and TNFR1 but not between TNF and TNFR2, PEG-R1antTNF might not have the undesirable side effect of inhibiting antiviral immunity. Accordingly, we investigated the effect of Etanercept and PEG-R1antTNF on viral immunity, using adenovirus infection as a model [27]. While viral clearance was indeed compromised by the administration of Etanercept, PEG-R1antTNF had little effect on this important parameter. This indicated that use of PEG-R1antTNF may reduce side effects such as increased susceptibility to viral infection due to its TNFR1 selectivity.

Conclusion Remarks

In this report, we have demonstrated the usefulness of DDS-based technology to selectively enhance desirable therapeutic activities of bioactive proteins without increasing their side effects. These important steps in site-specific PEGylation technology (polymeric DDS) were based on our strategy for the development of functional artificial mutants (biological DDS), especially as applied to the production of functional lysine-deficient mutants. Furthermore, the novel site-specific PEGylation technique described here can enhance the therapeutic effect of R1antTNF. Even if the mutant TNF displays an increased risk of unanticipated side effects and antigenicity, PEG will assist in neutralizing these unwanted characteristics by introducing steric hindrance.

Proteome drug development requires the production of a wide variety of proteins and protein structural mutant types as well as the establishment of a high-throughput screening procedure for analyzing their function (e.g., assessing the strength of ligand–receptor interactions). A thorough evaluation of the relationship between these factors and the protein structure is also required. In the future, bioinformatics may develop to the point where it will be capable of using genome sequencing information to predict the function and structure of unknown proteins. Such an advance will radically alter proteome drug development. Our strategy, as outlined above, may well become an essential basic technology for large-scale and high-throughput analysis of protein function.

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Chapter 52
A Proliferation-Inducing Ligand (APRIL): The Development of Antagonistic Agents as Potential Therapeutics and Deciphering the Role of Heparan Sulphate Proteoglycans (HSPGs) in APRIL Signalling

Fiona Kimberley, Marco Guadagnoli, Hans van Eenennaam, and Jan Paul Medema

Introduction

APRIL (a proliferation-inducing ligand) is a TNF ligand originally discovered for its ability to promote tumourigenesis [9]. APRIL not only binds two specific TNF receptors, TACI (transmembrane activator and CAML interactor) and BCMA (B-cell maturation antigen), but also interacts more promiscuously with the widely expressed heparan sulphate proteoglycans (HSPGs) (reviewed in [5]). Although APRIL is expressed as a transmembrane protein it is cleaved in the golgi apparatus by a furin convertase and secreted in a soluble form as a homo-trimer. It can also occur as a membrane-bound protein by virtue of an alternative splicing event which fuses the extracellular domain of APRIL with the transmembrane domain of TWEAK, creating a unique protein termed TWE-pril, although the abundance of this and its functional relevance is largely unknown [25].

APRIL can signal the activation of a number of transcription factors via the internal recruitment of TRAFs. The specificity of these interactions is not fully elucidated although from in vitro assays it appears that BCMA binds TRAFs 1, 2 and 3 and that TACI binds TRAFs 2, 5 and 6, leading to the activation of the canonical NF-κB pathway as well as several other transcription factors including p38, AP-1/JNK, MAPK, ERK and PI3-K/AKT [10, 31]. Circumstances under which different transcription factors are activated are not well understood.

The physiological role of APRIL is in the maintenance of humoral immune responses. The knockout mouse was found to suffer from a lack of circulating

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IgA, and APRIL has since been shown to mediate class switch from IgG to IgA, specifically via TACI signalling [3]. APRIL is also found in abundance within plasma cell niches in the mucosa, where it mediates a crucial class switch from protease-susceptible IgA1 to protease-resistant IgA2 [19, 30]. APRIL also supports the survival of plasma cells, specifically via BCMA [1, 2]. However, physiologically many of the most crucial functions of APRIL can be functionally substituted for by BAFF, which is central to B-cell maintenance (reviewed in [18]). Therefore, it is the role of APRIL in disease that has sparked most interest from a therapeutic perspective.

APRIL appears to be over-expressed and potentially involved in a number of autoimmune diseases, such as rheumatoid arthritis (RA), sjögren's syndrome, systemic lupus erythematosus (SLE) and multiple sclerosis (MS) (reviewed in [15]). APRIL is also implicated as an important pro-survival factor in several B-cell malignancies, where it is either over-expressed by the tumour itself or the tumour is responsive to APRIL that might derive from infiltrating cells. These effects appear to be in part mediated via APRIL interactions with BCMA and TACI, driving N activation (reviewed in [14]). Using an in vivo model for multiple myeloma, treatment with TACI-Fc prevented pro-survival signals from BAFF and APRIL [32]. In addition, high APRIL expression in the serum of CLL patients appears to correlate with a poor prognosis. This link with CLL was revealed by joint studies in our and Michel Hahne's laboratory using the APRIL transgenic mouse, in which we detected an abundance of CD5-positive B cells in the peripheral lymph nodes, and a phenotype reminiscent of that seen in human CLL [23]. Subsequent analysis of patient samples revealed over-expression of APRIL compared with normal controls [24].

The Development and Characterisation of Antagonistic Monoclonal Antibodies Against Human APRIL

To study the role of APRIL in separation from the homologous ligand BAFF, with which it shares binding to BCMA and TACI, and to target APRIL specifically for therapeutic purposes, we developed novel APRIL antagonistic antibodies. Monoclonal antibodies have proved effective therapies in several diseases to date due to their specificity and long half-life in comparison to other biologics. In the case of APRIL, a soluble receptor in the form of TACI-Fc has been developed commercially as atacicept and is in stage II/III clinical trials for a number of different autoimmune diseases and some B-cell malignancies (reviewed in [8]). This has proved effective in the treatment of several autoimmune disorders, where it is beneficial to target both BAFF and APRIL simultaneously. However, there may be cases where targeting either ligand individually is a more logical approach. Long-term administration of atacicept, as demonstrated using a TACI-Fc transgenic mouse, causes depletion of the mature B-cell populations and so in cases where APRIL plays a dominant role it may be of greater benefit to target APRIL specifically [28].

We are developing anti-human APRIL monoclonal antibodies with antagonistic properties to block binding to both BCMA and TACI. Antibodies will be further validated for their functionality in terms of their ability to block known APRIL responses both in vitro and in vivo. Reliable antagonistic activity may suggest promise for systemic activity in disease scenarios.

Since APRIL over-expression is associated with many malignancies and in some cases appears to be dominant over a role for BAFF, the next step in our research is to establish a suitable tumour model in which to test their activity. We started experiments with several multiple myeloma cell lines that have been reported to express and depend on APRIL for survival [21]. However, we have identified many inconsistencies in the literature with the reported expression of APRIL, BCMA and TACI, as well as differences in the extent to which, in our hands, certain cell lines depend on APRIL. This may be due to differences that occur with long-term culture of these cell lines or in the form of APRIL used, which could differ widely. We have previously reported our difficulty in obtaining a purified and consistent preparation of APRIL, due to its inherent hydrophobicity, which seems to be a practical difficulty shared with others [16]; Hahne, personal communication, 2008). Thus different forms may differ vastly in activity. Indeed, we have found that some commercial preparations are much less active than using APRIL in conditioned medium taken directly from transfected cells (unpublished observations).

Consequently we have looked to other models aimed at inhibiting APRIL in a more realistic scenario that would mimic the natural micro-environment. To this end we made use of PBMC-derived osteoclasts [32] or fresh primary cells from B-CLL donors to provide a source of functional APRIL. Inhibiting APRIL in B-CLL samples will also be studied using an in vivo approach. In addition, future experiments are directed towards proving that the antibodies can target the in vivo growth of solid tumours.

The Role of HSPGs in APRIL Signalling

Work by us, and others, confirmed that the promiscuous binding of APRIL to a number of different cell lines occurred via interactions with heparan sulphate proteoglycans (HSPGs), which form part of the extracellular matrix [11, 13]. Initially the precise role of this interaction was unclear. However, we and others mapped the HSPG binding domain to a specific site on APRIL, located away from the TNF receptor binding site. Using point mutants of APRIL that lack binding either to the HSPGs or to the TNF receptors, we were able to show that binding to HSPGs allows effective ligand cross-linking and acts as a platform to enable oligomerisation of the ligand [16]. This oligomerisation of the trimeric ligand is necessary for an effective signal to be transmitted via BCMA and TACI.

It is well known for the TNF superfamily that the receptors require clustered ligands for signalling, a situation normally achieved via membrane presentation of the ligand or the existence of multimeric forms, as has been shown for BAFF [3, 17].

For APRIL, the requirement for antibody cross-linking in vitro had been reported, but there was no formal explanation for how such cross-linking would occur in a physiological context [3]. We were able to show that loss of HSPG binding could be compensated for in vitro by the addition of a cross-linking antibody, indicating that the binding requirement is purely structural and that in this context HSPGs do not directly signal [16]. This is in contrast to the work by Sakurai et al. [27], who claim that TACI signalling requires two distinct signals – one from the receptor and another from HSPGs. However, this work was shown using human B cells, whereas our study addressed this point using mouse B cells, so it is still possible that there are some differences between the requirements in human and mice. The situation is also complicated by the fact that TACI also binds HSPGs.

In solid tumours the role of APRIL–HSPG interactions may be different, since many of these tumours appear to respond to APRIL yet do not express either BCMA or TACI [11, 26]. This could suggest the existence of a third APRIL receptor, that HSPGs signal directly in this context or that they function as co-receptors. Interestingly, this has been known now for more than 10 years yet we are no closer to identifying the mediators. When APRIL was originally identified, NIH cells over-expressing APRIL were reported to grow out faster in mice than the parental cell line, indicating that APRIL conferred a survival or growth advantage to these cells, which were subsequently shown to lack expression of both APRIL receptors [9]. APRIL was also shown to stimulate enhanced survival of a number of other cell lines in vitro, which again have also been shown to lack receptor expression. The mechanism for this has remained a mystery and no new receptors have so far been identified, although the task of fishing for new interactions is made difficult by the promiscuous HSPG binding. One problem is that without HSPG binding APRIL may be rendered non-functional and therefore the readout for ‘fishing’ is lost.

To study the role of HSPGs in tumour outgrowth we have generated stable cell lines that express either the WT-APRIL or the non-HSPG binding form and will compare the outgrowth following injection into nude mice. In order to decipher the mechanisms of action in solid tumour-derived cell lines we are also studying APRIL knockdown. This has been addressed to some extent using colon cancer-derived cell lines [6, 7]. Interestingly, the authors report a role for APRIL in both cell adhesion and migration, although the mechanism of action is still not known and is no doubt the focus of future studies by several groups. It will be interesting to see if blocking the activity of APRIL by external agents has a similar effect in these cells or if this is an intrinsic property.

Another interesting angle to the story of APRIL and HSPGs is that TACI also binds and can be activated by HSPGs, although it is not yet known in which context this occurs or if there is any specificity. This is potentially interesting in light of the high levels of syndecans produced by several B-cell malignancies and the TACI-high signature of several multiple myelomas [20]. It has been suggested that on these cells APRIL occurs in a complex with TACI, with syndecan-1 as a co-receptor [22].

It is now known that APRIL is not the only TNF ligand to bind HSPGs. EDA-1 is also known to bind HSPGs, but work also reported at this conference revealed

that this binding in fact decreases the activity of EDA-1 by limiting its distribution to syndecan expressing cells; it is the collagen domain of EDA-1 that confers multimerisation properties in this case [29]. The opposite is thought to be true for APRIL, in that HSPGs potentially aid distribution to sites where it is most required, such as plasma cell-rich niches in the mucosa [12]. Such non-protein–protein interactions potentially open up an exciting new arm of TNF receptor–ligand biology. It will be interesting to see to what extent these interactions combine with the known protein interactions to regulate activity and partake in signalling.

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Chapter 53
Genetic Engineering of Death Ligands for Improvement of Therapeutic Activity

Jeannette Gerspach, Britta Schneider, Nicole Müller, Tina Otz, Harald Wajant, and Klaus Pfizenmaier

Introduction

Ligands of the TNF family are transmembrane proteins, but also occur as soluble molecules which are derived by proteolytic processing or alternative splicing from the corresponding membrane-bound ligand. Both forms share the extracellular TNF homology domain (THD), which is responsible for ligand trimerization and receptor binding. Notably, TNF receptors respond in two quite distinct ways to ligand binding. In the first case, a particular TNF receptor does not discriminate between binding of the transmembrane and the soluble form of the ligand and stimulates associated signaling pathways with equal efficacy (Fig. 53.1). Examples are TNFR1 and TRAILR1/DR4, which are efficiently activated by both the membrane-bound and the soluble form of TNF and TRAIL, respectively [12, 26]. In the second case, interaction with the transmembrane ligand likewise results in robust receptor activation and downstream signaling, yet the soluble ligand, despite receptor binding, elicits no or only modest cellular responses (Fig. 53.1). The interaction of CD95L with CD95, TRAIL with TRAILR2/DR5, and TNF with TNFR2 are well-defined examples for this case [12, 17, 23, 26].

The deficiency or reduced capability of some TNF receptors to respond to soluble, trimeric TNF ligands can be overcome by two means. First, by oligomerization of the soluble TNF ligand trimers (Fig. 53.2) and, second, by spatial fixation of the ligand trimers, e.g., by binding to the extracellular matrix or by specific immobilization on the cell surface (see below). In addition, depending on the ligand type, stabilization of the trimeric assembly of the soluble molecule can represent an additional mean by which intrinsic bioactivity can be increased (see below).

Thus, inactive or poorly active soluble TNF ligand trimers are not devoid of genetically encoded structural information required for receptor activation, but obviously lack auxiliary functions related to membrane association. This is of particular

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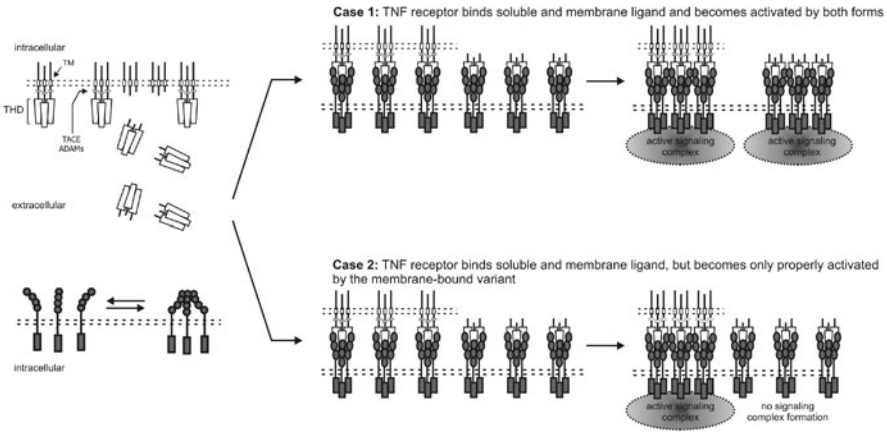
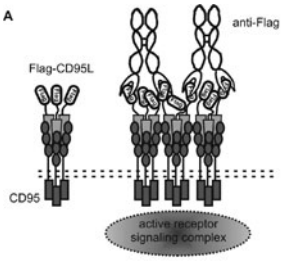


Fig. 53.1 With respect to activation of TNF receptors by TNF ligands two cases can be distinguished. In the first case, a TNF receptor (e.g., TNFR1 and TRAILR1) is robustly activated by the soluble and transmembrane variant of its ligand. In the second case, a TNF receptor (e.g., TNFR2, TRAILR2, 4-1BB, and OX40) interacts with the soluble and transmembrane variant of its ligand, but becomes only properly activated by the latter



B

Ligand	ED50			
	-	+M2	- / +M2	
CD95L	2000	0.1	20000	Berg et al., 2007
TRAIL[1]	>> 2000	0.1	20000	Berg et al., 2007
OX40L	2000	20	100	Müller et al., 2008
41BBL	>> 2000	100	>> 20	Wyzgol et al., 2009
CD27L	>> 2000	100	>> 20	Wyzgol et al., 2009
CD40L	1	0.3	3	Wyzgol et al., 2009
GITRL	1	0.2	5	Wyzgol et al., 2009
LIGHT	0.2	0.2	1	unpublished data
TNFR1-specific TNF mutant	2	2	1	unpublished data
TNFR2-specific TNF mutant	>> 2000	5	> 400	unpublished data

[1] with respect to TRAILR2 activation

Fig. 53.2 (**a**) Soluble TNF ligands, e.g., CD95L that bind to their cognate receptor, but fail to trigger robust signaling, convert into strong agonists upon oligomerization, e.g., via antibody crosslinking. (**b**) Effect of anti-Flag mAb crosslinking on the specific activity of trimeric Flag-tagged TNF ligands

importance for those TNF family members that bear the potential for clinical application, such as the death ligands TNF, CD95L, and TRAIL. A rational ligand design aimed at matching the requirements for optimal receptor activation should greatly improve the bioactivity of these ligands and moreover give the opportunity to unfold their activity in an antigen-restricted manner.

Stabilization of Trimer Formation

It is apparent that stabilization of trimer formation might first of all be achieved through inhibition of dissociation of ligand trimers into their monomers, which occurs rapidly at low concentrations of, for example, soluble TNF [8, 19]. Thus, we recently generated a single-chain variant of soluble human TNF, called scTNF, consisting of three human TNF monomers covalently linked via two glycine serine linkers. Compared to wild-type TNF, scTNF displayed similar maximum binding capacities and somewhat higher binding affinities for both TNF receptors as well as similar bioactivities in cell culture experiments, when freshly titrated. In contrast, upon incubation at 37°C in human serum, scTNF displayed strongly increased thermal and serum stability [14]. Furthermore, systemic toxicity of scTNF in a D-galactosamine-sensitized liver injury model was significantly lower, yet antitumor activity in two different mouse tumor models is at least similar or even enhanced compared to standard recombinant TNF [14]. Thus, by offering a wider therapeutic window compared to TNF, the scTNF format appears attractive for development of new TNF-based cancer therapeutics, including specifically tumor-targeted TNF fusion proteins. In an analogous way, a single-chain variant of human TRAIL as well as tumor-targeted TRAIL fusion proteins have been developed (see below).

In an independent approach for stabilization of trimeric TNF ligands, we made use of a small 30 amino acid motif of tenascin-C (TNC), which forms tightly packed trimers due to strong non-covalent interactions and disulfide bonding [13]. Fusion of this rigidly trimerizing domain to the N-terminus of soluble TNF family ligands resulted in an increase in intrinsic bioactivity in all studied TNF ligands, although to a widely varying extent [2, 16, 28]. The most obvious beneficial effect was seen with murine CD95L and human CD27L. Despite forming trimers, both TNF ligands failed to bind to an appreciable extend to their corresponding receptors when expressed as soluble Flag-tagged proteins and consequently failed to activate receptor signaling. In contrast, stabilizing the trimeric organization of these molecules by fusion with the TNC domain resulted in restoration of receptor binding and enabled efficient receptor activation, which, however, still required additional ligand oligomerization, e.g., by secondary crosslinking reagents [2, 28]. Similar results were seen with murine TRAIL. Genetic fusion of the TNC domain conferred efficient receptor interaction and robust TRAILR2 signaling after secondary crosslinking, while non-stabilized murine TRAIL only weakly interacted with TRAIL death receptors, accompanied by poor activity despite secondary oligomerization [2]. Even the THD encompassing variant of human TRAIL, already able to bind and activate its cognate receptors, displayed enhanced bioactivity upon introduction of the TNC domain [2]. Of note, while the cell death inducing capability increased only about fivefold for the non-crosslinked TNC-stabilized variant of human TRAIL, secondary oligomerization resulted in 100- to 200-fold increase in activity on tumor cells coexpressing TRAILR1 and 2. Apoptosis induction by TNC-stabilized human TRAIL in cells expressing only TRAILR2 showed that additional ligand crosslinking was still required to activate this receptor, thereby confirming that the TNC domain does not mediate formation of larger oligomers [2]. Thus, as

an alternative to the above-described single-chain format of TNF ligands, fusion of the tenascin-C domain might serve as an additional mean to increase the activity of recombinant TNF ligands through stabilization of trimer formation, potentially allowing lower doses of proteins to be applied in therapy.

Conversion of Inactive Death Ligands into Highly Active Molecules Through Tumor-Directed Targeting

Development of new therapeutics increasingly demands a strictly localized action in order to reduce unwanted side effects to a minimum. This aim has also to be met and pursued for the design of new therapeutics based on ligands of the TNF family. The systemic activation of CD95 or TNF receptors leads to severe, life-threatening side effects like hepatotoxicity or septic shock and multi-organ failure, respectively [15, 18, 23], necessitating strictly target-selective action. One approach to increase site-directed activity of TNF ligands is, for example, fusion with an antibody or a derivative thereof, recognizing a target tissue or target cell-specific antigen. In general, this results in improved localized action by enrichment of the TNF ligand at the target side, thereby lowering dose of the therapeutic and concomitantly its systemic action. Moreover, if those receptors with an inability/poor ability to respond to soluble TNF ligand trimers are intended to be activated, the fusion protein can potentially display particularly high, yet target restricted action. This requires the condition that the unbound fusion protein behaves like the soluble ligand, which is converted into a highly active variant upon cell surface targeting and functional presentation of the death ligand part of the antigen-bound fusion protein toward the corresponding death receptor. In fact, we and others have previously shown that death ligand fusion proteins comprised of a scFv-targeting domain and either CD95L or TRAIL only gain high bioactivity after cell surface immobilization by antigen binding, remaining essentially inactive (CD95L) or only poorly active (TRAIL) toward non-targeted cells or tissues (Fig. 53.3a, Table 53.1; [3–6, 24, 25]).

The modular design of the scFv death ligand fusion proteins opens up the possibility to use a nearly unlimited repertoire of target specificities that can be implemented through use of appropriate antibody fragments as targeting modules, as outlined above. In addition, we have shown that the principle of cell surface immobilization/presentation-dependent death ligand activation can also be achieved through use of other targeting molecules that undergo selective, high-affinity interactions with plasma membrane molecules. Thus, we generated EGF–CD95L fusion proteins for targeting EGFR (unpublished data) and used TNF receptors for membrane targeting of death ligands, too. Of note, aside from the important feature of presentation of the death ligand at the membrane and thereby its functional activation, use of appropriate targeting modules may encompass an independent action on the targeted membrane molecule, namely inhibition of binding of its natural ligand/receptor. Thus a dual action of such fusion proteins is conceivable. This appears

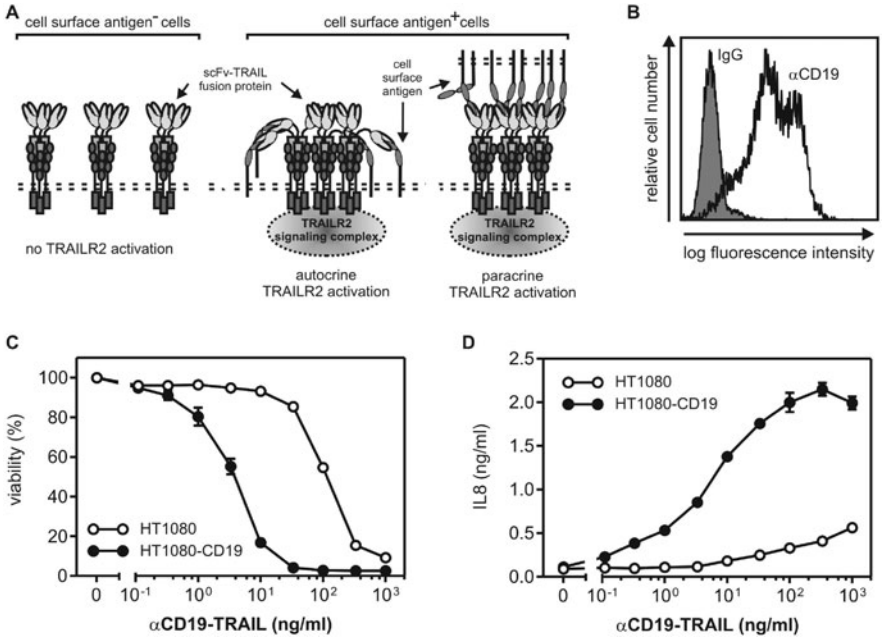


Fig. 53.3 (**a**) TRAILR2 does not or only poorly respond toward binding of soluble trimeric TRAIL fusion proteins with an N-terminal scFv domain recognizing a membrane-associated antigen when this antigen is not available. Binding of the scFv domain of the scFv-TRAIL fusion protein to its corresponding antigen, however, enables the TRAIL part of the molecule to stimulate efficient signaling via TRAILR2. For simplicity, TRAILR1, which is adequately stimulated by scFv-TRAIL fusion proteins irrespective of the presence of antigen, was omitted. (**b**) Flow cytometric analysis of mock and CD19-transfected HT1080 cells. (**c**) Mock and CD19-transfected HT1080 cells were stimulated in triplicates with the indicated concentrations of a scFv-TRAIL fusion protein with a CD19-specific scFv domain (α-CD19-TRAIL). After 18 h cell viability was determined by crystal violet staining. Cells were sensitized for cell death induction by treatment with 2.5 μg/ml CHX. (**d**) To demonstrate that the enhancing effect of cell surface antigen binding of α-CD19-TRAIL is not restricted to apoptosis induction, we analyzed IL8 induction by this molecule in the presence of the caspase inhibitor z-VAD-fmk (20 μM)

to be of particular relevance in a situation, where the targeted membrane molecule controls, through auto- or juxtatropic signaling, apoptosis sensitivity of the affected tumor cells. In the following, we review some of the salient features of such death ligand fusion proteins.

Targeting-Dependent Enhancement of TRAIL's Antitumoral Activity

Cells such as Jurkat T-cells that express the death receptor TRAILR2, but not the structurally closely related TRAILR1 molecule, are largely resistant toward

soluble TRAIL trimers but are readily killed upon stimulation with membrane TRAIL expressing cells or by oligomerized soluble TRAIL trimers [26]. In contrast, cells expressing in addition to TRAILR2 also TRAILR1, like, for example, HT1080 cells, already undergo apoptosis in response to soluble TRAIL trimers, although oligomerization of the latter still results in a shift of the corresponding ED50 dose toward lower concentrations. Furthermore, we observed that trimeric fusion proteins comprised of soluble TRAIL and a plasma membrane-targeting molecule distinct from the TRAIL receptors, e.g., a surface antigen-specific scFv, are highly active via TRAILR2 signaling on appropriately target antigen-positive cells, but not on target-negative cells (Fig. 53.3a). In contrast, TRAILR1 activation by such TRAIL fusion protein was found to be largely independent of binding of the specific membrane-target antigen. Using scFv of various specificities, we have successfully generated fusion proteins of all three death ligands targeted to distinct tumor-associated antigens (Table 53.1). For example, an α-CD19-TRAIL fusion protein consisting of a CD19-specific scFv antibody fragment fused N-terminally to the extracellular domain of TRAIL displayed about 30-fold higher cytotoxic activity on HT1080 cells stably transfected with CD19 compared to parental HT1080 cells (Fig. 53.3b, c). Furthermore, this enhanced bioactivity appears not restricted to apoptosis induction, which became evident in this cell model when apoptosis was blocked by caspase

Table 53.1 Bioactivity of cell surface antigen-targeted TNF ligand fusion proteins

	Increase in cell surface antigen-dependent cell death induction (*x*-fold)[a]	In vivo activity	Reference
α-CD19-TRAIL	~30	n.d.	Fig. 53.3
α-FAP-TRAIL	~30	n.d.	[26]
α-FAP-scTRAIL	>100	n.d.	Unpublished data
α-erbB2-scTRAIL	~5	erbB2-dependent higher antitumoral activity	[22]; Fig. 53.4
CD40-TRAIL	>100	n.d.	Fig. 53.6; [1]
α-FAP-CD95L	>100	No apparent toxicity in mice; FAP-dependent antitumoral activity	[21]
α-FAP-CD95L-PD	>100	No apparent toxicity in mice; FAP-dependent antitumoral activity	[27]
CD40-CD95L	>100	No apparent toxicity in mice	[1]
α-FAP-TNF-PD	>1000	n.d.	[10, 11]
α-FAP-scTNF	~30	FAP-dependent higher antitumoral activity	Unpublished data, Fig. 53.5

[a]Increase in cell death induction was revealed either by comparison of ED50 values on antigen-positive versus antigen-negative cells or by comparison of ED50 values of the targeted scFv-TNF ligand fusion protein versus the non-targeted TNF ligand; n.d.: not determined.

inhibitors. Under such conditions TRAIL-induced NFκB-mediated IL8 production can be demonstrated in HT1080 cells (Fig. 53.3d). Similar results had been shown by us before for a fibroblast activation protein (FAP)-specific TRAIL fusion protein using HT1080 cells stably transfected with FAP [26]. Particularly, blocking of TRAILR2 by neutralizing TRAILR2-specific Fab fragments confirmed dominant TRAILR2-dependent cell death in the latter cell model [26].

By analogy, we generated tumor stroma (FAP) and tumor (erbB2)-specific fusion proteins based on scTRAIL, one of the trimer-stabilized versions of TRAIL (see above). These fusion proteins contain only one antigen binding module compared to fusion proteins based on homotrimeric TRAIL comprising three scFv modules. Interestingly, the lower avidity, in respect of antigen binding, of an erbB2-specific scFv-scTRAIL fusion protein did apparently not negatively affect the bioactivity compared to its corresponding trivalent homotrimeric variant with conventional TRAIL (Schneider et al., unpublished data). Moreover, in a mouse tumor model using xenografted Colo205 tumors, the α-erbB2-scTRAIL molecule displayed superior antitumoral activity compared to the non-targeted scTRAIL (Fig. 53.4; [22]).

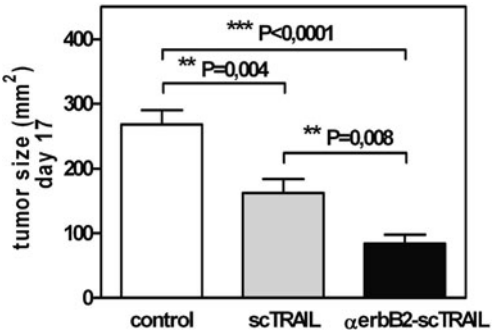


Fig. 53.4 ErbB2-dependent antitumoral activity of α-erbB2-scTRAIL. 3×10^6 Colo205 cells were subcutaneously inoculated at left and right dorsal sites of NMRI nu/nu mice. Treatment started when tumors reached about 25 mm^2 ($n = 8$ tumors per treatment group). Mice received eight daily intravenous injections of 1 nmol of the corresponding reagents. Shown are mean tumor sizes ± SEM at day 17 after first application. Statistical analysis was done by unpaired *t*-test (two-tailed *P* values)

Exploiting Therapeutic Potential of CD95L- and TNF-Based Reagents: Targeted Activation and Prodrugs

As mentioned before, soluble trimeric FasL/CD95L has no or only a low capacity to activate Fas/CD95 and might even act as an antagonist for membrane FasL/CD95L [23]. Accordingly, a very recent study with genetically modified mice has provided in vivo proof that membrane FasL/CD95L and not its soluble derivative is the

executioner of Fas/CD95-mediated cell death [17]. Yet soluble trimeric FasL/CD95L represents an ideal basic element for the development of systemically applicable therapeutics with tumor-targeted activity, as its apoptotic signaling capacity can be fully reconstituted, e.g., by membrane-assisted presentation. This has been previously shown by us with an α-FAP-CD95L fusion protein (sc40-FasL) consisting of a FAP-specific scFv fused to the extracellular domain of FasL/CD95L. This construct displayed highly increased cytotoxicity on FAP-expressing cells, resulting from FAP-dependent cell surface immobilization of the fusion protein enabling the conversion of the inactive into a fully active CD95L (Table 53.1; [21]). In vivo analyses revealed that sc40-FasL prevented outgrowth of FAP-expressing, but not target antigen-negative HT1080 tumor xenografts [21]. Noteworthy, systemic application of up to 90 μg of this CD95L fusion protein into mice (corresponding to ~10 × LD50 of conventional oligomerized CD95L) was well tolerated and subsequent histological analyses revealed no detectable toxicity, proving that the fusion protein was devoid of non-targeted activity [21]. More recently, similar results reported by others confirmed the usefulness and applicability of such FasL/CD95L fusion proteins. For example, a scFv fragment derived of the CD20-specific chimeric monoclonal antibody Rituximab and a CD7-specific scFv fragment were used to generate scFvRit:sFasL and scFvCD7:sFasL [6, 7]. Both fusion proteins displayed clear antigen-dependent cell death induction. Moreover, upon intraocular injection of scFvRit:sFasL into mice, no obvious systemic toxicity was observed [7].

Although fusion with an antibody fragment seems to be a rather simple approach, it has to be checked carefully that it adopts a strictly trimeric organization. This caveat stems from the observation that antibody fragments could have an intrinsic tendency to aggregate, thereby potentially leading to higher order complexes. In addition, secondary aggregate formation in vivo might occur, e.g., in the context of an immune response toward the fusion protein. If such an aggregation process does not disable the folding and receptor interaction sites of the THD, an oligomerized TNF ligand could result which potentially exerts unrestrained, off-target activity. Based on these considerations, the development toward genuine CD95L prodrugs, to be activated by proteolytic processing, has been pursued and verified. These prodrugs similarly exhibit antigen-dependent conversion into highly active cell surface immobilized CD95L variants provided that a tumor-specific protease is present and active in the vicinity of the targeted prodrug (Table 53.1; [27]). In the described prodrug molecules, the inhibitory domain consists of the extracellular domain of CD95 which is fused to the scFv-CD95L fusion protein by a protease-sensitive linker containing consensus sequences for matrix metalloproteinase-2 (MMP-2) or urokinase plasminogen activator (uPA). It functions to mask the CD95L domain until the antigen-bound and still inactive fusion protein is subsequently processed by the respective tumor-associated protease, thereby releasing the inhibitory domain and leaving the active cell surface immobilized fusion protein [27]. This prodrug format might be of particular relevance if the target antigen of choice is not strictly tumor specific, i.e., overexpressed on tumor cells, but also found on some normal tissues, where a targeted CD95L fusion protein could exert activity. In this situation, due to absence of MMP/uPA activity in normal tissue cells, the CD95L-prodrug

remains in a silent state despite membrane targeting and is likely to be removed by endocytosis before an accidental processing would occur. Thus, the prodrug format introduces another level of safety, which could be useful for all CD95L fusion proteins independent of the target antigen exploited.

Similar to soluble TRAIL and FasL/CD95L, soluble TNF can also be engineered to acquire target-dependent membrane TNF-like bioactivity leading to the activation of not only TNFR1 but also TNFR2. Although TNFR2 is not characterized as death receptor, activation in concert with TNFR1 might be of advantage for cancer therapy (reviewed in [11]). As TNF elicits systemic side effects already in its soluble form, which are expected to occur for scFv-TNF fusion proteins alike, we generated TNF prodrug fusion proteins. Analogous to the CD95L prodrugs, these are endowed with an inhibitory TNF receptor fragment linked to the core molecule via a protease-sensitive linker containing consensus cleavage sites for tumor-specific proteases like MMP-2 or uPA [9, 10]. In vitro, in the presence of target antigen negative, but MMP-2 and uPA-positive cells, TNF prodrugs remained intact and thus possessed only marginal activity compared to TNF. In contrast, upon binding on target antigen-positive cells, TNF prodrugs were efficiently processed by the respective proteases and converted into highly active TNF molecules. The antigen-bound, processed fusion proteins displayed activities comparable to the scFv-TNF fusion proteins on which the prodrugs were based and thus resemble the activity of membrane TNF (Table 53.1; [9, 10]). The design of a protein prodrug with targeting properties and, in addition, target-dependent activation, is an intriguing concept, but the complex modular composition of the prodrug and the size of the molecule may represent a technological hurdle when it comes to development of a cost-efficient, high-yield production process.

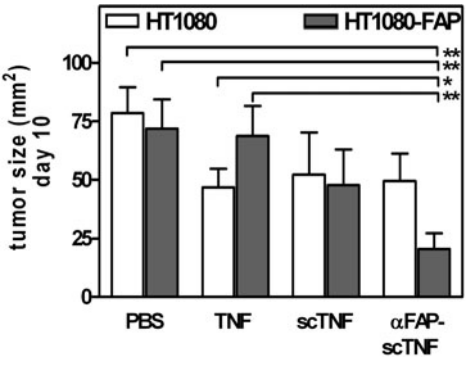


Fig. 53.5 FAP-dependent and FAP-independent antitumoral activities of α-FAP-scTNF. 1.5×10^6 HT1080 or HT1080-FAP cells were subcutaneously injected into NMRI nu/nu mice. Treatment began 5 days later when tumor size was about 25 mm^2 and was carried out by 7 daily intravenous injections with 10 μg α-FAP-scmuTNF, 7 μg scmuTNF, or 7 μg muTNF (equimolar doses) in 150 μl PBS. PBS-treated animals received 7 daily injections with 150 μl PBS. Shown are mean tumor sizes ± SEM of day 10 after first treatment (n=6). Statistical analysis was done by unpaired t-test (two-tailed P values)

In an independent attempt to improve TNF's antitumoral activity and potential use for systemic application, we made use of the single-chain format explained above for targeted TRAIL fusion proteins to develop similar TNF-based fusion proteins. To this end, a FAP-specific tumor stroma targeting scTNF fusion protein has been generated. Cell death induction of this α-FAP-scTNF was clearly increased on FAP-positive compared to FAP-negative cells as well as compared to scTNF lacking the scFv targeting domain (Table 53.1). First, in vivo application in a tumor transplantation model with HT1080 and HT1080-FAP tumor xenografts indicates that the α-FAP-scTNF fusion protein, when given as single agent, is superior to non-targeted scTNF as well as standard TNF and displays higher activity on target antigen expressing compared to target-negative tumors (Fig. 53.5).

Bifunctional Death Ligand Fusion Proteins

Using targeting domains not derived from antibodies, but instead representing, for example, the extracellular domain of a member of the TNF receptor family may also result in fusion proteins converting into highly active membrane-like TNF ligands after specific cell surface immobilization. Furthermore, in this case fusion proteins potentially display dual functions, as the receptor module not only mediates binding to the targeted transmembrane ligand but also may interfere with signaling emanating from this ligand. Although in principle dual function could also hold true for a scFv-TNF ligand fusion protein, this is only achieved when the antibody-derived scFv fragment possesses activating or inhibiting function upon binding to the target antigen, which is less common. Nevertheless, such examples do exist, e.g., the CD20 targeted scFvRit:sFasL described above represents a paradigm of a bifunctional apoptosis inducer, which on CD20 and Fas-positive malignant B cells mediates apoptosis via both modules. TRAIL and CD95L-based fusion proteins targeted through the extracellular domain of CD40 to CD40L are examples of dual function death ligand fusion proteins targeted not via an antibody module. Activation of CD40 by membrane CD40L in an autocrine/paracrine manner protects against death receptor-induced apoptosis, as has been observed, e.g., in breast cancer and non-Hodgkin lymphoma. Thus, blocking this signaling pathway should lead to sensitization for apoptosis induction. Indeed, T47D breast cancer cells are barely sensitive to cell death induction despite adequate stimulation of CD95 and TRAIL death receptors. In contrast, CD40-CD95L and CD40-TRAIL fusion proteins were highly cytotoxic on this cell type [1]. Immobilization of the CD40-death ligand fusion proteins to the cell surface via membrane CD40L binding results in robust triggering of CD95 and TRAILR2, which now transduces the signal to an appropriate effector level due to the concomitant neutralization of CD40L-induced survival signaling [1]. The model for the proposed underlying mechanism is shown exemplarily for CD40-TRAIL in Fig. 53.6. Of note, similar to the above-described scFv-CD95L fusion proteins, the homotrimeric CD40-CD95L fusion protein exerted no apparent toxicity at doses up to 100 μg/mouse upon i.v.

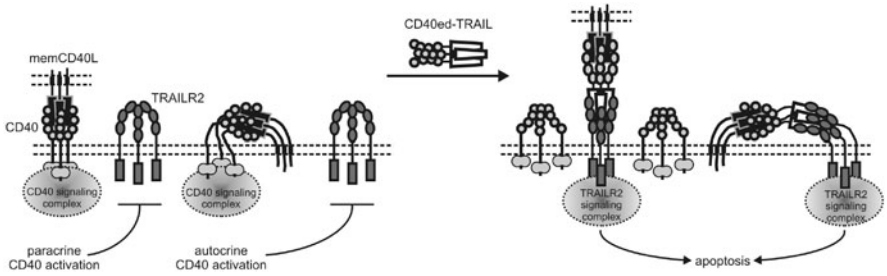


Fig. 53.6 Paracrine/autocrine activation of CD40 by membrane CD40L, as observed, for example, in breast cancer and non-Hodgkin lymphoma, protects against death receptor-induced apoptosis. CD40ed-TRAIL, a trimeric soluble fusion protein consisting of the extracellular domains of CD40 and TRAIL, on the one side inhibits this survival signaling loop by binding to membrane CD40L and on the other side achieves thereby the capability to robustly trigger TRAILR2 in the now sensitized cells

injection into mice. A further example for a dually active, non-antibody-based targeted fusion protein has recently been developed by Razmara et al. Fn14-TRAIL is composed of the extracellular domain of the TWEAK receptor Fn14 and TRAIL. Upon gene transfer, the dual function of this fusion protein, but not that of both components co-administered, led to significant attenuation of experimental autoimmune encephalomyelitis in mice by blocking the inflammatory action of TWEAK via the Fn14 domain and inhibiting activated, pathogenic T cells by TRAIL-induced apoptosis [20].

Conclusion

A series of examples now have accrued, clearly demonstrating that the full signaling capacity of soluble TNF ligands, that are inactive or only display activity on a subset of their receptors, can be reconstituted by genetic engineering. Such molecules provide an ideal platform for development of targeted protein therapeutics that meet clinically important criteria such as enhanced specific activity, stability, and adequate pharmacokinetic properties. Properly engineered fusion proteins based on the THD of these ligands and an antibody or non-antibody-derived targeting domain behave like the soluble ligand variants, but regain full signaling capacity upon cell surface immobilization by binding to the target antigen. Furthermore, in the case of TNF ligands already triggering side effects in the soluble version, generation of prodrug fusion proteins might be a promising additional strategy. Thus, side effects of such targeted TNF ligands may be alleviated not only by the reduction of dose due to target binding-dependent restoration of full bioactivity but also by the necessity for processing by tumor-associated proteases.

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Chapter 54
Computational Design of TNF Ligand-Based Protein Therapeutics

Almer M. van der Sloot and Wim J. Quax

Tumor necrosis factor (TNF) ligand family members and their cognate receptors activate several signaling pathways, eliciting activities ranging from cell proliferation to the induction of apoptosis. TNF-like ligands (TNF-Ls) and TNF-like receptors (TNF-Rs) are involved in a variety of biological processes, such as host defense, development, (auto)immunity, inflammation, and tumor surveillance [1, 2, 3]. Currently, 19 TNF ligands and 29 TNF receptors have been identified in humans [2, 4]. Several ligands have multiple receptors, and, conversely, some receptors also bind multiple ligands.

The TNF ligand family is characterized by a conserved C-terminal domain, the TNF homology domain (THD). This THD causes trimerization of the TNF-L and is responsible for receptor binding. All monomeric subunits of TNF ligands consist of antiparallel β-sheets, organized in a jellyroll topology, and these subunits self-associate in bell-shaped homotrimers, the bioactive form of the ligand. A trimer binds three subunits of a cognate receptor; each receptor subunit binds usually in the grooves between two adjacent monomer subunits. The ligands are type II transmembrane proteins (i.e., intracellular N-terminus and extracellular C-terminus), but the extracellular domain of some members can be proteolytically cleaved from the cell surface, yielding a bioactive soluble form of the ligand [1, 2, 5].

The distinguishing feature of the extracellular part of the TNF-like receptors is the cysteine-rich domain (CRD) and the number of CRDs in a particular receptor varies usually from one to four. Most of the TNF-R family members are type I or, in a few cases, type III transmembrane proteins. However, several exceptions exist, for example, some receptors are secreted as soluble receptors (OPG and DcR3) or are covalently linked to the cell membrane via a glycophosphatidylinositol (GPI) anchor (DcR1) [1, 2, 5]. The canonical model of TNF ligand-receptor signaling proposes that three receptor monomers are recruited upon binding of a trimeric ligand into a signaling complex with 3:3 stoichiometry (ligand monomer:receptor monomer)

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[6, 40, 76]. Receptor monomers cross-linked in this manner by the ligand subsequently transduce the signal into the cell. This simple model has been challenged by the fact that several TNF-R family members contain a pre-ligand assembly domain (PLAD) which allows the receptors to self-assemble in the absence of a ligand [6, 7]. Binding of a TNF ligand would then cause a rearrangement of the pre-assembled TNF-R complex and allow transduction of the signal. In addition, certain receptors are not fully activated upon stimulating with a soluble TNF-L but require the membrane-bound form of the ligand for activation or for full activation [8].

Signaling cascades downstream of TNF-Rs are mediated by various adaptor proteins that bind to intracellular domains of these receptors. These adaptors are generally recruited after binding of a TNF-L to its cognate receptor. Depending on the cellular context, some receptors are able to interact with multiple types of adaptor proteins: for example, TNF-R1 can bind to the TNF receptor-associated factor (TRAF2), mediating the activation of NF-κB and JNK, or to a death domain (DD) containing adaptor protein TRADD, regulating the activation of the caspase cascade, resulting in either cell proliferation or apoptosis. Deregulation of signaling pathways controlled by members of the TNF ligand family is implicated in the etiology and pathology of cancers and various autoimmune diseases.

Given this key involvement of several TNF ligands and receptors it is not surprisingly that this family is considered an attractive collection of therapeutic targets.

Currently, over 70 recombinant protein therapeutic products are on the market and approximately another 80 at various stages in clinical development [9]. The first recombinant protein therapeutics did have amino acid sequences identical to their natural counterpart. However, as natural occurring proteins have not evolved to be used as drugs, additional optimization might be required. Sequence/structure optimization using protein engineering has been an invaluable tool for the improvement of properties important for (putative) protein drugs, such as stability, affinity, specificity, solubility, immunogenicity, and pharmacokinetics, in order to obtain a variant with the desired characteristics.

Traditionally, the protein engineering tools of choice were “classical” rational design or directed evolution-based methods [10, 11]. Nowadays, a third tool has come of age: structure-based computational protein design (CPD). Progress in development and use of CPD has already resulted in impressive results such as several de novo designed proteins, as, for example, the design of a novel amino acid sequence able to fold into a predetermined structure [12] or the design of a new globular fold and structure [13].

From a more practical point of view, structure-based CPD algorithms have been extensively used to redesign (improve) properties of existing proteins. For example, these algorithms were successfully used to enhance the stability of various proteins [11, 14–20].

More recently, computational design has also been employed in the redesign of affinity and specificity of a wide variety of protein–protein interactions [21–32], which previously was the territory of phage display and related methods [33, 34]. In addition to redesign, CPD algorithms can also be employed in the analysis

of protein–protein interfaces, for example, using computational alanine scanning [35, 36]. Not only protein–protein interactions are subjected to design but also proteins with small molecule receptor functionality [37–40], enzyme–substrate [41, 42], and protein–nucleic acid interactions [43] have been modified – or were created – using computational protein design approaches [44].

Computational protein design (CPD) algorithms combine biophysical knowledge with in silico scoring to select optimal solutions, merging force fields [45] with efficient search algorithms [46, 47]. This permits screening of a much larger sequence space (up to 10^{80}) than is experimentally possible with selection methods or high-throughput screening techniques [48]. CPD algorithms employ usually an inverse protein folding approach, i.e., the algorithm determines which amino acid sequence is most compatible with a given protein three-dimensional backbone structure. A particular three-dimensional structure will have many sequences compatible with it while any given amino acid sequence only has one compatible three-dimensional structure. This makes inverse folding more tractable to solve computationally than protein folding problems [48]. The algorithm places discrete conformations (rotamers) of the naturally occurring amino acids at the positions considered for design. After simultaneous optimization of the conformations of the amino acids interacting with the substituted amino acid, the energy of the structure is determined, favorable substitutions are retained, and unfavorable substitutions are discarded (see Fig. 54.1). In many algorithms and depending on the particular design problem, the details of this basic scheme will differ (e.g., considering the protein backbone fixed or flexible).

In this review we will describe the application of computational protein engineering methods and their (potential) use in the design of TNF ligand-based protein therapeutics.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis in a variety of cancer cells but not in normal cells and therefore TRAIL draws considerable interest as a potential anticancer therapeutic [49–51]. TRAIL is a promiscuous ligand as it binds five receptors. Binding of TRAIL to death receptor 4 (DR4) or 5 (DR5) induces apoptosis, whereas decoy receptors DcR1, DcR2, and osteoprotegerin (OPG) can inhibit apoptosis induction (see Fig. 54.2).

We decided to modify the structural stability and receptor binding characteristics of TRAIL by employing protein design algorithms in order to improve its use as a future protein therapeutic. Availability of X-ray crystal structures or high-quality homology models is a prerequisite for the use of structure-based protein design algorithms. In the case of TRAIL this prerequisite is satisfied as several crystal structures are available of TRAIL or TRAIL in complex with DR5 [2, 3, 52]. However, currently no crystal structures exist of TRAIL in complex with its other receptors.

As mentioned above, improving the structural stability of a protein can be beneficial for its use as a protein therapeutic as it can improve biological activity, in vivo half-life, but also for storage and production purposes. This might be in particular the case for TRAIL: several studies showed that removing the zinc binding site located in the core of the TRAIL trimer, or removing zinc itself by extensive dialysis, had a severe negative effect on the structural stability and on the biological activity

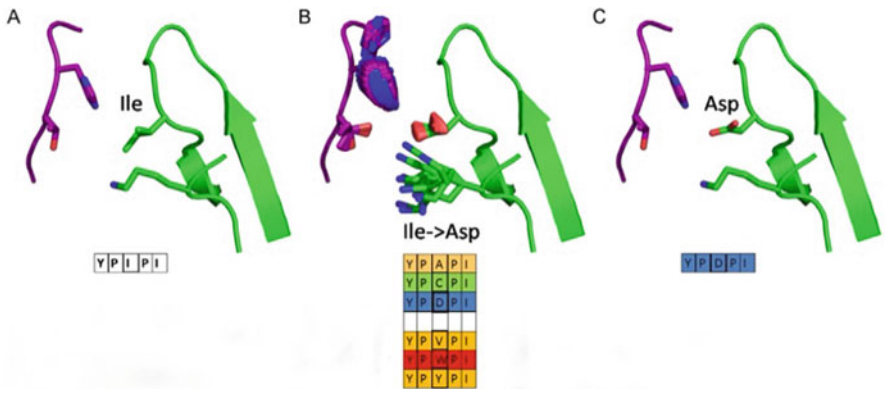

Fig. 54.1 The basic principles of a protein design algorithm. Structural overview of an interaction between a protein ligand (*green*) and its receptor (*purple*) at atomic resolution (1a). The CPD algorithm determines which amino acid sequence is most compatible with a given protein three-dimensional backbone structure. At the positions considered for design the algorithm "mutates" a residue to all other naturally occurring amino acids by replacing the original amino acid with discrete conformations of the substituting amino acid (rotamers). (As an example 1**b** depicts the different rotamers for the Ile to Asp mutation.) After simultaneous optimization of the side chain (and/or backbone) conformations of the substituted amino acid and the surrounding interacting amino acids the, energy of the structure is determined (**b**); favorable – low energy–substitutions are retained (**c**) and unfavorable – high energy–substitutions are discarded. An improvement in force field energy correlates directly with an increased structural stability, thus design of a lower energy variant in silico generally translates to a more stable protein in vitro. The strategy for improving affinity – or enhancing binding specificity – of a protein with one of its binding partners is similar to the one used for designing more stable proteins. The affinity of a certain interaction can be improved by optimizing the interaction with the target binding partner ("positive design"). On the other hand, in order to improve the specificity of an interaction one would also make interactions with any non-target partner less favorable ("negative design") [74]

of TRAIL. Moreover, the reported half-life of TRAIL in both mice (3.6 min) and non-human primates (27 min in chimpanzee) is very short [53]. Hence, improving the structural stability of TRAIL was considered a viable strategy to improve its therapeutic properties. As previous reports showed that exogenous N-terminal fusion histidine tags [54] or leucine zipper tags [55] for purification or stabilization purposes, respectively, could be toxic to normal cells, it was decided to only modify the soluble TRAIL domain (aa 114–281) and with a minimum of amino acid substitutions. A family alignment of TNF ligand members was constructed and used to focus the design toward non-conserved amino acid residues in TRAIL outside the receptor binding interface. As conserved residues are usually retained in a family for structural (or functional) reasons, any mutation at these positions is likely to compromise stability. In such cases, stability can be gained, but then usually requires extensive mutagenesis of surrounding residues in order to restore favorable interaction networks between these residues. On the other hand, amino acids at non-conserved positions are likely to be more tolerant toward substitution and stabilizing mutations might be easier to identify. Following this strategy, the number of mutations

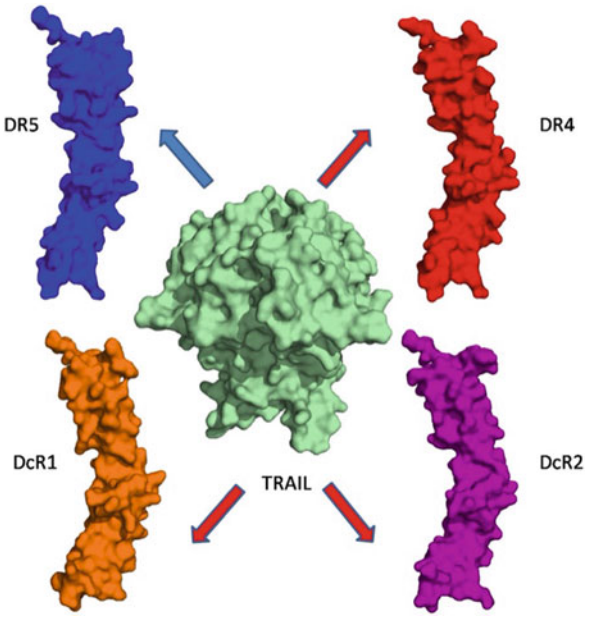

Fig. 54.2 TNF-related apoptosis-inducing ligand (TRAIL) exhibits extreme promiscuity in receptor binding. Binding of TRAIL to one of two death receptors (DR4 and DR5) leads to the induction of apoptosis. Binding of the very same TRAIL trimer to receptors DcR1 and DcR2, which lack a functional death domain leads to a non-signaling interaction and therefore DcR1 and DcR2 are named decoy receptors. OPG, not shown, is a soluble TRAIL decoy receptor

required to obtain a gain in structural stability could be minimized. Subsequently, a combination of the protein design algorithm PERLA [56] and the force field functionality of FoldX was used to determine stabilizing amino acid substitutions at the non-conserved positions of TRAIL. Stability characteristics and biological activity of the five highest scoring variants were experimentally verified. Four of the five tested variants showed a considerable improvement in structural stability without affecting biological activity: e.g., the highest scoring variant M1 (comprising substitutions E194I and I196S) could be incubated for 1 h at more than 70°C without affecting activity while TRAIL WT showed a large decrease in biological activity within 20 min of incubation [19]. This translates into a much improved resistance toward temperature-based unfolding/inactivation at more relevant temperatures such as room temperature or 37°C.

Next, receptor binding affinity and receptor binding promiscuity of TRAIL were addressed. Several studies indicate the need for such variants, for example: DR4 and/or DR5 were shown to be up-regulated after treatment with DNA damaging chemotherapeutic drugs and the response to TRAIL-induced apoptosis was significantly increased [57, 58]. In addition, irradiation appeared to specifically upregulate DR5 receptor expression and the combination of irradiation and TRAIL treatment had been demonstrated to have an additive or synergistic effect [59]. Colon and breast cancer cell lines were reported to signal primarily through DR5 [32, 60]. Other research demonstrated that primary cells isolated from patients with chronic lymphocytic leukemia and mantle cell lymphoma were almost exclusively sensitive to DR4-mediated apoptosis [61, 62]. Moreover, use of TRAIL

receptor-selective variants could permit better tumor-specific therapies through escape from the decoy receptor-mediated antagonism, resulting in a lower administrated dose, with possibly less side effects and as alternatives to existing agonistic receptor antibodies [63–65].

Using a crystal structure of the TRAIL/DR5 complex and homology models of TRAIL in complex with the other receptors, DR5-selective TRAIL variants were designed employing an improved version of the FoldX protein design algorithm [32]. Both positive design (improved affinity for DR5) and negative design (decreased binding to DR4, DcR1, and DcR2) were used. To prevent the complete redesign of the receptor binding interface, which would increase the chance of introducing immunogenic epitopes, only single amino acid substitutions were considered in the initial design, subsequently followed by double substitution variants generated by combining single substitution variants. Highest scoring mutants were constructed and expressed in *Escherichia coli* Bl21 DE3, and a fast in vitro surface plasmon resonance-based receptor binding screening assay of *E. coli* extracts was used to select variants with the most favorable DR5 specificity characteristics (see Fig. 54.3). Mutants showing the highest DR5/DR4 binding ratio were purified and more extensively characterized. In vitro receptor binding assays and biological activity assays demonstrated that the designed DR5 variants showed indeed an increase in affinity and a much improved specificity for the DR5 receptor, a double mutant variant (comprising substitutions T214R and D269H) was not capable of binding to DR4 at all [32] (Fig. 54.4). However, another variant (rhTRAIL-DR5, comprising substitutions E195R and D269H) showed a more potent apoptosis-inducing activity on DR5-responsive cancer cells and was chosen for follow-up studies. Both the maximum amount of cell death that could be achieved and the apoptosis-inducing activity (EC_{50}) in DR5-responsive cells were substantially improved when compared to rhTRAIL WT, while rhTRAIL-DR5 did not show activity in cancer cells exclusively responsive to DR4-mediated apoptosis. Importantly, and like TRAIL WT, rhTRAIL-DR5 did not show apoptosis-inducing activity or other toxicity toward non-cancer cells (HUVEC and fibroblast cells) [32]. In an in vivo ovarian carcinoma xenograft model rhTRAIL-DR5 was found to be superior to rhTRAIL WT, both as mono-therapy and in combination treatment with cisplatin [66].

Recently, rhTRAIL-DR5 was used to study TRAIL signaling. When measuring the rate of apoptosis induction, the DR5-specific variant exhibited a 17-fold increase compared to TRAIL WT, despite having only a threefold improvement in the association rate constant for DR5 receptor binding. With the aid of mathematical modeling it could subsequently be shown that receptor specificity was the most important factor for the observed increase in apoptosis induction rate: the tendency to form heteromeric receptor complexes was much reduced for receptor-specific rhTRAIL-DR5 than for promiscuous rhTRAIL WT. The formation rate of active homomeric DR5 complexes was significantly increased for this variant while rhTRAIL initially forms a large proportion of (inactive) heteromeric receptor complexes. This points toward a kinetically controlled antagonism-based mechanism by the decoy receptors (Szegezdi et al., in preparation).

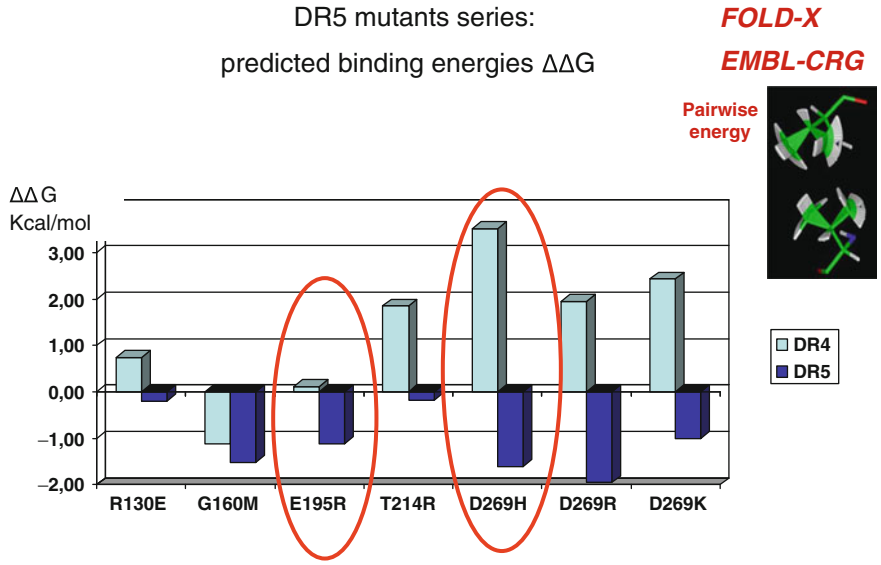

Fig. 54.3 Prediction of mutations contributing to DR5 selectivity of TRAIL. Following a systematic analysis of the interfaces between TRAIL and DR5 receptor and TRAIL and DR4 receptor (model) using FoldX a list of best mutations was proposed. The predicted differences in $\Delta\Delta G$ are shown. The combination of D269H and E195R turned out to be the mutant with the best specificity for the DR5 receptor

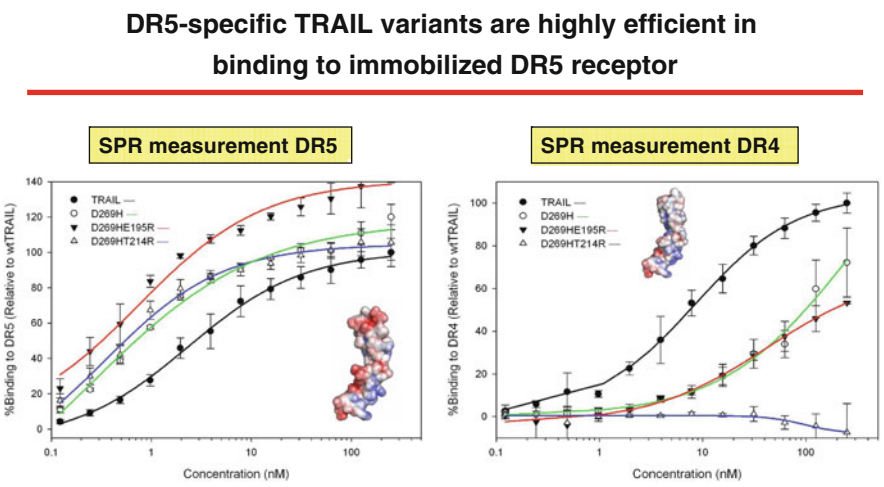

Fig. 54.4 Biophysical analysis of binding of single and double mutants to the two death receptors: DR4 and DR5. Mutants were constructed and purified as described [32] and the interaction with immobilized DR5 and DR4 receptors (Fc-fragments) was determined with surface plasmon resonance (BiaCore). It can be seen that all mutants have an improved binding to the DR5 receptor and a worsened binding to the DR4 receptor

The design of DR4-selective TRAIL variants was considerably more challenging than the design of DR5-selective TRAIL variants due to the lack of a crystallographic structure of TRAIL in complex with DR4. Because TRAIL WT has higher affinity for DR5 than DR4 [32, 67], the design of an effective DR4-selective TRAIL variant should preferably aim at both an enhanced affinity for DR4 and at decreased affinities for DR5 and decoy receptors. As for the design of DR5-selective TRAIL variants, it is essential to combine a positive design strategy strengthening the interactions between TRAIL and DR4 with a negative strategy that designs mutations disrupting interactions between TRAIL and the other receptors. Generally, it is less demanding to disrupt an existing interaction (or create an unfavorable one) by an amino acid substitution than to combine this with the creation of a new favorable interaction. Thus, the quality of the TRAIL-DR4 model for the purpose of designing a DR4-selective TRAIL is for that reason more important than for the design of the DR5-selective variants. A high-quality structural model containing all the relevant interactions between the interacting partners is therefore imperative. The homology model of TRAIL-DR4 was build using the protein design tools of FoldX [68]. First, the DR5 amino acid residues were mutated into the corresponding DR4 amino acids and, subsequently, all amino acid side chain interactions were optimized in order to accommodate TRAIL and receptor residues to their new interface. Subsequent design to identify amino acid substitutions conferring DR4 specificity was the same as described above for the DR5-specific TRAIL variants. Amino acid Asp 218 of TRAIL was identified as a "hot spot" for obtaining DR4 selectivity [68]. Receptor binding characteristics and biological activity of two mutants at position 218 (D218H and D218Y) were experimentally characterized. These DR4-selective rhTRAIL variants showed a lowered DR5 affinity in concert with increased DR4 specificity in receptor binding tests. In cell line assays the variants showed agonistic DR4 specificity. These results combined show that the variants D218H and D218Y have become DR4 selective. However, although specific for DR4, these variants did not show an increase in DR4-mediated apoptosis-inducing activity. It is likely that multiple mutations have to be combined in order to create a variant with a substantial gain in DR4 specificity and activity. Therefore, we designed two variants (G131R and G131K) that show improved affinity for DR4 and DR5, with the highest affinity increase for the DR4 receptor [69].

Interestingly, Kelley et al. used phage display to generate DR4- and DR5-selective TRAIL variants. Their best DR5-selective variant contained six amino acid substitutions. After a partial dissection to determine the role of each individual mutation in conferring selectivity of the phage display variants, Kelley et al. concluded that it was not possible to eliminate any mutation without compromising selectivity and/or losing biological activity [60]. The phage display approach did not identify any of the selectivity causing mutations as determined with the computational design approach. Subsequent analysis revealed that the DR4-selective TRAIL variant of Kelley et al. containing six mutations was biologically inactive [61]. Activity of this mutant could be restored after reverting one of the mutations back to the wild-type amino acid, but the effect on receptor selectivity was not examined [61].

Molecules that inhibit tumor necrosis factor-alpha (TNF-α) signaling have demonstrated clinical efficacy. The currently used molecules are antibodies or soluble TNF receptors which sequester TNF-α. Steed et al. used the CPD to design a dominant-negative TNF-α (DN-TNF, XPro-1595) variant that prevents formation of active TNF-α trimers and, consequently, inhibits TNF-α signaling [31]. By applying an in silico screen, only non-immunogenic amino acid substitutions were selected and considered for testing. Empirical validation showed both in vitro and in vivo a large reduction in TNF-α-mediated effects [31]. An added benefit of the DN-TNF inhibitor is that it does not compromise host immunity against *Mycobacterium tuberculosis* infections, a side effect observed when using anti-TNF-α antibodies or soluble TNF receptor [70, 71].

These studies demonstrate the power of the use of computational design algorithms and show that both computational design algorithms and directed evolution methods can be complementary.

Concluding Remarks

Several members of the TNF ligand and TNF receptor family are of considerable interest as therapeutic targets. The most famous examples are Etanercept, a soluble Ig-TNF-R2 receptor fusion protein, and antibody-based inhibitors of TNF-α in the treatment of modest and severe rheumatoid arthritis (e.g., Infliximab and Adalimumab). Moreover, several other therapeutics interfering with or modifying signaling of TNF ligand/receptor members are currently in various stages of clinical development, these include inhibitory antibodies against Baff/Blys (Belimumab) or Lymphotoxin-α, various agonistic antibodies against DR4 and DR5 and rhTRAIL WT (Dulanermin).

Both rational and directed evolution-based protein engineering methods have been extensively used in order to improve or alter properties such as receptor binding affinity, receptor selectivity, in vivo half-life, or other characteristics of TNF ligand members [60]. In here we reviewed the application of computational protein design in the modification of receptor binding specificity, binding affinity and stability of TRAIL, and the design of an inhibitory TNF-α variant. These examples show that computational protein design can be successfully used to obtain variants having favorable properties for future use as therapeutics and these properties are lacking in the native counterparts. For example, the DR5-specific TRAIL variant was shown to be a more potent inducer of apoptosis, both in vitro and in vivo, in DR5-responsive cancer cells and it was also shown to induce apoptosis 17-fold faster than TRAIL WT. In addition, aside from being a tool for designing improved protein therapeutics, computational protein design is also an excellent tool for studying and understanding biological systems.

However, despite the progress made, several challenges remain to be addressed. Structure-based algorithms require three-dimensional structural information, and in general the rule is that a higher resolution of the design target's (crystal) structure

yields a more reliable design outcome. Notwithstanding this requirement, several design exercises successfully relied (partly) on low resolution structural models or homology models as template structure. Moreover, structural genomics initiatives will make an increasing proportion of the protein sequence space amenable to protein design algorithms either by directly providing an experimentally determined structure or by providing structural templates to build more reliable homology models [72]. Recent progress in de novo protein structure prediction, by employing structure-based computational design algorithms, could in the future also contribute to make an even larger proportion of the sequence space amendable to structure-based design [73].

Furthermore, sequence-based algorithms and structural-based computational design algorithms can be interfaced to permit the design of protein therapeutics with enhanced clinical efficacy by optimizing their amino acid sequences for various clinical important properties such as potency, specificity, immunogenicity, and pharmacokinetics. By addressing these clinical properties of biopharmaceuticals early on in drug discovery and development as well as the optimization of its production properties, the use of computational methods will allow a faster transition from laboratory bench to clinic.

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Part XI
Roles of the TNF Family in Neuronal Development, Function, and Pathology

Chapter 55
Workshop Summary: Roles of the TNF Family in Neuronal Development, Function and Pathology

Malú G. Tansey and Tony Wyss-Coray

Once believed to be "immune privileged" and relatively protected from the effects and influence of the immune system, it is now well established that the brain is capable of mounting inflammatory responses in response to various stimuli. In addition, proteins once believed to be solely expressed in immune organs (and in particular TNF superfamily members and their receptors) have now been demonstrated to have important roles in normal central nervous system (CNS) function. Importantly, cross talk between the brain and the immune system and its regulation are critical determinants of normal central and peripheral nervous system (PNS) physiology and dysregulation of such is believed to underlie a number of chronic neurological disorders. While the role of inflammation in pathological conditions such as multiple sclerosis can be clearly appreciated, there are a number of other CNS and PNS disorders in which the role of the immune system is not well understood yet neuroinflammatory responses appear to be dysregulated and may be contributing to disease progression and neuronal loss. Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease fall within this category and affect a large number of individuals over age 65. Therefore, investigations aimed at gaining a better understanding of the role of TNF superfamily signaling cascades in normal brain function or in neurological disorders is of high importance and extremely timely in light of the rising number of aging individuals worldwide.

In the first paper, Wyss-Coray presents compelling new data on the signaling "communicome" in serum samples of patients with Alzheimer's which suggest clear involvement of inflammatory cascades in the etiology and progression of this neurodegenerative disease. In the second paper, Tansey presents a working model

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on the various genetic and environmental triggers which may chronically activate brain-resident microglia populations and induce overproduction of inflammatory mediators which enhance pro-apoptotic cascades within dopaminergic neurons and experimental evidence for the potential use of "dominant negative" TNF variants for blocking the neurotoxic effects of TNF on dopaminergic neurons in the ventral midbrain that are lost in Parkinson's disease. Taoufik implicates a role for caspase-8, a key executor protein of programmed cell death induced by Fas and TNF, in glutamate/NMDA receptor-mediated death and seizures in the CNS. Urlich Eisel and colleagues demonstrate a neuroprotective role for TNFR2 against glutamate-induced neuronal death mediated through activation of protein kinase B/Akt and NF-κB and the regulation of calcium-activated potassium channels which regulate the opening of calcium permeable NMDA receptors [38] Calcium flux through NMDARs is thought to play a critical role in *synaptic plasticity*, a cellular mechanism for *learning* and *memory*. The paper by Emmanouli reports that neuronal NF-κB may play a critical role in modulating the severity of autoimmune demyelinating disease, not only by enhancing neuroprotection but also by suppressing CNS immune responses. Laskey presents that it may be possible for TNF to interfere with NGF-induced Erk-mediated differentiation of neuroblastoma cells through activation of Akt. Moubarak presents data implicating cFLIP, a typically anti-apoptotic protein in TNF superfamily signaling cascades, in NGF/TrkA-mediated neuronal differentiation through activation of ERK and NF-κB activation. Stellwagen presents a role for glial-derived TNF in a form of synaptic plasticity termed homeostatic synaptic scaling. In the brain when neural activity is reduced, compensation for long-term reduction in neural activity can be achieved through increased glial release of TNF which increases excitation and reduces inhibition in neural circuits. Specifically, through activation of neuronal TNFR1 receptors, TNF induces exocytosis of AMPA-type glutamate receptors and results in an increase of excitatory synaptic strength. Concurrently, TNF also triggers endocytosis of GABA-A receptors and a decrease in inhibitory synaptic strength. The use of a TNF neutralizing antibody prevents the activity blockade-induced homeostatic plasticity, which is also absent in tissue from TNF-deficient mice. In addition, TNF may be required for visual cortical plasticity induced by monocular deprivation during the critical period of visual system development. Nikolaev presents compelling evidence that the orphan death receptor 6 (DR6) may have important roles in axonal degeneration during trophic factor deprivation. DR6 binds specifically to the soluble form of the amyloid precursor protein (APP) and is induced by it to activate a novel cell death pathway in peripheral nerve axons mediated by the pro-apoptotic master regulator protein Bax and caspase-6 in a caspase-3-independent manner.

Chapter 56
TNF: A Key Neuroinflammatory Mediator of Neurotoxicity and Neurodegeneration in Models of Parkinson's Disease

Melissa K. McCoy, Kelly A. Ruhn, Armin Blesch, and Malú G. Tansey

Microglia activation and overproduction of inflammatory mediators in the CNS have been implicated in Parkinson's disease (PD) [1]. Epidemiological studies suggest that chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) at lower doses is associated with lower incidence of idiopathic PD compared to non-NSAID users [2, 3, 4]. However, key molecular mediators of neurotoxicity that directly contribute to neurodegeneration have not been identified. A role for the pro-inflammatory cytokine tumor necrosis factor (TNF) has been implicated in PD (reviewed in [5]). Nigral midbrain dopaminergic (DA) neurons are extremely sensitive to TNF [6], and the CSF and post-mortem brains of patients with both diseases display elevated levels of TNF [7, 8]. Lastly, although no robust genetic association between TNF and development of PD has been demonstrated, a single nucleotide polymorphism (SNP) in the TNF promoter gene has been associated with a rare form of early-onset idiopathic PD [9]. Using engineered dominant-negative TNF variants (DN-TNFs) [10] and the decoy TNF receptor etanercept, we investigated the extent to which TNF-dependent mechanisms are required for loss of DA neurons in vitro and in vivo in two different models of parkinsonism.

Our results revealed that inhibition of TNF signaling in vitro with DN-TNF or etanercept attenuated DA neuron loss even after delayed administration. In vivo unilateral intrastriatal injections of 6-OHDA or intranigral chronic low-dose LPS infusion resulted in a 65–70% loss of ipsilateral nigral DA neurons, while co-administration of DN-TNFs on the lesion side reduced neuronal loss by half and attenuated ipsiversive circling behavior in 6-OHDA-lesioned rats [11]. To circumvent the inherent limitations of chronic infusion devices and the short half-life of peptides, we designed a lentiviral vector encoding DN-TNF and validated its ability to induce expression of DN-TNF protein (as measured by ELISA) in primary neuron–glia cultures transduced with the lentivirus. We next investigated the extent to which lenti-DN-TNF could rescue ventral midbrain DA neurons in a rat 6-OHDA model. Our results revealed robust transduction of glia in vivo, attenuated

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loss of nigral DA neurons, and a behavioral correlate that indicated reduced striatal DA depletion [12]. Experiments are underway to identify targets and signaling pathways that transduce the neurotoxic effects of TNF as well as molecular regulators of microglial TNF production in models of nigral degeneration. Multiple downstream effector pathways are likely to be involved in DA neuron death induced by TNF, including the JNK and ceramide signaling pathways.

In summary, rescue of nigral DA neurons achieved by in vivo administration of DN-TNF inhibitors and lentiviral vectors encoding DN-TNF has led us to conclude that TNF is a critical effector of ventral midbrain DA neuron dysfunction and subsequently degeneration. Results from these studies are providing new clues about the role of pro-inflammatory mediators in progression of PD in humans. Timely inhibition of the TNF pathway may slow the progressive loss of neurons in both PD and perhaps other neurodegenerative disorders associated with chronic inflammation.

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Chapter 57
The Contribution of TNFα to Synaptic Plasticity and Nervous System Function

David Stellwagen

The immune and nervous systems are generally thought to operate independently; however, evidence is accumulating proving that they interact in a variety of important ways. Recently, it has become clear that many immune molecules are used constitutively by the nervous system as signaling molecules, and disruption of this signaling through immunological challenge can contribute to neuronal dysfunction [1, 2]. One such example would be the pro-inflammatory cytokine tumor necrosis factor α (TNFα), principally known for its role in the inflammatory signaling cascade of immune cells.

Tumor necrosis factor α is a pleiotropic cytokine extensively characterized for its role in the immune system. While TNFα had been originally been thought to have a neuroendocrine effect on the nervous system, there is increasing evidence that TNFα may have an intrinsic neuro-modulatory function, in addition to its well-characterized role in the inflammatory response and effects on cell survival [3, 4]. However, a purely neuronal function for TNFα had not been hypothesized. Recently we demonstrated that TNFα regulates neurotransmitter receptor trafficking, with exogenous application of TNFα leading to a rapid exocytosis of glutamate receptors [5] and an increase in synaptic strength.

It is well established that glutamate is the main excitatory transmitter for the central nervous system and signals primarily through AMPA receptors (AMPARs) [6]. Synapses are highly regulated in terms of their content of neurotransmitter receptors, maintaining relatively constant receptor number at synapses despite the continual cycling of individual receptors. However, this relatively constant quantity of receptor content can change in response to specific stimuli [6]. This plasticity of glutamatergic synapses is one of the fundamental characteristics of the nervous systems and is thought to underlie learning and memory, including aberrant forms of learning such as drug addiction and neuropathic pain, as well as the activity-dependent

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refinement of connectivity seen during development. Further, over-activation of glutamatergic synapses contributes to the exocitotoxic neuronal cell death associated with many neuropathies.

Two general forms of synaptic plasticity that operate on very different timescales are thought to contribute to the activity-dependent refinement of neural circuitry: (1) Hebbian forms of plasticity, whose exemplars long-term potentiation (LTP) and long-term depression (LTD) involve rapid adjustments in the strengths of individual synapses in response to specific patterns of correlated synaptic activity [6] and (2) homeostatic synaptic plasticity which entails uniform adjustments in the strength of all synapses on a given cell in response to prolonged changes in the cell's electrical activity [7, 8]. Homeostatic plasticity is critical for the stability of neuronal circuits, providing negative feedback to counter the positive feedback nature of Hebbian plasticity, as well as adapting cells to the huge increase in input activity seen during development [9]. Both types of synaptic plasticity are believed to result, at least in part, from the alteration in the number of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type ionotropic glutamate receptors found at excitatory synapses [6, 10].

In the case of homeostatic synaptic plasticity, following a prolonged period of low activity, the excitatory synapses on primary neurons are strengthened due to insertion of additional AMPA receptors while the inhibitory synapses weaken due to removal of GABA receptors. The inverse occurs following prolonged periods of heightened cell activity. Thus the ratio excitatory-to-inhibitory drive on the cell is altered in such a way as to return the cell to a median firing rate. For example, such a mechanism appears necessary for the gain in responsiveness seen following visual deprivation (see below). Further, all the synapses on the cell are believed to be modified in a uniform, proportional manner, preserving the relative strength differences between synapses, while altering the overall excitatory drive on the cell. Due to this feature, homeostatic synaptic plasticity is often referred to as synaptic scaling [11].

Stability of neuronal firing rates is likely to be a fundamental and critical feature of neuronal circuits, and there are quite likely multiple, possibly redundant, forms of homeostatic plasticity [7, 12]. It seems probably that there are cell-autonomous [13] and non-cell-autonomous [14] forms, with distinct mechanisms. One of the first identified molecular mechanisms underlying synaptic scaling was the pro-inflammatory cytokine tumor necrosis factor alpha (TNFα) [14]. There has subsequently been a surge of interest in the molecular underpinnings of synaptic scaling, with recent work suggesting roles for the immediate early gene Arc [15, 16], retinoic acid [17, 18], and polo-like kinase 2 (PLK2) phosphorylation of the cytoskeletal protein SPAR [19]. The data on ARC and PLK2 are more suggestive of a role in the scaling down of excitatory synapses induced by increased levels of activity, with retinoic acid and TNFα more clearly involved in the scaling up of excitatory synapses sparked by prolonged activity blockades. The relationship between these various molecules, and the differentiation between modulators versus key signaling pathways, remains elusive, and much work remains to be done to develop a full mechanistic understanding of synaptic scaling.

TNFα remains the most attractive starting point for such an investigation. It is a tightly regulated signaling molecule that directly induces trafficking of both AMPA and GABA receptors. Further, unlike other candidate molecules, TNFα has no known role in Hebbian forms of plasticity [14], thus suggesting that TNFα does not have a general role in all forms of plasticity, but yet is still clearly required for at least one type of synaptic scaling. TNFα remains an excellent candidate to be a key mediator of the homeostatic synaptic plasticity observed following a global reduction in activity, and we are close to developing a mechanistic explanation for this important form of synaptic activity.

Recently we demonstrated that TNFα regulates neurotransmitter receptor trafficking, with exogenous application of TNFα leading to a dramatic and rapid exocytosis of AMPA receptors [5]. Within 15 min, application of exogenous TNFα to mature hippocampal cultured neurons resulting in a dramatic upregulation of the surface expression of AMPA receptors (Fig. 57.1) strongly suggests that TNFα is causing the exocytosis of an intracellular pool of existent receptors. Some of these

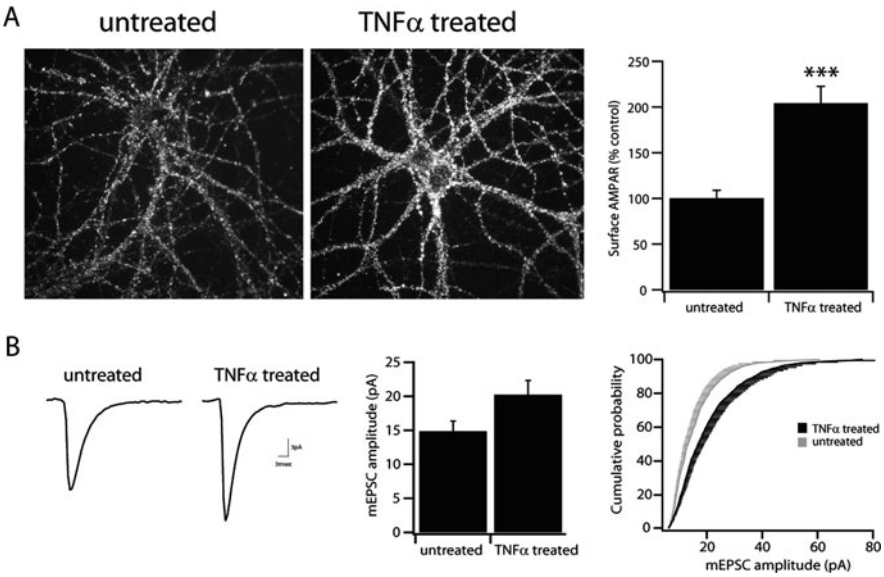

Fig. 57.1 TNFα treatment increases the surface expression of AMPA receptors and synaptic strength. (**a**) Representative micrographs from sister cultures of non-permeabilized isolated neurons immunostained for surface expression of the AMPA receptor subunit GluR1. The right-hand cells were treated with 100 ng/ml of TNFα for 15 min. Group data from 70 micrographs from untreated or TNFα-treated cultures showing a substantial increase in the surface area occupied by AMPA receptors. (**b**) Representative mEPSCs recorded from untreated and TNFα-treated cultures. The group data of average mEPSC amplitude of untreated (n=13) and TNFα-treated cells (n=15) show a significant increase in average mEPSC amplitude (p<0.05) in the TNFα-treated cells. The cumulative distribution of mEPSC amplitudes (*far graph*) demonstrates a significant rightward shift of TNFα-treated mEPSC amplitudes (*black*) compared with untreated cells (*gray*; p<0.002) (adapted from [20])

receptors are being inserted into synaptic sites, as we also observe an increase in miniature excitatory post-synaptic currents (mEPSCs; Fig. 57.1). mEPSCs result from the spontaneous fusion of glutamatergic vesicles at individual synapses and represents the unitary activation of single synapses. An increase in mEPSC amplitude represents an increase in synaptic strength and most likely is due to an increase in post-synaptic receptor number.

TNFα is not just inducing a pathological response in the neurons; rather, there is continual release of TNFα in both primary neuronal cultures and from acute hippocampal slices preparations, which is maintaining the surface expression of a population of AMPARs. Interfering with TNFα signaling, by bathing the tissue with a soluble form of the TNFα R1 receptor (s TNFR) for extended periods of time, results in the gradual reduction of the surface expression of AMPA receptors [5]. This suggests that TNFα is being constitutively released in neuronal tissue and not by infiltrating immune cells. The regulation of AMPA receptor trafficking occurs through activation of the TNF-R1 receptor, as activation of this receptor alone is as effective as TNFα in increasing the surface expression of AMPA receptors, while inhibiting this receptor prevents TNFα from causing the exocytosis of AMPA receptors (Fig. 57.2). Modulating the activity of TNF-R2 has no effect on AMPA receptors [20].

TNFα appears to be unique in its role in the nervous system, as other pro-inflammatory cytokines do not similarly regulate synaptic function. Exogenous IL-1β can drive the surface expression of AMPA receptors (Fig. 57.3a), in a similar fashion to TNFα, though with lower efficacy [20]. However, blocking IL-1β signaling does not reduce the surface expression of AMPA receptors, suggesting that IL-1β is not normally released in the nervous system (Fig. 57.3b). Exogenous IL-6 had no significant effect on AMPA receptor trafficking [20]. The TNFα −/− mouse also has distinct neuronal phenotypes, which also suggests that other pro-inflammatory cytokines do not compensate for TNFα in the nervous system. Anti-inflammatory cytokines, such IL-10, also do not seem to regulate AMPA receptor trafficking [20], although it remains to be seen if IL-10 can prevent the acute effects of TNFα on AMPA receptors.

TNFα also results in the insertion of a particular subtype of AMPA receptor, the GluR2-lacking AMPA receptor [20], which is Ca^{2+} permeable and therefore could also alter the threshold for synaptic plasticity or excitotoxicity. This subclass of receptor has also been implicated in a number of disease states [21]. AMPA receptors are thought to be tetramers comprised of the subunits GluR1-4, although GluR1-3 are the predominant subunits in the hippocampus. Most AMPA receptors on principal cells are GluR1/GluR2 or GluR2/GluR3 heteromers [6, 22], although GluR2-lacking AMPA receptors are observed in some circumstances (see [20, 23]). Critically, GluR2 confers calcium impermeability to the AMPA receptor as well as resistance to certain inhibitors, such as HPP-spermine [24, 25, 26]. Regulation of the surface expression of non-GluR2 containing AMPA receptors is important for a host of biological functions and dysfunctions [21, 27]. Availability of non-GluR2 containing AMPA receptors is critical to the pathogenesis of ALS [28, 29] and to the development of neuropathic pain [30]. Drugs of abuse also increase the

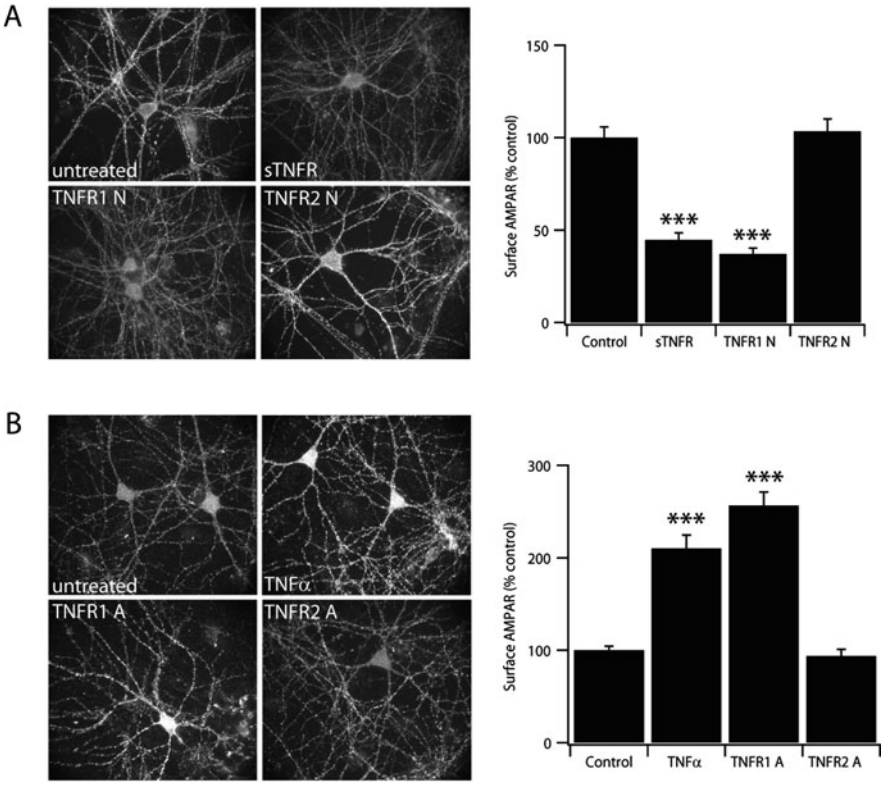

Fig. 57.2 TNFα acts through TNF-R1 to increase surface AMPARs. (**a**) Representative micrographs and composite data from cultures treated for 24 h with a soluble TNF receptor (sTNFR; n=54) decrease the surface expression of AMPARs relative to untreated control cells. Twenty-four-hour treatment with an neutralizing antibody for TNF-R1 (TNF-R1 N; n=66) also causes a similar decrease in AMPAR surface expression. Neutralizing antibodies for TNF-R2 (TNF-R2 N; n=84) were ineffective at decreasing AMPAR surface expression. (**b**) Sample micrographs and composite data demonstrating that an activating antibody for TNF-R1 (TNF-R1 Ag; n=81) increases the surface expression of AMPARs to a similar degree as sister cultures treated with TNFα (n=69), while an activating antibody for TNF-R2 (TNF-R2 Ag; n=88) was ineffective at increasing surface AMPARs. (From [20])

surface expression of non-GluR2 containing AMPA receptors in the reward pathway [31], and these receptors may exocytose during LTP ([32], but see [33]). Further, both exogenous TNFα and homeostatic synaptic plasticity cause the exocytosis of GluR2-lacking AMPA receptors [20, 23, 34, 35, 36], although Turrigiano has reported that GluR2 is necessary for synaptic scaling, using RNAi knockout of GluR2 in individual neurons [37].

These data give strong evidence that TNFα has a direct effect on neurons; however, our data indicate the continual release of TNFα is not from neurons themselves but from the neighboring glia [5]. An increasing body of literature supports the idea

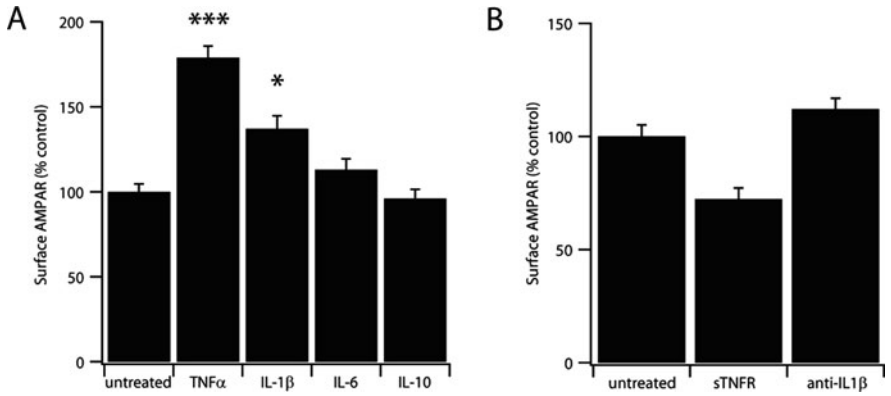

Fig. 57.3 Other cytokines do not compensate for TNFα in regulating AMPARs. (**a**) Composite data of surface AMPARs from cultures treated with TNFα (100 ng/ml, n=214), IL-1β (50 ng/ml; n=140), IL-6 (32 ng/ml, n=159), or IL-10 (40 ng/ml; n=130). (**b**) Group data of surface AMPARs from cultures treated for 24 h with sTNFR (10 μg/ml; n=59) or an antibody against IL-1β (40 μg/ml; n=72) (adapted from [20])

that glia are doing more than passively providing support for neurons [38, 39, 40, 41], but rather play an active role in modulating the behavior of neuronal circuits including promoting the formation of synapses and early synaptic function [42, 43]. The data on TNFα, as well as glial release of D-serine and ATP, extend these observations by demonstrating that glia are actively involved in mediating some forms of activity-dependent plasticity at established mature synapses [44]. Thus, glia may be actively involved in both the establishment of neural circuits as well as their subsequent activity-dependent regulation.

TNFα also regulates neuronal inhibition, by causing an endocytosis of the $GABA_A$ receptor, the principal mediator of fast inhibition in the brain [20]. Thus, TNFα drives AMPA receptors into synapses leading to an increase in excitatory synaptic strength, while simultaneously leading to an endocytosis of $GABA_A$ receptors and a decrease in inhibitory synaptic strength. The receptor trafficking induced by TNFα shares many similarities with the trafficking underlying homeostatic synaptic plasticity, suggesting the hypothesis that TNFα may underlie the homeostatic plasticity induced by chronic activity blockade. Interestingly, TNFα signaling is not required for Hebbian forms of plasticity [14]. However, there are compelling data that TNFα is a critical mediator of synaptic scaling. Interfering with TNFα signaling prevents the increase in strength of excitatory synapses (and also prevent the decrease in inhibitory synaptic strength) induced by chronic activity blockade (Fig. 57.4). The increase in excitatory synaptic strength in response to activity blockade is also absent in TNFα −/− mice. Curiously, the decrease in excitatory synaptic strength in response to increased neuronal activity is still present (Fig. 57.4), suggesting that a second signal mediates changes in opposition to TNFα, perhaps involving Arc signaling [15, 16] or PLK2 activation [19]. Moreover, the TNFα is of glial origin, as TNFα −/− neurons grown on wild-type glia demonstrate

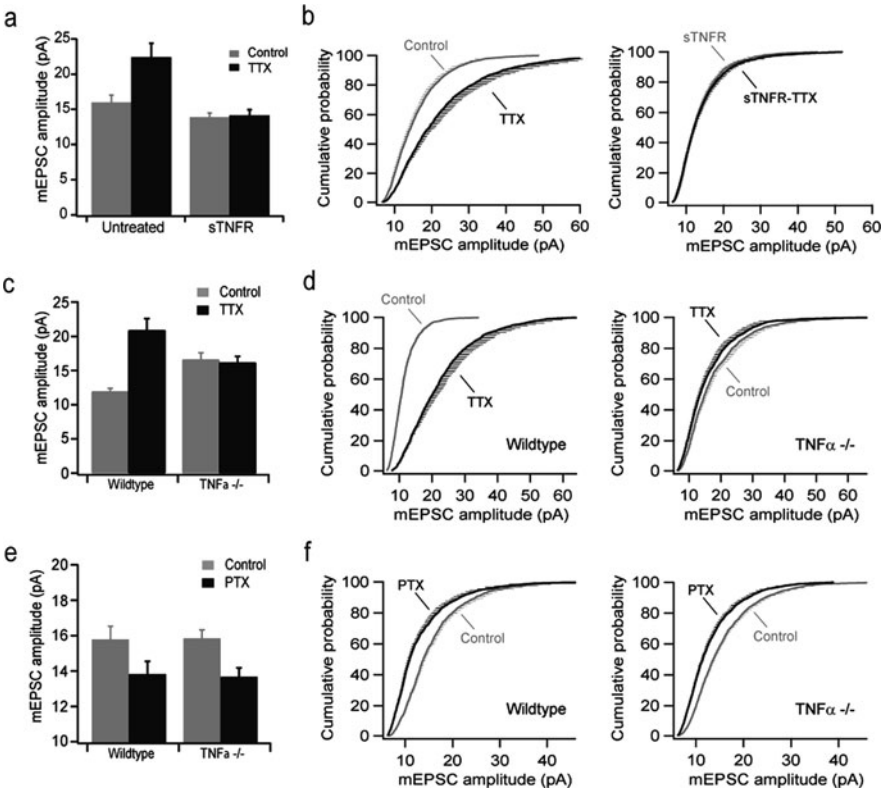

Fig. 57.4 TNFα mediates homeostatic synaptic plasticity during activity blockade. (**a**) Group data showing that addition of sTNFR during TTX treatment prevents the increase in mEPSC amplitude seen in TTX-treated rat cultures (control: n=15–17, p<0.05; sTNFR treated: n=16, p>0.7). (**b**) Cumulative amplitude distributions of control (*gray*) and TTX-treated (*black*) cells, showing a rightward shift in mEPSC amplitudes due to TTX and no shift in distribution when also treated with sTNFR. (**c**) Mean mEPSC amplitude of control and TTX-treated neurons from wild-type mouse cultures (n=12; p<0.0001) and TNFα −/− mouse cultures (n=15–17; p>0.7). (**d**) Cumulative distribution of mEPSC amplitudes of wild-type (*left* graph) and TNFα −/− (*right* graph) mouse cultures, for control cells (*gray*) or TTX-treated cells (*black*). (e) Mean mEPSC amplitude from control (*gray*) and PTX-treated (*black*) neurons, from wild-type mouse cultures (n=12–13; p<0.03) and TNFα −/− mouse cultures (n=17–20; p<0.002). (**f**) Cumulative distributions of the mEPSC amplitudes from wild-type (*left*) and TNFα −/− (*right*) mouse cultures (adapted from [14])

synaptic scaling, while wild-type neurons grown on TNFα −/− glia do not [14] (Fig. 57.5). These data support the hypothesis that glial TNFα mediates a form of homeostatic plasticity.

The release of TNFα from glia is regulated by activity. There are relatively low levels of TNFα in the conditioned media from untreated neuronal/glial cultures (roughly 25 pg/ml), but this level increases 4- to 5-fold following the chronic blockade of activity [14]. Similarly, glial cultures release relatively high levels of TNFα

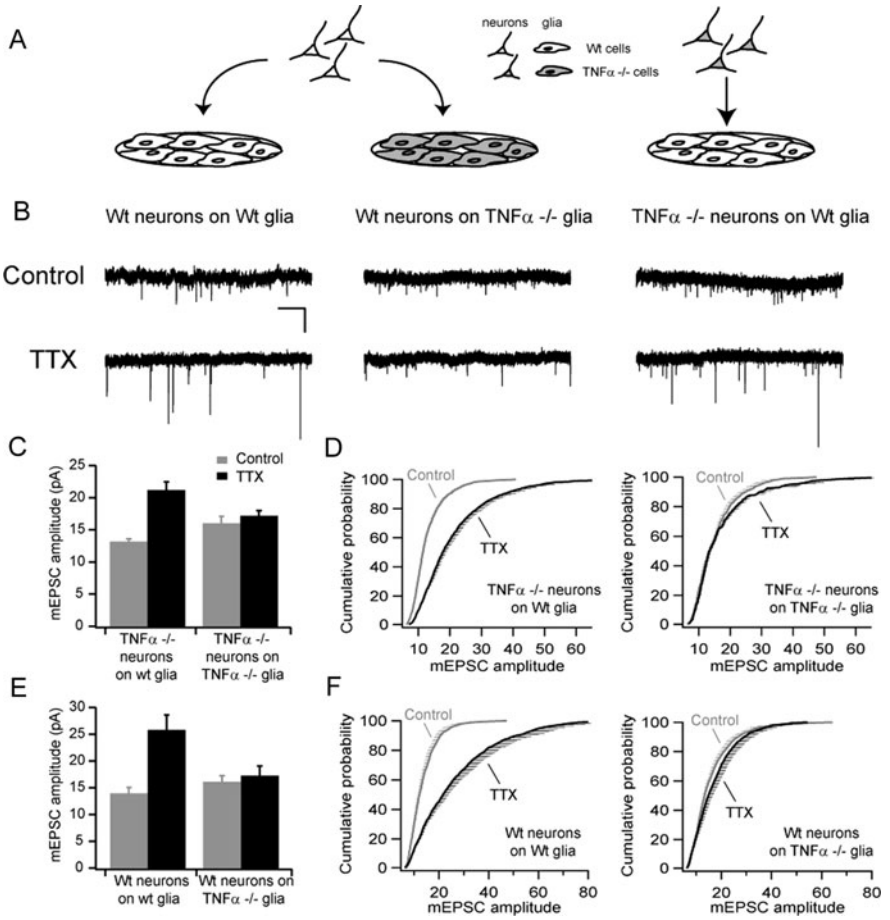

Fig. 57.5 Glia are the source of TNFα that is required for synaptic scaling during activity blockade. (a) Schematic depicting that wild-type (Wt) mouse neurons (*white*) were plated onto beds of either wild-type (*white*) or TNFα −/− (*gray*) mouse glia. Similarly, TNFα −/− neurons (*gray*) were plated onto wild-type mouse glia. (**b**) Representative recording of mEPSCs from control and TTX-treated cells, from neurons grown on different types of glia. Scale bar: 1 s; 20 pA. (**c**) Mean amplitudes of mEPSCs recorded from TNFα −/− neurons showing TTX-induced increases in mEPSC amplitude compared to control cells occur when TNFα −/− neurons are plated on wild-type glia (n=15–17; p<0.0001) but not on TNFα −/− glia (n=6, p>0.4). (**d**) Cumulative distribution of mEPSC amplitudes for TNFα −/− neurons grown on wild-type (*left*) or TNFα −/− glia (*right*). (**e**) Mean amplitudes of mEPSCs recorded from wild-type neurons showing TTX-induced increases in mEPSC amplitude compared to control cells occur when wild-type neurons are plated on wild-type glia (n=9–10; p<0.002) but not on TNFα −/− glia (n=12–14; p>0.6). (**f**) Cumulative distribution of mEPSC amplitudes for control and TTX-treated wild-type neurons plated on wild-type glia (*left* graph) or wild-type neurons plated on TNFα −/− glia (*right* graph) (from [14])

(presumably because these cultures lack neurons and therefore have no neuronal activity), but this is reduced by roughly half following the chronic administration of glutamate (which may mimic activity) [14]. Fully characterizing how glia sense neuronal activity and how this regulates TNFα production or release is an ongoing area of research.

The above data establish a role for TNFα in homeostatic plasticity in vitro. To extend these findings to the intact animal, we turned to a model for developmental plasticity, involving the re-adjustment of the visual system to a chronic decrease in activity. Critically, we find that mice deficient in TNFα have a deficit in visual cortical plasticity in precisely the manner theoretically predicted by loss of homeostatic synaptic scaling due to a reduction of neuronal activity [45]. Monocular deprivation during a critical period of development results in a profound shift in the responses in primary visual cortex, with an initial loss of responsiveness to the closed eye, followed by a gain of responsiveness to the open eye. TNFα-deficient mice have the normal loss of responsiveness to the closed eye, but are completely deficient in the gain of responsiveness to the open eye [45] (Fig. 57.6). This deficit can be phenocopied in wild-type mice receiving a cortical infusion of an inhibitor of TNFα signaling, indicating that this is a defect in plasticity and not a non-specific developmental defect [45]. This work demonstrates the TNFα has a role in the in vivo functioning of the nervous system and is not simply an artifact of culture systems.

We next investigated if TNFα could contribute to another form of neuronal adaptation – the behavioral response to anti-depressants. Major depression is a severe psychiatric condition whose biological underpinnings are not well understood and many of those afflicted are resistant to current treatments. Further, anti-depressants are not immediately clinically efficacious, but must be taken for a number of weeks, suggesting that the drugs themselves do not lead to clinical improvement, but are inducing a neural adaptation that is resulting in the improvement of mood. Recent evidence has suggested that synaptic plasticity mechanisms may contribute to depression [46]. There has been recent interest in a 'cytokine hypothesis of depression,' which suggests that pro-inflammatory cytokines may be causative in major depression [47, 48]. Pro-inflammatory cytokines, including TNFα, are elevated in patients suffering from major depression [49, 50, 51], and bipolar depression [52]. Genetic evidence suggests that polymorphisms in TNFα contribute to susceptibility to depression and bipolar depression [53, 54]. Treatment with various anti-depressants [50, 51, 55] or with electro-shock therapy [49] leads to a reduction in TNFα levels, but only in patients responsive to the therapy [49, 51]. The elevation in cytokines during depression may in fact be causative, rather than correlative. Medical conditions with a chronic inflammatory component are often accompanied by depression [47]. Cytokine elevation, due to exogenous application or acute inflammation, leads to depressive symptomology in rodents [56, 57, 58] and humans [59, 60], which can be alleviated by anti-depressants [61]. Further, TNFα receptor-deficient mice have reduced responsiveness during behavioral tests for depression-like states [62]. Lastly, depression may involve reduced neurogenesis in the dentate gyrus of the hippocampus (and anti-depressant treatment with increased neurogenesis) [63], and TNFα is known to negatively regulate

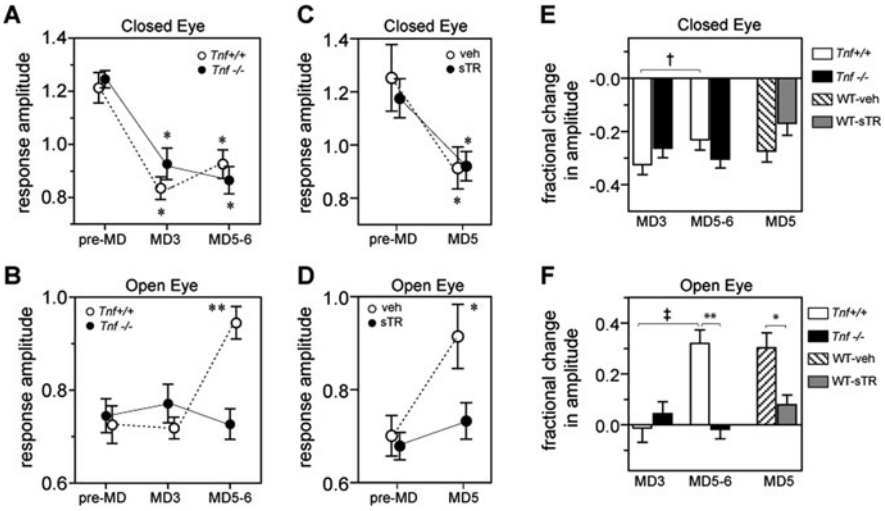

Fig. 57.6 TNFα signaling is required for one component of the visual cortical plasticity induced by monocular deprivation (MD). Repeated imaging of intrinsic signal reveals that lack of TNFα signaling impairs the delayed component of plasticity induced by MD. (**a**, **b**) Average visual response amplitude of the deprived-eye response (**a**) and open-eye response (**b**) in the binocular visual area, before deprivation (pre-MD), after 2.5–3 days of MD (MD3), and after 5–6 days of MD (MD5–6) in *Tnf*+/+ (n=6) and *Tnf*−/− (n=7) mice. All data from longitudinal measurements are in the same individual animals. (**c**, **d**) Average visual response amplitude of the deprived-eye response (**c**) and open-eye response (**d**) in the binocular visual area in inbred C57Bl6 wild-type mice treated with cortical infusion of soluble TNF receptor1 (sTR) (n=7) or vehicle solution (n=5), before the deprivation and after 5 days of MD. Values in **a**–**d** present mean ± SEM of the group. $^{**}p<0.01$, $^{*}p<0.05$ compared with baseline (pre-MD), repeated measure ANOVA, and Bonferroni multiple comparisons (adapted from [45])

hippocampal neurogenesis [64]. Taken together, the data suggest that the response to elevated levels of pro-inflammatory cytokines may be important in the pathophysiology of depression, and decreasing TNFα levels may help alleviate symptoms.

However, it remains unclear how TNFα and other cytokines could be impacting the function of the nervous system to induce depression. The role of TNFα in homeostatic plasticity suggests that disruption of the on-going release of TNFα in the brain could have profound effects on neuronal network activity, which could underlie changes in mood or behavior. Interestingly, anti-depressants also directly reduce the stimulated release of TNFα from glia [65], and changes in hippocampal synaptic strength, in a manner suggestive of homeostatic plasticity, correlate with depressive behavior in mice and these changes are reversed by anti-depressant treatment [66]. These changes mirror the changes observed following homeostatic plasticity in hippocampal slice cultures [67]. Our data suggest that TNFα signaling is required for the behavioral effects of chronic anti-depressant treatment.

TNFα −/− mice have normal locomotion, and their behavior in two widely used models of depressive behavior (the tail suspension test (TST) and Porsolt forced swim test (FST)) is indistinguishable from wild-type mice. Further, TNFα −/− mice respond normally to acute treatment of large doses of two different classes of anti-depressant (fluoxetine and desipramine). Critically, however, these mice do not respond to the chronic low-dose administration of the same anti-depressants. While TNFα was not required for the development of depressive-like behavior in mice, nor in the acute behavioral response to anti-depressants, it was required for the behavioral response to chronic anti-depressants. This suggests that chronic administration of anti-depressants induces a homeostatic neuronal adaptation, dependent on TNFα, that underlies the improvement in mood.

Overall, our data demonstrate that TNFα is an endogenously released glial factor that regulates synaptic function, and that TNFα signaling is required for some forms of developmental plasticity as well as the behavioral response to anti-depressants. This suggests that disruption of the release of TNFα in the brain has profound effects on neuronal network activity and could therefore result in the neural adaptations underlying changes in development or behavior.

TNFα-dependent homeostatic plasticity defines an intrinsic neuronal function for an immune signaling molecule. Combined with recent work on a neuronal role of major histocompatibility complex (MHC) class I [68] and the complement cascade [69], it suggests that immune signaling molecules serve novel functions in the normal functioning of the nervous system. It also suggests that infiltration of immune cells into neuronal tissue, during disease or insult, could lead to unexpected dysfunctions due to disruption of the normal neuronal functions by the immune cell released cytokines. TNFα and other pro-inflammatory cytokines are believed to be critically involved in a variety of neuronal insults. Increases in TNFα and the associated neuro-inflammation contribute to the damage from neuronal insults, such as stroke and head/spinal trauma, and neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS), as well as contributing to neuropathic pain [70, 76, 77]. Understanding the mechanism by which TNFα contributes to these disorders is a necessary step in mitigating the ensuing damage. ALS, a neurodegenerative disease featuring selective cell death of motor neurons and currently without effective treatment, is a particularly attractive starting point for such an investigation, as it involves inflammation, glial factors, calcium-permeable AMPARs, and resulting excitotoxic cell death.

Amyotrophic lateral sclerosis (ALS) is a motor neuron degenerative disease. The disease is thought to involve glia [78, 79, 80]. Two recent studies suggest that in mouse models of ALS, the spinal glia release a soluble factor that induces cell death in the motor neurons [81, 82], confirming earlier studies using chimeric animals [83]. ALS is also believed to involve inflammatory cytokines including TNFα [84, 85, 86]. TNFα levels increase before symptoms appear [85, 87] and glia from ALS model mice release higher levels of TNFα [88] than control glia. TNFα alone can kill motor neurons [89] and spinal TNFα levels correlate with the degree of

motor neuron death [87, 90]. ALS also involves excitotoxic damage through activation of calcium-permeable AMPA receptors [28, 29, 91, 92]. Astrocytes control expression of GluR2 in spinal motorneurons [93], and GluR2 expression regulates sensitivity to excitotoxicity in ALS model mice [91]. Our data on the control of calcium-permeable AMPA receptor trafficking by glially released TNFα [20] suggest a model that could unify these disparate elements. ALS may involve a dysregulation of homeostatic synaptic plasticity, due to excessive cytokine release from activated glia, leading to the exocytosis of non-GluR2 containing AMPARs and an increased vulnerability to excitotoxicity. ALS model mice have altered AMPA receptor trafficking [29], potentially due to increased release of homeostatic plasticity signaling molecules (including but not limited to TNFα) from glia, leading to excessive surface expression of GluR2-lacking AMPA receptors [92, 94, 95].

The potentiation of excitotoxicity by the TNFα-driven increase in surface expression of GluR2-lacking AMPA receptors may be a common cell death pathway. The laboratories of Phil Barker and Adrianna di Polo have recently demonstrated a nice example of this concept [96]. Injection of either NMDA or pro-NGF into the retina leads to the cell death of retinal ganglion cells (RGCs), but this effect turns out to be indirect. The injection into the retina leads to the activation of retinal glia, which upregulate their production and release of TNFα. The glial release of TNFα in turn results in increased surface expression of GluR2-lacking AMPA receptors on the neighboring RGC neurons, and the resulting calcium influx results in excitotoxic cell death of RGCs. The RGC cell death can be substantially rescued by blocking TNFα signaling or by inhibiting AMPA receptor function [96]. This unexpected finding suggests that TNFα-dependent AMPA receptor trafficking may contribute to the cell death seen in many neuropathies.

TNFα-mediated plasticity is at the intersection of four exciting areas of neuroscience – synaptic plasticity, glia–neuronal interactions, the use of immune signaling molecules for distinct neuronal functions, and mechanisms of damage during inflammatory insult. TNFα is endogenously released by glia in an activity-dependent manner, but TNFα will be released at a much higher level by activated astrocytes, microglia, and infiltrating immune cells during neuro-inflammatory insults. This suggests that TNFα not only plays a role in normal neuronal function, but this function may be dysregulated during inflammation, contributing to the associated neuronal damage.

There is strong overlap of synaptic scaling and inflammation, both of which employ TNFα and possibly other similar signaling molecules. Inflammation, which is an important contributor to pathology in various neurodegenerative diseases such as ALS, may strongly activate this plasticity mechanism, leading to increased sensitivity to excitotoxicity and contributing to the neuronal cell death. This work could provide insights into novel mechanisms underlying neurodegeneration in ALS as well as other neurodegenerative diseases, chronic pain, stroke, and traumatic head and spinal injuries. Many of these dysfunctions are without effective therapies, and a fundamental understanding of the novel role of TNFα in the nervous system will be necessary to develop more effective regimens and could lead to potential therapies for a variety of neuronal disorders and neurodegenerative diseases.

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Chapter 58
Intracellular and Intercellular Cross Talk Between NGF and TNF

Yoshinori Takei and Ronald Laskey

Introduction

Nerve growth factor (NGF) is a member of the NGF neurotrophin family that can promote neural cell survival and maturation [13, 14]. During development, NGF is secreted from target tissues of neurones, and neurones expressing NGF receptors, $p140^{trkA}$ and $p75^{NTR}$, extend neurites in the direction of the secreted NGF. Only neurones that obtain a sufficient level of NGF can survive and interact with the target tissues, and neurones that fail to obtain NGF die through an apoptotic pathway [7, 13, 14, 37].

TNFα is a cytokine that contributes to this neural cell death during development [2]. Some neurones, including NGF-dependent neurones, express TNFα [2, 5, 16, 17, 33, 34]. When TNFα signalling through a TNFα receptor (TNFR) 1 is inhibited, death of the neurones after withdrawal of NGF is decreased. The number of NGF-dependent neurones in the TNFα-deficient mouse embryos was greater than in normal mouse embryos [2]. These findings indicate that TNFα can show pro-apoptotic effects on neurones through TNFR1 and that the pro-apoptotic effects contribute to their selection of NGF-dependent neurones during development.

TNFα has another receptor TNFR2 in addition to the pro-apoptotic TNFR1 [15]. Signals from TNFR2 appear to promote neuronal survival [8, 25, 36, 40]. In our recent study, we indicated that endogenous TNFα in neuronal cells contribute to their NGF-dependent survival through TNFR2 [39].

TNFα Signalling Through TNFR2 Modulates NGF Signalling

Recently, we found that NGF induces expression of TNFα in undifferentiated SH-SY5Y and BE(2)-C human neuroblastoma cell lines as well as in a human

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non-transformed cortical neural cell line HCN-1A [39]. NGF induces TNFα expression through Akt kinase and NF-kB transcription factor, and the TNFα induced by NGF re-activates Akt through TNFR2 in neurones [39]. Thus, a positive feedback loop of Akt activation is composed of TNFα, TNFR2, Akt and NF-kB (Fig. 58.1).

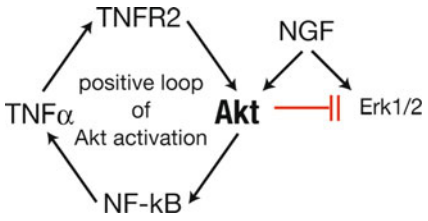

Fig. 58.1 An intracellular cross talk between NGF and TNFα signalling. Intracellular cross talk between NGF and TNFα signalling in neural cells. NGF induces TNFα expression through NF-kB. The TNFα induced by NGF binds to TNFR2 and activates Akt. Thus, a positive feedback loop of Akt activation is constructed, which consequently inhibits Erk activation by NGF

Although NGF alone can induce only faint and transient activation of Erk, inhibition of the Akt loop augments Erk activation by NGF [39]. Thus, endogenous TNFα can change downstream signalling of NGF from the MEK-Erk path to the PI3-Akt path, through a positive feedback loop of Akt activation including TNFα, TNFR2, Akt and NF-kB (Fig. 58.1).

NGF Appears to Change the Effects of Endogenous TNFα on Neuronal Survival

The PI3-Akt path is well established in pro-survival signalling pathways [4]. Consistent with this, NGF-induced TNFα contributes to NGF-dependent survival, through the positive feedback loop of Akt activation. When the loop is inhibited, NGF-dependent survival of neurones from dorsal root ganglia is decreased [39]. Inhibition of TNFR1 failed to show significant effects on the survival.

Taking this finding together with the report by Barker et al [2], endogenous TNFα induced by NGF appears to activate TNFR2 preferentially in the presence of NGF, supporting survival [39], but activate TNFR1 in the absence of NGF, contributing apoptosis [2]. One possible mechanism for this receptor preference of endogenous TNFα might be that NGF changes the ratio of these receptors at the surface of neurones. Another possibility is that, although TNFα could activate both TNFR1 and TNFR2, signalling through TNFR1 might be antagonised by cooperative signalling by NGF and TNFα through TNFR2. While signalling through TNFR2 alone can inhibit pro-apoptotic signals through TNFR1 in some NGF-independent neurones [8, 25, 40], the cooperation between NGF signalling and TNFα signalling through TNFR2 is essential for NGF-dependent survival of neurones from rat dorsal root ganglia [39]. This suggests differences between various types of neurones.

NGF changes effects of endogenous TNFα in neurones on their survival, from pro-apoptotic to anti-apoptotic.

Possible Contributions of Endogenous TNFα to Tumourigenesis of Neuroblastomas

Neuroblastoma is a malignancy derived from the sympathetic nervous system and observed in infancy and childhood [12]. While overall cure rate is only 40%, neuroblastomas in the early stages sometimes show spontaneous regression, and high expression of $p140^{trkA}$ is correlated with the regression [18]. Neuroblastoma cell lines expressing low levels of $p140^{trkA}$ fail to differentiate into neurone-like cells in response to NGF. However, upon overexpression of $p140^{trkA}$, NGF can induce their differentiation through activation of Erk [20, 26, 27, 32]. Furthermore, xenotransplanted *trkA*-transfected neuroblastoma cells in mice form tumours that differentiate upon treatment with NGF [27]. These reports suggest that inadequate $p140^{trkA}$ fails to activate Erk sufficiently, causing the insensitivity of neuroblastoma cells to NGF-dependent differentiation.

However, our report indicate that NGF-dependent activation of Erk is augmented by inhibition of the positive loop of Akt activation and that the augmentation is sufficient for neural differentiation of neuroblastoma cell lines expressing low levels of $p140^{trkA}$, SH-SY5Y and BE(2)-C [39]. Thus, the positive loop of Akt activation causes insensitivity of neuroblastoma cells to NGF-dependent differentiation, and overexpression of $p140^{trkA}$ is not necessary to induce NGF-dependent differentiation of neuroblastoma cells. The overexpression of $p140^{trkA}$ appears to overcome the inhibition of Erk activation by the TNFR2 signalling pathway.

Furthermore, recent papers indicate that basal levels of active Akt in neuroblastomas are correlated with their insensitivity to chemotherapy [22, 35] and that Akt activation could be a prognostic indicator of overall survival in the cancer [31]. Thus, NGF could contribute to their insensitivity to chemotherapy through the positive loop of Akt activation, although NGF can induce regression of neuroblastomas in the early stages [18]. A combination of NGF and inhibition of TNFα signalling might provide a new approach to the treatment of neuroblastomas.

Where Is the Endogenous Source of NGF in the Adult Nervous System?

While NGF is produced during development, almost all cells stop or decrease NGF synthesis in the adult body [13, 14]. Therefore, an obvious question is which cells can provide NGF to neurones in the adult body.

Glial cells are the other major cell group in the nervous system and they have essential roles in the development, maintenance and function of the nervous systems [24, 41]. While glial cells in development produce NGF, the cells in the adult

nervous tissues stop or decrease expression of NGF to undetectable levels. However, pro-inflammatory cytokines can augment NGF synthesis in Schwann cells, the glial cells forming myelin sheaths around axons of neurones in the peripheral nervous system (PNS) [9, 23, 29]. NGF expression in astrocytes, the glial cells covering neural cell bodies and synapses in the central nervous system (CNS), is also augmented by inflammation [1, 10, 11, 19, 28] .

Under inflammatory conditions, immune cells, including mast cells, macrophages/monocytes/microglia and leukocytes, can produce NGF in the adult body [3, 6, 11, 21, 28, 30]. Thus, both immune and glial cells can provide NGF in the adult body at least under inflammatory conditions.

A Possible Intercellular Loop of NGF and TNFα Expression

Since neurones and glial cells are closely associated, it is possible that NGF synthesised by glial cells could induce TNFα expression in neurones. Reciprocally, TNFα produced by neurones could induce NGF expression in glial cells. Furthermore, since TNFα is a potent inflammatory cytokine, TNFα synthesised by neurones could attract immune cells, macrophages in the PNS and microglia in the CNS. These immune cells can produce both TNFα and NGF. Thus, a positive loop of NGF and TNFα synthesis could be established among neural cells, glial cells and immune cells [38] (Fig. 58.2). This positive loop of TNFα and NGF expression could promote neuronal survival during inflammation, minimising inflammatory damage to the nervous system.

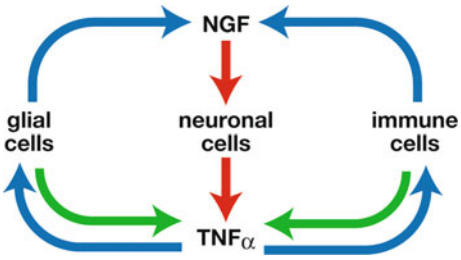

Fig. 58.2 An intercellular positive feedback loop of NGF and TNFα expression. Neural cells produce TNFα in response to NGF. The TNFα induced by NGF could induce NGF expression in glial cells and immune cells. Both immune and glial cells, but not neuronal cells, can produce TNFα in response to TNFα. Thus, it appears that a positive feedback loop of NGF and TNFα expression could be composed among immune, glial and neuronal cells

Conclusion

We propose a positive loop of TNFα and NGF expression between neurones and glial cells (Fig. 58.2). NGF signalling and TNFα signalling modulate each other, and their balance is essential for proper cell responses to them. The NGF–TNFα

cross talk could promote survival of not only NGF-dependent neurones but also some NGF-independent neurones, since TNFα can promote survival of some NGF-independent neurones through TNFR2 [8, 25, 36, 40]. Once their balance is disturbed, the cross talk could contribute to either excessive neuronal death or excessive proliferation of immature neural cells, i.e. neurodegenerative diseases or cancer.

Although inhibition of inflammation shows positive effects on both cancers and neurodegenerative disorders, findings summarised in this article suggest that total inhibition of inflammation could cause another problem. Inhibition of inflammation can block TNFα signalling, and absolute inhibition of TNFα signalling could induce cell death of some portion of neurones. Instead of the total inhibition of TNFα signalling, a combination of specific inhibitors of the TNFR1 signalling and specific activators of the TNFR2 signalling might be optimal for promotion of neuronal survival in neurodegenerative disorders. Reciprocally, a combination of activation of TNFR1 signalling and inhibition of TNFR2 signalling could be effective for cancer cell killing. Inflammation appears to involve both positive and negative survival signalling. Control of specific signalling rather than total inhibition of inflammation could be safer and more effective for treatment of these diseases.

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Chapter 59
TNFR2 – Target for Therapeutics Against Neurodegenerative Diseases?

Ingrid M. Nijholt, Ivica Granic, Paul G.M. Luiten, and Ulrich L.M. Eisel

TNF and Its Receptors

Tumor necrosis factor α (TNFα) is a proinflammatory cytokine which acts via its two receptors TNFR1 and TNFR2. It is involved in many cell types in various cellular functions of cell growth, differentiation, and cellular apoptosis. Whereas soluble TNF has the highest affinity for TNFR1 and is therefore its main activator, the membrane standing precursor form of TNF was found to be the strongest activator of TNFR2. This may be also the simple technical reason why for a very long time hardly any TNFR2-specific response was known. In fact for a very long time TNFR2 was believed to function as ligand-passing receptor to TNFR1 or to be just a costimulator of TNFR1 signaling events. In fact under certain conditions this might be true yet a specific and TNFR1-independent signaling role was until recently not established [24].

TNF and TNFRs in Neurodegenerative Disease

In many neurodegenerative diseases such as stroke, Alzheimer's disease, Parkinson syndrome, and multiple sclerosis TNF plays an essential role in the neuroinflammatory response to the disease (for review see [5]).

Increased levels of TNF can be found in lesioned tissue areas (Fig. 59.1a) as well as extensive upregulation of both TNFR1 and TNFR2 (Fig. 59.1b and c) [6]. This led to investigations as to whether TNF and its receptors are involved in the pathological process or rather play a more protective role. Depending on the neurodegenerative processes analyzed, however, research by us and others led to different conclusions (reviewed in [5]). In our own studies we had investigated the

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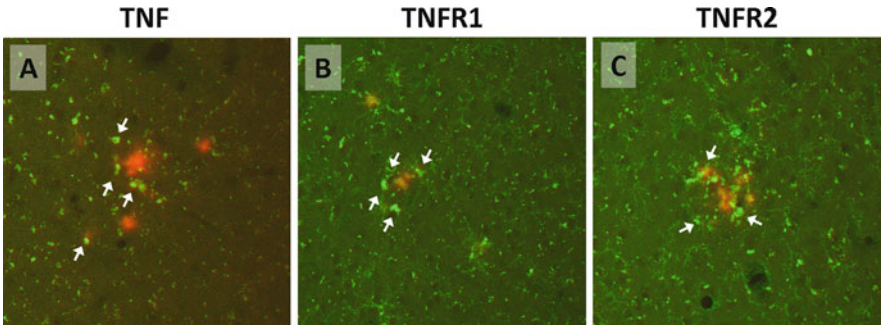

Fig. 59.1 Double immunolabeling for cortical amyloid-β plaques in *red* and in *green* TNF (**a**), TNFR1 (**b**), or TNFR2 (**c**) in aged $APP_{S,L}$/PS1mut (M146L) transgenic mice. *White arrows* indicate an upregulation adjacent to the amyloid deposits

role of TNF and TNFRs by using in vivo knock-out mouse models of the respective cytokine and cytokine receptors in retinal ischemia models and in in vitro cell culture models of glutamate-induced excitotoxicity in primary cortical neurons. In both models we could establish a strong neuroprotective function of TNFR2. It turned out that the neuroprotective signals via TNFR2 are mediated through activation of PI3 kinase and nuclear factor-kappa B (NF-κB). NF-κB is a transcription factor which induces the expression of genes involved in the regulation of several cellular processes, including cellular survival (Bcl-2, inhibitor of apoptosis proteins [IAP], TNF or TNFR2 genes) and immune response (TNF, interleukins [IL]-2, IL-6 genes). In addition, the TNFR2 gene contains the consensus elements for transcription factors, such as nuclear factor-kappa B (NF-κB) in the 5′-flanking region suggesting receptor self-promotion [14, 17]. In neuronal cells sustained activation of NF-κB was shown to induce neuroprotection [11, 12]. This beneficial outcome was in part the result of PI3-kinase-PKB/Akt pathway activation, increase in mitochondrial antioxidant enzymes Cu/Zn-SOD, Mn-SOD, and the induction of anti-apoptotic proteins Bcl-2 and Bcl-x. Furthermore, NF-κB cellular survival functions underlie cytokine-induced neuroprotective mechanisms, including transforming growth factor-beta1 (TGF-β1) and TNF [25].

Kinetic studies revealed that activation of NF-κB by TNFR1 and TNFR2 differ in their length of activation with peak activity of NF-κB upon TNFR1 stimulation within 60 minutes whereas stimulation of TNFR2 lasted in much longer (up to 24 h) NF-κB activity [11]. Interesting with respect to neuronal processes underlying neurodegeneration, coactivation of the ionotropic glutamate receptor (*N*-methyl-D-aspartate receptor) NMDAR resulted in an enhancement of PKB/Akt phosphorylation and NF-κB activation. This is remarkable, as the NMDAR is widely seen as the major inducer of apoptosis upon overstimulation by glutamate in various neurodegenerative disorders. It seems that an inherent and cooperative salvage pathway (see Fig. 59.2) emanates from both the cytokine receptor TNFR2 and neurotransmitter receptor NMDAR rendering neurons less sensitive toward injurious glutamate challenges. Besides regulation of the expression of several genes

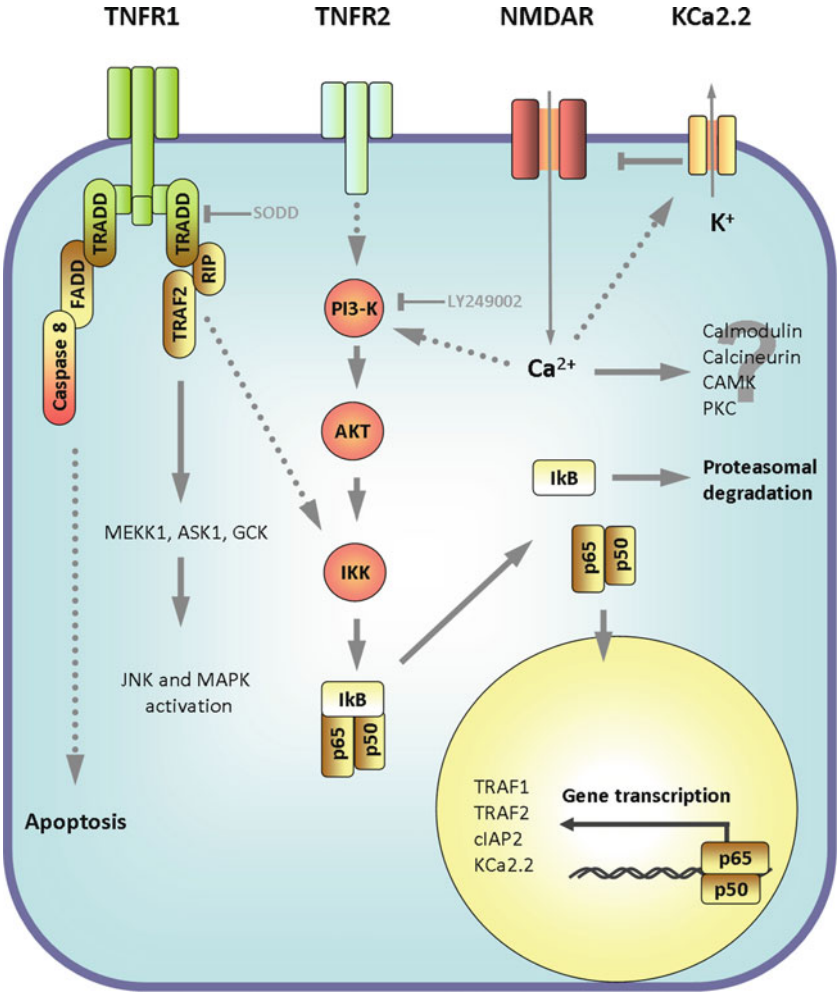

Fig. 59.2 Schematic representation of the neuroprotective TNFR2 signaling pathway. AKT, protein kinase B/AKT; IKK, inhibitor of κB kinase; IκB, inhibitor of κB; PI3-K, phosphoinositide-3 kinase; TNFR, tumor necrosis factor α receptor; FADD, fas-associated protein with death domain; TRADD, TNF receptor-associated death domain; RIP, receptor interacting protein; p65 and p50, subunits of the nuclear transcription factor -κB; TRAF 1 TNF receptor-associated factor; MEKK, mitogen-activated protein kinase kinase kinase; ASK1, apoptosis signal-regulating kinase 1; GCK, glucokinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NMDAR; LY249002, inhibitor of PI3-K; SODD, silencer of death domains; cIAP2, inhibitor of apoptosis. *Dashed arrow*: indirect effect; *full arrow*: direct effect; *blunt arrow*: inhibitory effect

involved in the inflammatory process, NF-κB can also interfere with ion homeostasis by controlling the level of certain ion channels. Among these are the subunits of NMDA receptors but also small conductance Ca^{2+}-activated potassium (SK or KCa2) channels.

Activation of TNFR2, can directly, lead to sustained activation of NF-κB (p50/p65). Upon phosphorylation, IκB becomes degraded and NF-κB can translocate into the nucleus to initiate transcription of NF-κB-dependent anti-apoptotic genes (e.g., TRAF1, TRAF2, cIAP, and KCa2.2).

TNF and SK Channels

SK channels are voltage-independent potassium channels that link intracellular calcium transients to membrane potential changes. The three identified SK channel subtypes, SK1 (KCa2.1), SK2 (KCa2.2), and SK3 (KCa2.3), each has a different pharmacology and distribution in the central nervous system. Whereas SK1 and SK2 are frequently expressed in the same neurons, SK3 shows a complementary distribution [15, 16]. Overall, SK2 appeared to be have the highest expression in the central nervous system. Splice variants have been identified for all three SK channel genes. In the mouse brain, for example, splice variants have been identified that may lead to up to 16 different SK1 polypeptides [18] and three SK2 isoforms [13, 21]. Three splice variants have been described for SK3, two of which can be found in the human brain [22, 26]. SK channels regulate neuronal firing by contributing to the afterhyperpolarization following action potentials and affecting interspike intervals during a burst of action potentialsas well as the length of the burst (reviewed by [20]). Since these channels reduce neuronal excitability, it could be expected that activation of these channels yields neuroprotection against neuronal overstimulation. Indeed, overexpression of KCa2.2 channels was shown to prevent kainic acid- and glutamate-induced excitotoxicity [9].

In a study which characterized the intronic promoter region of the most common mouse SK2 isoform [7], two functional NF-κB response elements at positions −1652 and −1586 bp were identified in this SK2 promoter region [8]. These two NF-κB binding sites appeared to have distinct implications for SK2 promoter activity. Promoter deletion analysis revealed that from both NF-κB binding sites only the −1586 bp site was essential for full transcriptional activity [8].

Since the SK2 gene contains NF-κB binding sites and SK channels might have neuroprotective properties, we recently investigated in our lab whether SK2 channels are involved in TNFα-mediated neuroprotection in mouse primary cortical neurons [1]. A long-lasting (6–24 h) neuroprotective pretreatment with TNFα was shown to be paralleled by an increase in the expression of SK2 channels whereas the expression of SK1 and SK3 channels remained unaltered. Experiments in primary cortical neurons from TNFR1 and TNFR2 k.o. mice revealed that the upregulation of SK2 channels during TNFα treatment was TNFR2 dependent. The TNFα-mediated neuroprotective effect could be blocked via downregulation of SK2 channel expression with specific SK2 siRNA probes and the NF-κB inhibitor BAY11-7082. Taken together, it was concluded that treatment of primary cortical neurons with TNFα leads to a TNFR2 and NF-κB-dependent increase in SK2 channel expression, which renders neurons more resistant to excitotoxic cell death [1].

Statins and the TNFR2 Neuroprotective Pathway

Recently we and others reported that statins, a class of drugs originally used to lower cholesterol levels in people with or at risk of cardiovascular disease, can also act as neuroprotective agents (reviewed, e.g., by [19, 23]. Most interestingly, lovastatin, one of the naturally occurring statins, was shown to selectively increase the cell surface expression of TNFR2 without affecting the expression level of TNFR1 in human epithelial cells (HUVEC) [14]. These results together with the finding that TNFR2 activation has a neuroprotective effect in mouse primary cortical neurons led us to investigate whether lovastatin might act as a neuroprotective agent by sensitizing primary cortical neurons toward TNFα-induced signaling via upregulation of TNFR2 expression [3]. In mouse primary cortical neurons lovastatin (24 h treatment) indeed had a neuroprotective effect against an excitotoxic glutamate challenge in a concentration-dependent manner (0.1–100 μM). Moreover, lovastatin treatment of neurons with or without glutamate and/or TNFα increased TNF receptor 2 (TNFR2) expression in cortical neurons. Additional evidence for a role of TNFR2 in the neuroprotective effect of lovastatin came from experiments with neurons isolated from TNFR1–/– or TNFR2–/– mice. We found that lovastatin is neuroprotective in TNFR1–/– neurons, while protection is completely absent in TNFR2–/– neurons. Consistent with our data that TNFR2 signaling involves PKB/Akt phosphorylation and NF-κB activation [11], lovastatin treatment increased PKB/Akt phosphorylation in primary cortical neurons, and the neuroprotective effect of lovastatin could be blocked by the PKB/Akt inhibitor LY294002 and the NF-κB inhibitor BAY11-7082. We also found that pretreatment of glutamate challenged primary cortical neurons with lovastatin and a TNFα neutralizing antibody (V1q) [4] resulted in a substantially reduced neuroprotective effect as compared to lovastatin treatment alone. This finding suggest that endogenous TNFα and here most likely the membrane-bound form of TNFα in an auto- or juxtatropic way may be involved in stimulating TNFR2 upon lovastatin treatment. Overall, our study provided a novel neuroprotective mechanism of statins via the activation of the TNFR2 signaling pathway [3]. It should, however, be noted that we could not exclude the involvement of other lovastatin-induced neuroprotective mechanisms besides activation of TNFR2 signaling such as cholesterol depletion, inhibition of the lipid peroxidation product, 4-hydroxynonenal (HNE), or prevention of small G-proteins isoprenylation. To confirm our in vitro data, we also investigated the neuroprotective action of lovastatin in an in vivo mouse model for neurodegeneration. Intraperitoneal injections of lovastatin (20 mg/kg) on five consecutive days were shown to significantly protect cholinergic neurons and their cortical projections against an excitotoxic injection of *N*-methyl-D-aspartate in the magnocellular nucleus basalis of mice [2]. This neuroprotective effect could be blocked by the injection of the PKB/Akt inhibitor LY294002. The functional relevance of this finding was provided by the assessment of behavioral alterations in the mice. The NMDA-induced cholinergic lesion led to a characteristic decrease in memory performance which could be reverted by pre-lesion treatment with lovastatin [2]. Although the involvement of TNFα and its receptors was not directly investigated in this study, the lovastatin-induced

upregulation of TNFR2 expression observed in primary cortical neurons and the finding that the neuroprotective effect of lovastatin both in vitro and in vivo involves PKB/Akt activation, suggests a common neuroprotective mechanism of lovastatin via TNFR2 upregulation and PKB/Akt activation. Future experiments, e.g., with TNFR1–/– and TNFR2–/– mice should confirm this hypothesis.

Overall Discussion and Conclusion with Future Perspectives

In conclusion our data suggest that in contrast with TNF receptor 1, TNF receptor 2 is in a key position to mobilize neuroprotective intracellular processes upon activation by the cytokine TNF. TNF–TNFR2 activation has its own temporal dynamics and is subject to additional neuroprotective influences, e.g., exerted by exposure to statins. Since neurodegenerative diseases are often the outcome of excitotoxic overstimulation by glutamate, the impact of TNFR2 activation on neuronal hyperpolarization through small conductance calcium-dependent potassium channels may explain some of the protective underlying mechanisms, next to intracellular signaling pathways that link this receptor to anti-apoptotic cell survival. It can be concluded that selective activation of TNFR2 or downstream signals of TNFR2 can be a potent strategy for the treatment of neurodegenerative diseases.

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Chapter 60
A Role for Neuronal NF-κB in Suppressing Neuroinflammation and Promoting Neuroprotection in the CNS

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Introduction

Experimental autoimmune encephalomyelitis (EAE) is an animal model for multiple sclerosis (MS) that reflects several aspects of MS [1]. In experimental animals immunization with myelin antigens such as myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) triggers autoimmune, demyelinating disease. Critical participants and mechanisms of the immune response in MS are also detected in EAE, but there are also discrepancies that should be considered [2]. EAE is a useful model to study mechanisms that can restrain detrimental inflammatory pathways leading to demyelination and pathways that promote neuronal protection in the CNS. Current strategies to treat MS aim at selective suppression of immune functions related to T cells, B cells, cytokines, and adhesion molecules or apply non-selective immunosuppressive drugs [3]. In this context, an imperative need to boost neuroprotection and repair for MS treatment is recognized.

Transcription Factor NF-κB in EAE Pathology

NF-κB activation is a key event during EAE pathology indispensable for activation of peripheral immune cells [4, 5], for progression of the immune response within the CNS [6, 7] and maybe for resolution of inflammation, as it was shown for other inflammatory diseases [8]. Impairment of the canonical pathway in NF-κB1/p50-deficient mice leads to significant resistance to EAE [4], and furthermore NF-κB inactivation specifically in T cells causes complete resistance to EAE [5]. Moreover, the deletion of IKKγ/NEMO or IKKβ from neurons/astrocytes [6] or the inactivation of NF-κB in astrocytes [7] resulted in significant amelioration of EAE showing that NF-κB-mediated astrocyte activation has deleterious effects in this model of chronic

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CNS inflammation. In the normal CNS, NF-κB is constitutively active in neurons and is further activated by basal synaptic input and glutamate in a Ca^{2+}-dependent manner in hippocampal neurons; it is involved in the regulation of spatial memory formation, synaptic transmission, and plasticity in mice [9–11]. In CNS pathology, however, the role of NF-κB is much less clear. It is believed that NF-κB signaling plays a crucial role during inflammatory, demyelinating disease and that becomes evident through its opposing proinflammatory and neuroprotective functions [12] that depend on the different cell types where is activated.

In the canonical pathway of NF-κB activation, dimers containing RelA or cRel are held in the cytoplasm through interaction with specific inhibitors of the IκBs. Inducible phosphorylation of the IκBs leads to ubiquitination and subsequent degradation by the proteasome and allows NF-κB dimers to translocate to the nucleus. The canonical NF-κB pathway is essential for innate immunity, whereas the alternative pathway is more important for lymphoid organ development and adaptive immunity [13]. Both pathways depend on the Iκβ kinase (IKK) complex which consists of two catalytic subunits, IKKα (IKK1) and IKKβ (IKK2), and the regulatory subunit NF-κB essential modulator (IKKγ/NEMO) [13]. IKKβ and IKKγ are important for Iκβ inhibitor phosphorylation and degradation leading to unmasking of NF-κB nuclear translocation signals (NLS) whereas IKKα is mostly involved in the alternative pathway [14].

Conditional Gene Targeting of IKKβ Kinase During Autoimmune, Demyelinating Disease

As IKKβ kinase is the catalytic subunit of the IKK complex, its function is essential for the activation of the canonical NF-κB pathway (Fig. 60.1a). We applied conditional gene targeting techniques to produce a mouse with deleted IKKβ kinase from CamkIIα-expressing CNS neurons to study the role of NF-κB canonical pathway in the CNS during pathological conditions. We used $IKK\beta^{F/F}$ mice [15, 16] and CamkIICre mice [17] to produce nIKKβKO mice (Fig. 60.1b). CamkIIα is expressed in the forebrain, including cortex, hippocampus, and striatum, and in the Purkinje cells of the cerebellum as previously described [17]. Additionally, apart from its expression in the brain, CamkIIa is also expressed in the spinal cord leading to deletion of IKKβ from NeuN-positive neurons as detected by confocal microscopy [18].

As recently reported by Emmanouil et al. [18], after inducing $MOG_{35–55}$-EAE in nIKKβKO and control mice in a C57Bl6 background, nIKKβKO mice have similar disease initiation compared to control but develop significantly more severe clinical disease characterized by a non-resolving course (Fig. 60.2a). Histological analysis showed extensive inflammatory infiltration and demyelination in the spinal cord of nIKKβKO mice at day 34 of disease (Figs. 60.2b and 60.2c). Furthermore, there is significantly more axon loss in the nIKKβKO spinal cord compared to control at day 34 of the chronic phase, indicating severe neurological deficit in nIKKβKO mice (Fig. 60.2d).

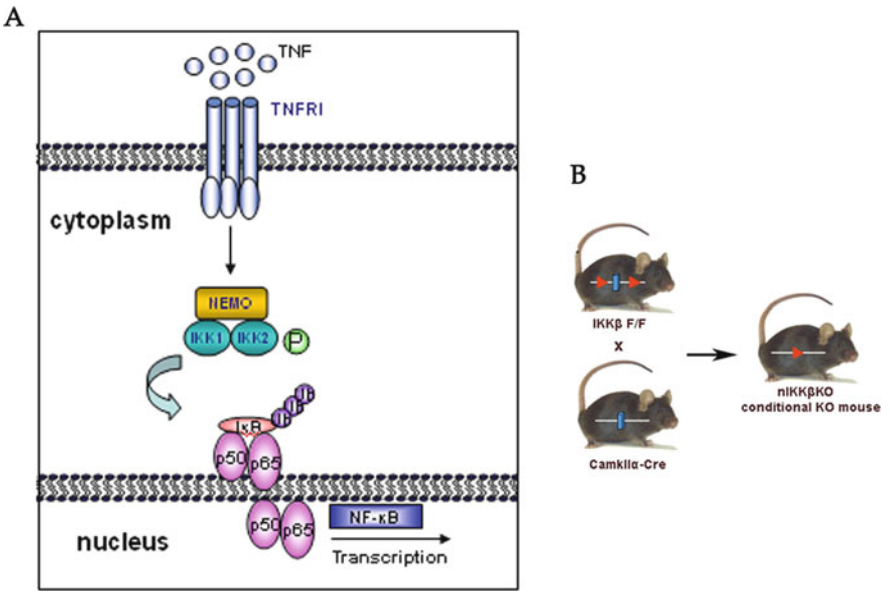


Fig. 60.1 IKKβ, the catalytic subunit in canonical NF-κB pathway (**a**), is selectively deleted in CNS neurons of nIKKβKO mice (**b**)

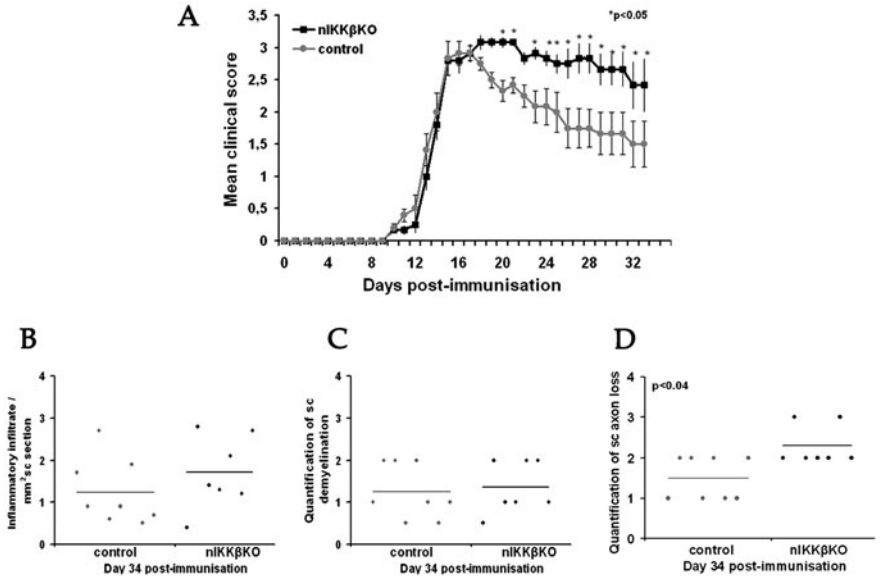


Fig. 60.2 nIKKβKO mice develop a severe, non-resolving form of $MOG_{35–55}$-EAE with enhanced inflammation and significantly more axon loss in the spinal cord compared to control mice. (**a**) Mean clinical scores for nIKKβKOand control mice after immunization with $MOG_{35–55}$ peptide. (**b–d**) Quantitative analysis of inflammatory infiltrates, demyelination, and axon loss in spinal cord sections taken at day 34 after immunization [18]

Proinflammatory and Neuroprotective Effects During EAE in nIKKβKO Mice

We performed a cytokine antibody array analysis in spinal cord lysates from both groups of EAE mice at day 22 post-immunization, when clinical scores start to show a significant difference and show that a number of key cytokines, chemokines, and neuroactive molecules that have been previously associated with EAE are differentially regulated in nIKKβKO mice compared to control (Table 60.1). There is a significant upregulation in cytokines responsible for increasing inflammation in the CNS, like IL-12 and IL-17, TNF and TNF receptors and chemokines secreted by T cells, microglia, astrocytes, and others. Moreover, several proteins are downregulated at nIKKβKO mice, such as VEGF involved in neuroprotection and IL-10 involved in immunomodulation.

Table 60.1 Enhanced production of pro-inflammatory cytokines and chemokines and reduced production of neuroprotective factors in the spinal cord of nIKKβKO EAE mice

Cytokines	Mean fold change ±SEM	Cytokines	Mean fold change ±SEM	Cytokines	Mean fold change ±SEM
CD30L	1.19 ±0.09	IL-6	1.28 ±0.07	CXCL16	2.50 ±0.42
IL-12 p40/p70	2.52 ±0.69	sTNF receptor II	1.20 ±0.07	MCP-5/CCL12	1.27 ±0.14
IL-12 p70	1.29 ±0.05	TNF	2.04 ±0.16	MIP-1α/CCL3	1.10 ±0.02
IL-17	2.29 ±0.61	sTNF receptor I	1.46 ±0.10	TARC/CCL17	1.62 ±0.13
IL-10	0.78 ±0.07	KC/CXCL1	1.84 ±0.01	TCA-3/CCL1	1.47 ±0.14
GM-CSF	1.20 ±0.02	MCP-1/CCL2	1.11 ±0.001	MIG/CXCL9	0.85 ±0.002
Thrombopoietin	1.62 ±0.02	RANTES/CCL5	1.12 ±0.001	Eotaxin/CCL11	0.80 ±0.06
VEGF	0.42 ±0.02	CTACK/CCL27	1.21 ±0.003	Leptin receptor	1.78 ±0.20
Axl	0.67 ±0.01	TECK/CCL25	1.63 ±0.03	TIMP-1	1.45 ±0.17

Differential production of immune mediators in protein lysates from nIKKβKO compared to control spinal cord taken on day 22 post-immunization as measured by a mouse cytokine antibody array. Statistically significant results are presented as the mean fold change ± SEM in cytokine production in spinal cords ($p < 0.05$).

The enhanced production of proinflammatory molecules was further validated at the mRNA level by RT-PCR. TNF (Fig. 60.3a), CCL2 (Fig. 60.3b), and CXCL16 (Fig. 60.3c) mRNA levels are significantly increased at day 22 after immunization at nIKKβKO spinal cord compared to control. Furthermore, FLIP is an NF-κB-inducible neuroprotective protein that was shown by Taoufik et al. [19] and others [20] that is sufficient to protect TNFRKO neurons after ischemic injury through the TNFR-NF-κB axis in the CNS. We show here that FLIP mRNA levels are significantly reduced in the spinal cord of nIKKβKO mice during EAE (Fig. 60.3d).

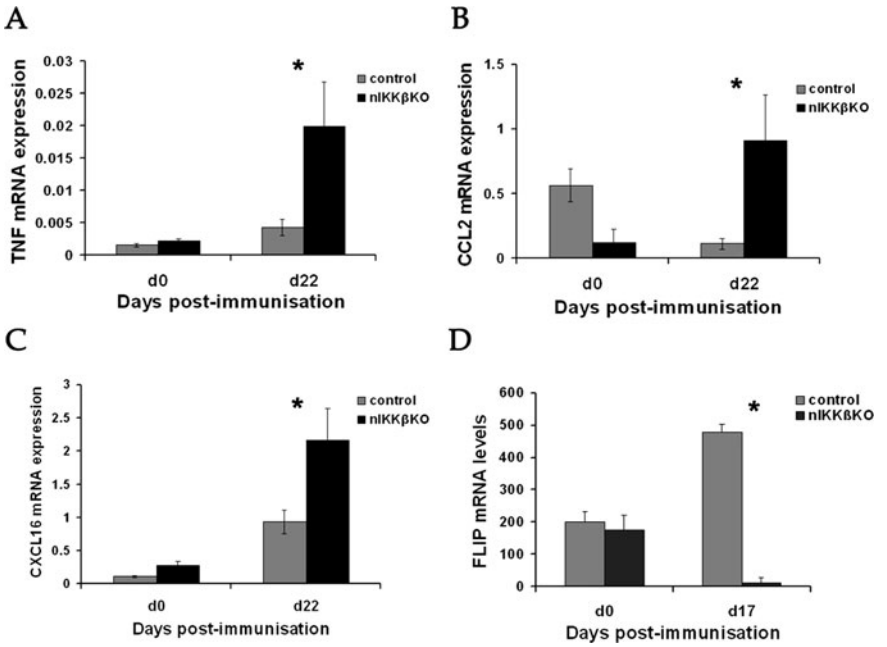
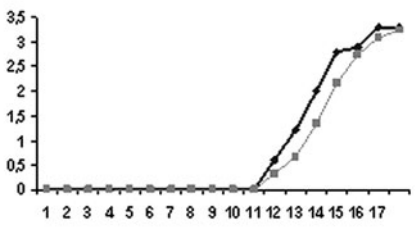


Fig. 60.3 Differential expression of immune genes in total mRNA isolates taken from nIKKβKO and control spinal cord prior to (d0) and 22 days after EAE induction as measured by qRT-PCR. nIKKβKO mice express increased levels of proinflammatory cytokines TNF (**a**), CCL2 (**b**), and CXCL16 (**c**) and fail to maintain or induce production of the neuroprotective protein FLIP (**d**) in the spinal cord during EAE

A



B

Cell markers	nIKKβKO	control	P value
$CD4^+$	20.08±3.17 %	28.08±3.36 %	**0.006**
$CD8^+$	4.18±1.20 %	2.73±0.25 %	NS
$NK1.1^+$	46.24±3.36 %	32.54±2.05 %	**0.3**
$CD11b^+$	18.28±1.40 %	20.43±0.50 %	NS
$CD4^+IFN\gamma^+$	9.08±1.09 %	16.75±2.09 %	**0.002**
$CD4^+IL17^+$	6.7±1.02 %	7.79±1.79 %	NS

Fig. 60.4 CNS-infiltrating monocytes isolated at the peak of EAE, that is equivalent for both groups (**a**), show enrichment of $CD4^+$ and $CD4^+IFN\text{-}\gamma^+$ T cells and reduction in $NK1.1^+$ cells in the spinal cord of nIKKβKO mice (**b**)

To investigate whether the increased levels of proinflammatory cytokines measured in spinal cord extracts from nIKKβKO EAE mice were reflected by differences in CNS-infiltrating immune cell populations, we isolated mononuclear cells from the spinal cords of mice with active EAE at the peak of disease (clinical score 3.5–4 in both strains) (Fig. 60.4a) and measured the proportions of the major immune cell populations known to be associated with EAE. No differences in the proportions of spinal cord $CD8^+$ or $CD11b^+$ macrophage were detected between the two strains (Fig. 60.4b). A significant increase in the proportion of $CD4^+$ T cells and a significant decrease in the proportion of $NK1.1^+$ cells were observed in the spinal cord compared to control (Fig. 60.4b). Further, intracellular staining of cytokines within the pool of CNS-infiltrating monocytes at the peak of EAE revealed a significant increase in the proportion of $CD4^+$IFN-γ^+ cells in the nIKKβKO compared to control spinal cord (Fig. 60.4b), but not $CD4^+$IL-17^+ cells (Fig. 60.4b).

Conclusion

The role of NF-κB in CNS neurons and its contribution to neurodegeneration during disease is important to define, particularly in view of current efforts to develop more selective blocking reagents for NF-κB and one of its major inducers, TNF, for the treatment of a variety of inflammatory conditions and cancer. In the study by Emmanouil et al. [18], we addressed the specific contribution of IKKβ in CamkII-expressing neurons to the development of EAE, an autoimmune model for MS that is characterized by CNS inflammation, demyelination, and early axonal damage. We showed that neuronal IKKβ does not affect the initiation of EAE but plays important roles in inducing the production of neuroprotective molecules, in modulating the CNS inflammatory response, and in regulating the accumulation of specific immune cell populations in the spinal cord after the induction of disease with the result of protecting mice against the development of severe neurologic impairment. Our findings demonstrate that neurons play an important immunoregulatory role during the development of autoimmune demyelination and identify IKKβ as a critical neuronal molecule that links neuroprotection with neuron-mediated suppression of CNS inflammation.

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Part XII
Cell Death Mechanisms Controlled by the TNF Family

Chapter 61
Workshop Summary: Cell Death Mechanisms Controlled by the TNF Family

John E. Eriksson and Peter Vandenabeele

Introduction

In this introduction we provide a short chronological overview of the presentations that have been given in the session on "Cell death mechanisms controlled by the TNF family." **Peter Vandenabeele** (VIB and University of Ghent, Belgium) started with a brief overview of the signal transduction pathways of three major cellular responses induced by TNF: cell survival through NF-kappaB activation, apoptosis, and programmed necrosis or necroptosis. Recruitment and activation of caspases play a crucial role in the initiation and execution of TNF-induced apoptosis. However, experimental inhibition of caspases reveals an alternative cell death pathway, namely necrosis, also called necroptosis, suggesting that caspases actively suppress the latter outcome. TNF-induced necrotic cell death crucially depends on the kinase activity of receptor interacting protein serine-threonine kinase 1 (RIP1) and RIP3. It was recently demonstrated that ubiquitination of RIP1 determines whether it will function as a pro-survival or pro-cell death molecule. Deeper insight into the mechanisms that control the molecular switches between cell survival and cell death will help us to understand why TNF can exert so many different biological functions in the etiology and pathogenesis of human diseases.

Martin Krönke (University of Cologne, Germany) reported on the molecular mechanisms of TNF-induced reactive oxygen species (ROS) produced by NADPH oxidase function as defence and signaling molecules related to innate immunity and various cellular responses. The activation of NADPH oxidase in response to plasma membrane receptor activation depends on the phosphorylation of

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cytoplasmic oxidase subunits, their translocation to membranes, and the assembly of all NADPH oxidase components. TNF binding to TNFR1 leads to the recruitment of riboflavin kinase (RFK, formerly known as flavokinase) that physically and functionally couples TNFR1 to NADPH oxidase. In mouse and human cells, RFK binds to both the TNFR1-death domain and the p22(phox), the common subunit of NADPH oxidase isoforms. RFK-mediated bridging of TNFR1 and p22(phox) is a prerequisite for TNF induced but not for Toll-like-receptor-induced ROS production. Exogenous flavin mononucleotide or FAD was able to substitute fully for TNF stimulation of NADPH oxidase in RFK-deficient cells. RFK is rate limiting in the synthesis of FAD, an essential prosthetic group of NADPH oxidase. The results suggest that TNF, through the activation of RFK, enhances the incorporation of FAD in NADPH oxidase enzymes, a critical step for the assembly and activation of NADPH oxidase.

Zhenggang Liu (NIH, USA) reported on the identification of a novel anti-apoptotic gene, ATIA (anti-TNF-induced apoptosis), which protects cells against TNF-induced apoptosis. ATIA forms a complex with TRAF2 and localizes on the plasma membrane and in the mitochondria. The latter localization apparently is essential for the protection against TNF-induced apoptosis and requires association with mitochondrial thioredoxin-2 (TRX-2). The anti-apoptotic function has been confirmed in vivo using the recently developed ATIA knockout mice.

Francis Chan (University of Massachussets, USA) reported on the role of RIP3 in TNF-induced necrosis. He showed that during TNF-induced necrosis a RIP1/RIP3 complex is formed and RIP3-mediated phosphorylation of RIP1 occurs. Necrosis is also an important antiviral immune defence mechanism. Using vaccinia virus infections in mice as models, he demonstrated a critical role for RIP3-dependent programmed necrosis in virus-induced inflammation and innate immune responses.

Uwe Bertsch (University of Kiel, Germany) reported on the internalization of the TNFR1 complex and how subsequent apoptotic signaling involves generation of ceramide by acidic sphingomyelinase (A-Smase) and subsequent activation of cathepsin D resulting in the cleavage of Bid. Confocal microscopy and immunomagnetically isolated TNF receptosomes revealed the colocalization of caspase-8 and A-Smase. A functional link between the latter two was suggested by the absence of A-Smase activation in caspase-8-deficient cells. However, caspase-8 is not a direct activator of pro-A-Smase but requires the effector caspase-7 in order to activate A-Smase in the TNF receptososomes.

Timothy R. Billiar (University of Pittsburgh, USA) reported on the identification of death-inducing signaling complex (DISC) components in cultures of primary hepatocytes following exposure to TNF or TNF/actinomycine D (ActD) using a combination of confocal and electron microscopy as well as cell fractionation, immunoprecipitation, and western blotting. The studies showed a rapid formation of a DISC involving TNFR1, TRADD, FADD, and caspase-8, which is increased by ActD. Interestingly, different DISC components were found at various subcellular localization: TNFR1/TRADD was found in the cytosol; TNFR1/FADD/caspase-8 in the mitochondria; and TNFR1, TRADD, FADD, and caspase-8 in the nucleus. The presence of ActD enhanced DISC components localization in the mitochondria

and the nucleus. The functional implications of the subcellular localization of DISC components remain enigmatic.

Adrian T. Ting (New York, USA) elaborated on how TNFR1 ligation can result in cell survival or cell death. The current model suggests that it is the activation of the NF-κB pathway and its induction of prosurvival genes, or the lack thereof, which determines the outcome. NEMO-deficient cells are highly sensitive to apoptosis, and as NEMO is essential for NF-κB activation, it has been assumed that this is due to the lack of NF-κB. This assumption was incorrect and NEMO has apparently another anti-apoptotic function independent of its role in the NF-κB pathway. NEMO prevents RIP1 from engaging caspase-8 before NF-κB-mediated induction of anti-apoptotic genes. Without NEMO, RIP1 associates with caspase-8 resulting in rapid tumor necrosis factor TNF-induced apoptosis. These results suggest that there are two cell death checkpoints following TNF stimulation: an early transcription-independent checkpoint whereby NEMO restrains RIP1 from activating the caspase cascade, followed by a later checkpoint dependent on NF-κB-mediated transcription of prosurvival genes.

Lester Lau (University of Illinois, Chicago, USA) gave a presentation on how the cell matrix may give cues to death receptor signaling pathways. A novel matrix-associated protein, CCN1 (CYR61), synergizes with apoptotic signaling from death receptors by elevated production of reactive oxygen species (ROS). CCN1 interacts with integrins and heparin sulphate proteoglycans to generate ROS from several sources, including mitochondria and 5-lipoxygenase. CCN1 is likely to be of importance during developmental processes and wound healing, providing a mechanism for integrating dynamic changes in the extracellular matrix with control of death receptor signaling.

Han-Ming Shen (University of Singapore, Singapore) reported on the mechanism how caspase inhibitors such as z-VAD-fmk induce necrotic cell death in L929 cells. Apparently, the cytotoxic mechanism involves autocrine produced TNF. This was demonstrated by using transcription or translation blockers and antagonists of TNF or TNFR1. z-VAD-fmk induction of TNF involves RIP1-mediated non-canonical NF-κB. This mechanism may represent a general example on how cellular stress induced by pharmacological agents may evoke cytotoxicity through autocrine TNF production. A similar observation was done for PMA and IAP antagonists.

Bruce R. Ksander (Harvard Medical School, Boston, USA) presented a mechanism by which death receptor interactions are likely to be operative in glaucoma, a disease characterized by uncontrolled cell death of retinal ganglion cells. Increased eye pressure is characterized by elevated levels of TNF-a, which in turn causes expression of FasL on microglia and/or astrocytes. During these conditions, the RGCs are sensitized to the FasL signaling and will undergo apoptosis unselectively. The study identifies FasL expression mediated by genetic or other factors as an important cause for glaucoma and, furthermore, outlines a number of therapeutic targets that could be used for treatment of glaucoma.

Lisa Sedger (Westmead Millenium Institute, Sydney, Australia) linked together lymphoproliferative diseases with the interaction between TRAIL and FasL by employing mice double deficient for these death ligands. The double-deficient mice died early due to uncontrolled lymphoproliferative disease and displayed obvious

autoimmune symptoms due to accumulation of autoimmune antibodies. The results demonstrate that the signaling pathways regulated by these ligands cooperatively control lymphocyte homeostasis in vivo, by modulating the activation and execution phases of activation-induced cell death. It remains to be seen whether this approach could be employed for pharmacological purposes to define molecular targets in immune diseases.

Najoua Lalaoui (Univ. of Borgogne, Dijon, France) presented a surprising effect by decoy receptor 2 (DcR2), which has previously been shown to protect cancer cells against TRAIL-mediated apoptosis. While this inhibition may be a result of direct competition for TRAIL, the presented results indicate that there is another measure by which DcR2 is able to mediate protection is the through activation of PI3K/Akt pathway. Hence, DcR2 has two functional modalities, one at the membrane level and one at the level of survival signaling by affecting PI3K/Akt signaling through an unknown mechanism.

Martin Zörnig (Georg-Speyer-Haus, Germany) employed a novel concept in deciphering FasL signaling by using an approach that involved the knockin of the FasL, which lacked the intracellular domain. The question of reversed FasL signaling has remained enigmatic and the hope is that this approach should provide an answer to this question. While it was already clear that the truncated FasL is still able to induce cell death in Fas-sensitive cells, the experiments deciphering the effects on T and B-cells from the generated mice were ongoing. Preliminary results indicated an inhibitory role for the intracellular Fas ligand on ERK1/2 signaling during lymphocyte activation.

Philippe J. Jost (The Walter and Eliza Hall Institute, Melbourne, Australia) presented results indicating that XIAP would be a critical factor in the switching between type I and type II signaling modes of Fas responses. While the type II signaling involves participation of the BH3 domain only protein Bid, gene targeting of XIAP and Smac/Diablo mimetic inhibitory drugs made hepatocytes independent of Bid. If XIAP indeed is the key determinant between type I and type II signaling, as the results indicate, the study implies that cancer therapies using general IAP inhibitors should be planned carefully, bearing this cautionary aspect in mind.

John E. Eriksson (Åbo Akademi University, Turku, Finland) reported on the deciphering post-translational modifications that determine the turnover of cellular FLICE-inhibitory proteins (c-FLIP). The three c-FLIPs are crucial regulators of the death-inducing signaling complex (DISC) and caspase-8 activation. Ser-193 was identified as a novel in vivo phosphorylation site of all c-FLIP proteins and demonstrated to selectively influence the stability of the short c-FLIP isoforms. Surprisingly, this site did not affect the stability of the long isoform c-FLIP(L), indicating that S193 phosphorylation has a different function in c-FLIP(L). This site was shown to be operated by the protein kinase C (PKC). The results revealed that the differential stability of c-FLIP proteins is regulated in an isoform-specific manner by PKC-mediated phosphorylation. Taken together, the identified c-FLIP phosphorylation sites are likely to generate a signaling hub, which integrates numerous different extracellular and intracellular signals in a way that is consequential for the fate decisions of the cell.

Chapter 62
A RNA Interference Screen Identifies RIP3 as an Essential Inducer of TNF-Induced Programmed Necrosis

YoungSik Cho, Sreerupa Challa, and Francis Ka-Ming Chan

Introduction

Cell death plays an important role in homeostasis of multi-cellular organisms. TNF-like cytokines and their cognate receptors are important activators of cell death. In recent years, it has become clear that TNF-like cytokines can trigger multiple forms of cell death that are distinct in morphologies and mechanisms. Programmed necrosis (also known as necroptosis) is a caspase-independent, non-apoptotic form of cell death whose molecular mechanism is poorly defined [1]. Until recently, the only identified molecular component of programmed necrosis is the protein serine/threonine kinase Receptor Interacting Protein 1 (RIP1). Morphologically, programmed necrosis is marked by organelle swelling and extensive intracellular vacuolation. The disruption of osmotic balance within the dying cell eventually led to rupture of plasma membrane. The release of cellular "adjuvants" from the necrotic cells promotes inflammation [2]. These features distinguish cell death by programmed necrosis from apoptosis, which is generally considered to be non-inflammatory and tolerogenic.

Identification of RIP Kinases as Critical Mediators for Programmed Necrosis

RIP1 is a pleiotropic adaptor for TNF receptor signaling. Genetic "knock-out" and biochemical studies indicate that RIP1 is an essential adaptor for TNF-induced NF-κB activation. Interestingly, the kinase function of RIP1 is not required for RIP1-mediated NF-κB activation. Rather, the RIP1 kinase activity is essential for induction of programmed necrosis [3–5]. We reasoned that since RIP1 can activate multiple downstream signals, additional cues must be involved to turn on its

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pro-necrotic function. In order identify the molecular component(s) that control the pro-necrotic activity of RIP1, we performed a targeted RNA interference (RNAi) screen. We focused our screen on kinases based on the assumption that the pro-necrotic function of RIP1 is either turned on by an upstream kinase or mediated through a downstream kinase.

FADD-deficient Jurkat cells were chosen for our screen because these cells undergo programmed necrosis exclusively in response to TNF stimulation [4]. We introduced 21-mer small interference RNAs (siRNA) into FADD-deficient Jurkat cells and found that out of 691 kinases, siRNA against RIP3 along with RIP1 conferred the strongest protection against TNF-induced programmed necrosis. siRNA-mediated silencing of RIP1 and RIP3 specifically inhibited TNF-induced programmed necrosis, but not apoptosis or NF-κB activation [6]. Thus, RIP3 fulfills the requirement as a specific inducer of programmed necrosis.

RIP3 Is Recruited to the Cytoplasmic Signaling Complex and Interacts with RIP1 via the RHIM

TNFR-1 signaling is mediated by two spatially and temporally distinct complexes. The TNFR-1 associated "Complex I" is made up of the receptor, RIP1, TRAF2, and TRADD. Complex I is a transient complex and is rapidly internalized within the first hour of stimulation. Upon internalization, TNFR-1 dissociates from Complex I to allow binding of additional adaptors including FADD and caspase-8 to the complex [7]. This cytoplasmic signaling complex, termed "Complex II," is responsible for induction of apoptosis. Unlike RIP1, which was present in both Complex I and Complex II, RIP3 was only recruited to the caspase-8 associated Complex II, but not Complex I. Interestingly, RIP3 was recruited to caspase-8 associated complex under both apoptotic (TNF alone) and pro-necrotic conditions (TNF + zVAD-fmk). However, when RIP1-associated complexes were examined, it became clear that RIP3 only interacted with RIP1 when cells were stimulated with TNF in the presence of caspase inhibition [6]. Thus, we conclude that the interaction between RIP1 and RIP3 is specifically induced in cells undergoing programmed necrosis.

The RHIM and Kinase Domains of RIP1 and RIP3 Are Essential for Programmed Necrosis

The necrosis-specific interaction between RIP1 and RIP3 requires an intact RIP homotypic interaction motif (RHIM) for both RIP kinases, since tetra-alanine substitutions abolished their interaction. Interestingly, RIP1 and RIP3 underwent necrosis-specific phosphorylation. Inhibition of RIP1 kinase activity with the RIP1-specific inhibitor necrostatin-1 [8] abolished TNF-induced RIP3, but not RIP1 phosphorylation [6]. Necrosis-specific phosphorylation of RIP1 was also absent in $RIP3^{-/-}$ cells [6]. These experiments indicate that both RIP1 and RIP3 are required

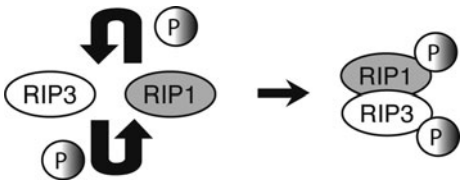

Fig. 62.1 Trans-phosphorylation of RIP1 and RIP3 stabilizes their interaction during programmed necrosis

for their necrosis-specific phosphorylation, possibly through trans-phosphorylation (Fig. 62.1). Necrosis-specific phosphorylation of RIP1 and RIP3 is important for stable RHIM-mediated RIP1 and RIP3 interaction, since inhibition of RIP1 activity by necrostatin-1 abolished their interaction under pro-necrotic stimulation [6]. Functionally, phosphorylation and assembly of the RIP1–RIP3 pro-necrotic complex is important for activation of Complex II kinase activity, since necrostatin-1 inhibited the induction of necrosis-specific Complex II kinase activity [6]. These results indicate that RIP1 and RIP3 phosphorylation plays a critical role in the early induction of programmed necrosis by promoting formation and activation of the pro-necrotic signaling complex. Further experiments are required to definitively determine the hierarchy of activation of RIP1 and RIP3.

RIP3 Controls ROS Production

Reactive oxygen species (ROS) are critical mediators of necrosis in many cell systems [9]. Recently, the Nox-1 [10] and riboflavin kinase [11] were identified as critical upstream regulators for ROS production during TNF-induced programmed necrosis. Both Nox-1 and riboflavin kinase were implicated to signal via the TNFR-1 membrane associated complex, suggesting that ROS is generated early during programmed necrosis to mediate cell death. Interestingly, we found that RIP3 also controls production of ROS during programmed necrosis [6]. As we have mentioned above, RIP3 is recruited to the cytoplasmic Complex II, but not the TNFR-1 associated Complex I. Thus, our results suggest that multiple pathways might control the generation of ROS during programmed necrosis, perhaps in a cell-type specific manner.

The Role of RIP3 in T-Cell Death

In recent years, many of the components of apoptosis pathway have been shown to participate in other signaling pathways. For example, the death receptor adaptor FADD and the initiator caspase caspase-8 were shown to be required for T-cell activation [12, 13, 14]. FADD$^{-/-}$ or caspase-8$^{-/-}$ T-cells failed to proliferate in response to T-cell receptor stimulation [15]. Rather, these cells underwent cell death that was inhibited by the RIP1 kinase inhibitor necrostatin-1 or RIP1-specific small interference RNA [16]. Consistent with these observations, we found that RIP3$^{-/-}$

T-cells exhibited enhanced protection against restimulation-induced T-cell death when caspases are inhibited by the broad-specificity caspase inhibitor zVAD-fmk or by vaccinia virus-encoded caspase inhibitor B13R/Spi2 [6]. These results indicate that RIP1/RIP3-dependent programmed necrosis may control antigen-specific T-cell expansion when caspases are inhibited, such as that during certain viral infections.

RIP3 Participates in Anti-viral Innate Immune Responses

The fact that TNF can also induce apoptosis raises questions about the physiological function of programmed necrosis. As we have discussed already, optimal induction of programmed necrosis requires caspase inhibition. Importantly, many viruses encode caspase or apoptosis inhibitors. Thus, infection by viruses that inhibit caspase might skew the cell death response toward programmed necrosis. Indeed, TNF induces cell death with classical necrotic morphology in vaccinia virus-infected cells [6, 17]. In contrast, vaccinia virus-infected RIP3$^{-/-}$ mouse embryonic fibroblasts were protected from TNF-induced programmed necrosis [6]. Similarly, vaccinia virus-infected RIP3$^{-/-}$ T-cells were also protected from T-cell receptor-induced cell death.

Like most poxvirus infections, vaccinia virus infection causes florid inflammation. The resistance of vaccinia virus infected RIP3$^{-/-}$ cells suggests that RIP3-dependent necrosis might contribute to anti-viral responses. We examined the response of RIP3$^{-/-}$ mice to vaccinia virus infections and found that necrosis in the liver and visceral fat pad was conspicuously absent in RIP3$^{-/-}$ mice. The lack of necrosis in RIP3$^{-/-}$ mice correlated with a lack of inflammation in the infected tissues [6]. Consequently, the RIP3$^{-/-}$ mice failed to control viral replication and succumbed to the infection [6]. These results are consistent with a model in which programmed necrosis controls virus infection by eliminating the viral factory and to promote inflammation through the release of "endogenous adjuvants" from the necrotic cells [2] (Fig. 62.2). In addition to virus-induced inflammation, RIP3 might play a wider role in controlling other inflammatory processes. For instance, RIP3$^{-/-}$ mice were protected from cerulein-induced pancreatitis [18, 19]. Taken together, our results show that RIP3-dependent programmed necrosis is important for controlling inflammation in certain pathological situations.

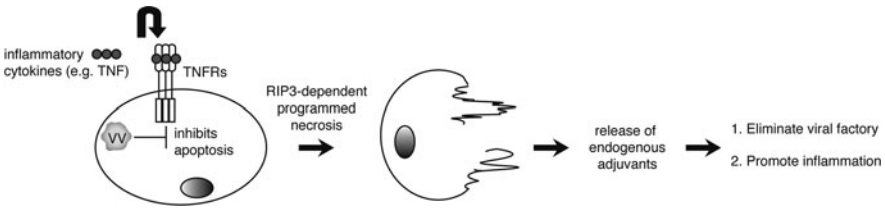

Fig. 62.2 Programmed necrosis controls the viral factory and promotes inflammatory through the release of endogenous adjuvants

In summary, we have shown that a RIP1–RIP3 complex is critical for driving programmed necrotic cell death. It will be of interest in the future to determine whether a common RIP1/RIP3-dependent pathway regulates other physiological and pathological processes that involves necrotic cell injury.

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Chapter 63
The Extracellular Matrix Protein CCN1 Dictates TNFα and FasL Cytotoxicity In Vivo

Chih-Chiun Chen, Vladislava Juric, and Lester F. Lau

Introduction

Extensive analysis of the apoptotic activity of TNFα has revealed elegant details in its apoptotic pathway, and expression of TNFα is required for or strongly implicated in apoptosis in certain contexts in vivo [1–3]. Yet TNFα by itself is unable to induce apoptosis in normal cells in culture, but requires the blockade of de novo protein synthesis or NFκB signaling to be cytotoxic [4, 5]. How might TNFα induce apoptosis in vivo? Here we present evidence that the extracellular matrix (ECM) microenvironment, specifically the presence of the CCN family of matricellular proteins, can dictate the cytotoxicity of TNFα and related cytokines.

The CCN Family of Extracellular Matrix Proteins

The CCN family of ECM proteins consists of six structurally conserved members in vertebrates, and is named after the first three members identified: *C*yr61 (cysteine rich 61, CCN1), *c*onnective tissue growth factor (CTGF, CCN2), and *n*ephroblastoma overexpressed (Nov, CCN3) [6–8]. CCN proteins share a similar modular structure, characterized by an N-terminal secretory peptide followed by structural domains with homologies to insulin-like growth factor binding proteins (IGFBP), von Willebrand factor type C repeat (vWC), thrombospondin type I repeat (TSR), and a C-terminal domain (CT) that contains a "cysteine knot" motif found in some growth factors (Fig. 63.1). These dynamically expressed proteins regulate diverse aspects of cellular behavior, including cell adhesion, migration, proliferation, survival, and differentiation in many cell types. As such, CCNs are recognized as "matricellular" proteins to draw distinction from classical ECM proteins whose primary roles are critical for the structural integrity of tissues [9, 10].

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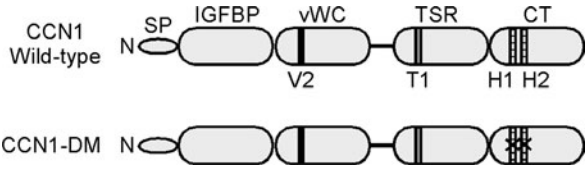

Fig. 63.1 A schematic diagram of CCN1 protein structure**.** At the N-terminus (N) of CCN1 is a secretory signal peptide, followed by four modular domains with sequence homologies to insulin-like growth factor binding protein (IGFBP), von Willebrand factor type C repeat (vWC), thrombospondin type 1 repeat (TSR), and a C-terminal domain (CT) that contains a cysteine knot motif. All other CCN proteins share the same domain structure except CCN5, which lacks the CT domain. The receptor-binding sites identified in CCN1 are V2, an $\alpha_v\beta_3$ integrin-binding site in the vWC domain [51]; T1, an $\alpha_6\beta_1$ integrin-binding site in the TSR domain [52]; and H1 and H2, binding sites for $\alpha_6\beta_1$ and HSPGs in the CT domain [20]. The CCN1-DM mutant protein is disrupted in the H1 and H2 sites, thereby abrogating $\alpha_6\beta_1$-HSPGs-dependent activities while preserving $\alpha_v\beta_3$-dependent angiogenic functions [21]

Like other ECM cell adhesion molecules, CCNs function through direct binding to integrin receptors, utilizing cell surface heparan sulfate proteoglycans (HSPGs) as co-receptors in some contexts [6].

Accumulating evidence indicate that CCN proteins regulate the development of the cardiovascular and skeletal systems during embryogenesis and participate in wound healing and tissue repair in adults [6–8]. Correspondingly, targeted disruptions of *Ccn1* and *Ccn2* in mice result in embryonic and peri-natal lethality due to cardiovascular and skeletal defects, respectively [11–13], although *Ccn3*-null mice are viable and exhibit only modest and transient skeletal phenotypes [14]. In adults, CCNs are highly induced at sites of injury repair and inflammation where TNF cytokines are often co-expressed, providing the opportunity for their interaction.

CCN Proteins and TNF Cytokines Synergize to Induce Apoptosis In Vitro and In Vivo

TNFα is a potent activator of the NFκB pathway, which drives the expression of many pro-inflammatory and anti-apoptotic genes, thereby promoting cell survival. Paradoxically, TNFα can also activate a powerful apoptotic pathway if NFκB signaling or de novo protein synthesis is blocked [4, 5]. Indeed, mice that are deficient in NFκB signaling die in utero from TNF-dependent apoptosis of liver cells [15]. Thus, the apoptotic activity of TNFα is thought to be contextual, subject to sensitizing viral infection or IFN-γ that perturbs NFκB signaling or protein synthesis. LTα binds the same receptors as TNFα and is thought to act similarly. In contrast, FasL and TRAIL are weak inducers of NFκB and do not require inhibition of NFκB signaling to induce apoptosis, but their cytotoxicity is nevertheless regulated by environmental factors.

Our recent studies showed that the extracellular matrix microenvironment, as reflected by the presence of the dynamically expressed CCN proteins, can profoundly regulate TNF cytokine cytotoxicity. The matricellular proteins CCN1, CCN2, or CCN3, either in a soluble form or as cell adhesion substrates, enable TNFα and LTα to induce apoptosis and enhance the cytotoxic effects of FasL and TRAIL without perturbation of NFκB signaling or protein synthesis, leading to rapid apoptosis [16–18]. Whereas TNFα and LTα did not induce cell death on their own, each of the three CCN proteins enables them to induce apoptosis in ~25% of cells, more than 2-fold higher than the presence of 10 μg/ml cycloheximide within 4–6 h (Fig. 63.2), further suggesting that CCNs work through a mechanism distinct from inhibition of protein synthesis. These activities appear unique to CCN proteins and are not found in other ECM proteins tested, including collagen, fibronectin, laminin, and vitronectin [16]. Thus, although TNFα alone promotes cell proliferation in fibroblasts by inducing the expression of PDGF [19], the presence of CCN proteins can unmask its apoptotic activity and turn it into a cytotoxic factor.

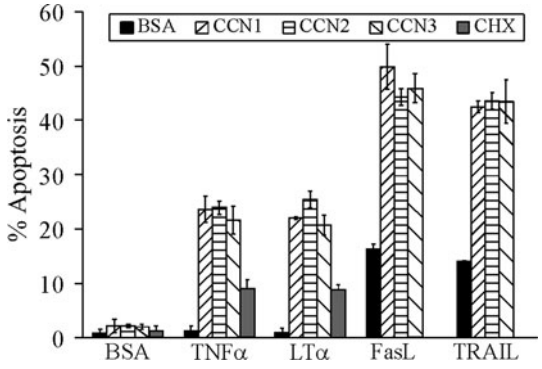

Fig. 63.2 Apoptotic synergism between the CCN and the TNF protein families. Normal human skin fibroblasts were serum-starved overnight before being treated for 6 h at 37°C with serum-free media containing purified recombinant CCN1, CCN2, or CCN3 proteins (5 μg/ml each), with or without TNFα (10 ng/ml), LTα (10 ng/ml), FasL (50 ng/ml), or TRAIL (20 ng/ml). Where indicated, cycloheximide (CHX, 10 μg/ml) was added to cells 15 min prior to addition of TNFα. Apoptotic cells were scored by DAPI staining as described [16]

Since *Ccn1*- and *Ccn2*-null mice suffer pre- and peri-natal lethality, respectively, these animal models are not suitable for studying the effects of CCNs on TNF cytokine functions in vivo. To circumvent this problem, we created knockin mice in which the genomic *Ccn1* was replaced by an allele that encodes a mutant CCN1, DM, which is disrupted in the two $\alpha_6\beta_1$-HSPG binding sites in the CT domain (Fig. 63.1), leaving the other integrin-binding sites in CCN1 intact [20, 21]. The CCN1-DM mutant protein is unable to synergize with TNFα or FasL to promote fibroblast apoptosis [16, 17], but is fully active in mediating integrin $\alpha_v\beta_3$-dependent angiogenic activities [21]. In contrast to *Ccn1*-null mice, *Ccn1*$^{dm/dm}$ mice are viable, fertile, and without any apparent abnormality [16]. CCN1/TNFα synergism was

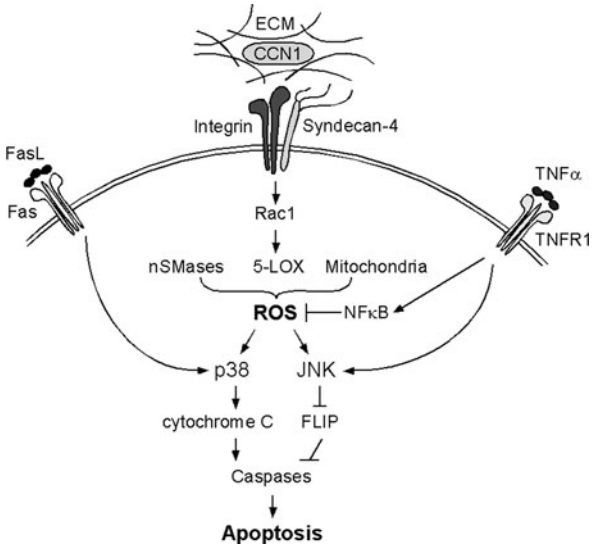

Fig. 63.3 Pro-apoptotic signaling cross talk between CCN1 and TNFα and FasL. The matricellular protein CCN1 binds to integrins, $\alpha_v\beta_5$, and $\alpha_6\beta_1$, and the HSPG syndecan-4 to generate a high level of ROS through several mechanisms involving nSMases, 5-LOX, and the mitochondria [16, 17]. The level of CCN1-induced ROS is sufficient to override the anti-apoptotic effects of NFκB by enhancing and maintaining the activation of JNK, which targets c-FLIP for degradation and allows activation of caspases-8/10, leading to apoptosis. In the presence of FasL, CCN1-induced ROS allows the hyperactivation of p38MAPK, which promotes Bax activation and cytochrome *c* release

first tested in *Ccn1*$^{dm/dm}$ mice by a subcutaneous injection of a bolus (50 μl) of high concentration TNFα (0.5 μM), which rapidly induced cutaneous apoptosis at the injection site. Consistent with the notion that CCN1 is critical for TNFα cytotoxicity, the number of apoptotic cells in *Ccn1*$^{dm/dm}$ mice was reduced by >60% compared to wild-type mice [16].

We further tested the *Ccn1*$^{dm/dm}$ mice in three different models of toxin-induced hepatitis to examine TNF cytokine-mediated apoptosis in vivo. First, intravenous delivery of the plant lectin concanavalin A activates inflammatory cells in the liver, inducing hepatocyte apoptosis in a TNFα-dependent process that is abrogated by neutralizing antibodies against TNFα or genetic ablation of TNFR1 or TNFR2 [22, 23]. Second, tail vein injection of the agonistic anti-Fas monoclonal antibody Jo2 activates the Fas receptor and leads to massive hepatocyte apoptosis that is completely Fas dependent [24]. Finally, intragastric administration of ethanol gavage mimics binge drinking and leads to FasL-mediated hepatocyte apoptosis that is prevented by neutralizing antibodies against FasL [25]. In all three experimental models, *Ccn1*$^{dm/dm}$ mice consistently show >60% reduction in hepatocyte apoptosis compared to wild-type mice [16, 17]. These results support the notion that CCN1 is a physiologic regulator of TNFα- and Fas-mediated apoptosis in vivo and that its interaction with $\alpha_6\beta_1$/HSPGs is critical for this activity. Thus, the extracellular

matrix microenvironment, specifically the presence of CCN1, can profoundly affect the cytotoxicity of TNF cytokines. These results do not exclude the participation of other factors such as IFNγ, which can also regulate TNF cytotoxicity in certain contexts [26].

ROS Mediates Signaling Cross Talk Between CCNs and TNFα or FasL

Upon TNFα treatment, activated TNFR1 recruits the adaptor protein TRADD through its intracellular death domain, leading to the recruitment of RIP and TRAF2 to form a complex (complex I) that is critical for activation of NFκB and the stress kinase JNK. This signaling complex subsequently dissociates from TNFR1 and recruits FADD and procaspases-8/10 to form complex II, which triggers the activation of the caspase cascade and apoptosis [2, 5]. However, the anti-apoptotic c-FLIP, which exists in the cell and is also induced by NFκB, can compete with procaspases-8/10 for binding to complex II and thereby prevent caspase activation by TNFα [27, 28]. Since JNK can phosphorylate the ubiquitin ligase ITCH, which targets c-FLIP for proteosome degradation, a sustained level of activated JNK leads to elimination of c-FLIP and allows the apoptotic pathway to proceed [29]. However, TNFα-activated JNK in normal cells is rapidly inactivated by MAPK phosphatases, which are also induced by NFκB [30], thus protecting c-FLIP from JNK-mediated degradation. MAPK phosphatases are, in turn, sensitive to the redox state in the cell and are inactivated by cellular reactive oxygen species (ROS) through oxidation of the critical cysteine residues in their active sites [31]. NFκB also induces anti-oxidant proteins, including Mn^{2+}-superoxide dismutase and ferritin heavy chain, which reduce cellular ROS levels [32, 33]. Thus, NFκB suppresses the apoptotic activities of TNFα in normal cells by inducing de novo synthesis of c-FLIP, MAPK phosphatases, and anti-oxidant proteins, all of which serve to limit JNK activity and therefore apoptosis.

CCN proteins cross talk with TNF signaling by inducing a high level of ROS, apparently sufficient to override the anti-apoptotic effects of NFκB, without inhibiting NFκB activity [16, 17]. CCN1-induced ROS allow TNFα-induced JNK activation to be sustained despite NFκB function, leading to JNK-dependent apoptosis [16]. ROS are also critical for CCN1/FasL synergism, where they trigger the hyperactivation of p38 MAPK, Bax activation, and mitochondrial localization, leading to cytochrome *c* release and apoptosis [17].

Mechanistically, CCN1 induces ROS generation through binding to integrins, $\alpha_v\beta_5$, $\alpha_6\beta_1$, and the HSPG syndecan-4 [16], leading to activation of the small GTPase RAC1 [34]. RAC1 regulates ROS generation through multiple mechanisms, including 5-lipoxygenase (5-LOX), certain isoforms of NADPH oxidase (NOX), and the mitochondria [35, 36]. In CCN1/TNFα synergism, RAC1-dependent ROS generated through 5-LOX and mitochondria are critical [16]. Although TNFα induces ROS generation through a NOX-dependent mechanism [16, 37], this

pathway of ROS generation is not required for CCN/TNFα synergism [16]. CCN1 also induces neutral sphingomyelinase 1 (nSMase1) activity [17], which leads to the production of the lipid second messenger ceramide and the generation of ROS [38]. Studies using chemical inhibitor and siRNA showed that nSMase1-dependent generation of ROS is critical for p38 hyperactivation in CCN1/FasL synergism [17]. Thus, CCN1 is able to induce high level of ROS through multiple pathways that override the antioxidant effects of NFκB.

Future Prospects

Our recent studies unravel a novel mechanism by which the tissue microenvironment directs or reinforces the apoptotic activity of TNF cytokines. In what biological contexts is this pro-apoptotic CCN/TNF synergism important? We have shown that CCN1 is critical for TNFα and FasL-mediated apoptosis in the liver, suggesting that CCNs may participate in hepatocyte injury in a diverse array of liver diseases in which TNF cytokines play a role. For example, TNFα and FasL levels are elevated in hepatic diseases in which apoptosis is induced by toxin exposure, alcohol abuse, and microbial infection. Accordingly, inhibition of TNFα and FasL by means of neutralizing antibodies, soluble decoy receptors, or genetic disruption resulted in reduction of hepatocyte cell death [22, 25, 39–45]. TNFα and FasL are also critical in cardiomyocyte apoptosis after cardiac ischemia and infarction, conditions where overexpression of CCN1 and CCN2 has been observed, suggesting that CCN/TNF apoptotic synergism may be important in cardiac injury as well [46–50]. Beyond apoptosis, CCNs may also regulate other aspects of TNF cytokine function. These possibilities are currently under investigation.

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Chapter 64
Compartmentalization of TNF-Receptor 1 Signaling: TNF-R1-Associated Caspase-8 Mediates Activation of Acid Sphingomyelinase in Late Endosomes

Uwe Bertsch, Bärbel Edelmann, Vladimir Tchikov, Supandi Winoto-Morbach, and Stefan Schütze

TNF-Receptor Signaling

TNF-α is the prototypic cytokine of the TNF superfamily. TNF was first described as a cytokine that exhibits antitumor effects in mouse models, but it is now recognized as a highly pleiotropic cytokine that triggers a variety of cellular responses. These range from induction of inflammation, through differentiation, and control of cell proliferation to the initiation of apoptosis in target cells expressing specific receptors for this cytokine. TNF is known to bind to two receptors of the TNF-receptor superfamily: TNF-receptor 1 (TNF-R1, also known as p55, p60, or CD120a) and TNF-receptor 2 (TNF-R2, also known as p75, p80, or CD120b). TNF-R1 is a member of the death receptor subgroup within the TNF-receptor superfamily. This subgroup comprises CD95 (also known as Fas or APO-1) and TNF-related apoptosis-inducing ligand (TRAIL) receptors 1 and 2 (also known as DR4 and DR5, respectively) as well as DR3, DR6, and p75NTR. These death receptors are distinguished by a "death domain" (DD), a conserved, 80 amino acid sequence in their cytoplasmic tail which is necessary for direct activation of the apoptotic program elicited by some (TNF-R1, CD95, TRAIL-R1, and TRAIL-R2) of these receptors. TNF-R1 is responsible for most of the cellular processes that are triggered by TNF. In particular, TNF-R1 is the only receptor that mediates apoptotic signals in response to TNF stimulation.

Non-apoptotic Signaling of TNF-Receptor 1

As already mentioned, binding of TNF to TNF-R1 can activate different signaling pathways. In any case ligand-bound TNF-R1 recruits the TNF-R-associated death

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domain (TRADD) protein to the cytoplasmic DD of the receptor [22]. This adaptor protein serves as assembly platform for further signaling proteins. Within minutes after TNF stimulation complexes of TNF-R1 and TRADD recruit receptor interacting protein-1 (RIP1) and TNF-receptor-associated protein-2 (TRAF2) as well as the inhibitor of apoptosis protein 1 (c-IAP1) to form a signaling complex at the cell surface. Micheau and Tschopp [37] termed this assembly "complex I." They showed that it activates NF-κB through recruitment of the inhibitor of κB (IκB) kinase (IKK) "signalosome" [8]. The signalosome is a high molecular weight complex that assembles three main proteins: two IκB kinases (IKK1 and IKK2) and the scaffold protein IKKγ (also known as NEMO). Apart from the role of activated NF-κB as a main transcription factor required for the expression of inflammatory cytokines, it also regulates the expression level of proteins involved in the intracellular processing of apoptosis signals. Thus the formation of complex I positively regulates the cellular levels of the FLICE-inhibitory protein $cFLIP_L$ and cIAP [36]. These proteins all function as inhibitors of caspase-8 activation in apoptosis signaling, leading to an increased cell survival.

TRADD binding to TNF-R1 also leads to the induction of the c-Jun N-terminal kinase (JNK) cascade [31].

Apoptosis-Inducing Signals from TNF-Receptor 1

In the model of Micheau and Tschopp [37] TNF-R1 signaling proceeds through the consequential assembly of two distinct signaling complexes that differ in molecular composition and spatial distribution. These sequentially activate NF-κB first and later on caspases. Subsequent to the formation of complex I at the plasma membrane this model states that at later time points and after internalization of TNF-R1, RIP1, TRAF2, and TRADD become modified by ubiquitinylation and thereby dissociate from the receptor. The liberated DD of TRADD can then form a heteromeric complex with the DD of FADD. FADD in turn recruits pro-caspase-8 through its death effector domain (DED) to form the death-inducing signaling complex (DISC). Recruitment of pro-caspase-8 to the DISC results in the activation of this initiator caspase by autoproteolytic self-cleavage. Depending on the cell type-specific extent of DISC generation cell death can be reached by two distinct pathways. While Type I cells produce enough activated caspase-8 for direct cleavage of caspase-3 in order to reach the effector phase of apoptosis induction [4, 46], Type II cells need to activate an amplification loop via the mitochondrial apoptosis pathway that involves caspase-8-mediated cleavage of the pro-apoptotic Bcl-2 family member Bid. The resulting tBid fragment liberates cytochrome *c* from mitochondria, which leads to apoptosome assembly in the cytoplasm and activation of caspase-9 resulting in proteolytic activation of caspase-3 and caspase-7.

Mechanisms of Receptor Internalization

Internalization of cell surface receptors after binding to their cognate extracellular ligand has formerly been regarded as a mechanism merely used by cells to

terminate signaling via receptor degradation. However, there is now increasing evidence from a variety of biological systems that many receptors remain active even after endocytosis and that signaling by these receptors continues along their way through the endocytic compartments [34, 35, 55, 57]. It has become clear and is now widely accepted that compartmentalization of signaling pathways may ensure their precise spatial and temporal regulation in order to evoke a unique, receptor-specific response. Receptors and ligands can internalize from the cell surface by different routes. The mechanism of endocytosis that has been studied most extensively is the clathrin-mediated endocytosis (CME). In CME the selective recruitment of transmembrane receptors and their associated ligands into specialized membrane microdomains, called clathrin-coated pits (CCPs), represents the first step. Several adaptor protein complexes participate in this process. The binding of the adaptor protein complex-2 (AP2) to the plasma membrane through its lipid-binding domains and to specific transport sequences within the intracellular domain of the activated receptor initiates internalization. The best-characterized endocytosis motifs of cargo proteins are the tyrosine-based YXXΦ motif (where Y represents tyrosine, X any amino acid, and Φ a bulky hydrophobic amino acid) and dileucine-based motifs. Tyrosine-based endocytosis motifs can be found in the C-terminal intracellular domain of TNF-R1 as well as those of CD95 and DR6. Interaction of AP2 with the GTPase dynamin then leads to the formation of a dynamin ring around the neck of a deeply invaginated CCP. Shrinkage of the dynamin ring results in membrane fission and generation of free clathrin-coated vesicles (CCVs). After uncoating during intracellular trafficking, CCVs fuse with early endosomes from which ligand–receptor complexes are sorted to various intracellular compartments. Fusion of early endosome vesicles with trans-Golgi vesicles leads to the formation of late endosomes, also known as multivesicular bodies (MVBs). Receptor complexes that are destined for degradation finally fuse with lysosomes, where they undergo proteolysis. Clathrin-independent pathways also play important roles in endocytosis (reviewed in [14, 27, 33]). One form of clathrin-independent endocytosis that involves cholesterol and sphingolipid-enriched membrane domains, so-called lipid rafts, as well as specialized membrane invaginations, so-called caveolae, is termed raft/caveolar endocytosis (RCE). Raft association tends to concentrate specific proteins within these plasma membrane microdomains, thereby affecting receptor signaling pathways (recently reviewed in [26]). In addition, several different dynamin-independent endocytosis mechanisms distinct from both CCPs and caveolae have been identified (recently reviewed in [20, 41, 54]).

DISC Assembly Occurs on Internalized TNF-Receptosomes

The idea that TNF-receptor internalization may play a role in mediating TNF cytotoxicity was suggested initially almost two decades ago [25, 42]. Blockade of CCP formation by monodansylcadaverine (MDC) in U937 cells demonstrated that selected TNF-R1 DD signaling pathways, including the pathway that leads to apoptosis, were dependent on TNF-receptor internalization, while others were not [50]. This treatment inhibited activation of A-SMase and JNK as well as TNF-induced

cell death. By contrast, the interaction of TNF-R1 at the cell surface with the adaptor molecule factor associated with neutral sphingomyelinase (FAN) and TRADD was undisturbed. Thus, normal activation of plasma membrane-associated neutral sphingomyelinase (N-SMase) as well as the stimulation of proline-directed protein kinases (PDPK) was still possible [50].

We recently demonstrated the importance of internalized TNF-R1 as the essential platform for recruiting the DISC to TNF-R1 receptosomes [47, 48]. DISC formation occurred within 3 min after TNF stimulation, and DISC association with TNF-R1 remained stable for at least 60 min on TNF-R1 receptosomes. Inhibition of TNF-R1 internalization blocked DISC recruitment and apoptosis, while it still allowed the recruitment of RIP-1 and TRAF-2 to TNF-R1 for unchanged NF-κB activation. For these experiments a novel experimental approach was used: Biotin-TNF coupled to streptavidin-coated magnetic nanobeads was utilized to isolate intact TNF–TNF-receptor complexes within their native membrane environment with the help of a specialized magnetic device [52]. Immunomagnetic isolation of morphologically intact vesicles revealed trafficking and maturation of TNF-receptosomes along the endocytic pathway and fusion of TNF-receptosomes with trans-Golgi membranes resulting in the formation of multivesicular endosomes [47]. Deletion of the TNF-R1 internalization domain (TRID) within the cytoplasmic tail of TNF-R1 or point mutations within the YQRW internalization motif resulted in complete elimination of TNF-R1 internalization. These mutations abolished TRADD, FADD, and caspase-8 recruitment and provoked an almost entire inhibition of TNF-induced apoptosis [47]. A residual DISC-independent cell death was observed in cells that express a ΔTRID deletion mutant of TNF-R1 after prolonged TNF treatment. This may result from an increased local production of ceramide by overactivated N-SMase [40]. However, in adenovirus-infected cells as well as cells transduced with expression vectors for the adenoviral E3-14.7 K protein inhibition of TNF-R1 endocytosis correlated with a complete block of TNF-induced apoptosis [48]. On the other hand, RIP-1 and TRAF2 were still recruited to the DD of the internalization-deficient ΔTRID deletion mutant of TNF-R1. Therefore TNF-dependent activation of NF-κB still occurred, even in the absence of TRADD as an assembly platform [47, 48]. This latter observation is in line with previous reports on a direct interaction of RIP-1 with TNF-R1 and CD95 [23, 56]. In addition, Zheng et al. [63] and Jin and El Deiry [24] showed that TRADD and RIP-1 can independently and competitively associate with TNF-R1. Recently TRADD-deficient mice have been shown to be completely TNF insensitive with regard to apoptosis. In contrast, they retained a partial responsiveness with regard to TNF-induced NF-κB and MAPK activation [10, 43]. It was concluded that the main functions of TRADD are the recruitment of TRAF2 or FADD. RIP-1 binding to TNF-R1 may occur independent of TRADD, but full activation of RIP-1 requires activating ubiquitinylation by TRAF2 within complex I.

How the formation of the distinct TNF-receptor complexes and the subsequent signaling events are regulated during TNF-R1 endocytosis still deserves explanation. As the receptor-associated TRADD, RIP-1, and TRAF-2 proteins are co-internalized along with TNF-R1, a full propagation of DISC-mediated

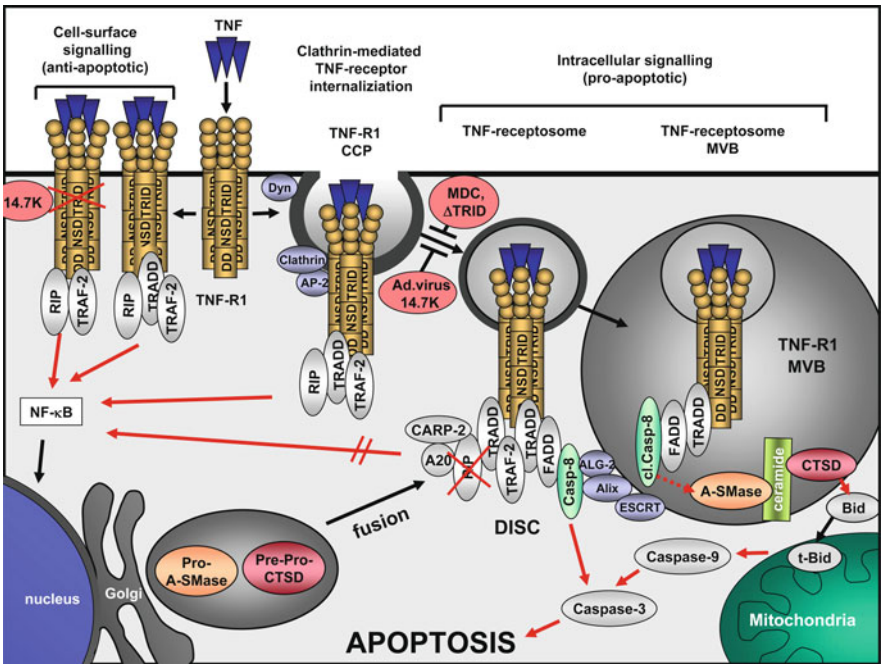

Fig. 64.1 Compartmentalization of TNF-R1 signaling and A-SMase activation. Binding of TNF ligand to TNFR1 initializes the clathrin-dependent endocytosis and the recruitment of TRADD, FADD, and caspase-8. Within the receptosome-bound DISC, caspase-8 is activated and can induce caspase-3 activation. Along the endocytotic pathway, TNF-receptosomes fuse with trans-Golgi vesicles that contain pro-acid sphingomyelinase (pro-A-SMase) and pre-pro-cathepsin D (pre-pro-CTSD) to form multivesicular bodies (MVBs). Within the MVB, activated caspase-8 mediates activation of A-SMase. Ceramide production activates pre-pro-CTSD leading to the cleavage of BID which triggers the mitochondrial intrinsic apoptosis pathway. Translocation events are denoted by *black arrows*, activation events by *red arrows*. Inhibitors of internalization (monodansylcadaverine (MDC), adenovirus 14.7 K protein, and the ΔTRID mutation) are on *red* background. Components of the clathrin endocytosis machinery are in *light blue*. TNF is in *dark blue* and TNF-R1 in *gold*

pro-apoptotic signals can only occur, if components of complex I are removed from TNF-R1 during endocytosis and NF-κB signaling is therefore downregulated. The ubiquitin protein ligase CARP-2 was identified as a constitutive negative regulator of TNF-induced NF-κB activation [29]. CARP-2 is localized to endocytic vesicles. There it interacts with internalized TNF-receptosomes and mediates ubiquitinylation of RIP-1 in concert with the ubiquitin ligase A20. CARP-2 thereby promotes degradation of RIP-1 leading to a downregulation of NF-κB activity. Recently the involvement of Alix/AIP and apoptosis-linked gene-2 (Alg2) in the recruitment of pro-caspase-8 to TNF-R1 on endosomes has been reported [32]. Using the same experimental approach introduced by us [47], magnetically labeling TNF-receptors for purification in a magnetic field, they demonstrated that in isolated TNF-R1 receptosomes both proteins are instrumental for linking pro-caspase-8 and TNF-R1

as well as the endosomal sorting complex required for transport (ESCRT) resulting in the activation of caspase-8 and subsequent execution of apoptosis. These findings strongly support our model of compartmentalized TNF-R1 signaling and provide an explanation how caspase-8 becomes activated in endosomes.

In summary, two temporary and spatially distinct TNF-R1 signaling complexes are formed: one at the cell surface with the capacity to activate NF-κB signaling and a second assembled on internalized receptosomes that promotes apoptosis (see Fig. 64.1). Therefore TNF-R1 compartmentalization has an important role in the diversification of TNF-mediated biological responses (see [51] for review). The discrepancies between our findings of TNF-R1-associated DISC and the results of Harper et al. [17] and Micheau and Tschopp [37] might be due to the different detergents used for solubilization of TNF-R1 from endosomes and the fact that we used a different labeling and isolation protocol for TNF-R1 receptosomes. Furthermore, early DISC formation at TNF-receptosomes occurs rapidly and transiently and may be detectable only when the internalization is synchronized by pre-labeling the receptors with TNF at 4°C followed by a rapid increase of the temperature to 37°C.

A further confirmation of the importance of TNF-R1 internalization for signaling comes from non-phagocytic cells, where TNF-receptor internalization is required for TNF-induced production of reactive oxygen species (ROS) and activation of MAPKs and AKT/protein kinase B (PKB), but not for activation of NF-κB [61].

Of note, the role of receptor internalization is not restricted to TNF-R1, since compartmentalization is also important for transducing pro-apoptotic signaling of CD95 in Type I tumor cells [2, 3, 11, 28] and TRAIL-R2 in hepatocellular carcinoma cells [1].

A-SMase Activation by TNF

It has been established by several reports that TNF-receptor triggering activates the endolysosomal enzyme SMPD1, also known as A-SMase [49, 53, 59, 60]. This enzyme generates the potent pro-apoptotic lipid second messenger ceramide from sphingomyelin (reviewed in [30, 39]). A role of A-SMase in transmitting apoptotic signals of death receptors has been reported not only for TNF-R1 [13, 19, 38] but also for CD95 [5, 6, 7, 21] and TRAIL-receptors [9, 58]. A-SMase activation has been reported in conjunction with protein kinase C (PKC) activation. First, diacylglycerol (DAG), which is generated by hydrolysis of phosphatidylcholine by phosphatidylcholine phospholipase C (PC-PLC) in response to TNF, activates PKC as well as A-SMase [49]. Second, phosphorylation of A-SMase at Ser 508 by PKC delta (PKCδ) has been reported to cause activation and translocation of A-SMase [62]. Induction of A-SMase is linked to the death domain of TNF-R1, since activation of A-SMase after TNF stimulation was found to be mediated through TRADD and FADD [53]. Moreover, A-SMase activation is prevented by deletion of the death domain of the TNF-R1 [47]. As mentioned before, the recruitment of TRADD and FADD to TNF-R1 upon TNF binding is inhibited in the ΔTRID deletion mutant of

TNF-R1 lacking the domain responsible for receptor internalization, implying that internalization of the TNF-R1 is necessary for A-SMase activation as well.

Downstream of A-SMase the generated ceramide has been shown to activate the Asp-protease cathepsin D (CTSD) within the same endolysosomal compartment [18, 19, 47]. CTSD-positive vesicles colocalize with pro-apoptotic Bid, and after TNF stimulation both proteins are located in Rab5-positive vesicles, indicating that Bid is located at the subcellular site of CTSD activation. Ceramide-mediated activation of CTSD leads to its translocation through the endosomal membrane. In the cytosolic compartment CTSD is capable of cleaving Bid to the pro-apoptotic tBid fragment. As described above, tBid then initiates the intrinsic apoptotic signaling pathway via activation of caspase-9 and caspase-3.

One initial experiment in the study of Heinrich et al. [19] indicated that caspase-8 might be involved in the activation of A-SMase. Still the molecular mechanism of activation of A-SMase after TNF stimulation remains to be elucidated. Therefore, we set out to clarify the role of caspases in this process.

Caspase-8 Deficiency Prevents TNF Activation of A-SMase and Cathepsin D

We compared caspase-8-deficient Jurkat cells with wild-type cells and observed a strict dependence of TNF-induced A-SMase activation on the presence of functional caspase-8. TNF treatment leads to a transient increase in A-SMase activity in lysates from wild-type Jurkat cells, while there was no change in A-SMase activity in caspase-8-deficient cells. Transfection of caspase-8-deficient Jurkat cells with an expression plasmid for caspase-8 restored the ability of these cells to respond to TNF stimulation by an increase in A-SMase activity. A-SMase could also be activated in vitro by the addition of exogenous, purified caspase-8 to lysates from caspase-8-deficient Jurkat cells. In caspase-8-deficient Jurkat cells not only the A-SMase activation is unresponsive to TNF treatment but also the production of the active 32 kDa fragment of CTSD is not increased upon TNF treatment. Thus TNF-induced ceramide production is required for activation of CTSD.

Active Caspase-8 Colocalizes with Internalized TNF-Receptosomes and A-SMase

We further addressed the intracellular, spatial distribution of the molecular components of a potential signaling cascade leading form TNF-R1 to A-SMase via caspase-8. In synchronized internalization experiments using biotinylated TNF and streptavidin FITC for TNF-receptor labeling, the localization of ligand-bound TNF-R1 was compared to that of fluorescently labeled active caspase-8 and A-SMase. In confocal laser scanning microscopy immunofluorescence detection of TNF-receptors and cleaved caspase-8 revealed a time-dependent appearance of endocytic

vesicles that were positive for both fluorescent labels. However, we also detected cytoplasmic spots stained with the label for cleaved caspase-8 that did not colocalize with TNF-receptosomes. These labeled entities may reflect the proportion of TRADDosome-associated active caspase-8 that has already dissociated from TNF-R1 and most likely represents the cytosolic complex II in line with the model proposed by Micheau and Tschopp [37]. Nevertheless, the observed partial colocalization of both labels confirms that a fraction of activated caspase-8 is also associated with TNF-receptosomes, further supporting our previous findings of TNF-R1-associated DISC [47].

Due to a lack of specific antibodies against human A-SMase, we had to rely on the transfection of HeLa cells with expression vectors for either EGFP- or HA-tagged A-SMase for colocalization experiments. Immunofluorescence co-staining of several intracellular marker proteins revealed that the majority of the EGFP- as well as HA-tagged recombinant A-SMase becomes mainly localized to the trans-Golgi network, indicating a preserved functionality of the recombinant fusion protein. Simultaneous staining of HA-A-SMase expressing cells for active caspase-8 revealed partial colocalization of both proteins after TNF treatment. Such colocalization was almost absent in cells not treated with TNF. This indicates the possibility of a direct interaction between caspase-8 and A-SMase in the same cellular compartment.

Proteolytic Cleavage of Pro-A-SMase Correlates with Enhanced Enzymatic Activity

In A-SMase-EGFP expressing HeLa cells we observed a protein cleavage fragment, correlating with the induction of A-SMase activity after TNF stimulation. We assume that this protein might represent a truncated A-SMase-EGFP fusion protein that may have been proteolytically clipped at its N-terminus and may have been activated thereby. In fact, the addition of purified recombinant caspase-8 to whole cell lysates of A-SMase-EGFP expressing cells led to an increase of both, the intensity of the band representing the A-SMase fragment and the A-SMase activity of the same preparations in a kinetically related fashion. However, caspase-8 may not be directly responsible for A-SMase cleavage and activation, since recombinant caspase-8 treatment in vitro did not show any effects on activity and fragmentation pattern of A-SMase-EGFP fusion protein that had been purified by immunoprecipitation. This leaves the open question, which target(s) of caspase-8 might be responsible for the observed cleavage of recombinant A-SMase fusion proteins in overexpressing cells. This target protease(s) might be the missing link that completes the signaling cascade between TNF-R1 and A-SMase. Meanwhile we could identify this missing link downstream of caspase-8 and these new findings will be published in the near future elsewhere.

The involvement of caspase-8 has already been demonstrated for A-SMase activation by CD95 signals in a variety of cells [12, 15, 16, 19, 44, 45]. The requirement

of caspase-8 for A-SMase activation we have found in this study for TNF-stimulated Jurkat cells is therefore in good agreement with the results obtained for CD95 signaling. This points to a general signaling pathway that couples death receptors via caspase-8 to A-SMase activation. Based on our data, we propose a novel mechanistic model for the apoptotic signaling of TNF-R1: that is the sequential activation of caspase-8 leading to increased A-SMase activity within TNF-receptosomes, as depicted in Fig. 64.1. The resulting ceramide production in these organelles will ultimately lead to the activation of the mitochondrial caspase amplification loop culminating in the apoptotic response.

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Chapter 65
TNF/TNF Receptor 1-Mediated Apoptosis in Hepatocytes

Hyun-Ae Eum and Timothy R. Billiar

Introduction

Apoptosis is a controlled mechanism of cell death that is involved in a large range of physiological as well as pathological events. It is characterized by the elimination of single cells without the disruption of the biological function of the tissue. However, sustained exposure to TNF can lead to excessive apoptosis. Overproduction of TNF leading to tissue toxicity has been implicated in a wide spectrum of human disease, including sepsis, ischemic disease, autoimmune disorders, and many types of cancer.

The majority of cells in the liver are hepatocytes, which constitute at least two-third of the liver mass, and hence their susceptibility to TNF is of relevance in disease progression. Hepatocyte apoptosis is a prominent feature of many forms of liver diseases. Indeed, a wide range of injurious stresses can lead to hepatocytes apoptosis manifested as liver damage.

The administration of large doses of TNF [1] causes acute liver failure which is lethal within hours due to the induction of apoptosis through the TNFR1 signaling pathway. TNF signaling in hepatocytes can lead to the activation of inflammatory pathways or pathways leading to cell death. There remains a gap in our understanding of the molecular mechanisms that determine which type of signaling will predominate. Many of the details of TNF signaling have been established in cell lines. Thus, the molecular mechanisms of TNF signaling in hepatocytes are only pathway understood and represent another major gap in our knowledge.

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The Biology of TNF and TNF Receptor 1 System in the Liver

TNF is produced predominantly by activated macrophages and other immune cells as a 26 kDa protein. This pro-TNF is expressed on the plasma membrane, where it can be cleaved in the extracellular domain by matrix metalloproteinases, which result in the release a soluble 17 kDa form [2, 3].

TNF functions as a two-edged sword in the liver. Physiologically, TNF is an important cytokine that regulates hepatocyte proliferationduring liver regeneration. The inhibition of TNF signaling can prevent liver regeneration following injury [4]. In contrast, the overproduction of TNF by inflammatory cells residing or infiltrating the liver is a critical step in the development of several liver diseases associated with liver damage [5]. Therefore, an understanding of the sensitivity of hepatocytes to TNF represents an area of great interest in hepatology. Inhibition of toxic TNF-signaling pathways could be used to modulate the course of some acute and chronic liver diseases [5].

Two different TNF receptors have been identified in cells: TNFR1 and TNFR2. The intracellular domains of TNFR1 and TNFR2 are completely different, suggesting that these two receptors are involved in distinctly different signaling pathways. The intracellular domain of TNFR1 has a death domain, whereas TNFR2 does not. Direct signaling through TNFR2 occurs less extensively and its role in the liver biology is less well characterized and will not be discussed further (Table 65.1).

Table 65.1 Functions attributed to the TNF/TNFR1 system in liver

	TNF function	References
Enhanced apoptotic pathway	– Acute and chronic viral hepatitis	[33–37]
	– Alcoholic hepatitis	[33–35]
	– Graft rejection	[38, 39]
	– Cholestatic liver disease	[44]
	– Fulminant liver failure	[40–42]
Non-apoptotic pathway	– Liver regeneration	[43–45]
	– Proliferation	[46, 47]

The binding of TNF to TNFR1 is thought to cause the apoptotic cell death seen in TNF-associated liver pathology [6]. In most cell lines, binding of TNF to TNFR1 results in receptor trimerization and clustering of TNFR1 intracellular death domains. This allows binding of an intracellular adaptor molecule called TNFreceptor-1-associated death domain (TRADD) via interactions between death domains. TRADD binding can trigger two separate intracellular signaling pathways. On one hand, TRADD may interact with TNF receptor-associated factor (TRAF-2) leading to activation of NF-κB which supports survival of the cell [7]. On the other hand, TRADD can also associate with Fas-associated death domain (FADD) [8]. FADD contains a death effector domain by which it subsequently recruits procaspase-8 [9], thus constituting the death inducing signaling complex (DISC) [10]. Clustering of caspase-8 in the DISC results in its auto-activation and

initiates the caspase cascade ultimately leading to apoptotic cell death. In hepatocytes, however, the amount of caspase-8 generated at the DISC is limited, and the activation cascade does not propagate directly but is instead amplified via the mitochondria. This process is initiated by the cleavage of Bid by caspase-8, followed by the translocation of the truncated Bid (tBid) to the mitochondria. This, in turn, induces the release of proapoptotic molecules to the cytoplasm, which results in effective induction of apoptosis.

The Signaling and Function of TNF Receptor 1 in the Liver

Paradoxically, TNF signaling through TNFR1 can mediate concurrent signals associated with both cell survival and cell apoptosis in liver and other tissues. However, the molecular mechanism that determines which of these two opposing signaling pathway is preferentially activated is unclear. Based on studies in HT1080 (human fibrosarcoma) and 293T (human embryonic kidney cell line), Mischieau et al. proposed a dual signaling complex and this model is widely accepted [11, 12]. The rapidly formed complex I is assembled on the cytosolic death domain of the receptor and composed of the adaptor molecule TRADD, the protein kinase receptor-interacting protein-1 (RIP1), and the signal transducer TRAF2. This complex I promotes cell survival through the Iκ-B kinase-dependent activation of NF-κB. Thus, complex I triggers activation of NF-κB and cell survive. In this paradigm, once the receptor complex is internalized, TRADD, RIP, and TRAF2 become modified and dissociate from TNFR1 [13]. According to Schneider et al., TRADD and RIP then binds to FADD, resulting in caspase-8 recruitment in the cytosol (Complex II). This complex lacks TNFR1 but contains the proapoptotic molecules. This interaction between the death effector domains of FADD and pro-caspase-8 leads to apoptosis through protein cleavage and activation of the downstream caspase cascade as well the cleavage and activation of pro-apoptotic molecules such as Bid. It is thought that the activation of NF-κB through complex I can antagonize apoptotic signaling through the expression of several genes present the pro-death potential of complex II assembly. Therefore, the activation of NF-κB promotes cell survival by inducing proteins that prevent or block the proapoptotic consequences of complex II at different levels [12, 14]. In the absence of a productive signal from complex I, complex II formation can dominate and lead to death.

It is important to stress that the paradigm detailed above was described mostly in transfected cell lines. Whether these events pertain to hepatocytes is uncertain. In vivo studies have shown that NF-κB inhibition increases TNF-induced liver damage [15]. This along with the well-established observation that TNF-induced apoptosis in cultured hepatocytes requires the addition of a sensitizing agent such as actinomycin D [16] or cyclohexamide [16] suggests that the NF-κB survival pathway predominates in hepatocytes (Fig. 65.1). The molecular mechanisms that account for the shift toward apoptosis following sensitization are not known.

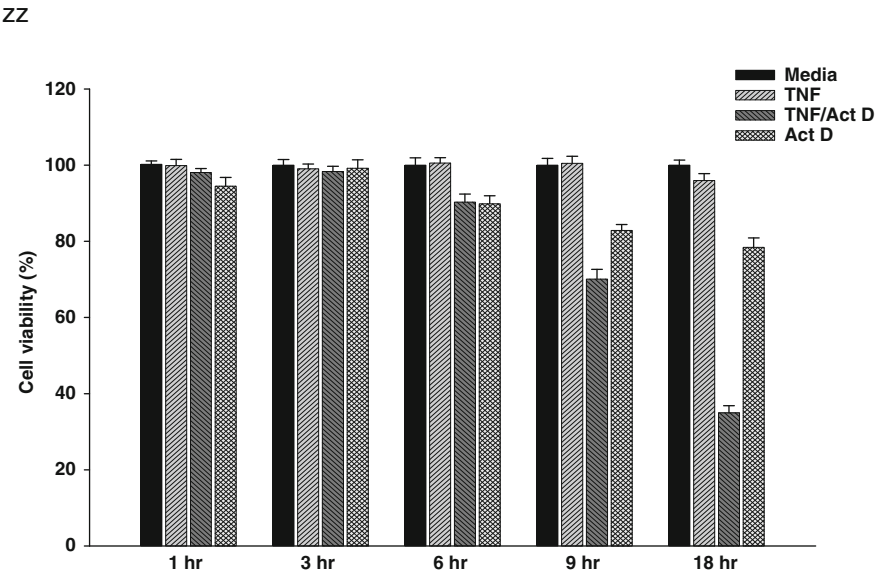

Fig. 65.1 Primary rat hepatocyte cultures were exposed to TNF, TNF/Act D, or Act D alone and cell viability was assessed by crystal violet staining. None of these assays revealed significant cell death in hepatocytes treated with TNF alone at any time point. However, the co-treatment of TNF with Act D led to significant hepatocyte death in a time-dependent manner

Previous investigations have demonstrated that the binding of TNF to TNFR1 will induce an apoptotic response mediated by JNK and caspase-8 in sensitized hepatocytes [17]. As in other cells this can be suppressed by NF-κB activation.

We have had a long-standing interest in the signaling pathways that regulate TNF-induced hepatocyte apoptosis. This stemmed from an observation that nitric oxide (NO) is a potent inhibitor of TNF/Act D-induced hepatocyte cell death [18]. Mechanistic studies revealed that NO could block caspase-8 activation [19]. This occurred both through a cGMP-dependent mechanism and through the S-nitrosylation of death effector caspase [20]. In an effort to delve more deeply into the signaling events associated with TNF-induced cell death in hepatocytes we made a number of unique and perhaps hepatocyte-specific observations.

In hepatocytes, FADD has an essential role in the most proximal steps to activate TNF-induced apoptosis. The relationship between FADD levels and apoptotic cell death has been previously described [21]. We found that FADD protein levels vary dramatically in hepatocytes and proposed that hepatocyte apoptosis could be controlled by intracellular changes in FADD levels. FADD levels increased in cultured primary hepatocytes exposed to TNF/Act D in a time-dependent manner. Somewhat unexpectedly we also observed that not only caspase-8 but also caspase-3 cleavage is required for the increase in FADD protein levels [22] and the levels of FADD protein expression in whole cell cultures as well as individual cells correlated with the extent of apoptotic cell death following treatment of hepatocyte with TNF/Act D. There was also a slight increase in TRADD levels with TNF/Act D treatment.

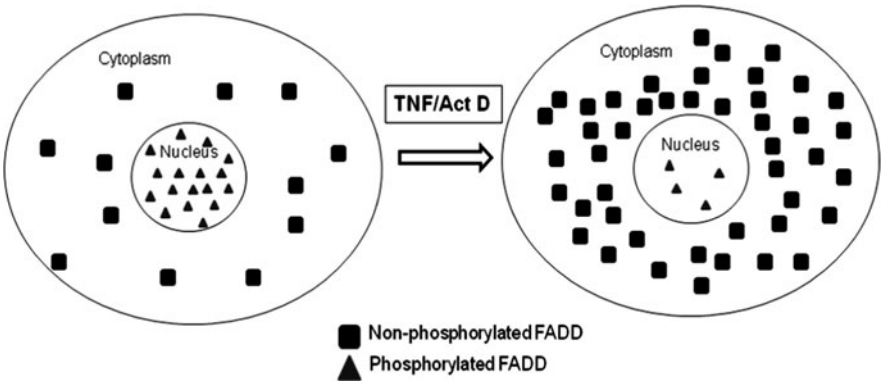

Fig. 65.2 Cellular distribution of FADD in hepatocyte following TNF/Act D treatment. Cytoplasmic FADD up-regulation by TNF/Act D stimulation is coupled with nuclear phosphorylated FADD down-regulation

The increase in FADD as well as the activation of caspase-8 and cell death could be blocked by cyclic nucleotides supporting the conclusion that FADD upregulation is downstream of caspase-8. We have proposed that FADD upregulation is an important feed forward mechanism in promoting hepatocyte apoptosis in response to TNF.

FADD is known to shuttle between the nucleus and the cytosolic cellular compartments and to be regulated by phosphorylation and homotypic interaction [23]. In detailed studies on the distribution and phosphorylation status of FADD in TNF-treated hepatocytes we found that dimeric non-phosphorylated and dimeric phosphorylated FADD localized predominately in the cytoplasm and remained constant with the TNF/Act D stimulation (Fig. 65.2). Trace amounts of dimeric non-phosphorylated and dimeric phosphorylated FADD were present in nuclear faction of non-stressed hepatocytes but disappeared upon treatment with TNF/Act D. However, the level of cytosolic non-phosphorylated, monomeric FADD increased in hepatocytes exposed to TNF/Act D. This was associated with a decline in nuclear phosphorylated FADD [23]. This observation suggests that the change in FADD localization and abundance occurs as the result of the activation of proximal signaling events in apoptosis.

Other Unique Observations on TNFR1 in Hepatocytes

It is well known that induction of apoptosis by the TNF/TNFR1 system involves the mitochondrial amplification pathway in hepatocytes. Among the many death-inducing mechanisms triggered by TNF, the role of Bid is a particular note. The importance of caspase-8-induced cleavage of Bid has been confirmed in hepatocytes [24]. Truncated Bid, tBid, translocates from the cytosol to the outer mitochondrial membrane and causes the release of apoptogenic factors from mitochondria.

Although deletion of Bid leads to nearly complete blockage of Fas-mediated hepatocyte apoptosis and liver injury [25, 26], deletion of Bid only delays and does not block TNF-mediated hepatocyte apoptosis and liver injury [27–29] or the activation of mitochondria. These results indicate that bid is not essential to the induction of apoptosis in sensitized hepatocytes exposed to TNF in vitro.

TNFR1 is traditionally regarded as a cell surface receptor, although studies in endothelial cells by Bradley et al. [30] and Jones et al. [31] have shown that TNFR1 is predominantly expressed in the *trans* Golgi network, with only low levels being detected at the plasma membrane. However, there is controversy in the literature concerning the localization of TNFR1 in various cell lines. Cottin et al. have reported that TNFR1 was predominantly localized to the lipid raft [32]. Furthermore, there is no information in hepatocytes on the distribution of the TNFR1 either at baseline or under stress conditions.

Hepatocytes have a proportionately higher numbers of mitochondria than many of the other cell types often studied in apoptosis research. Given the importance of the mitochondria in TNF signaling of hepatocytes we assessed the relationship between DISC components and the mitochondria in hepatocytes in the resting state and following TNF exposure. Using a combination of biochemical and cell-imaging modalities, including immune fluorescence, immune-gold labeling, and EM microscopy, and cell fractionation with immunoblotting, we characterized the subcellular distribution of TNFR1 and key DISC components. TNFR1 is predominantly localized in the cytosolic fraction with only a minor subpopulation of TNFR1 presented in the mitochondria in the resting hepatocyte in culture. Interestingly, TNFR1 accumulates in the mitochondria following either TNF or TNF/Act D. The addition of Act D alone also caused TNFR1 to appear within mitochondria (unpublished observation). FADD and caspase-8 were seen to associate with TNFR1 in the mitochondria in the stimulated cells.

How TNFR1 moves to the mitochondria and the functional significance of the mitochondria association of TNFR1 following TNF exposure remains unknown. Further elucidation of role of TNFR1 in the mitochondria may be an important factor in determining how a cell chooses between life and death. This process may thus represent a novel mechanism to regulate TNF signaling in hepatocyte.

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Chapter 66
Regulation of Cell Death by c-FLIP Phosphorylation

Tomoko Asaoka, Aura Kaunisto, and John E. Eriksson

Introduction

The adult human body consists of approximately 10^{14} cells and millions of cells undergo growth and division daily. To maintain homeostasis, comparable numbers of irrelevant or damaged cells die in return. All cells bear the ability to die through programmed cell death, or apoptosis, and there are two major apoptotic pathways that can be regulated in a stimulus-specific manner. While the intrinsic pathway is triggered by cellular stresses within the cell, the death signals that initiate the extrinsic pathway are mediated by the ligands and receptors of the TNF superfamily.

Activation of Apoptosis by Death Receptors

The extrinsic apoptotic pathway is initiated by the binding of a death ligand to its cognate death receptor on the cell surface (Fig. 66.1a); examples include CD95, TRAIL-R, and TNF-R1. Upon ligation, death receptors recruit adaptor proteins and form the death-inducing signaling complex (DISC) to pass on the signal downstream. Fas-associated death domain (FADD) protein is a molecule that contains one death domain (DD) and one death effector domain (DED), enabling interactions with the death receptor and in turn forms a platform for other DED-containing proteins [3]. Initiator caspase-8 and caspase-10 precursors are such DED-containing proteins with a catalytic caspase domain, allowing them to be recruited to the DISC via homophilic binding with FADD. This promotes interactions between the procaspases via N-terminal DEDs [1]. The assembly forms zymogens, which in turn induce two-step proteolytic processing converting procaspases to form the proteolytically active caspase-8 and caspase-10 heterotetramers. Mature initiator

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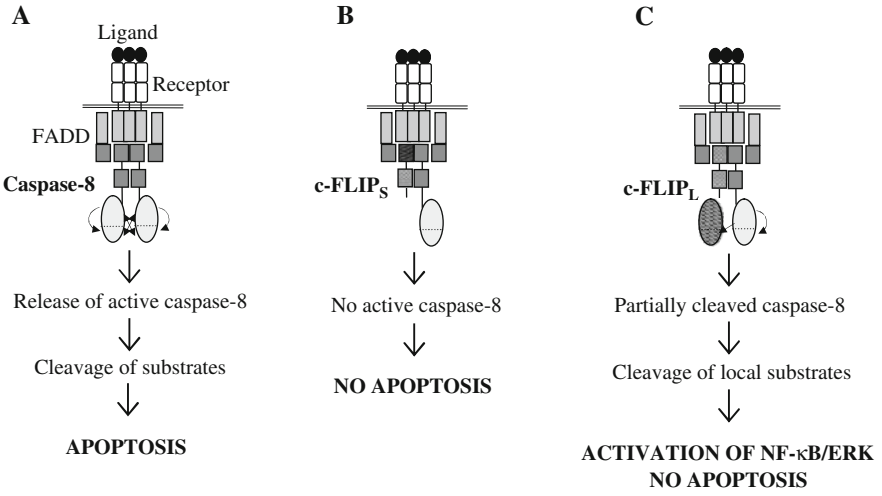

Fig. 66.1 Regulation of c-FLIP in the DISC activity. Upon activation by the corresponding death ligands (*black*), DDs (*light grey*) of the death receptors assemble to rapidly transduce the pro-apoptotic signal. FADD is then engaged to the intracellular part of the receptor by homophilic DD-mediated recruitment. (**a**) When caspase-8 molecules are recruited to the DISC, it induces auto- and transproteolytic cleavage of these initiator procaspases. Active caspases dissociate from the DISC to the cytoplasm and cleave executioner procaspases and induce apoptosis. (**b**) When c-FLIP$_{S/R}$ (*dotted*) is recruited to the DISC, procaspases remain unprocessed and apoptosis is not triggered. (**c**) c-FLIP$_L$ forms a heterodimer with procaspase-8 and generates intermediates of the caspase. These cleaved fragments, however, do not form mature caspases and remain in the DISC to regulate cell survival processes

caspases dissociate from the DISC to the cytosol and cleave effector procaspases, which subsequently cleave other substrates, leading to the destruction of cellular components and formation of the apoptotic bodies (reviewed by [4]).

c-FLIP as a Regulator of the Cell Fate

The initiation of death receptor-mediated apoptosis is controlled by a delicate balance between pro- and anti-apoptotic intracellular proteins to avoid accidental activation of the cell death machinery. The activation of the initiator caspases can be encountered by another member of the DED family proteins, called cellular FLICE-inhibitory (c-FLIP). Fourteen mRNA c-FLIP splice variants have been detected [5], of which three are identified at the protein level. c-FLIP long (c-FLIP$_L$), 55 kDa in size, is a homologue of procaspase-8, whereas c-FLIP short (c-FLIP$_S$) is a considerably smaller, 26 kDa protein [11, 19]. Another short isoform was later identified from Raji cells, hence called c-FLIP$_R$ [7]. All three c-FLIP isoforms are identical in their 202 N-terminal amino acids, which include two tandemly repeated DEDs that mediate recruitment to the DISC and caspase-8 binding [11]. In comparison,

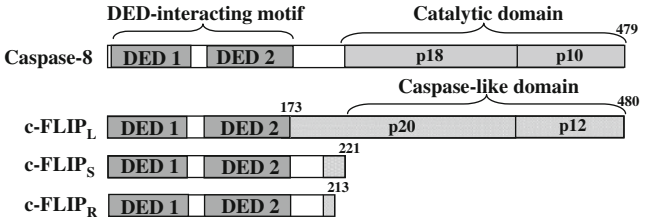

Fig. 66.2 The c-FLIP proteins and caspase-8 share similar domain structures. All N-terminal prodomains contain two conserved DEDs. Long isoforms of DED-containing proteins have large and small subunits (caspase-like domain for c-FLIP$_L$). c-FLIP$_S$ and c-FLIP$_R$ have truncated C termini

the C termini are completely different (Fig. 66.2). The short isoforms have unique splicing tails, whereas c-FLIP$_L$ contains a C-terminal inactive caspase-like domain, in which the catalytic cysteine residue is absent.

c-FLIP$_S$ acts as a potent caspase inhibitor by competing with caspase-8 and caspase-10 in binding to the DISC and by inhibiting processing of the procaspases (Fig. 66.1b) [17]. c-FLIP$_R$ only differs from c-FLIP$_S$ in its C terminus and the roles of two short isoforms in death receptor-mediated apoptosis are comparable to each other [20]. Although an anti-apoptotic function of c-FLIP is commonly described, c-FLIP$_L$ is also capable of partially cleaving procaspase-8 [23] (Fig. 66.1c). Thus, while first considered to be comparable inhibitors, the c-FLIP isoforms have now been established as DISC proteins with distinct functions and by carefully monitoring the c-FLIP levels, a cell can engage on an appropriate response to a given death ligand.

Regulation of c-FLIP by Post-Translational Modifications

Pro- and anti-apoptotic proteins determine the death receptor sensitivity of a cell and c-FLIP is an example whose activity can be regulated in an isoform-specific manner. The post-translational modifications most commonly connected to the regulation of c-FLIP are phosphorylation and ubiquitylation. Phosphorylation involves the covalent attachment of a phosphate group to the substrate and it is regulated by the rapid counteracting actions of kinases and phosphatases. Earlier observations reported that phosphorylation can either stabilize c-FLIP$_L$ in the DISC, thereby providing protection against death receptor-mediated apoptosis, or degrade both c-FLIP proteins, resulting in sensitization to extrinsic apoptosis pathway [9, 21, 22] .

Ubiquitin tagging signals the proteasomal degradation of a protein and c-FLIP proteins are no exception. Diverse stimuli can ubiquitinate c-FLIP, including p53 activation [6] and adenoviral infection [15]. Previously, we reported that although both c-FLIP$_L$ and c-FLIP$_S$ are actively degraded through the ubiquitin–proteasomal pathway, their regulation is isoform specific and displays different protein stabilities [16]. This is due to different ubiquitin acceptor lysines between the isoforms

and that the unique splicing tail of c-FLIP$_S$ possessed a destabilizing role, a mechanism demonstrated to be crucial for death receptor responses upon erythroid differentiation and lymphocyte persistence during hyperthermia [14, 16].

PKC-Mediated Phosphorylation Regulates the Stability of the Short c-FLIP Isoforms

We have recently identified serine 193 (S193) as a novel in vivo phosphorylation site of all c-FLIP isoforms [12]. S193 is found within a protein kinase C (PKC) consensus sequence, a family consisting of several serine/threonine kinases and many are known to be regulating apoptosis (reviewed by [8]). As expected, an inducer of the classical PKC markedly enhanced the phosphorylation of S193 and the phosphorylation was decreased by the kinase-specific inhibitors, as well as overexpression of kinase-dead PKCα and PKCβ mutants. Furthermore, S193 is located in a region we previously shown to regulate c-FLIP$_S$ ubiquitylation, thus inspiring us to explore the potential interplay between the two post-translational modifications. The disruption of S193 phosphorylation caused increased ubiquitylation in all isoforms, while the phosphor-mimetic S193D mutants were more resistant to ubiquitylation. The study showed that S193 phosphorylation regulates the ubiquitylation of c-FLIP, which selectively affects the half-lives of the short isoforms. Our results demonstrate that although the phosphorylation in itself does not characterize the anti-apoptotic potential of c-FLIP, it affects the protein stability of c-FLIP$_S$ and c-FLIP$_R$, thereby connecting PKC to DISC signaling (Fig. 66.3).

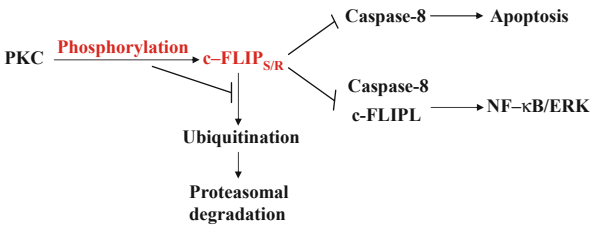

Fig. 66.3 Phosphorylation-mediated regulation of c-FLIPS/R provides a switch for the cell fate. Classical PKC-modulated phosphorylation on c-FLIPS/R S193 can prevent ubiquitylation and proteasomal-dependent degradation of these proteins. Stabilized short isoforms of c-FLIP can inhibit caspase-8 activation, or hinder the heterodimerization of c-FLIP$_L$ and procaspase-8, thus blocking the survival signaling pathway

Shortly after this study was presented at the 12th TNF International Conference, another research group published c-FLIP$_L$ S273 as a PI3K/Akt-phosphorylation site whose phosphorylation makes c-FLIP$_L$ prone for degradation during macrophage activation [18]. In addition, the murine short c-FLIP isoform was reported to be phosphorylated on S4 and Y211 by p38 and c-Abl, respectively, which was then recognized by c-Cbl for ubiquitylation and degraded [13]. Together, these reports

show that c-FLIP is phosphorylated on numerous residues by a range of kinases and the consequences depend on physiological circumstances.

Pursuing Interest on c-FLIP Ubiquitylation

Interestingly, signals that activate a death receptor are not always comprehended as a preeminent message for cell death. Given the remarkable functional differences between the long and short isoforms, c-FLIP expression is an important determinant for cells to adjust their responsiveness to extracellular signals. Ubiquitin conjugation is an extremely versatile mode of post-translational modification and resulting proteasomal degradation is only one among many variable outcomes of ubiquitylation (reviewed by [10]). It can be hypothesized that c-FLIP proteins are ubiquitylated in an isoform-specific manner by providing unique binding platform for E3 ubiquitin ligases on their C-terminal tails. This speculation is supported by the identification of Itch as the E3 ligase that specifically targets c-FLIP$_L$ to ubiquitylation via interacting with the caspase-like domain [2].

Responses to death ligand stimulation will depend upon a summing-up of the pro- and anti-apototic signaling that will be integrated by the c-FLIP isoforms. The c-FLIP isoforms will thereby constitute an integrating unit for survival and death signaling. Our continuing investigations focus on identifying different types of ubiquitin chains the c-FLIP isoforms accommodate, emphasizing the biological functions of these modifications, and on the specific roles of the isoform and site-specific phosphorylation events.

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Appendix II: Abstracts of Talks and Posters Presented at the 12th TNF Conference

KEYNOTE LECTURE

ORCHESTRATION OF THE TNF RESPONSE BY MESSENGER RNA STABILITY AND MICRORNAS

Baltimore, D., Hao, S., Boldin, M., Taganov, K., O'Connell, R., Rao, D. and Chaudhuri, A

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TNF is a powerful inducer of inflammatory activities but the particular responses evolve over time after induction and they are subject to secondary regulation. We can separate the genes activated in the TNF response into 3 quite discrete time-courses: Group I of fast reacting genes that are then down-modulated; Group II of slower reacting genes with mRNAs that stay elevated as long as inducer is present and Group III of genes that take hours to begin induction but whose mRNA accumulates continually for more than 12 hours. While transcriptional activities are important for these differences, a major determinant is mRNA stability. Group I RNAs are particularly unstable, Group ii intermediate and Group III very stable. The number of AU-rich sequences in the 3′-UTRs of the mRNAs correlates strongly with the stability of the particular mRNA.

We have also been examining the role of microRNAs in the inflammatory response. We have concentrated on two, mir-146a and mir-155. Both are strongly induced by TNF, LPS and TLRs. One role of mir-146a appears to be feedback inhibition of the induction. However, in knockout animals we were surprised to see a hyperproliferative disease that we are presently characterizing. Mir-155 induction leads to a myeloid hyperplasia. It has many apparent target genes but one, SHIP 1, appears to be the dominant cause of the phenotype. The powerful regulatory role of these microRNAs is remarkable.

LECTURES
ROLES OF THE TNF FAMILY IN TISSUE HOMEOSTASIS AND NORMAL DEVELOPMENT

BEYOND BONES - THE MULTIPLE FUNCTIONS OF RANKL-RANK

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Bone-related diseases, such as osteoporosis or rheumatoid arthritis, affect hundreds of millions of people worldwide and pose a tremendous burden to health care. By deepening our understanding of the molecular mechanisms of bone metabolism and bone turnover, it became possible over the past years to devise new and promising strategies for treating such diseases. In particular, three tumor necrosis factor (TNF) family molecules, the receptor activator of NF-κB (RANK), its ligand RANKL, and the decoy receptor of RANKL, osteoprotegerin (OPG), have attracted the attention of scientists and pharmaceutical companies alike. Genetic experiments evolving around these molecules established their pivotal role as central regulators of osteoclast development and osteoclast function. RANK-RANKL signaling not only activates a variety of downstream signaling pathways required for osteoclast development, but crosstalk with other signaling pathways also fine-tunes bone homeostasis both in normal physiology and disease. In addition, RANKL-RANK have essential roles in lymph node formation, establishment of the thymic microenvironment and development of a lactating mammary gland during pregnancy. I will also discuss a novel function for RANKL/RANK in the brain.

HAIR FOLLICLE RANK-LIGAND REGULATES EPITHELIAL GROWTH VIA BCL-3

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The hair follicle is receiving increased attention for its renewal capacity and source of readily accessible stem cells. It regulates the local immune and epidermal homeostasis and is source of tumour cells. Rank-ligand, a tnf-family member, is

expressed by osteoblasts and is required for the formation of osteoclasts, specialized bone-absorbing macrophages. in view of certain similarities between bone and skin, and because rank-ligand is involved in mammary gland development, we investigated if rank-ligand and its receptor rank play a role in hair follicle formation and epithelial homeostasis of the mouse skin.
We found that rank is expressed at low levels in epithelial cells but rank-ligand is strongly expressed in growing hair follicles. Mice deficient in rank-ligand are unable to enter the hair follicle anagen (growth) phase and display reduced epidermal thickness. On the other hand, mice transgenic for rank in the hair follicle show increased hair renewal cycles and epidermal hyperplasia. To understand the signalling pathway triggered by rank, we performed immunolabelling and western blot analyses and found that rank induces nuclear translocation of bcl-3. Bcl-3 is a member of the nf-kappa b inhibitor family, but is capable of promoting gene transcription when bound to p50 or p52 homodimers. As cylindromas show bcl-3 nuclear translocation, it is possible that rank-ligand is implicated in the growth of these tumours.

TNFs IN HAIR AND MAMMARY GLAND DEVELOPMENT

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Organs that form as appendages of the embryonic ectoderm include hair follicles, teeth and mammary glands. Although skin appendages vary considerably in adult form and function, their early stages of development are notably similar both morphologically and molecularly. In addition to conserved signaling molecules of the Wnt, FGF, TGFβ, and hedgehog families, two TNF pathways, Edar and Troy, are known to have an essential role at multiple steps of ectodermal organogenesis. In humans, mutations in the Eda pathway genes result in hypohidrotic ectodermal dysplasia, a developmental disorder characterized by sparse hair, missing teeth, and defects in several exocrine glands including the mammary gland. The analysis of Eda gain- and loss-of-function mice has revealed that Eda/NF–κB has a critical role in the initiation of skin appendage development and it regulates both their number and size. However, the exact function and downstream targets of Eda have remained largely unknown. Here, I will discuss the role of the Eda pathway in hair and mammary gland development in light of some recently identified putative target genes of Edar.

TNF PRIMES ENDOTHELIAL CELLS FOR ANGIOGENIC SPROUTING BY INDUCING A TIP CELL PHENOTYPE

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Pathological angiogenesis associated with wound healing often occurs subsequent to an inflammatory response that includes the secretion of cytokines such as Tumor Necrosis Factor (TNF). Controversy exists on the angiogenic actions of TNF, with it being generally pro-angiogenic *in vivo*, but anti-angiogenic *in vitro*. We find that whereas continuous administration of TNF *in vitro* or *in vivo* inhibits angiogenic sprouting, a 2-3 day pulse stimulates angiogenesis by inducing an endothelial "tip cell" phenotype. TNF induces the known tip cell genes Platelet-Derived Growth Factor B (PDGFB) and Vascular Endothelial Cell Growth Factor Receptor-2 (VEGFR2), while at the same time blocking signaling through VEGFR2, thus delaying the VEGF-driven angiogenic response. Notch signaling regulates tip cell function and we find that TNF also induces the notch ligand jagged-1, through an NFκB-dependent mechanism. Enrichment of jagged-1 in tip cells was confirmed by immunofluorescent staining as well as by laser capture microdissection/qRT-PCR of tip cells sprouting *in vitro*. Thus, in angiogenesis the temporal expression of TNF is critical: it delays angiogenesis initially by blocking signaling through VEGFR2, but in addition by inducing a tip cell phenotype through an NFκB-dependent pathway, it concomitantly primes EC for sprouting once the initial inflammatory wave has passed.

LTalpha CONTRIBUTES TO LYMPHATIC VESSEL DEVELOPMENT AND FUNCTION

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LT and TNF play crucial roles in inflammation and lymphoid organ development. Lymphangiogenesis occurs in states of inflammation. Here, we demonstrate that LTα contributes to the function of lymphatic vessels and participates in lymphangiogenesis in inflammation. LTα deficient mice exhibited altered lymph flow velocities and an increased interstitial fluid pressure. When expressed ectopically, LTα led to the development of LYVE-1 and Prox1-positive lymphatic vessels within tertiary

lymphoid organs in pancreata and kidneys of RIPLTα mice that was independent of LTβ. More lymphangiogenesis occurred in the tracheas of LTβ-/- than WT or LTα-/- mice following airway infection with M. pulmonis. Similarly, more lymphangiogenesis occurred in the skin draining an OVA-CFA immunization site of LTβ-/- mice than WT and LTα-/- animals. Macrophages and B cells were prominent in the skin surrounding the immunization site, as were LTα, LTβ and TNFα. These data suggest that LTα3, rather than the LTα1β2 complex, plays a direct role in lymphatic vessel function and in inflammation-induced lymphangiogenesis.
Current studies concern regulation of lymphatic vessels in lymph nodes after immunization where interaction occurs between these vessels and high endothelial venules (HEVs). To evaluate these effects in vivo, we produced mice with green fluorescent HEVs and are developing mice with red fluorescent lymphatic vessels.

TNF SIGNALING AND ITS PHYSIOLOGICAL ROLES IN DROSOPHILA

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Eiger, a Drosophila ortholog of tumor necrosis factor (TNF) that causes cell death when it is overexpressed in fly tissues. The c-Jun N-terminal kinase (JNK) and caspases are crucial mediators of the TNF signaling. To investigate the genetic pathway and physiological roles of the TNF-mediated signaling, we conducted a genetic screen using Drosophila for suppressors of the cell death induced by Eiger. The results from the screen, combined with subsequent genetic and biochemical analyses, indicate that Eiger-stimulated signaling is transduced through the Wengen/DTRAF2/dTAK1 complex. Intriguingly, the screen identified mutations in a group of genes involved in mitochondrial energy production. Our findings suggest the connection between Eiger-signaling and the energy homeostasis.

ROLES OF THE TNF FAMILY IN CANCER

THE EGFR PATHWAY: RECENT LESSONS IN SIGNAL TRANSDUCTION

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Growth factors and their transmembrane receptors contribute to all steps of tumor progression, from the initial phase of clonal expansion, through angiogenesis to

metastasis. An important example comprises the epidermal growth factor (EGF) and the respective receptor tyrosine kinase, namely ErbB-1/EGFR, which belongs to a prototype signaling module that drives carcinoma development. The extended module includes two autonomous receptors, EGFR and ErbB-4, and two non-autonomous receptors, namely: a ligand-less oncogenic receptor, HER2/ErbB-2, and a kinase-dead receptor (ErbB-3). This signaling module is richly involved in human cancer and already serves as a target for several cancer drugs. From an evolutionary point of view, the four receptors and dozen ligands evolved from a single ligand-receptor pair, which throws light on network evolution and features conferring robust operation.
Due to inherent complexity and a large amount of experimental data, we propose a systemic approach to understanding ErbB signaling in mammals. EGF - to - ErbB signaling is envisioned as a bow-tie configured network, sharing modularity, redundancy and control circuits with robust biological and engineered systems. Our work concentrates on system controls, a plethora of positive and negative feedback loops, which include rapidly synthesized ligands of ErbBs (e.g., transforming growth factor alpha), E3 ubiquitin ligases (e.g., Cbl and Nedd4), receptor endocytosis and newly transcribed genes. Because network fragility is an inevitable tradeoff of robustness, systems level understanding is expected to identify therapeutic opportunities for targeting aberrant activation of the network in human pathologies. Specific examples include anti-receptor monoclonal antibodies, such as Trastuzumab, as well as dual-specificity kinase inhibitors, such as Lapatinib, drugs targeting essential network hubs. Combinations of targeted therapies and chemo- or radio-therapy are relatively effective in clinical settings, but the underlying molecular mechanisms remain incompletely understood. Another fragile aspect of oncogenic networks, especially those incorporating protein kinases, comprises reliance on chaperones. Accordingly, chaperone inhibitors, such as blockers of the nucleotide binding site of heat shock protein 90, may develop into effective drugs. Upon inhibition of HSP90 or the binding site at the kinase domain of HER2, the oncogenic kinase is directed to degradation in proreasomes. Mechanisms underlying response to approved, as well as experimental drugs, and evolvement of secondary patient resistance will be discussed.

STUDY OF APRIL EXPRESSION AND APRIL-MEDIATED SIGNALING IN BREAST CANCER

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APRIL (a proliferation-inducing ligand) is a TNF family protein expressed by hematopoietic cells and other cell types such as epithelial cells and osteoclasts. In

addition to its expression in physiological conditions, increased levels of APRIL mRNA and protein are found in tumor cell lines and in human primary tumors of diverse origin. In B cell malignancies, APRIL promotes tumor cell survival and protects malignant cells from apoptosis.
Preliminary data from our group suggested that APRIL is implicated in breast cancer. We found that 30% of the human primary breast cancers analyzed had high APRIL mRNA levels, and that APRIL stimulates proliferation of breast tumor cell lines. Based on these observations, we sought to characterize the relevance of APRIL in human breast cancer.
We analyzed APRIL protein expression in several human breast cancer cell lines with distinct phenotypic characteristics (location within the tumor, estrogen and progesterone receptor expression, invasiveness), and found that all tumor lines showed APRIL protein expression at different levels. A study of the regulation of APRIL expression in response to various stimuli indicated that some Toll-like receptor ligands stimulate APRIL production in breast tumor cell lines. We tested the APRIL effect on cell survival and proliferation, characterizing the signal transduction pathways activated by APRIL in our panel of breast tumor lines. Our in vitro results have led us to investigate APRIL participation in breast tumor development in vivo, using APRIL transgenic mice that develop spontaneous breast tumors.

ROLE OF RECEPTOR ACTIVATOR OF NF-κB LIGAND (RANKL) IN TUMORIGENESIS AND METASTASIS

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RANKL, a member of the tumor necrosis factor superfamily, binds to its cognate receptor RANK expressed on osteoclast progenitor cells and mature osteoclasts. RANKL is an essential mediator of osteoclast formation, function, and survival. Tumor cell-mediated osteolysis is regulated through RANKL within the bone stroma. In experimental models of bone metastasis, RANKL blockade has been shown to prevent tumor-induced osteolysis and to decrease progression of established skeletal tumors or significantly delay de novo formation of skeletal metastases. Denosumab, a fully human monoclonal antibody that specifically inhibits RANKL, inhibits osteoclastogenesis and osteoclast mediated bone destruction. Both phase I and phase II clinical trials have shown that denosumab can suppress bone resorption in patients with malignant bone disease from multiple myeloma, breast cancer, and other solid tumors. Denosumab is currently being investigated in clinical trials for the treatment of postmenopausal osteoporosis and the prevention of skeletal-related events in patients with bone lesions from multiple myeloma, and other solid tumors including breast and prostate cancer.

In addition to the role of this pathway in osteoclastogenesis, RANK and its ligand (RANKL) are crucial for the development of mouse mammary gland during pregnancy. The functional activity of RANK on cancer cells has been demonstrated in human and mouse tumor cell lines (Holstead Jones et al., 2006; Armstrong et al., 2008) and in the transgenic murine prostate (TRAMP) cancer model (Luo et al., 2007). In the latter study, induction of RANKL within the prostate tumor mass was correlated with increased numbers of soft-tissue metastases. These studies suggest that RANKL may play a role in tumorigenesis and metastasis independent of its role in tumor-induced osteolysis. Transgenic mice overexpressing RANK via the MMTV promoter in the mammary gland show a higher incidence of ductular hyperplasias (HP), mammary intraepithelial neoplasias (MIN), and mammary adenocarcinomas than wild-type (WT) mice in both spontaneous and hormone- and carcinogen-induced models of mammary tumorigenesis (progesterone/MPA+DMBA). The median time to palpable mammary tumors after the last DMBA treatment in WT mice is 132 days and 86 days in MMTV-RANK transgenic mice. Using immunohistochemistry, we observed that RANKL expression increased after MPA treatment in mammary epithelia and is induced in epithelial cells at the stage of early preneoplastic MIN lesions and in adenocarcinomas. RANK is highly expressed in the epithelial component of HP and is also observed in MIN lesions and adenocarcinoma. MMTV-RANK mice treated with a RANKL inhibitor (RANK-Fc) at initiation of DMBA treatment and continuing throughout the experiment demonstrated a significantly delayed time to palpable mammary tumors. Histopathological analysis confirmed that RANKL inhibition decreased the incidence of mammary adenocarcinomas per mouse assessed at necropsy. At early timepoints in the MPA- and DMBA-induced mammary tumor model, the presence of preneoplastic lesions (as measured by whole mount analysis) and proliferation (as measured by BrdU) were increased in MMTV-RANK mice relative to WT. Inhibition of RANKL with RANK-Fc significantly decreased the presence of preneoplastic lesions and proliferation of mammary epithelial cells in MMTV-RANK mice. In addition, treatment of either MMTV-RANK or WT mice with RANK-Fc delayed the onset of palpable mammary tumors. Treatment of the MMTV-RANK mice with RANK-Fc decreased the incidence of mammary adenocarcinoma. These data are the first to define a role for RANKL in mouse mammary tumor development and support additional studies to determine whether RANKL inhibition will delay tumor progression and metastases.

TNF-MEDIATED MYELOID CELLS SURVIVAL IS NECESSARY FOR TUMOR GROWTH

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The CD11b+Gr1+ myeloid cells belong to an important immune-regulatory cell population that inhibits T cell activity and promotes tumor growth in mice. Here we show that the development of such myeloid-derived suppressor cells (MDSCs) is impaired in the absence of TNF receptors (TNFR). Accordingly, tumor growth is inhibited in TNFR knockout mice and in wild type mice treated with neutralizing anti-TNF mAb. Adoptive transfer experiment shows that TNFR positive, but not negative MDSCs are able to rescue tumor growth in TNFR deficient mice. Further experiment shows that TNFR-expression on MDSCs is necessary for their survival through a NF-kB signaling pathway. Our results demonstrate that the endogenous TNF may promote tumor growth by increasing MDSC numbers. This may shed new light for cancer therapy to prevent T cells from MDSC-induced suppression.

FAS SIGNAL PROMOTES LUNG CANCER GROWTH BY RECRUITING MYELOID-DERIVED SUPPRESSOR CELLS VIA CANCER CELL-DERIVED PGE2

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Tumor cells consistently release many kinds of immunosuppressive (TGF-β, VEGF, PGE2 etc) and proinflammatory (TNF-α, IL-1β, IL-6 etc) factors which facilitate tumor immune escape and tumor growth. More and more evidences suggest that chronic inflammation contributes to cancer development and progression. Fas/FasL system has been extensively investigated with respect to its capacity to induce cellular apoptosis. However, accumulated evidences show that Fas signaling also exhibits non-apoptotic functions such as induction of cell proliferation and differentiation. Lung cancer is one of cancers refractory to the immunotherapy, however, the underlying mechanisms remain to be fully understood. In this study, we show that Fas overexpression does not affect in vitro growth of 3LL cells, but promotes lung cancer growth in vivo. However, such tumor-promoting effect is not observed in FasL-deficient (gld) mice, and also not observed in the immune competent mice once inoculation with domain-negative Fas-overexpressing 3LL cells, suggesting the critical role of Fas signal in the promotion of lung cancer growth in vivo. More

accumulation of myeloid-derived suppressor cells (MDSC) and Foxp3+ regulatory T cells (Treg) is found in tumors formed by inoculation with Fas-overexpressing 3LL cells, but not domain-negative Fas-overexpressing 3LL cells. Accordingly, Fas-ligated 3LL lung cancer cells can chemoattract more MDSC but not Treg cells in vitro. Furthermore, Fas ligation induces 3LL lung cancer cells to produce proinflammatory factor PGE2 by activating p38 pathway, and in turn, 3LL cells-derived PGE2 contribute to the Fas ligation-induced MDSC chemoattraction. Furthermore, in vivo administration of COX-2 inhibitor can significantly reduce MDSC accumulation in the Fas-overexpressing tumor. Therefore, our results demonstrate that Fas signal can promote lung cancer growth by recruiting MDSC via cancer cell-derived PGE2, thus providing new mechanistic explanation for the role of inflammation in cancer progression and immune escape.

NF-KAPPAB INHIBITION AND SKIN CANCER: A VISCOUS CIRCLE OF CHRONIC INFLAMMATION AND IMMUNOSUPPRESSION DRIVEN BY TNFR1 IN KERTINOCYTES

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Inhibition of NF-kappaB in keratinocytes (K5-IkappaBalpha mice) causes chronic inflammation, hyperplasia and spontaneous development of squamous cell carcinomas (SCCs). TNFR1-signalling has been shown to be crucial both for the development of inflammation and cancer in this model. We here show that TNFR1 expression in keratinocytes mediates the inflammatory circuit elicited by inhibition of NF-kappaB. TNFR1 expression in all other cell types, including the immune cells, is surprisingly redundant. Further, we show that the keratinocytes takes an active part in shaping the inflammatory response by upregulation of the immunosuppressive cytokine IL-10, creating an environment that prevents an effective anti-tumour response. Indeed, the macrophages in inflamed K5-IkappaBalpha skin display a phenotype consistent with an alternative type II (M2) activation and display similarities to tumour associated macrophages (TAMs) already at the onset of inflammatory changes. Hence, inhibition of NF-kappaB in keratinocytes elicits a very distinct type of inflammation that can favour tumourigenesis.

ENDOGENOUS LIGAND-INDUCED ACTIVATION OF TLR4 IN PRE-METASTATIC PHASE IS BOTH DOWNSTREAM AND UPSTREAM OF TNF SIGNALING

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The S100A8/A9 heterodimer was initially found as an abundant and potentially pathogenic molecule in the synovial fluid of rheumatoid arthritis (Nature, 1987). We have reported that TNFa produced from distant primary tumors prepares soil in the pre-metastatic lungs by inducing S100A8/A9 expression in pulmonary endothelial cells and macrophages (Nat. Cell Biol., 2006). S100A8/A9 showed chemoattractant activity toward tumor cells as well as Mac1+myeloid cells from bone marrow. Anti-TNFa neutralizing antibody could inhibit the S100A8/A9 induction and anti-S100A8 antibody could block lung recruitment of tumor cells in pre-metastatic phase. Given that S100A8/A9 can in turn stimulate TNFa production via NFkB and p38, tumor-derived TNFa triggers and S100A8/A9 amplifies the TNFa/S100A8 paracrine cascade in the lungs. We have very recently showed that S100A8/A9 up-regulates expression of another strong chemoattractant serum amyloid A3 (SAA3) in lung macrophages in a paracrine fashion and that both S100A8 (KD 11nM) and SAA3 (KD 0.356nM) are an endogenous ligand for Toll-like receptor (TLR) 4 (Nat. Cell Biol., 2008) that has been recognized as a sensor for extrinsic pathogens. Anti-SAA3 neutralizing antibody or TLR4 gene knockout could dramatically abrogate lung metastasis. A 500-bp SAA3 promoter fragment contains two NFkB-binding sites and SAA3 activates NFkB. Therefore SAA3, once stimulated, is auto-amplified in the lungs through TLR4. The interplay between TNFa receptor and TLR4 suggests a close linkage between inflammation and metastasis.

MECHANISMS OF THE TUMOR-PROMOTING ACTION OF TNF-α IN EPITHELIAL TUMORS

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TNF-α was first identified for its ability to induce rapid haemorrhagic necrosis of experimental cancers. As efforts to harness this anti-tumor activity in cancer treatments were underway, a paradoxical pro-tumor role of TNF-α became apparent. Now that links between inflammation and cancer are appreciated, data are accumulating that TNF-α may be a target in some malignant diseases.
TNF-α and its receptors are involved in tumor promotion and progression in some experimental cancers and both are found in human cancer biopsies. Mice deficient in

TNF-α or TNFR1 are resistant to skin carcinogenesis and mechanisms of the tumor-promoting action of TNF-α involve the host immune system. TNF-α drives a cancer cell-autonomous cytokine network in ovarian cancer that stimulates production of other cytokines by host immune cells. Evidence from animal models and expression array datasets from patient biopsies, shows that TNF-α signalling pathways are intricately linked with other pathways involved in host tumor communication, including CXCR4, CXCL12, IL-6, Notch receptors and ligands. High levels of some of these pathways have prognostic significance in the human disease.
Because TNF-α was a major player in cancer promotion and progression, we hypothesised that neutralising its activity would be of therapeutic benefit. We tested this in Phase I/II clinical cancer trials of TNF-α antagonists and obtained a signal of clinical activity, with stable disease and some partial responses achieved in patients with advanced renal and ovarian cancer, and identification of possible biomarkers of response.
We believe that optimal treatment of cancer will be achieved by targeting not only the pathways that drive oncogenesis but also those pathways that control communication between malignant cells and the rest of the tumor micro-environment. Targeting TNF-α may be one way of inhibiting this communication.

NOVEL ASPECTS OF SIGNALING ACTIVATION BY THE TNF FAMILY

NON-CANONICAL MODES OF UBIQUITINATION IN REGULATION OF TRANSCRIPTION: PROCESSING OF THE NF-κB p105 AND ACTIVATION OF POLYCOMB REPRESSIVE COMPLEX 1

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Activation of NF-κB is regulated via numerous ubiquitin- and proteasome-mediated steps - an important one is processing of the precursor p105 to the p50 active subunit. The mechanisms involved in this exceptional case, where the substrate is processed in a limited manner rather than destroyed completely, are largely unknown. We have shown that proteasomal processing of p105 requires ubiquitin, but not generation of polyubiquitin chains. *In vitro*, ubiquitin species that cannot polymerize mediate processing. In yeast that express non-polymerizable ubiquitins, processing proceeds normally, whereas degradation of substrates that are dependent on polyubiquitination is inhibited. Similar results were obtained in mammalian cells. Interestingly, processing requires multiple monoubiquitinations, as progressive elimination of lysines in p105 is accompanied by gradual inhibition of p50 generation. Last,

the proteasome recognizes the multiply monoubiquitinated p105. These findings suggest that a proteolytic signal can be comprised of a cluster of single ubiquitins, and not necessarily of a chain.
Polycomb complexes mediate gene silencing, in part by modifying histones. Ring1B is a RING finger protein that is a member of the Polycomb Repressive Complex 1. It is an E3 - that like many other Ring finger E3s - mediates its own polyubiquitination and catalyzes ubiquitination of an exogenous substrate – in this case it monoubiquitinates histone H2A. We have shown that unlike other RING finger proteins that are believed to mediate their own ubiquitination and degradation, Ring1B is degraded by an exogenous E3, independent of its RING domain. Consistent with the non-proteolytic self ligase activity of Ring1B, it we found that it generates atypical mixed Lys6, Lys27, and Lys48-based self polyubiquitin chains, that require the presence of all these Lys residues on the same ubiquitin molecule. This unique modification is necessary for Ring1B ability to monoubiquitinate H2A, unraveling a novel mechanism for ligase activation via self ubiquitination.

SIGNALING LESSONS FROM DEATH RECEPTORS

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It was thought that receptors signaled either by serving as ion channels or by altering phosphorylation-dephosphorylation events. Work in my laboratory in the mid-nineties conclusively showed that death domain-containing receptors signaled by an entirely new mechanism, specifically, adapter (FADD)-mediated recruitment and direct activation of a death protease (FLICE/caspase-8) (1,2). Subsequent studies by my own and other laboratories showed that this was a universal mechanism deployed by death receptors (3).
Further study of the NF-kB inducing death receptor, TNFR1, has recently unveiled another new signaling mechanism, that we have termed “ubiquitin editing.” Ubiquitin editing is mediated by a highly conserved single polypeptide protein, termed A20, which contains both a ubiquitin hydrolase and a ubiquitin ligase domain (4). Initially, this seemed counterintuitive as it was akin to a molecule possessing both kinase and phosphatase activities – the ultimate futile cycle! We found, however, that these seemingly contradictory activities actually act in concert to attenuate cytokine signaling. It has recently become apparent that components of a number of signaling pathways are activated by an unusual form of ubiquitin polymerization involving isopeptide linkage through K63. This is in marked contrast to the well-known degradative linkage that involves K48. We showed that the ubiquitin hydrolase domain first removes the activating K63-linked poly-ubiquitin, following which the ubiquitin ligase domain adds on degradative K48-linked poly-ubiquitin, targeting the signaling component for proteosomal destruction. Since there was

no precedence for the existence of such an enzymatic system, we asked if this phenomenon of ubiquitin editing where K63 linked poly-ubiquitin is edited to a K48 linked polymer is observed in other signaling pathways. Indeed, ubiquitin editing, was found to be used by other receptors of the innate immune system to limit NF-kB signaling. Finally, the prospect of targeting DUBs as a therapeutic modality will be discussed (5).

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IN VIVO STUDY OF THE (PATHO)PHYSIOLOGICAL ROLE OF THE ANTI-APOPTOTIC AND ANTI-INFLAMMATORY PROTEIN A20, USING GENETICALLY MODIFIED MICE

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The ubiquitin-editing enzyme A20 is a critical negative regulator of NF-κB-dependent inflammation and apoptosis. A20 deficient mice spontaneously develop multiorgan inflammation, cachexia and premature lethality, and cells derived hereof are hypersensitive to TNF-induced NF-κB activation and apoptosis, demonstrating the strong anti-inflammatory and anti-apoptotic properties of this protein. A20 may also play important roles in human autoimmune and inflammatory diseases. Recent genetic studies suggest that polymorphisms in or near the human A20/tnfaip gene are associated with Crohn's disease, rheumatoid arthritis and systemic lupus erythematosus. Other inflammatory diseases, such as atherosclerosis, were also shown tot involve A20. As A20-deficient mice die early after birth, we generated conditional knockout mice for A20, in order to study the role of A20 in the adult organism. Conditional gene inactivation was achieved through use of the Cre/LoxP recombination system in which the targeted gene is flanked by LoxP consensus sites.

Cell-specific deletion of A20 can now be obtained in a spatially and temporally controlled manner, by crossing with transgenic mice expressing Cre recombinase in a tissue specific or/and inducible pattern. These tissue-specific A20 knockout mice are currently used in different mouse models of inflammatory and degenerative diseases in order to clarify the role of A20 in the regulation of inflammation, apoptosis and immune responses involved in these pathologies.

REGULATION OF TNF-INDUCED JNK ACTIVATION AND INFLAMMATION BY SMOR1

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TNF-α induces the recruitment of TRADD, TRAF2/5, RIP1 and cIPA1/2 to TNF-R1 to activate downstream signaling pathways including JNK, p38 and IKK. Yet, the underlying control mechanisms are incompletely understood. We have recently identified that TNF-α-induced site-specific K48-linked polyubiquitination and proteosomal degradation of SMOR1, a novel TNF-Specific Modulator Or Regulator, is required for activation of JNK but not p38 or IKK. Mass spectrometry and yeast two-hybrid screening identified SMOR1 as a novel component of the JNK signalsome through interaction with JNK1 and TRAF2. Genetic disruption of SMOR1 alleles augments TRAF2 E3 ligase activity and K63-linked polyubiquitination, thereby accelerating TNF-α-induced JNK1 activation, cell death and inflammatory responses. Upon TNF-α stimulation, SMOR1 undergoes K48-linked polyubiquitination and proteosomal degradation, thereby relieving the suppression by SMOR1. Thus, our results provide a novel mechanism by which the TNF-R1 Complex is selectively activated by polyubiquitination-dependent degradation of SMOR1, leading to JNK1 activation for cell death or inflammation.

CULLIN3-BASED POLYUBIQUITINATION AND P62-DEPENDENT AGGREGATION OF CASPASE-8 MEDIATE CELL EXTRINSIC APOPTOSIS SIGNALING

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Death receptors trigger apoptosis from the cell surface through a death-inducing signaling complex (DISC) that recruits the apical protease caspase-8. Apoptosis commitment requires efficient activation and auto-proteolytic release of caspase-8 into the cytoplasm to engage effector caspases. While DISC recruitment initiates

stimulation, full caspase-8 activation depends on further molecular events that are not fully understood. Death receptor engagement induced polyubiquitination of caspase-8, through interaction of the DISC with a cullin3 (CUL3)-based E3 ligase. CUL3 augmented polyubiquitination of caspase-8 via the RING box protein RBX1, while the deubiquitinase A20 reversed this modification. The ubiquitin-binding protein p62/sequestosome 1 promoted aggregation of CUL3-modified caspase-8 within p62-dependent aggresomes, leading to full activation and processing of the enzyme and commitment to apoptosis. These results identify a novel mechanism that positively controls apoptosis signaling through polyubiquitination and aggregation of a key initiator caspase.

SELECTIVE BINDING OF LINEAR UBIQUITIN CHAINS TO NEMO (NF-κB ESSENTIAL MODULATOR) IN NF-κB ACTIVATION

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The transcription factor NF-κB (nuclear factor-κB) regulates critical cellular processes during development, skin homeostasis and immunity. A key step in activation of the NF-κB is binding of NF-κB essential modulator (NEMO) to ubiquitinated substrates. In this report we provide structural and functional evidence for selective binding of NEMO UBAN (Ubiquitin Binding in ABIN and NEMO) motif to linear (head to tail) rather than Lys63-linked ubiquitin chains. Crystal structures of UBAN motif reveal parallel coiled-coil dimer which binds to two linear diubiquitin molecules to form a hetero-tetrameric complex. All four ubiquitin moieties contact UBAN motif to render efficient NF-κB activation. Binding of the ubiquitin moieties to UBAN dimer occurs via Ile44 surface on distal and a novel surface on proximal ubiquitins thereby providing specificity for linear chain recognition. Residues of NEMO involved in binding to linear ubiquitin chains are essential for NF-κB activation by TNFα and other agonists, which explains the detrimental effect of NEMO mutations in patients suffering from X-linked ectodermal dysplasia and immunodeficiency.

A NOVEL UBIQUITIN LIGASE RECRUITED TO THE TNF-R1 SIGNALLING COMPLEX IS REQUIRED FOR EFFICIENT TNF-INDUCED NF-κB ACTIVATION AND GENE INDUCTION

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Despite much progress over the last decade in understanding the signalling pathways emanating from TNF receptor 1 (TNF-R1), especially some of the proximal signalling events are not yet fully explained. We reasoned that this may be due to the fact that not all components of the TNF-R1 signalling complex (TNF-RSC) have been identified. To elucidate the composition of the native TNF-RSC we developed a modified tandem affinity purification (moTAP). Using this approach we identified two new components of the native TNF-RSC, HOIL-1 and HOIP, which together form a linear ubiquitin chain assembly complex (LUBAC), i.e. a ubiquitin ligase (E3) which had been shown to catalyse head-to-tail ligation of ubiquitin on existing ubiquitin chains. Overexpression of HOIL-1/HOIP activated NF-kB and ablation of HOIL-1/HOIP expression severely reduced TNF-induced activation of NF-kB, JNK and, consequently, gene expression. Consistent with LUBAC-dependent NF-kB activation, absence of HOIL-1/HOIP sensitised cells to TNF-induced apoptosis. Identification of HOIL-1 and HOIP as components of the TNF-RSC allows for a better understanding of the early events of TNF signalling. We found that LUBAC is required for stable formation of the pro-survival TNF-RSC and that association of various known components of this protein complex including RIP1 and TRAF2 is reduced in the absence of HOIL-1 and HOIP. Further insight into the biochemistry and function of LUBAC in TNF-R1 signalling gained by examining cells in which known components of the TNF-RSC are individually ablated will be presented.

TRANSCRIPTIONAL NETWORKS AND SYSTEMS BIOLOGY

CHARACTERIZATION OF NOVEL TNFα/NF-κB SIGNALING PATHWAY COMPONENTS BY GENOME-WIDE RNAI

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Signaling pathways are crucial for the regulation of cell proliferation and differentiation, as well as for mediating adequate immune responses. Aberrant signaling is involved in the pathogenesis of many human diseases. Thus dissection of important signaling pathways is essential to develop new diagnostic and therapeutic approaches.
Nuclear factor κb (NF-κB) transcription factors are central coordinators of cell proliferation, apoptosis, innate and adaptive immune responses. Aberrantly active NF-κB signaling is thought to play a role in carcinogenesis and chronic inflammatory diseases. NF-κB is activated by different stimuli. One of these is tumor necrosis factor alpha (TNFα), a multifunctional cytokine activating NF-κB by the canonical pathway.
Here, we use RNAi to identify and characterize novel regulators of TNFα/NF-κB signaling. Using genome-wide RNA interference (RNAi) screens in human cells we identified approximately 50 previously not described regulators of TNFα/NF-κB signaling. After performing secondary screens to assess the most promising candidates among these 50 proteins, we are currently characterizing 8 top candidates using different functional assays. We will present the latest results of our on-going work.

RNA-INTERFERENCE BASED SCREEN IDENTIFIES NEW FACTORS IMPORTANT FOR NF-kappaB ACTIVATION AND TERMINATION

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Research efforts over the past decades have led to a more and more detailed understanding of NF-kappaB signaling but the picture is not yet complete. Especially the activation of NF-kappaB by bacteria and the termination of NF-kappaB activation

are not well understood. To identify new key regulators of NF-kappaB we have conducted an RNA-interference based screen. For this purpose, we have developed a new model for high throughput analysis using a human epithelial cell line stably expressing a p65-GFP-fusion construct. The nuclear translocation of p65-GFP can be quantified by automated microscopic analysis. Three different stimuli were compared: the cytokines TNFalpha and IL-1beta and the gastric pathogen *Helicobacter pylori*. Screening a library of siRNAs targeting 646 kinases and associated proteins, we identified 24 factors, which also included known factors such as IKKalpha and IKKbeta. Interestingly, we found general NF-kappaB regulators as well as factors specifically acting in one pathway. Amongst the newly discovered regulators, an ubiquitin E3 ligase could be shown to be necessary for termination of NF-kappaB activation: Upon knockdown of this E3, base level of IkappaBalpha is reduced and nuclear translocation of p65 as well as IKK activity is prolonged. The screen is currently expanded to a genome wide scale.

CASEIN KINASE 1α, NF-κB, AND HUMAN LYMPHOMA CELL SURVIVAL

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The transcription factor NF-κB is required for lymphocyte activation and proliferation as well as the survival of certain lymphoma types. Antigen receptor stimulation assembles an NF-κB activating platform containing the scaffold protein CARMA1/CARD11, the heterodimer BCL10/MALT1 (CBM complex), linked to the inhibitor of NF-κB kinase (IKK) complex. We conducted parallel screens involving a mass spectrometry analysis of CARMA1 binding partners and an RNAi screen for growth inhibition of the CBM-dependent "activated B cell-like" (ABC) subtype of diffuse large B-cell lymphoma (DLBCL), and identified casein kinase 1α, (CK1α) as a bifunctional regulator of NF-κB. CK1 α dynamically associates with the CBM complex upon T-cell receptor engagement to allow proper cytokine production and lymphocyte proliferation. However, CK1 α kinase activity plays a counterposing role by subsequently promoting the phosphorylation and inactivation of CARMA1. CK1 αhas thus a dual "gating" function, which first promotes and then terminates receptor-induced NF-κB. ABC DLBCL cells required CK1α for constitutive NF-κB activity indicating that CK1α functions as a "conditionally essential malignancy" (CEMal) gene - a member of a new class of potential cancer therapeutic targets.

PATHOGENIC ROLE OF IL-6 WITH TNF-α or IL-1 ON THE INDUCTION OF ACUTE PHASE PROTEINS IN CHRONIC INFLAMMATORY STATUS OF AUTOIMMUNE DISEASE

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IL-6 develops inflammatory status in autoimmune disease. IL-6 blocking therapy with an anti-IL-6 receptor antibody (Tocilizumab) for Castleman's disease (CD), rheumatoid arthritis (RA), and Juvenile Idiopathic Arthritis (JIA) improved the most of clinical symptoms and laboratory findings. Especially, CRP and SAA levels were decreased and completely normalized, even if another cytokines and chemokines were activated in the patient with RA. On the other hand, single use of TNF-α blockade, such as infliximab or etanercept, decreased the SAA and CRP levels, but hardly reached them into normal range. Therefore, we analyzed this different mechanism between IL-6 and TNF-α in vitro utilizing hepatoma derived cell lines. In result, activation of STAT3 through IL-6 signal was essential for induction and augmentation of SAA and CRP expression via assembling the transcription factors complex with NF-κB p65, and with HNF-1α and c-fos, respectively. TNF-α or IL-1 complementally contributed the augmentation of SAA and CRP through the activation of NF-kB p65 and c-fos, respectively. Recently, the essential contribution of IL-6 on the induction of hepcidin, an acute phase protein, was found and further confirmed by our investigation in patient with inflammatory anemia. However TNF-α did not induce hepcidin. Our findings explained the different role of IL-6 from TNF-α in the pathogenesis of RA, an autoimmune disease with chronic inflammation. The clinical results of IL-6 blocking therapy were expected to contribute to the development of a therapeutic strategy for autoimmune disease besides TNF-α blockage.

NEW INSIGHTS IN LTBR-MEDIATED NF-κB ACTIVATION

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Several members of the TNFR family have been recently described for their intrinsic capabilities of activating distinct NF-κB complexes through the classical and the alternative pathway. How ligation of a single receptor activates both pathways is still poorly understood.
By performing a yeast-two-hybrid screening using the cytosolic tail of the LTβR, we have identified μ adaptin proteins as new interacting partners. We showed

that dominant negative AP2μ2 expressing-cells that are incompetent for clathrin-mediated endocytosis were deficient for LTβR-mediated activation of the classical NF-κB pathway.
Conversely, LTβR-mediated activation of the alternative NF-κB pathway requires its internalization but independently of AP2μ2 or dynamin. This mechanism relies on a specific cytosolic region within LTβR playing a dual function by controlling AP2μ2-independent internalization of the receptor and TRAF3 recruitment through an unconventional TRAF binding site. The internalization route taken by LTβR is controlled through its level of oligomerization by a phenomenon we named LEO (Lymphotoxin-betaR-Elicited Oligomerization).

THE NF-κB SIGNALING SYSTEM AS AN INTEGRATOR OF DIVERSE SIGNALS

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Every member of the diverse TNFR superfamily activates the NF-kB signaling system but produces specific cellular responses. The NF-κB signaling system is capable of producing up to 15 specific dimers that show diverse mechanisms of activation and inactivation. We have undertaken a systematic biochemical and genetic study of the mechanisms that generate NF-κB dimers and those that control it. We have quantified known mechanisms, and identified novel factors, complexes and mechanisms. These inform the construction of a mathematical model that recapitulates dimer formation and regulation via IkB proteins and proteolytic processing. Combined computational and experimental studies illuminate cases of synergy between stimuli, antagonistic interactions between pathways, and signaling that is surprisingly insulated from crosstalk mechanisms. We have explored the relevance of these characteristics in diverse biological contexts.

MATHEMATICAL MODELING OF THE TNF-a SIGNALING PATHWAY

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Among a wide range of cellular activities, TNF-a can promote apoptosis, cell survival or tumorigenesis. Maintaining the balance between the apoptotic and survival

pathways plays a crucial role in deciding cell fate in response to inflammation and infection, and there is an extensive crosstalk between these pathways. In silico models of signal transduction have been developed as supportive tools for simplifying complex signaling pathways and gaining detailed insight into biological dynamics. Development of more integrated representations for cellular systems would be very useful for studying the function and control of biological processes. The fundamental theme of our modeling research is to produce biologically sound predictions and suggest novel directions for future experiments in the TNF signaling. In addition, by constructing dynamic mathematical models for TNF-a signaling transduction pathways, the project will contribute to a reliable development of targets for effective therapeutic intervention in breast cancer and eventually translate the acquired information to diagnostics and therapeutics. One approach to the illustration of simultaneous system is based on a mathematical model called Petri Net. This quantitative analysis model simulates and analyses the time-dependent properties of a network. Using invasive and immortalised breast cell lines, MCF7, MCF10A, and K562 erythroleukaemia cell line as models, quantitative and kinetic parameters involved in TNF-a induced NF-kB and caspase activation signaling were measured. The profile from the experimental data from these cells has been applied to simulate TNF-a signaling Petri Net model.

DYNAMICS WITHIN THE CD95 DEATH-INDUCING SIGNALING COMPLEX DECIDE LIFE AND DEATH OF CELLS

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This study explores a highly significant dilemma in cell biology, namely how triggering of CD95 (Fas/APO-1) in some situations results in cell death and in others circumstances leads to the activation of NF-kappaB resulting in cell growth. We established an integrated kinetic mathematical model of CD95-mediated NF-kappaB and apoptosis signalling. Systematic model reduction resulted in a surprisingly simple model well approximating experimentally observed dynamics. The model suggested a new link between c-FLIPL cleavage in the Death-Inducing Signalling Complex (DISC) and the NF-kappaB pathway. We validated experimentally that CD95 stimulation results in binding of p43-FLIP to the IKK complex followed by its activation. Furthermore, we demonstrated that the apoptotic and NF-kappaB pathway diverge already at the DISC. Model and experimental analysis of DISC formation showed that a subtle balance of c-FLIPL and procaspase-8 determines life/death decisions in a non-linear way. This is the first mathematical model explaining the complex dynamics of CD95-mediated apoptotic and non-apoptotic signalling.

TNF-α SENSITIZES PRIMARY MOUSE HEPATOCYTES TO FAS/CD95-INDUCED APOPTOSIS: A SYSTEMS BIOLOGY APPROACH

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TNF-α plays a pivotal role in the apoptosis/survival switch in primary hepatocytes. We demonstrated that TNF-α and ActD induced apoptosis occurs via type II pathway requiring the protein Bid and the mitochondrial apoptotic mechanism. In contrast, Fas/CD95 mediated death signaling shows no Bid dependency and proceeds via type I apoptosis pathway. Interestingly, TNF-α is able to sensitize primary hepatocytes to Fas/CD95-induced apoptosis. We prestimulated the cells with TNF-α followed by FasL treatment and observed synergistically induced apoptosis. This effect had a threshold value of appr. 2 h indicating a mechanism involving processes such as protein biosynthesis or receptor upregulation. However, neither Fas nor FasL were upregulated after TNF-α prestimulation and experiments in both Fas-/- and FasL-/- hepatocytes did not reveal any influence. The observed effect is specific for TNF-α as FasL followed by TNF-α stimulation does not lead to increased apoptosis. Interestingly, experiments in Bid-/- hepatocytes showed a complete decreased sensitizing effect suggesting a mechanism involving a switch to type II apoptosis pathway. In addition, Bid mRNA was upregulated after TNF-α stimulation confirming an involvement of Bid. XIAP KO did not influence the observed effect. Furthermore, JNK was phosphorylated due to TNF-α stimulation. JNK inhibition resulted in decreased apoptosis. Hence, JNK activation and Bid cleavage seem to be crucial for the sensitizing effect of TNF-α. To gain detailed insights into the crosstalk of TNF-α and Fas/CD95 in enhancing hepatocyte apoptosis a quantitative dynamic mathematical model is being generated.

UNIQUE PERSONALITIES WITHIN THE NF-κB FAMILY: DISTINCT FUNCTIONS FOR P65 AND RELB IN THE OSTEOCLAST

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The osteoclast (OC) is the resorptive cell of the skeleton, responsible for both normal homeostasis and pathological bone loss. NF-κB is a critical signal for OC

differentiation downstream of RANKL, and its global disruption protects mice from pathological bone loss. Differently from other TNF family members, RANKL activates both the classical NF-κB pathway, activating both p65 and cRel, and the alternative pathway, inducing expression and activation of RelB. In order to determine whether the classical and alternative NF-κB pathways have distinct roles in OC differentiation, we examined mice lacking p65, RelB, or cRel.
p65 controls cell survival during a critical stage of commitment to the OC lineage by modulating JNK activation. Although JNK is required for activation of NFATc1, a critical differentiation factor, in the absence of p65, expression of JNK inhibitors MKP5 and Gadd45β is decreased, JNK is hyperactivated, and apoptosis ensues. Partial blockade of JNK or its downstream mediator Bid prevents apoptosis and allows OC formation. Only p65 transmits anti-apoptotic signals since p65-/- cells have intact cRel and RelB activation in response to RANKL, and cRel deficient cells show normal survival and differentiation in vitro. Furthermore, overexpression of RelB in p65-/- precursors does not prevent apoptosis, confirming the unique role for p65 in OC survival.
Absence of RelB restricts OC differentiation, but without RANKL-induced apoptosis. Retroviral expression of RelB, but not p65, rescues the relB-/- defect, again indicating that these subunits have distinct functions. Precursors lacking NIK, the upstream kinase in the alternative pathway, also have a non-apoptotic block in differentiation. Expression of either p65 or RelB in NIK-/- cells confirms that only RelB rescues differentiation.
Thus, we have shown that p65 and RelB have critical but distinct roles in the OC.

THE INTERRELATIONSHIP BETWEEN NF-kappaB, Nrf2 AND KEAP1 IN CONTROLLING TNF INDUCED INFLAMMATORY RESPONSES

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Monocytes play a central role in the immunopathological effects of autoimmune disease. This role is mediated mainly by the production of tumor necrosis factor α (TNF). The transcription factor NF-E2-related factor 2 (Nrf2) regulates innate immune responses in various experimental disease models. We have previously investigated the anti-inflammatory effects of Nrf2 activation in LPS treated human monocytes and have described coordinated induction of heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) expression. This coordinated induction dampens LPS induced TNF and IL-1β responses.
Here we investigate the regulation of Nrf2 transcription in response to TNF and the consequent effects to inflammation and apoptosis. We find that in the human

monocytic cell line THP-1, TNF induces Nrf2 transcription in an NF-kappaB dependent manner. We also examined the role of the Nrf2 inhibitor KEAP1 in regulating the activation of TNF induced Nrf2. Our data suggest that KEAP1 regulation of Nrf2 is more complex than previously thought. We find that KEAP1 doesn't play a role in regulating Nrf2 that has been transcriptionally induced by TNF. However, TNF activation of THP-1 cells also increases reactive oxygen species, which in turn induces Nrf2 stabilisation and release from KEAP1. Silencing Nrf2 increases TNF-induced inflammation but has little effect on apoptosis. The data reveals a regulatory network integrating NF-kappaB, Nrf2 and KEAP1 function with the regulation of TNF-induced inflammatory responses.

AATF IS A NOVEL ACTIVATOR OF THE C-JUN TRANSCRIPTION FACTOR UPON UV-LIGHT EXPOSURE

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AATF/Che-1 is an evolutionary highly conserved protein that was originally characterized as an interacting protein for RNA polymerase II. It exhibits a dual role in cell cycle control as it is able to modulate both cell proliferation and growth arrest by interacting with various important components of cell survival machinery, such as pRb, DLK kinase, HDACs, Tau, Par4 (Ref1, 2). Recently, Che-1 was found to play an important role in the DNA damage response and cell-cycle checkpoint control, by inducing the expression and stabilization of p53 and p21. (Ref 3,4). The activation of "safeguard" regulators gives the cell time to repair the DNA damage or, if the damage is too extensive, they trigger apoptosis. Here we identify AATF as a new positive regulator of c-Jun transcription factor. c-Jun is implicated in several physiological processes including, apoptosis, proliferation and differentiation. In response to various stress stimuli, JNK/SAPKs kinases phosphorylate c-Jun, ATF2 and ATF3 which, in turn, enhance AP-1 transcriptional control of specific gene expression. Our results show how, upon UV-light exposure, AATF overexpression strongly increased c-Jun and ATF2 activation compared to the mock transfected cells. Interestingly the activation of c-Jun and ATF-2 is correlated to the translocation of AATF protein from the nucleolus to the nucleus, suggesting a tight regulation of the spatial-temporal dynamics of the protein upon different cell signals. Moreover we find that AATF has a connection to the CD95 signaling machinery, which connects it both to survival and death signaling.
AATF is a novel key stress-sensor factor, which regulate the cell response upon a genotoxic stress. In particular our preliminary results suggest that AATF, being able to enhance the activation of c-Jun upon a stress, can heavily affect "the weights on the balance" between a "stop and repair" or a "stop and die" response.

MICRORNA miR-146 IS A KEY REGULATOR OF AUTOIMMUNITY AND INFLAMMATION

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Excessive and inappropriate activation of the immune system can be quite deleterious to the organism, therefore nature employs multiple mechanisms to control and properly terminate the immune responses. MicroRNAs, ~22nt long non-coding RNAs, have recently emerged as key post-transcriptional regulators controlling diverse biological processes. Previously, we have identified miR-146a/b as endotoxin-responsive genes and proposed a role for these miRNAs in the control of Toll-like receptor and cytokine signaling through a negative feedback regulation loop involving down-regulation of IRAK1 and TRAF6 target genes.
To establish the physiological role of miR-146a gene we have utilized loss- and gain-of-function mouse models. miR-146a knockout (KO) mice are born normal, but starting at the age of 6 month they develop an autoimmune disorder, characterized by splenomegaly, lymphoadenopathy and multiorgan inflammation, and as the result die prematurely. Enlarged spleens of miR-146a KO animals display massive myeloproliferation and exhibit signs of follicular hyperplasia. Furthermore, lack of the miR-146a gene results in defective thymocyte development with a block at the DN1/DN2 stage, while peripheral T cells from miR-146 KO mice display an activated, effector status. Moreover, miR-146a is also required for normal development of 'innate-like' B cells, because we observed a significant reduction in the number of B-1 and marginal zone B cells in miR-146 null animals. We have also found that primary macrophages from miR-146 KO mice overproduce proinflammatory cytokines (e.g. TNF□ and IL-6) in response to LPS challenge, while conversely, cells with enforced expression of miR-146a gene showed reduced production of proinflammatory cytokines. These findings taken together suggest that miR-146a plays a central role in regulation of inflammation and in the development and function of immune cells. We will discuss these findings as well as our current efforts to understand the molecular mechanisms of miR-146 action.

MicroRNA-146a PROTECTS FROM TNFα-INDUCED APOPTOSIS

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MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules acting as key regulators of immune cell differentiation and innate immune responses. miRNA-146 expression is induced by activation of the Toll-like/interleukin-1 receptor pathway (TIR-pathway), where it targets essential adaptor and signaling molecules, thus serving as a regulator preventing the cells from an exacerbated pro-inflammatory response. Since TNFα also up-regulates the expression of miRNA-146a, we decided to explore whether this miRNA is involved in the regulation of apoptosis. To this end, we used the HeLa human epithelial cell line as a model system for TNFα signaling. Following TNFα and cycloheximide (CHX) treatment miRNA-146a transfected cells showed significantly reduced levels of the active pro-apoptotic caspases 8 and 3 (CASP8/3). In line with this, miRNA-146a conferred enhanced protection against TNFα-induced DNA fragmentation and mitochondrial potential drop-down. This effect was specific for miRNA-146, since it could be blocked by co-transfection of a complementary miRNA-inhibitor. Our results demonstrate that miRNA-146a is a regulator of receptor-mediated apoptosis. Similar to the TIR-pathway, miRNA-146a seems to be part of a negative feedback mechanism of the TNFα signaling cascade. Ongoing research focuses on the identification of the specific pro-apoptotic molecules targeted by miRNA-146a. Furthermore, we are exploring the relevance of our observations for the mycobacterial infection of human macrophages, where the regulation of apoptosis is critical.

miRNA REGULATION OF ENDOTHELIAL CELL RESPONSES TO TNF

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miRNAs are short (∼22 nt) noncoding double stranded RNAs that target mRNAs by binding partial complementary binding to sequences in the 3′ UTRs of mRNAs and repress protein synthesis. miRNAs are processed from primary transcripts by the sequential actions of Drosha and Dicer and may be expressed in a tissue-specific and/or inducible manner. We have previously shown that human endothelial cells (ECs) express miRNAs that can be inhibited by siRNA-mediated knock down of

Dicer. In the present work we have found that pro-inflammatory responses of TNF, such as adhesion molecule induction, are increased by Dicer knock down in human ECs. Specifically, in the absence of Dicer, TNF treatment showed enhanced the expression of ICAM-1 and E-selectin indicating that these adhesion molecules are under the control of miRNAs. To explore the possibility that TNF might induce miRNAs, human umbilical vein ECs were treated with TNF for 0, 2 and 24 hrs and the expression of miRNAs quantified using microarrays. We found that TNF regulated the levels of several miRNAs. TNF induced expression of hsa-miR-17, -31, -155 and -191 (confirmed by Northern blotting and qRT-PCR) with little change in hsa-miR-20a, -126 and -222. Among the stimulated miRNAs, we found (Target Scan, miRanda and RegRNA) 2 prediction sites for hsa-miR-17-3p in ICAM-1 3′UTR. The authenticity miRNA/mRNA target interaction was validated by using a reporter system where the 3′-UTR of ICAM-1 was located immediately downstream of the luciferase open reading frame sequence contained in the reporter plasmid. Furthermore, mimetics of this miRNA inhibit ICAM-1 expression whereas specific miRNA antagonists enhance ICAM-1 expression. In summary, we find that TNF responses of ECs are modulated by TNF-induced miRNAs, providing an additional level of control over TNF responses.

ROLES OF THE TNF FAMILY IN INFECTIOUS DISEASES AND INTERRELATIONSHIP OF THE TNF FAMILY AND PATTERN RECOGNITION RECEPTOR SIGNALING

THE IL-1 RECEPTOR / TOLL-LIKE RECEPTOR SUPERFAMILY: 10 YEARS OF PROGRESS

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The IL-1R/TLR superfamily was first defined in detail 10 years ago. Since then, there has been remarkable progress in our understanding of both branches of the superfamily. Ligands have been described for most receptors. Within the IL-1R subfamily, notable examples include IL33 for ST2 and IL-1F6 for IL-1Rrp2. The role of TLRs in the sensing of microbial products has led to a renaissance of interest in innate immune mechanisms. For investigators interested in signal transduction, the area has proved very fruitful in terms of the discovery of new signalling pathways and processes. MyD88 is the universal adapter for the superfamily and its central role in inflammation, host defence and even in certain cancers, has been confirmed from studies in knockout mice. We now have a good understanding of the major components activated by TLRs, notably the TIR domain- containing adapters that initiate signalling following recruitment to TIR domains within the TLRs themselves, the IRAK family of protein kinases that are then recruited, and a series of

ubiquitination and phosphorylation reactions that ultimately lead to the activiation of transcription factors such as NF-kappaB and IRF family members. The structural basis for signalling is still poorly understood however, and we have no appreciation of the kinetics involved in the pathways. Additional components and regulatory cross-talk from multiple signals also continue to be discovered. Genetic variation in signalling components such as in IRAK4, Mal and Unc93b however highlight the importance of these pathways in human health and disease. I will discuss our recent findings of a novel component in TLR4 signaling termed TAG, which specifically inhibits TLR4 signaling from endosomes, and the emerging role of miRNAs as key regulators of TLR signalling events.

ACTIVATION OF INNATE IMMUNE REACTION BY MAMMALIAN DNA THAT ESCAPED FROM DEGRADATION, LEADING TO ANEMIA AND ARTHRITIS

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A large amount of DNA is degraded during programmed cell death and definitive erythropoiesis. DNase II is the enzyme that digests DNA of apoptotic cells and nuclei expelled from erythroid precursor cells after macrophages engulf them. *DNase II*$^{-/-}$ mice are embryonic lethal due to severe anemia. Whereas, when *DNase II* gene is inactivated after birth, the mice develop polyarthritis in an age-dependent manner. In *DNase II*$^{-/-}$ mice, engulfed but not digested DNA accumulates in macrophages in various tissues such as the thymus, spleen, and bone marrow where programmed cell death and erythropoiesis take place. These macrophages constitutively produce interferon (IFN) β and tumor necrosis factor (TNF). Blockage of the IFN-signaling rescues the lethality of *DNase II*$^{-/-}$ embryos by preventing the anemia, while blockage of the TNF-signaling prevents the development of polyarthritis, suggesting that IFNβ and TNF are responsible for anemia and polyarthris, respectively. The production of IFNβ by the macrophages in *DNase II*$^{-/-}$ mice is independent from Toll-like receptor (TLR) system, but depends on interferon regulatory factor (IRF) 3/IRF7. When *DNase II*$^{-/-}$ fetal liver macrophages were subjected to engulfment of apoptotic cells, they produced IFNβ, indicating that there is a TLR-independent system to sense the DNA accumulated in lysosomes.

MALARIAL HEMOZOIN IS A Nalp3 INFLAMMASOME ACTIVATING DANGER SIGNAL

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Characteristic symptoms of malaria include recurrent fever attacks and neurodegeneration, signs that are also found in patients with a hyperactive Nalp3 inflammasome. *Plasmodium* species produce a pigment called hemozoin that is generated by detoxification of heme after hemoglobin degradation in infected red blood cells. We will present data showing that hemozoin acts as a proinflammatory danger signal through activation of the Nalp3 inflammasome, causing the release of IL-1β. Similar to other Nalp3-activating particles, hemozoin activity is blocked by inhibitors of phagocytosis, K^+ efflux and NADPH oxidase. *In vivo*, injection of hemozoin results in acute peritonitis, which is impaired in Nalp3- and IL-1R-deficient mice. Moreover, the pathogenesis of cerebral malaria is reduced in caspase-1-deficient mice infected with *Plasmodium berghei* sporozoites, while parasitemia remains unchanged. Thus, *Plasmodium*-generated hemozoin may act as a danger signal resulting in an uncontrolled proinflammatory host response and thereby contributing to the cerebral manifestations seen in malaria.

CASPASE-8 REGULATES CELLULAR RESPONSE TO PATTERN RECOGNITION RECEPTORS

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Several signaling proteins activated by receptors of the TNF/NGF family, including FADD/MORT1, TRADD, RIP1, TRAF3 and TRAF6, have been shown also to participate in signaling activation by both membrane-associated and cytosolic receptors for pathogen-associated molecular patterns. Analysis of pathogen induced signaling in the liver of mice with caspase-8 deficient hepatocytes indicated that this enzyme, whose association with the TNF/NGF receptors initiates cell death, also participates in the regulation of signaling activation by pattern recognition receptors (PRR). This role of caspase-8 was confirmed in further studies of cultured cells made deficient of caspase-8 either by its knockout or knockdown. Moreover, we found that caspase-8 is recruited to the signaling complexes activated by the PRR. These findings indicate that caspase-8 serves a general role in regulation of cellular responses to infecting pathogens, of which apoptotic destruction of the infected cells by killer cells that

activate the extrinsic cell-death pathway is just one of several possible alternative options.

CELL-AUTONOMOUS ROLE OF TNFR AND TLR SIGNALING PATHWAYS IN ANTI-BACTERIAL IMMUNITY

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Tumor necrosis factor (TNF) is essential for antibacterial host defense, and mice lacking TNF or TNF receptor 1 (TNFR1) are highly susceptible to infection with Listeria monocytogenes. L. monocytogenes infection activates toll like receptors (TLRs), downstream signaling of which involves activation of the transcription factor NF-κB and mitogen-activated protein kinases (MAPKs). This leads to immediate expression of TNF in the cell upon infection. Mice with conditional knockout of specific signaling mediators of the TNFR and TLR pathways in myeloid cells and hepatocytes were used to study the cell-autonomous role of these cascades upon L. monocytogenes infection in vivo. Our findings suggest that TNFR-associated factor-6 (Traf6) mediated signaling in macrophages is important for host survival against L. monocytogenes infection. Traf6-deficient macrophages are characterized by reduced NFκB activation upon TLR stimulation by lipopolysaccharide (LPS) and reduced TNFα release in vivo and in vitro. However, TNFR1-associated death domain (Tradd)-mediated signaling in the same cells is dispensable while the Fas-associated death domain (Fadd)-mediated signaling in hepatocytes protected the host against systemic L. monocytogenes infection. TNFR1 signals played primary role for hepatocyte cell survival upon L. monocytogenes infection in vivo. Our findings also re-enforce the role of hepatocyte specific NFκB in anti-bacterial responses and the involvement of macrophage specific IκB kinase (IKK) 1-mediated signaling in increased host susceptibility against infection. Activation of NFκB is essential to protecting cells from TNFR1-induced apoptosis. These results contribute to our understanding of the mechanisms by which TLR and TNFR1 signaling act at the single cell level to coordinate the anti-bacterial response leading to effective host defense and clearance of infection.

BEYOND TNFR SIGNALING: THE MULTIFUNCTIONALITY OF TRADD

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Despite decades of study on tumor necrosis factor (TNF) receptor 1 signaling, the definitive role of TRADD, a core adaptor protein in this pathway, has been delineated only recently, with the generation of the TRADD knockout mouse. We are among one of the three groups that have concurrently generated this knockout animal. Employing this model, we have constituted a global view of the functions of this molecule. Primarily, we have validated the current model of TNFR1 signaling, which positions TRADD as a central mediator of TNFR1 dependent cellular functions. In particular, we demonstrated that TRADD deficiency compromises TNFα-mediated apoptosis, as well as NF-κB, JNK and ERK activation in vitro. As well, we also showed that TNFα-mediated inflammatory responses in vivo, and germinal center formation, a phenomenon that requires TNFα signaling, are both TRADD-dependent.

In addition to TNFR1 signaling, we have also explored other potential cellular functions of TRADD. TRADD has been implicated previously to act downstream of the death receptor 3 (DR3), which is a member of the TNF receptor superfamily. Indeed, we found that TRADD is important for DR3-mediated co-stimulation of T cells. On the contrary, despite previous speculations of a role of TRADD in IFNγ-induced signaling, no significant defect in this pathway was observed in TRADD deficient cells. A highlight of our study is the identification of a novel role of TRADD in TLR3 and TLR4 signaling. TRADD participates in the TLR4 complex formed upon LPS stimulation, and TRADD-deficient macrophages show impaired cytokine production in response to TLR ligands in vitro. Thus, TRADD is a multifunctional protein crucial for TNFR1 and other signaling pathways relevant to immune responses. Extending from these findings, we contemplate on further exploring the role of this protein in other receptor signaling pathways.

THE FUNCTION OF TRADD IN SIGNALING THROUGH TUMOR NECROSIS FACTOR RECEPTOR 1 AND TRIF-DEPENDENT TOLL-LIKE RECEPTORS

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TRADD plays a critical role in TNF signaling. However, the physiological role of TRADD in TNFR1 signaling remains unclear and controversial due to the unavailability of a TRADD genetic deletion. Here, by generating TRADD knockout mice, we demonstrated that TRADD is essential for TNFR1-mediated TNF signaling, including the activation of NF-kB, the activation of MAP kinases, and the induction of necrotic and apoptotic cell death in mouse embryonic fibroblast (MEF) cells. Our data demonstrate that TRADD is necessary for the recruitment of TRAF2 and the optimal recruitment of RIP to the receptor complex, where TRADD is also responsible for RIP ubiquitination in which TRAF2 and TRAF5 have a redundant role. Surprisingly, while TRADD is essential for TNFR1 signaling in MEFs, abundant RIP in macrophages is sufficient to mediate modest signals without TRADD. TRADD-/- mice are morphologically normal, but resistant to TNF, LPS and poly(I:C) cytotoxicity in the presence of N-galactosamine. TRADD may also play a role in TLR3/4 signaling in MEFs although LPS and poly(I:C) signaling is normal in macrophages. Clearly, TRADD plays a much broader role in mediating different biological processes than just as an effector protein of TNFR1. In summary, our study definitively establishes the physiological role of TRADD in TNFR1 signaling.

ROLE OF 4-1BB AND RELATED TNFR FAMILY MEMBERS IN CD8 T CELL RESPONSES TO VIRUSES

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The immune system must be precisely regulated to allow protection from infection while preventing immune pathology. Inducible TNFR family members such as 4-1BB allow the immune system to fine tune the duration of the CD8 T cell response, so as to sustain responses for pathogens that take longer to clear. For

example, during severe respiratory influenza virus infection, the expression of 4-1BB and 4-1BBL is increased and sustained in the lung and the presence of 4-1BBL correlates with improved CD8 T cell response, increased virus clearance, better lung function and increased mouse survival. In contrast, during mild influenza infection, 4-1BB expression by the T cells is more transient and fully dispensable for the primary CD8 T cell response and disease outcome. However, if other costimulatory signals are limiting, such as in CD28-deficient mice, 4-1BB can play a key role in primary CD8 T cell responses to influenza and a single dose of agonistic anti-4-1BB during primary can program T cells for secondary expansion. 4-1BB is also induced by some common gamma chain cytokines on memory but not naïve CD8 T cells in the absence of antigen and 4-1BB deficiency in the T cells or 4-1BBL deficiency in the host results in decreased maintenance of CD8 T cell memory. 4-1BB induced survival signals in T cells are critically dependent on TRAF1 and TRAF2. TRAF1 is required for ERK dependent Bim modulation downstream of 4-1BB. This signal can play a critical role in survival of influenza and HIV specific T cells and the ability of 4-1BB to downmodulate Bim in a TRAF1-dependent fashion distinguishes it from CD27. GITR, a TNFR family member that also binds TRAF1, is expressed on resting T cells and further upregulated with activation. It also plays an intrinsic role in survival of CD8 T cells in response to influenza virus, with effects observed earlier in the response compared to 4-1BB signals. Taken together, these studies suggest that sequential use of TNFR family members may fine-tune the level of response to infection at different stages of the response.
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CRITICAL ROLE OF MEMBERS OF TNF OF MYCOBACTERIAL INFECTION REACTIVATION OF INFECTION BY TNF INHIBITORS

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Tumor necrosis factor (TNF) is crucial for the control of mycobacterial infection as TNF deficient (KO) die rapidly of uncontrolled, disseminated disease upon aerosol infection with *M. tuberculosis* (*Mtb*, H37Rv, 100cfu). We investigated the role membrane TNF in the absence of secreted TNF using a knock-in mouse with a non-cleavable and regulated allele (mem-TNF). Mem-TNF mice recruited normally T cells and macrophages, developed mature granuloma in the lung and controlled acute *Mtb* infection. Further, tissue specific inactivation suggests that the T cell derived TNF may be required to control infection.

TNF neutralization used clinically to treat severe rheumatoid arthritis is associated with reactivation of latent tuberculosis infection. Ongoing studies suggest that TNF neutralizing antibodies abrogate protection in mem-TNF mice suggesting that the antibodies neutralize also membrane expressed TNF. However, a dominant negative mutant TNF, which neutralizes only soluble TNF, had no effect on host resistance. Further, we established a model of latent tuberculosis infection to investigate reactivation of tuberculosis by TNF inhibitor therapy. Reactivation by neutralizing TNF antibodies or induced inactivation of the tnf gene of chronically infected BL6 mice will be reported.
In conclusion, the ongoing investigations suggest that membrane expressed TNF may be sufficient to allow cell-cell signaling and control acute *Mtb* infection and reactivation of chronic/latent infection. Therefore, therapies sparing mem-TNF may reduce infectious complication.

TUMOUR N ECROSI FACTOR ALPHA PRODUCED BY MACROPHAGES AND NEUTROPHILS IS REQUIRED FOR INITIAL PULMONARY PROTECTIVE INNATE IMMUNE RESPONSES BUT IS DISPENSABLE FOR PROTECTIVE IMMUNITY DURING PERSISTENT M. tuberculosis INFECTION

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Tumour Necrosis Factor alpha (TNF) is a pro-inflammatory cytokine critical for the induction of protective immune responses against *M. tuberculosis* infection in humans and mice. TNF is produced primarily by activated macrophages. In this study we investigated the contribution of TNF produced by macrophages and neutrophils in a murine tuberculosis model. Mice deficient for TNF expression by macrophages and neutrophils (MN-TNF KO) were challenged by aerosol inhalation with a low dose of 50 – 100 colony forming units of *M. tuberculosis*. MN-TNF KO mice revealed a susceptible phenotype similar to TNF KO mice during the early stages of *M. tuberculosis* infection with bacterial burdens in the lungs and spleens in MN-TNF KO mice similar to TNF KO mice at 3 and 4 weeks postinfection.

Subsequently, MN-TNF KO mice were able to control infection and had bacterial burdens similar to that of WT mice. MN-TNF KO mice therefore has a transient susceptibility to *Mycobacterium tuberculosis* H37Rv and survived the 6 month experimental period, similar to WT mice. Granuloma formation was independent of macrophage and neutrophil derived TNF, as well defined granulomas were present in the lung of MN-TNF KO mice. Early susceptibility was not ascribed to a lack of IL-12p70 or IFNγ levels because similar levels were present both in MN-TNF KO and WT mice. Recruitment of activated $CD4^+$ and $CD8^+$ T cells in the lungs of MN-TNF KO were capable of inducing TNF levels similar to that of WT mice. The absence of macrophage and neutrophil derived TNF did not reduce the frequency of IFNγ producing $CD4^+$ and $CD8^+$ T cells after mycobacterial specific antigenic stimulation in MN-TNF KO. In contrast $CD4^+$ IFNγ producing T cells was increased in MN-TNF KO mice in comparison to WT mice. In TNF KO mice $CD4^+$ and $CD8^+$ producing IFNγ T cells were significantly less compared to WT and MN-TNF KO. This study shows that macrophage and neutrophil derived TNF is crucial during the acute tuberculosis infection.

A CRITICAL ROLE OF THE POXVIRUS CrmD PROTEIN, A TNF AND CHEMOKINE BINDING PROTEIN, IN IMMUNE EVASION AND PATHOGENESIS

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Poxviruses are a family of complex enveloped dsDNA viruses including several human and animal pathogens which encode a large number of genes with immunomodulatory potential. Amongst these are a number of secreted cytokine binding proteins including a family of secreted tumour necrosis factor receptors (TNFRs) with similarity to the cellular TNFRs.To address the role of these viral TNFRs in vivo we have used ectromelia virus (ECTV), a natural mouse pathogen belonging to the orthopoxvirus family that causes mousepox, a severe disease which is considered as a model for acute viral infections in general and human smallpox in particular. In ECTV, only one predicted active TNFR named CrmD is found. We have shown that this protein is able to block both TNF and chemokine activity in vitro through its N-terminal and C-terminal domains, respectively. To determine the role of TNF and chemokine blockade in mousepox pathogenesis we cosntructed a recombinant ECTV lacking the CrmD gene. We found that in the absence of CrmD, the LD50 in susceptible mice was elevated by at least six orders of magnitude, rendering the virus practically avirulent. Moreover, absence of CrmD was accompanied by a strong and quick inflammatory response at the initial infection site and reduced

viral spread to the main target organs, spleen and liver. To determine the differential role of the TNF and chemokine binding domains, an ECTV recombinant expressing only the TNF binidng activity of CrmD was constructed. It was found that this virus was highly attenuated; too, showing that chemokine blockade by CrmD is essential for mousepox progression. Moreover, in the absence of chemokine binding activity, the inflammatory reaction at the infection site was found to be delayed, showing that chemokines may potentiate the proinflammatory activity of TNF in vivo. In summary, we have found that the secreted EV protein CrmD, which inhibits both chemokines and TNF is an essential virulence factor in vivo.

HEME SENSITIZATION TO TNF-MEDIATED PROGRAMMED CELL DEATH DICTATES THE OUTCOME OF PLASMODIUM INFECTION IN MICE

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Under homeostasis, heme acts as a prosthetic group in hemoproteins. Under inflammatory conditions however, non-covalently bound heme can be released and generate cytotoxic free heme. We have previously shown that free heme released from hemoglobin (an hemoprotein) can dictate the lethal outcome of Plasmodium infection (malaria) in mice (Pamplona A. et al., Nat. Med. 2007, 13, 703). We now provide a molecular mechanism underlying this pathogenic effect. Namely, free heme sensitizes cells to undergo TNF-mediated programmed cell death. This cytotoxic effect, which occurs independently of newly gene transcription and/or protein synthesis, relies on the unfettered generation of free radicals in response to TNF, as demonstrated in hepatocytes in vitro. When exposed to free heme in vitro, hepatocytes respond to TNF by sustaining the activation of the c-jun N-terminal kinase (JNK), which leads to further accumulation of free radicals and to apoptosis, i.e. caspase-8 and -3 activation, DNA condensation. Inhibition of free radical accumulation by N acetylcysteine (NAC) or Butylated hydroxyanisole (BHA), inhibition of JNK activation (pharmacologic) or JNK expression (shRNA) as well as inhibition of caspase -8 and 3 activation (pharmacologic) suppress the cytotoxic effects of free heme plus TNF in hepatocytes. Expression of the heme catabolyzing enzyme heme oxygenase 1 (HO-1) or the iron sequestering protein H Ferritin in hepatocytes acts in an anti-oxidant manner to afford cytoprotection against heme plus TNF in vitro as well as in vivo, providing complete protection against Plasmodium infection in mice. In conclusion, this data reveals a novel mechanism via which free heme sensitizes hepatocytes to TNF mediated programmed cell death, an effect countered by the expression of cytoprotective genes that prevent the lethal outcome of Plasmodium infection.

TNF/TNFR1 SIGNALING UP-REGULATES CCR5 EXPRESSION ON CD8+ T LYMPHOCYTES AND PROMOTES HEART TISSUE DAMAGE IN TRYPANOSOMA CRUZI INFECTION: BENEFICIAL EFFECTS OF TNF BLOCKADE

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In Chagas disease, the comprehension of the dual participation of the immune response controlling the parasite growth but also leading to heart damage might contribute to design new therapeutic strategies. TNF is important in resistance to acute Trypanosoma cruzi infection, however in chronic patients plasma TNF levels correlate with cardiomyopathy. Recent data support that chagasic myocarditis formation involves CCR1/CCR5-mediated cell migration. Herein, the contribution of TNF, specially signaling via its receptor TNFR1/p55, in the pathophysiology of T. cruzi infection focusing on myocarditis formation and heart dysfunction. T. cruzi-infected C57BL/6 mice present increased frequencies of TNFR1/p55+ and TNF+ splenocytes. Although TNFR1-/- mice exhibited reduced myocarditis in absence of parasite burden they succumbed the acute infection. Akin C57BL/6, Benznidazole-treated TNFR1-/- mice survived the acute infection. In TNFR1-/- mice the reduced CD8-enriched myocarditis was associated with disturbance in activation of CD44+CD62Llow/- and CCR5+ CD8+ lymphocytes. Also, anti-TNF reduced the frequency of CD8+CCR5+ circulating cells and myocarditis, though parasite load was unaltered in C57BL/6 infected mice. TNFR1-/- and anti-TNF-treated infected mice showed regular expression of connexin-43 and reduced fibronectin deposition, respectively. Furthermore, anti-TNF treatment resulted in lower levels of CK-MB, a cardiomyocyte lesion marker. Our results suggest that TNF/TNFR1 signaling promotes CD8-enriched myocarditis formation and heart tissue damage, raising the possibility of considering the TNF/TNFR1 signaling pathway as potential therapeutic target to control T. cruzi-elicited cardiomyopathy.

A LYMPHOTOXIN-DRIVEN PATHWAY TO HEPATOCELLULAR CARCINOMA

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Hepatitis B and C viruses (HBV, HCV) cause chronic hepatitis and hepatocellular carcinoma (HCC) by poorly understood mechanisms. Here we show that the cytokines lymphotoxin (LT) a, b and LIGHT, their receptor (LTbR) and respective target genes are drastically increased in HBV or HCV induced human hepatitis and HCC. We demonstrate that LTa and LTb expression on hepatocytes causes chronic hepatitis-driven HCC in transgenic mice. HCC formation depends on lymphocytes and the IKappa B kinase b expressed by hepatocytes, but is independent of tumor necrosis factor 1 receptor signaling. In vivo LTbR stimulation identified hepatocytes as the major LT responsive liver cell and mirrored transcriptional changes of LTab-overexpressing livers. Thus, sustained LT signaling on hepatocytes represents a hitherto unknown pathway involved in chronic hepatitis-induced HCC formation.

NOVEL ASPECTS OF IMMUNE REGULATION BY THE TNF FAMILY

TL1A (TNFSF15): A MASTER REGULATOR OF MUCOSAL INFLAMMATION

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The pathogeneses of Crohn's disease (CD) and ulcerative colitis (UC) relate to an inappropriate and exaggerated mucosal immune response to constituents of the

intestinal flora. Antigen-presenting cells (APCs) such as dendritic cells (DCs) are likely to play a central role in the host response to intestinal flora, both in innate responses to bacteria and by shaping the character of the host's adaptive immune response. Furthermore, $CD4^+$ T cells activated by APCs also have been shown to be involved in the pathogenesis of inflammatory bowel disease (IBD). A dysregulated T cell response leads to alterations in mucosal cytokine expression. CD has been characterized as having a T-helper (T_H) 1 and T_H17 cytokine pattern, and antibodies to both IFN-g and p40 (IL-12/23) can treat subsets of CD patients.

TL1A (TNFSF15) is a member of the TNF superfamily and is a strong costimulator of T cells. TL1A binds to death domain receptor 3 (DR3, TNFRSF25) and induces NF-κB activation in cells expressing this receptor. TL1A increases IL-2 responsiveness and enhances IFN-γ and GM-CSF release in anti-CD3- and anti-CD28-stimulated peripheral blood T cells. We have previously shown that DR3 expression in $CCR9^+CD4^+$ T cells is up-regulated following stimulation with IL-12 and IL-18. Furthermore, TL1A augments IFN-γ production by IL-12/IL-18-stimulated peripheral blood and mucosal $CD4^+CCR9^+$ T cells. Several studies have implicated the TL1A/DR3 pathway in the pathogenesis of IBD. We have shown that surface expression of TL1A and its receptor DR3 by mucosal T cells is increased in mucosal inflammation in CD. These findings were supported by Bamias et al, who showed that TL1A is expressed on $CD3^+$ T cells and macrophages in the mucosa of patients with CD, and also expressed on plasma cells in patients with UC. They also indicated that DR3 was expressed on $CD4^+$ T cells and $CD8^+$ T cells. The same group showed that both TL1A and DR3 were up-regulated in the inflamed intestinal mucosa in two different models of chronic murine ileitis.

We recently identified the FcγR signaling pathway as a major inducer of membrane and soluble TL1A while neither TLR ligands nor IFN-g were able to induce TL1A in antigen-presenting cells. Furthermore, TL1A production in FcγR activated monocytes leads to enhancement of IFN-g production by IL-12/IL-18 primed $CD4^+$ T cells. Because of the important role TL1A plays as a T cell activator and its up-regulation in inflammatory conditions the elucidation of signaling pathways that potentially inhibit TL1A induction and expression is of great potential therapeutic importance.

In our investigation of the mechanisms and functional significance of TL1A induction by microorganisms, we found that the induction of TL1A by microbial components in antigen presenting cells suggests a role for TL1A in host response to enteric microorganisms. TL1A mediates modulation of T cell effector function and may be a significant mechanism of mucosal immune regulation in the immunopathology of human IBD.

In animal models of chronic colitis resembling human CD, we have demonstrated that TL1A is important to activate both T_H1 and T_H17 $CD4^+$ T cells and that neutralization of TL1A attenuates colitis in a DSS chronic colitis and Gαi2 T cell transfer model. TL1A strongly enhances both IFN-g and IL-17/IL-6 production from mucosal $CD4^+$ T cells induced by IL-12 and IL-23 respectively. Neutralization of TL1A reduced the infiltration of mucosal T cells and cytokine production and attenuated DSS-induced chronic colitis. Additionally, anti-TL1A treatment

efficiently diminishes established DSS-induced chronic colitis. These results suggest that TL1A is a central immune modulator and is important for activation of mucosal T_H1/ T_H17 $CD4^+$ T cells that is critical for the development of chronic colitis. Together with our previous studies in humans and the importance of IFN-g and IL-17 in the pathogenesis of CD, our findings suggest that neutralization of TL1A could be a novel, highly specific approach for therapeutic intervention in CD.
Recent genome-wide association studies revealed a highly significant association of single nucleotide polymorphism haplotypes of the *tl1a* gene with CD, in Japanese, European, and US cohorts. We have demonstrated presence of TL1A gene haplotype B increases risk in Jewish CD patients with antibody titers for the E. coli outer membrane porin C (OmpC+). Our findings suggest that TL1A gene variation exacerbates induction of TL1A in response to FcgR stimulation in Jewish CD patients and this may lead to chronic intestinal inflammation by overwhelming T cell responses. Thus, TL1A may provide an important target for therapeutic intervention in this subgroup of IBD patients.
These data show that TL1A affects multiple, important cytokine pathways central to mucosal homeostasis, and protection against pathogens and that upregulated expression of TL1A may be involved in IBD pathogenesis.

TL1A-DR3 INTERACTIONS IN T-CELL MEDIATED AUTOIMMUNITY

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DR3 (death receptor-3; TRAMP, LARD, WSL-1, TNFRSF25) is a close homolog of TNFR1 expressed mainly on lymphocytes, with the highest levels expressed on T cells. Like TNFR1, DR3 recruits TRADD and can mediate dual signals that lead to inflammation through NFκB signaling or cell death through caspase activation. The sole known ligand for DR3 is the TNF superfamily member TL1A (TNF like cytokine 1A; TNFSF15). In vitro, exogenous TL1A can co-stimulate T cell activation and cytokine production through DR3. TL1A expression can be induced in innate immune and endothelial cells by pro-inflammatory stimuli, including LPS, Fc-receptor crosslinking, and TNF. Using DR3 knockout mice, we have recently shown that TL1A-DR3 interactions play a key role in experimental autoimmune encephalomyelitis (EAE) and in an ovalbumin-induced asthma model, facilitating local T cell accumulation at the site of inflammation, while not affecting systemic T cell priming, T cell polarization or host defense against *Toxoplasma gondii* (Meylan et al, 2008 *Immunity* 29:79). Other studies have suggested a role for TL1A

and DR3 in various diseases including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD).
To further study the role of TL1A-DR3 interactions in vivo, we have generated TL1A transgenic mice in which either T cells or dendritic cells constitutively express TL1A. Multiple founders of both transgenic lines were born apparently healthy with expected frequency but develop spontaneous IBDwhich spares the colon after 3 weeks of age. Development of this syndrome is strictly dependent on DR3. Inflammatory pathology is found primarily in the ileum and duodenum in all transgenic lines expressing TL1A more than eight-fold over baseline levels. Consistent with the role of TL1A as a T cell costimulator, T cells isolated from TL1A transgenic mice had an activated phenotype and an increased percentage of memory cells. We found no defects in numbers or function of FoxP3+ regulatory T cells in these mice. Interestingly, both IL-13 and IL-17 expression is elevated in the intestines of TL1A transgenic mice. Ectopic expression of TL1A appears to be able to overcome T-cell self-tolerance and drive IBD with no other overt stimuli. Blocking TL1A-DR3 interactions may be an attractive therapeutic strategy for autoimmune disease. We have generated anti-TL1A antibodies to explore this possibility, and will present data on the ability of these antibodies to block diverse T-cell dependent mouse models of autoimmunity.

TNFR25/TL1A CONTROL CD4 REGULATORY CELLS, TH17/TH2 POLARIZATION AND NKT-IL-13 ACTIVITY IMPORTANT IN MUCOSAL AND SYSTEMIC IMMUNITY

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TNFRSF25 (TNFR25) and its ligand TL1A are important regulators of the immune response by controlling the function of CD4+ CD25+ T regulatory cells (Treg), Th17 CD4 cells and NKT cells. We have shown previously that a TL1A blocking antibody abrogates allergic lung inflammation by interfering with IL-13 production by NKT and CD4 T cells. Here we show that TNFR25 agonists (TL1A or agonistic antibody 4C12) strongly inhibit the functional activity of Treg, exceeding the inhibitory activity of GITR and OX40-L. Th17 polarization mediates potent upregulation of TL1A expression on CD4 cells while also supporting TNFR25 expression. Th17 cells are known to suppress Treg. This function is at least in part mediated by TL1A. TNFR25 signals initiated by TL1A or agonistic antibody also suppress the biogenesis of antigen specific Treg by CD103+ CD11c+DC isolated from mesenteric lymph nodes. Thus TNFR25 inhibits the activity of existing Treg and blocks the generation of new Treg while supporting Th17 and Th2 polarization. TNFR25 signals are neutral in Th1 polarization.

The inhibition of Treg activity and biogenesis by TNFR25 signals prompted the study of inflammatory bowel disease (IBD) and the use of TNFR25 agonists as adjuncts for cancer and HIV vaccines. We show that anti TL1A completely blocks IBD in the SCID model, acting analogous to the function Treg. Using cancer vaccines (based on secreted gp96-Ig) we demonstrate that TNFR25 agonistic 4C12 antibody increases tumor rejection from 30% of vaccine alone to 80% with anti TNFR25 as adjunct associated with increased antigen specific CD8 CTL expansion. Similarly agonistic anti TNFR25 increases the frequency of HIV specific CD8 CTL in the mucosal lamina propria and intraepithelial space by a factor of 3-5 upon HIV-gp96-Ig immunization.
The data show that TNFR25/TL1A have broad and important function especially in mucosal but also in immunity by controlling Treg, Th17, Th2 and NKT function. We propose that the observed TNFR25/TL1A activity in IBD, Asthma and in cancer vaccines is primarily mediated via the Treg/Th17/ NKT axis.

IDENTIFICATION OF NEW CANDIDATES AS MUCOSAL VACCINE ADJUVANT IN TNF SUPERFAMILY CYTOKINES

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Mucosal vaccination is a fundamental strategy for inducing both systemic and mucosal immune responses to prevent viral infection. However, almost all mucosal vaccines were not applied for clinical situation, because of the lack of mucosal adjuvant with effectiveness and safety. To overcome this problem, we have applied for cytokines to mucosal adjuvant. Previously, we showed that TNF-α induced strong mucosal immune responses. In this study, we compared the potential of TNF superfamily (TNFSF) cytokines to identify new candidates of mucosal adjuvants. BALB/c mice were intranasally immunized with ovalbumin (OVA) alone or together with 16 different TNFSF cytokines. Seven days after the last immunization, the levels of OVA-specific antibody responses in serum and mucosal secretion were determined by ELISA. In TNFSF cytokines, TL1A and APRIL induced significantly higher level of OVA-specific IgG responses in serum and IgA responses in mucosal secretion than OVA alone or TNF-α. Characterization of immune responses immunized with TL1A and APRIL revealed the elevated OVA-specific Th2-type cytokine production in splenocytes from immunized mice. These results provide the first evidence that TL1A and APRIL may be new candidates of mucosal vaccine adjuvants.

ROLE OF TWEAK/Fn14 PATHWAY IN INTESTINAL INFLAMMATION AND TISSUE REPAIR

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TWEAK is a unique, multifunctional TNF family cytokine that signals through its highly inducible receptor, Fn14. Fn14 expression is relatively low in healthy tissues, but is dramatically upregulated on epithelial and mesenchymal cell types after injury and in disease. In contexts of chronic inflammatory disease, TWEAK mediates pathological tissue remodeling by amplifying inflammation, promoting tissue damage and potentially impeding endogenous repair mechanisms. Prior studies demonstrated a pathological role of TWEAK in murine models of arthritis and lupus. The role of the TWEAK/Fn14 pathway in the intestine was investigated using the TNBS-induced colitis model. We show that colitis severity and histological scores are significantly reduced in TWEAK or Fn14 knockout mice or after treatment with a TWEAK blocking mAb. In addition, neutrophil and macrophage infiltrates, chemokines, cytokines and MMP expression are reduced in the TWEAK deficient colon after TNBS administration, however, systemic adaptive immune responses to TNP are not altered. Fn14 is expressed by colon epithelial cells in TNBS colitis and TWEAK induces their production of pathological mediators. TWEAK also regulates intestinal epithelial turnover, as evidenced by reduced cell death after gamma-irradiation injury in TWEAK and Fn14 KO mice. Thus our studies elucidate a nonredundant TWEAK-intestinal epithelial cell axis and suggest that blocking TWEAK may dampen chronic intestinal inflammation and allow normal epithelial repair. Taken overall, TWEAK/Fn14-mediated tissue remodeling may be an important, universal mechanism leading to various end-organ pathologies associated with autoimmune and inflammatory disorders. The highly localized nature of its pathogenic contribution makes the TWEAK/Fn14 pathway a unique and promising therapeutic target.

TWEAK INDUCES PROLIFERATION IN RENAL TUBULAR EPITHELIUM: A ROLE IN RENAL HYPERPLASIA

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The new TNF family member, TNF-like weak inducer apoptosis (TWEAK) activates the Fn14 receptor and has proapoptotic, proliferative and proinflammatory actions that depend on the cell type and the microenvironment. We have now explored the proliferative actions of TWEAK on cultured tubular cells and on renal tubules in healthy animals in vivo. To test the relevance of this effect in clinical settings, we examined the involvement of TWEAK pathway in the compensatory proliferation in a unilateral nephrectomy murine model.

TWEAK increased the proliferation and cell number of cultured tubular cells and induced their expression of expression of cyclin D1. Serum increased TWEAK and Fn14 expression in these cells and their proliferative response to TWEAK. TWEAK activated the mitogen-activated protein kinases ERK and p38, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and NFkB. TWEAK-induced proliferation was prevented by inhibitors of these protein kinases and by the NFkB inhibitor parthenolide. TWEAK induced proliferation of cultured tubular cells was diminished in the presence of IFNgamma and TNFalpha.

TWEAK administration in vivo induced cell proliferation and CyclinD expression in the kidney tubules of healthy mice. Consistent with the proliferative effects of TWEAK on cultured tubular cells, TWEAK knock-out mice displayed lower tubular cell proliferation in the remaining kidney following unilateral nephrectomy.

In conclusion, our data shows that TWEAK has proliferative actions on tubular epithelial cells, both in vitro and in vivo. TWEAK proliferative effects on tubular cells play a role in the compensatory renal hyperplasia following unilateral nephrectomy.

THE EFFECTS OF TWEAK AND TNF ON HUMAN OSTEOBLASTS: MAPK DEPENDENT INDUCTION OF SCLEROSTIN EXPRESSION

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We showed recently that TNF-like weak inducer of apoptosis (TWEAK), is a novel mediator in a mouse model of inflammatory bone destruction.[1] We sought to investigate a role for TWEAK in human osteoblast biology, and how TWEAK might interact with TNF in this context. Human primary osteoblasts (NHBC) expressed TWEAK and its receptor, Fn14. Both TWEAK and TNF were mitogenic for NHBC. TWEAK dose- and time-dependently suppressed the transcription of the master osteoblast transcription factor, RUNX2 and promoted the expression of osterix. TWEAK inhibited in vitro mineralisation whereas TNF had a positive effect. Notably, TWEAK, alone and in conjunction with TNF, induced the expression of the osteoblast differentiation inhibitor and product of the SOST gene, sclerostin, in a JNK and Erk1/2-dependent manner. TWEAK induction of sclerostin was sensitive to cycloheximide, implying the requirement for new protein synthesis. Exposure of NHBC to exogenous sclerostin mimicked the TWEAK and TWEAK/TNF effects on RUNX2 and osteocalcin expression. Our results suggest that TWEAK and TNF act in part by promoting sclerostin expression, which in turn regulates the expression of key osteoblast transcription factors. Our results suggest that the persistent presence of TWEAK may be anti-anabolic, and that TWEAK and TNF need to be considered together in the aetiology of inflammatory bone remodelling. Our results also demonstrate for the first time that sclerostin, described as a product of mature, mineral-embedded osteocytes, may be induced under inflammatory conditions in osteoblasts at earlier stages of their differentiation.

1. Perper S, et al. (2006) J Immunol 177:2610.

TWEAK REGULATES PROINFLAMMATORY TNF RECEPTOR-1 SIGNALING

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Outcome of TNFR1 signaling is critically modulated by concomitantly occurring external stimuli and cytosolic proteins like the TNF receptor associated factors (TRAFs). TRAF2 holds a crucial role in proinflammatory, antiapoptotic TNFR1 signaling. Upon stimulation with soluble TNF-like weak inducer of apoptosis (TWEAK) TRAF2 translocates to a Triton X100 insoluble compartment without changes in the total cellular TRAF2 content. TWEAK-induced TRAF2 translocation is paralleled by a strong increase in p100 processing to p52 indicating that TRAF2 redistribution is already sufficient for activation of the alternative NFkappaB pathway. Furthermore, TWEAK-primed cells display a reduced capacity to activate the classical NFkappaB pathway or JNK in response to TNF. Additionally, TWEAK stimulation sensitizes cells for TNFR1-mediated induction of apoptotic and necrotic cell death.
Together, our data demonstrate that soluble TWEAK depletes cytosolic TRAF2 and shifts proinflammatory TNFR1 signaling towards cell death induction

Fn14: A COMMON STRUCTURAL FRAMEWORK FOR SPECIES SEQUENCE DIVERGENCE

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TWEAK and Fn14 are members of the TNF ligand/receptor superfamily that are present in species ranging from zebrafish to men. Despite a relatively low sequence homology within the ligand-binding domain between human and xenopus proteins, the finding that both can bind human TWEAK prompted us to determine the three-dimensional structure of the extracellular domains by NMR and MS-based disulfide mapping methods. The structures reveal a remarkable conservation of

the tertiary fold and binding interface and structural features that distinguish Fn14 from other small TNF receptors such as BCMA, TACI and BAFF-R. A model of the Fn14-TWEAK complex was computed based on these high-resolution structures and validated by site-directed mutagenesis. These findings shed light on how TNF ligand/receptor interactions have evolved over time, and provide clues on the diversification of cystein-rich domains of TNF receptors.

CONTROL OF LYMPHOCYTE FUNCTION AND REPERTOIRE BY THE TNF FAMILY

UNCONVENTIONAL LIGAND ACTIVATION OF HVEM MEDIATES CELL SURVIVAL

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The tumor necrosis factor superfamily member, herpesvirus entry mediator (HVEM, TNFRSF14), activates both stimulatory and inhibitory pathways, serving as a molecular switch by engaging two distinct classes of molecules: the TNF-related cytokines, and members of the Ig superfamily. HVEM engages the canonical TNF-related ligand, LIGHT (TNFSF14) and lymphotoxin (LT)-α. LIGHT initiates a strong costimulatory signal promoting inflammation and enhancing immune responses. By contrast, HVEM engagement of the Ig superfamily members, B and T lymphocyte attenuator (BTLA) and CD160, activates inhibitory signaling in lymphoid cells. However, emerging evidence indicates BTLA also promotes T cell survival, yet its structural differences from LIGHT intimated BTLA was unlikely to function as an activator of HVEM. We demonstrate BTLA and CD160, as well as herpes simplex virus envelope gD, function as activating ligands for HVEM that promote NFκB activation and cell survival. Membrane expressed BTLA and CD160, and the soluble dimeric receptor surrogates BTLA-Fc and gD-Fc specifically activated HVEM-dependent NFκB. BTLA and CD160 engagement induced TRAF2, but not TRAF3, recruitment to HVEM that specifically activated RelA but not the RelB form of NFκB in a mucosal epithelial tumor cell line. Moreover, Btla-/- T cells survive poorly following activation, but were rescued with BTLA-Fc, indicating

HVEM-BTLA bidirectional signaling may serve as a critical cell survival system for lymphoid and epithelial cells. The importance of the HVEM-BTLA pathway in cell survival revealed here illuminates the nature of the selective pressures guiding the evolution of herpes viruses and retroviruses, which so efficiently target this TNF Superfamily signaling pathway.

LYMPHOTOXIN-β RECEPTOR SIGNALING LICENSES DENDRITIC CELLS TO PRIME CD8⁺ T CELL RESPONSES

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During an immune response, antigen-bearing dendritic cells (DC) migrate to the local draining lymph node and present antigen to $CD4^+$ helper T cells. Activation of $CD4^+$ T cells provokes the expression of Tumour Necrosis Factor (TNF) family members including CD40-ligand and Lymphotoxin (LT)-αβ. Although it is well accepted that CD40 stimulation on dendritic cells (DC) is required for DC licensing and cross-priming of $CD8^+$ T cell responses, it is likely that other signals are integrated into a comprehensive DC activation program. Here we show that expression of LTαβ on $CD4^+$ helper T cells as well as LTβ-Receptor (LTβR) on hematopoietically derived antigen presenting cells is necessary for optimal $CD8^+$ T cell expansion, but not effector function. In contrast, CD40 signaling appears to be more critical for $CD8^+$ T cell IFNγ production. Therefore, different TNF family members provide integrative signals that shape the licensing potential of antigen-presenting DC.

THE TNF-FAMILY RECEPTORS RANK AND CD40 COOPERATIVELY REGULATE CENTRAL TOLERANCE IN THYMUS

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Clonal deletion is one of the main mechanisms maintaining T-cell tolerance to prevent autoimmunity. Several lines of evidence suggest that establishment of T-cell tolerance requires medullary thymic epithelial cells (mTECs) in thymus.

mTECs have a unique property to "promiscuously" express peripheral tissue specific antigens (TSAs). It is proposed that mTECs present TSAs to T-cells in order to eliminate self-organ specific T-cells. The TSA expression in mTECs is in part regulated by autoimmune regulator (Aire), which is responsible for human autoimmune disorder. In previous study, we showed that the development of Aire- and TSAs-positive mTECs depends on TNF receptor-associated factor 6 (TRAF6)(Science, 2005). However, the receptor to regulate mTEC development via TRAF6-dependent manner remained to be determined. In this meeting, we describe that receptor activator of NF-κB (RANK) and CD40 cooperatively regulates mTEC development to induce self-tolerance. Whereas defect of mTEC development is partial in each single mutant mice deficient in RANK ligand (RANKL) or CD40, mTEC development and expression of Aire and TSAs were abolished in RANKL and CD40 doubly deficient mice (DKO). In addition, transfer of splenocytes from DKO induced severe autoimmunity in recipients. We also found that ligation of RANK or CD40 with recombinant ligand is sufficient for the development of mTECs in thymic stroma culture. Furthermore, the mTEC development via RANK or CD40 ligation depends on TRAF6, NF-κB inducing kinase, and RelB. Therefore, our study delineates the signal pathway required for the mTEC development (Immunity, 2008).

THE DUAL ROLE OF TNF ON T CELL ACTIVATION

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TNF is a major inflammatory cytokine mediating its effects via two types of receptors: TNFR1 and TNFR2. Increasing evidence indicates that TNF also has immunosuppressive effects. TNF interacting with TNFR2 exerted the well documented initial co-stimulatory effect when added to activate T cell cultures and vice versa lack of TNF - TNFR2 interaction reduced T cell proliferation. TNFR2 is predominantly expressed by a subset of CD4+CD25+FoxP3+ Treg and identified mouse Treg with an activated/memory phenotype and maximal suppressive activity. In synergy with IL-2, TNF expanded Treg in vitro and markedly up-regulated expression of CD25 and Foxp3 resulting in the subsequent enhanced suppressive activity of Treg. Thus, TNFR2 can serve as a marker for functionally active mouse Treg and potentially also has a co-stimulatory function on the activation of Treg. However, the expression of TNFR2 is not a prerequisite for the development of functional Treg since Treg from both wild type as well as TNFR2-deficient mice suppressed proliferation of activated CD4+ T cells equally well. Addition of human TNF which only interacts with mouse TNFR1 but not with mouse TNFR2 did not augment this Treg-induced suppression of T cell proliferation of co-culture. In contrast, addition of mouse TNF interacting with both TNF receptors partially

abrogated Treg induced suppression of T cell proliferation during the first 48 hours. Presumably at this early time point the exogenous mouse TNF exerts the TNFR2-dependent co-stimulatory effect on both effector as well as regulatory T cells and in addition neutralizes inhibitory soluble TNFR2. Subsequently, the induction of functional Treg overcomes the co-stimulatory effect of TNF. Further work is required to dissect the temporal and context dependent activating versus immunosuppressive effects of TNF and its receptors on T cell activation and its relevance in vivo.

THE ROLE OF CD40 T CELLS IN AUTOIMMUNE DIABETES: THE IDENTIFICATION OF AUTOAGGRESSIVE T CELLS AND A MECHANISM TO THWART PERIPHERAL TOLERANCE

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CD40, tnfsf5, is classically associated with antigen presenting cells, but we described the expression of CD40 on a unique subclass of T cells. These T cells have proven to be pathogenic and phenotypically are described as CD4loCD40+, which we have termed Th40. In the mouse model for type 1 diabetes, T1D, Th40 cell percentages are drastically expanded relative to control mice and this cell subset rapidly transfers diabetes to non-diabetic NOD.scid recipients. Th40 cell numbers expand concurrently with progressive insulitis leading to hyperglycemia. We have detected these T cells directly in pancreata of diabetic and pre-diabetic mice. Th40 cells isolated from pre-diabetic mice transfer T1D suggesting that Th40 cell levels are predictive for T1D onset.

Mechanistically CD40 induces the recombinase proteins RAG1 and RAG2 and altered TCR expression in peripheral Th40 cells, a process described as TCR revision. This is the first demonstration of a cellular mechanism to induce RAG1 and RAG2 directly. Importantly, this mechanism suggests a means of breaking peripheral tolerance. Further relative to the role of Th40 cells in autoimmunity, CD40 induces pro-survival factors in Th40 cells from diabetes – prone mice specifically NOD mice but does not induce survival in Th40 cells from control mice. Th40 cells from NOD mice are not susceptible to Fas-mediated cell death and undergo much more rapid homeostatic expansion than Th40 cells from non-autoimmune mice. CD40 signals Th40 cells from autoimmune conditions inducing cFLIP-p43, a pro-survival factor, as well as Bcl-XL.

Th40 cells are present in human subjects and T1D subjects have significantly expanded numbers of Th40 cells. Th40 cells from T1D subjects but not controls respond to diabetes – associated self antigens leading to the production of pro-inflammatory cytokines including IFNg, TNFa and IL-6. T1D expansion of Th40 cells occurs independently of age, gender and duration of disease. Importantly Th40

cell expansions occur independently of HLA haplotype. Th40 cells are expanded in T1D subjects carrying diabetes - associated DR3 or DR4, but importantly are also expanded in T1D subjects who carry neither DR3 nor DR4. Control subjects including those with HLA-DR3 and or DR4 do not have expanded Th40 cell levels. Thus Th40 cells prove to be an important risk factor. We further found that expansion of Th40 cells is not limited to T1D, but also occurs in multiple sclerosis.

APRIL RESTORES B CELL SURVIVAL AND LPS ANTIBODY RESPONSE IN BTK-DEFICIENT MICE

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Lack of Bruton's tyrosine kinase (Btk) causes the X-linked immune defect (Xid) in mice. Btk is critical for B cell development and Xid mice show reduced mature B cell populations. In addition, peripheral Xid B cells have a high spontaneous apoptosis rate and are hyporresponsive to various mitogenic stimuli, partially due to defects in expression of anti-apoptotic molecules (Bcl-2, Bcl-xl) and of cytokines (IL-10, TNF). Xid mice also fail to respond to T cell-independent antigens and have low levels of natural IgM antibodies. Moreover, a role for Btk in B cell homing and migration has been recently reported.
APRIL (a proliferation-inducing ligand) is a member of the TNF family, with important functions in B cell survival and activity. We previously reported that APRIL-Tg mice show an expanded peritoneal B-1 B cell population, elevated natural antibody levels, as well as enhanced TI humoral responses. These data prompted us to test whether APRIL ameliorated any of the B cell defects caused by Btk deficiency, and to evaluate the implication of Btk in APRIL-mediated signaling. We generated Xid/APRIL-Tg mice and characterized the B cell compartment and function. Our results show that APRIL overexpression does not increase B cell number in Xid mice, but enhances natural antibody levels and restores the LPS antibody response (IgM). In adittion, we detected improved B cell survival and activation, accompanied by upregulation of Bcl-2 and of activation markers. We are currently characterizing the signal transduction pathways implicated. Our data allow us to conclude that APRIL can promote antibody production to TI-type1 antigens in a Btk-independent manner, partially restoring X-linked immunodeficiency in mice.

REGULATION OF HEMATOPOIESIS BY CD137

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CD137 (TNFRSF9, 4-1BB, ILA) is involved in the regulation of activation, proliferation, differentiation and cell death of most types of leukocytes. Bidirectional signaling exists for the CD137 receptor/ligand system as CD137 ligand which can be expressed as a transmembrane protein, can also transduce signals into the cells it is expressed on.
Recently we found that CD137 is expressed within the bone marrow, and CD137 ligand is present on small subsets of hematopoietic progenitor cells in man (Jiang et al., 2008; Stem Cells, 26:2372-81) and mouse (Jiang et al., 2008; J. Immunology, 181:3923-32). Interaction of CD137 with CD137 ligand induces prolongation of survival and proliferation of hematopoietic progenitor cells. It further induces differentiation to myeloid cells, specifically to monocytes/macrophages.
In ongoing work we investigate how the CD137 receptor/ligand system regulates differentiation of granulocytes and dendritic cells, and maturation of dendritic cells. Also, we aim to determine under what physiological or pathological situations CD137-induced hematopoiesis takes place.
CD137 and its ligand have been mainly known for their functions in regulating activities of mature immune cells, in particular costimulation of CD8+ T cells. This study identifies a novel and unsuspected activity of the CD137 receptor/ligand, system, i.e. the regulation of hematopoiesis.

THE LTβR-PATHWAY PROMOTES B-CELL ACCUMULATION LARGELY VIA CXCL-13 EXPRESSION IN ECTOPIC LYMPHOID AGGREGATES IN NOD MOUSE LACRIMAL GLANDS

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We are investigating the importance of the LTBR-axis in NOD mouse Sjogren's-like disease, with particular attention to leukocyte accumulation in lacrimal glands. Antagonism of LTBR with LTBR-Ig was found to alter the expression of many genes in Affymetrix Chip analysis, among which CXCL13 was found to be highly affected. Reduction of CXCL13 mRNA expression in lacrimal glands by LTBR-Ig treatment of mice was confirmed by real-time PCR. Similar results were obtained at the CXCL13 protein level by ELISA analysis of homogenates of lacrimal glands.

A concomitant reduction of CXCL13 expression and the number of B-cells was observed after both prophylactic and therapeutic modes of LTBR-antagonism. The majority of B-cells in the massive infiltrates are conventional follicular B-cells, as determined by FACS analysis. FDC networks are rare in lacrimal-gland infiltrates, and little cell-proliferation was detected by BrDU labeling. The sources of CXCL13 protein are currently under investigation by multicolor fluorescence immunohistochemistry, and our preliminary results suggest that follicular dendritic cells in the massive B-cell aggregates that develop in lacrimal glands of NOD mice are not primary source of CXCL13, but rather a different population of cells that do not display the classic FDC marker CD21.

DIFFERENTIAL REQUIREMENT OF LTBR DURING THE MATURATION OF LYMPH NODE ANLAGEN STROMA

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Our understanding of lymph node (LN) development has improved a lot since a growing numbers of studies have described the role of lymphoid tissue inducer cells (LTi's) in the development of secondary lymphoid organs. Recruitment to the LN anlagen of LTi's, expressing the TNFα family molecule LTα1β2, allows the engagement of Lymphotoxin Beta Receptor (LTBR) on stromal cells. Subsequent activation of the NF-κB pathways induces high-level expression of cell adhesion molecules, chemokines and cytokines by the stromal organiser cells. However, our knowledge concerning the early events regulating LN anlagen formation are rather limited and the origin of the LN stromal cells remains unknown.

Here we show that the LN anlagen in the mouse embryo consist of two stromal cell populations: a central blood-lymphatic endothelium core surrounded by layers of mesenchymal cells. The latter gives rise to stromal organiser cells through a two-step activation process. The first step doesn't require LTi's and LTBR and allows expression of low levels of cell adhesion molecules, chemokines and IL-7 that are necessary for the attraction and recruitment of LTi's. At this stage, the stroma starts to express RelB and thus can respond to LTBR engagement. The second step is dependant on the expression of LTBR and LTi's, as the development of LNs is blocked at the earlier step in LTBR- and RORγt-deficient embryos. Engagement of LTBR leads to high expression of ICAM and VCAM, chemokines and RANKL that are characteristic of stromal organiser cells.

In order to identify the mechanisms responsible for the first step of stromal development we analysed the first cells to enter the inguinal and mesenteric LN. We show that a population of macrophages expressing Lyve-1 and high levels of TNFα is present in the developing LN anlagen before the arrival of LTi's. The Lyve-1+ macrophages are able to induce MadCAM-1 expression on mouse embryonic fibroblasts.

ANALYSIS OF B LYMPHOCYTE TUMOR NECROSIS FAMILY MEMBER SIGNALLING UTILIZING MEKK1ΔKD TRANSGENIC MICE

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Map3k1 kinase domain deficiency (Mekk1ΔKD) in mice provides a valuable means for evaluating the biological role of MEK kinase 1 (MEKK1) in immune signalling networks initiated by Tumor Necrosis Factor Receptor (TNFR) family members. Mekk1ΔKD mice have a B cell intrinsic signalling defect in TNFR family members CD40 and BAFF receptors c-Jun N-terminal kinase (JNK) and p38 Mitogen-activated Protein kinase (MAPK) activation. A result of defective MAPK signalling is Mekk1ΔKD B cell deficiency in: CD40-mediated gene expression, proliferation and immunoglobulin class switching. Following immune system challenge, Mekk1ΔKD mice are defective in thymus-dependent (TD) immune responses, and exhibit reduced germinal center formation and TD antibody production. Genetic analysis of Mekk1ΔKD mice has facilitated the molecular understanding of MEKK1 and TGF-β-activated kinase 1 (TAK1) signalling from TNFRs, revealing the novel two-stage MAPK signalling model.

NOVEL ASPECTS OF THE FUNCTIONS OF THE TRAFS AND CIAPS

PROTEIN UBIQUITINATION CASCADES AND SIGNALING BY TNF AND TOLL RECEPTORS

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Innate immune responses are triggered upon recognition of microbial products, known as pathogen associated molecular patterns (PAMPs) and so called “danger signals” by membrane-associated and cytoplasmic pattern recognition receptors (PRRs). A major class of membrane-associated PRRs is the Toll-like receptor (TLR) family, whose members transduce signals via adaptor proteins with which they associate through Toll-IL-1 Receptor (TIR) domains. One of the adaptors, MyD88, mediates activation of signaling pathways that lead to induction of proinflammatory cytokines and chemokines, whereas another adaptor, TRIF, mediates activation

of a signaling pathway that culminates in induction of interferons (IFNs) and IFN-related genes. Both adaptors interact with members of the TRAF family of signaling proteins, namely TRAF3 and TRAF6. We previously found that TRAF3 is required for activation of the TRIF-dependent IFN response. However, TRAF3 also associates with MyD88 and functions as a negative regulator of inflammatory cytokine production as well as an attenuator of MAPK activation downstream of TNF receptor (TNFR) family members such as CD40. We used a biochemical approach to understand the enigmatic ability of TRAF3 to function both as a positive and negative regulator of innate immune signaling. We found that the decision whether TRAF3 acts positively or negatively depends on its differential ubiquitination which is dictated by its subcellular distribution. During MyD88-dependent signaling from plasma membrane localized TLRs, as well as during TNFR signaling, TRAF3 is subjected to degradative K48-linked polyubiquitination that depends on TRAF6 (or TRAF2 in the case of TNFRs) and a pair of related ubiquitin ligases, cIAP1 and cIAP2. The latter are directly responsible for TRAF3 ubiquitination after they are activated by TRAF2 or TRAF6. The degradation of TRAF3 is essential for cytoplasmic translocation of plasma membrane receptor associated signaling complexes that is needed for MAPK activation via TAK1 and MEKK1. However, during TRIF-dependent signaling from endosomal TLRs, TRAF3 is subjected to activating K63-linked polyubiquitination. Interference with cIAP1/2-dependent TRAF3 degradation prevents the induction of inflammatory cytokines without affecting the IFN response.

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A SIGNALING PATHWAY FROM TNF RECEPTOR TO CELL DEATH

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Apoptotic cell death is executed by intracellular caspase-3/-7 activities, which are activated by upstream caspase-9 after mitochondrial damage or casapse-8 when cell membrane death receptors are activated by their respective ligands. The damaged

mitochondria also release Smac-like proteins to relieve the caspase inhibition imposed by inhibitor of apoptosis proteins (IAP). We have studied the biochemical pathway of apoptosis initiated from mitochondria in details and recently designed a cell permeable small molecule peptide mimetic that mimics the activity of Smac protein in cells. This Smac mimetic compound induces apoptosis in a variety of human cancer cell lines both in tissue culture and in mouse xenografic tumor models. It does so in combination with other apoptosis-inducing agents, or as a single agent alone. The cells responding to Samc mimetic alone turn out to secrete TNF-α in an autocrine fashion. We also characterized the mechanism through which Smac mimetics and TNF-α induce apoptosis. We have found that Smac mimetic induces apoptosis by triggering auto-degradation of cIAP1 and cIAP2 (cIAP1/2), leading to the release of receptor interacting protein kinase (RIPK1) from the activated TNF receptor complex to form a caspase-8-activating complex consisting of RIPK1, FADD, and caspase-8.
This defined a new caspase-8 activating pathway and should provide guidance to clinical application of Smac mimetics for cancer therapy.

CRYSTAL STRUCTURE OF TRAF6 REVEALS SPECIFICITY AND MECHANISM OF TRAF6-MEDIATED UBIQUITINATION

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TNF receptor associated factor 6 (TRAF6) is a RING-type ubiquitin ligase (E3) and a critical mediator in immune receptor signal transduction. Together with the dimeric ubiquitin conjugating enzyme (E2) Ubc13/Uev1A, it mediates Lys63 (K63)-linked polyubiquitination of self and downstream signal transducers to induce activation of the IkappaB kinase and the NF-kappaB transcription factors. TRAF6 contains an N-terminal RING and zinc finger domains and a C-terminal coiled coil and TRAF-C domains. Here we report the crystal structures of the N-terminal region of TRAF6 and its complex with Ubc13. The RING and zinc fingers of

TRAF6 assume a rigid, strikingly elongated dimeric structure. The interaction of TRAF6 with Ubc13 requires not only the RING, but also the first zinc finger and residues preceding the RING. Only TRAF6 is capable of direct interaction with Ubc13 while other TRAF family members may elicit K63-linked polyubiquitination via alternative mechanisms. Surprisingly, the specific TRAF6 dimerization observed in the crystal and confirmed in solution is critical for its E3 activity. By combining a ubiquitin-loaded TRAF6/E2 dimeric complex with another TRAF6/E2 dimer using the known interaction between ubiquitin and the acceptor ubiquitin binding site, a tetramer model of TRAF6 can be constructed. This model could explain the promotion of polyubiquitin synthesis and is supported by model-directed mutagenesis. While the N-terminal region of TRAF6 is sufficient for polyubiquitin synthesis, the coiled coil domain is also required for TRAF6 auto-ubiquitination, suggesting the role of higher order aggregation. Together with the trimeric arrangement of the C-terminal region of TRAF6, we propose that TRAF6 activation results in creation of an "infinite" scaffold. Transient tetramerization and possibly other forms of oligomerization within this aggregation scaffold may underlie the robust E3 activity of TRAF6 and provide a common molecular mechanism for polyubiquitination.

TRAF2 BALANCES THE LEVELS OF cIAP1 AND cIAP2 THEREBY PROTECTING CANCER CELLS FROM TNFα INDUCED CELL DEATH

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One of the hallmarks in cancer is the acquired ability of the cancer cell to resist apoptosis. Inhibitor of Apoptosis (IAP) proteins are frequently over-expressed in cancer and their expression levels correlate with poor prognosis. Using RNAi-mediated knockdown of IAPs, we have addressed the contribution of individual IAPs in protecting cancer cells from the cytotoxic effect of TNFα. We find that coordinated knockdown of cIAP1 and cIAP2 is required to sensitise cells. Interestingly, depletion of cIAP1 results in TRAF2- and NF-κB-dependent compensatory induction of cIAP2 gene expression. Therefore, triple-knockdown of cIAP1/TRAF2/cIAP2 was required to maximally sensitize MDA-MB-231 and BE cells to TNFα. Our data indicate that cIAP2 responds to NF-κB signalling and acts in a positive feedback loop to provide additional protection from the cytotoxic effects of TNFα. Although

XIAP is a potent inhibitor of apoptosis, knockdown of XIAP on its own or in combination with either cIAP1, cIAP2 or TRAF2, did not significantly sensitise to cell death. Coordinated knock-down of XIAP with cIAP1/cIAP2 was required to sensitise cancer cells to TNFα. Further, we have analysed the sensitivity of a panel of knockout MEFs (cIAP1, cIAP2, TRAF2 and, DKO of cIAP1/2). Interestingly, while single knockout MEFs were highly sensitive to the cytotoxic effect of TNFα, targeting more then one of these proteins had a much stronger effect on cell viability. Together, our data indicate that IAPs and TRAF2 contribute to treatment failure by cooperatively protecting cancer cells from the cytotoxic effect of TNFα.

TUMOR NECROSIS FACTOR (TNF) RECEPTOR-ASSOCIATED FACTOR 1 (TRAF1) ENHANCES PROINFLAMMATORY TNF RECEPTOR-2 (TNFR2) SIGNALING AND MODIFIES TNFR1-TNFR2 COOPERATION

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It has been demonstrated that TNFR2 stimulation leads to degradation of TRAF2 and inhibition of TNFR1-induced activation of NFkB and JNK. Here, we show that TRAF1 inhibits TNFR2-induced proteasomal degradation of TRAF2 and relieves TNFR1-induced activation of NFkB from the inhibitory effect of TNFR2. TRAF1 co-recruited with TRAF2 to both TNF receptors. Despite lacking an amino-terminal RING/zinc finger domain, TRAF1 did not interfere with TNFR1-induced activation of JNK and NFkB. Notably, physiological expression levels of TRAF1 enhanced NFkB activation and IL8 production induced by TNFR2. Thus, TRAF1 shifts the quality of integrated TNFR1-TNFR2 signaling from apoptosis induction to proinflammatory NFkB signaling.

CELLULAR INHIBITORS OF APOPTOSIS (c-IAPs) ARE CRITICAL REGULATORS OF TNF FAMILY MEDIATED SIGNALING

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Inhibition of apoptosis enhances the survival of cancer cells and facilitates their escape from immune surveillance and cytotoxic therapies. Inhibitor of apoptosis (IAP) protein family members block cell death in response to diverse stimuli. IAP proteins are expressed at elevated levels in the majority of human malignancies, which makes them attractive targets for developing a novel class of cancer therapeutics. c-IAP1 and c-IAP2 are recruited to tumor necrosis factor receptor 1 (TNFR1)-associated protein complexes where they regulate receptor-mediated signaling. Through their ubiquitin E3 ligase activities, c-IAP1 and c-IAP2 regulate canonical and non-canonical NF-kB pathways by promoting ubiquitination of RIP1 and NIK. We have designed small-molecule IAP antagonists that bind with high affinities to select baculovirus IAP repeat (BIR) domains of IAPs resulting in a dramatic induction of c-IAP auto-ubiquitination activity and rapid proteasomal degradation. Furthermore, the IAP antagonists induce cell death that is dependent on TNF signaling and de novo protein biosynthesis. Additionally, IAP antagonists combine with agonistic death receptor antibodies to stimulate cell death in a synergistic fashion. Our mechanistic studies reveal the significance of antagonizing individual IAP family members in this synergistic pro-apoptotic activity. Finally, our IAP antagonists inhibit tumor growth in vivo, both as single agents and in combination with other stimulators of cell death pathways. Understanding the molecular mechanisms of protein stability and death receptors-mediated pathways for inhibition of apoptosis by IAP proteins is important for the design of potent IAP-directed compounds for the treatment of human malignancies.

THE cIAP1 AND cIAP2 INTERACTING DOMAIN OF TRAF2 IS IMPORTANT FOR TNF AND TWEAK INDUCED NF-κB AND PROTECTION AGAINST TNF INDUCED APOPTOSIS

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TNF receptor associated factor-2 (Traf2) binds cIAP1 and cIAP2 and can interact with members of the TNF receptor superfamily, including TWEAK-FN14 and TNF-TNFR1 ligand-receptor complexes. It is clear that both TRAF2 and cIAPs are required to protect cells from TNF induced death, but the roles of each protein

within this complex in particular is unclear. To address this question, we identified a cIAP interacting domain (CID) within Traf2 and complemented Traf2 and Traf2/Traf5 knockout cells with Traf2 CID mutants. Both TRAF2 and cIAP1 knockout MEFs have elevated activation of non-canonical NF-κB due to stabilisation of NIK. Consistent with this, cIAP/TRAF2 interaction was required to restore non-canonical NF-κB equilibrium in Traf2 knockout cells. Similarly, both Traf2 and cIAP1 knock-outs are sensitive to TNF and Traf2 CID and TRAF2 RING domain mutants failed to restore TNF resistance in Traf2 knockout cells. An intact TRAF2 CID, but not, surprisingly, the TRAF2 RING domain, was also required for proper TNF induced NF-κB, and Traf2/Traf5 double knockout cells complemented with Traf2 CID mutants displayed increased RIP1 recruitment to TNFR1 and altered RIP1 ubiquitination. Our results demonstrate that the RING domain of Traf2 is required to protect cells from TNF induced apoptosis but plays no significant role in TNF induced NF-κB. I will discuss the implications of these findings and compare and contrast these results with other data analysing the role of the cIAP1 RING in resistance to TNF and activation of NF-κB in response to TNF.

ADVANCES IN APPLYING OUR KNOWLEDGE TO THERAPY

AN UPDATE ON LESSONS LEARNED FROM ANTI-TNF THERAPY OF RHEUMATOID ARTHRITIS

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In the 1980's new technology and concepts of pathogenesis stimulated a paradigm shift in the development of biological targeted therapies for rheumatoid arthritis (RA). With Marc Feldmann and our colleagues at The Kennedy Institute, we established the concept that over-production of TNF-alpha was of pivotal importance in driving the imbalance in the cytokine network and consequential inflammatory and tissue-damaging pathways. In a programme of academic-led clinical trials we then demonstrated the clinical validity of TNF blockade therapy.

In this presentation I discuss some of the lessons learned from studies on the mechanism of action of anti-TNF in patients which illuminate the biological role of TNF in RA. The focus will be on cell recruitment into joints, modulation of cytokine, chemokine and immune networks and downstream effects on matrix of cartilage and bone biology. These mechanisms likely hold true for other chronic immune-mediated inflammatory diseases, such as Crohn's disease, psoriasis and ankylosing spondylitis.

Anti-TNF therapy, especially as co-therapy with low-dose methotrexate, is effective in controlling signs and symptoms, improving quality of life, preventing structural damage and, in early disease, inducing remission in the 'responder population'. Over 1 million patients world-wide have been exposed to anti-TNF biologicals with long-term benefit in 50-60%. Serious infections and the possibility that some cancers occur in increased frequency remain a concern and mandate careful selection and monitoring of patients receiving this treatment.

BLOCKADE OF THE LYMPHOTOXIN-LIGHT PATHWAY AND THE TREATMENT OF AUTOIMMUNE DISEASE

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The lymphotoxin-β receptor (LTBR) and herpes virus entry mediator (HVEM) signaling pathways are effectively inhibited by the receptor based Ig fusion protein, LTBR-Ig. Disruption of LTBR/HVEM signaling will have multiple immunological consequences and presents a rather complex picture. The non-developmental components of LTBR/HVEM can be separated into at least three areas: a). effects on stromal/FDC networks and chemokine release with consequences for germinal center function, b). the dialog between activated T cells and mature dendritic cells, as well as DC homeostasis and c). control of addressin expression and possibly angiogenesis and lymphangiogenesis in the lymph nodes. In this talk, we will explore the impact of LTBR-Ig on GC reactions and HEV status in monkeys in mesenteric and peripheral LN, and the spleen. These studies are complex due to the non-synchronized exposure to antigenic stimuli but do provide some insight into the length of a GC reaction during normal antigen exposure and the dependence on FDC networks. While the role of the LT system in modulating lymphoid architecture and tertiary lymphoid tissue development in murine autoimmune disease has been often assessed, the impact on human autoimmune disease remains poorly defined. Here, we will explore parallels between mouse, monkey and man and attempt to overlay this picture onto the recent clinical trials in rheumatoid arthritis.

EXPLORATION OF THE ROLES OF SOLUBLE AND TRANSMEMBRANE TNF IN ANIMAL MODELS OF INFLAMMATION AND IMMUNITY: COMPARATIVE STUDIES OF SELECTIVE INHIBITORS OF SOLUBLE TNF TO NONSELECTIVE ANTI-TNF BIOLOGICS

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We have used protein engineering to create novel "dominant-negative" versions of native human TNF (DN-TNFs) that eliminate active TNF trimers in vitro and in animal models through a mass-action subunit-exchange mechanism (Steed et al., *Science* 301, 1895). DN-TNF biologics specifically inhibit only the soluble form of TNF, unlike antibodies and decoy receptors which bind to and inhibit both soluble (sol) and transmembrane (tm) TNF. Selective inhibition of solTNF, by sparing tmTNF activity, results in a biologic that is anti-inflammatory without suppressing immunity to infection (see Zalevsky et al., *J. Immunol.* 2007). These contrasts markedly with nonselective anti-TNF antibodies and decoy receptors, which can suppress immunity both in the clinic and in animal models (e.g., see Plessner et al., *J. Inf. Dis*. 2007).

Given a current hypothesis that solTNF is pro-inflammatory, while tmTNF is essential for maintaining innate immunity (e.g., see Jacobs et al., *Eur. Cytokine Net*. 2007; Saunders et al., *J.Immunol*. 2005), the selectivity of DN-TNFs for solTNF has interesting implications regarding the efficacy and safety of this class of anti-TNF biologics. In particular, recent use of DN-TNF biologics vs. antibodies and soluble receptors to compare selective vs. nonselective TNF inhibition supports extensive literature using genetic models to demonstrate the differing roles of the two TNF ligands in inflammation and immunity.

My presentation will broadly describe efforts by our lab and our collaborators to exploit DNTNF biologics as research tools to understand the roles of solTNF and tmTNF in many disease models, including rheumatoid arthritis (e.g., Zalevsky et al. *J. Immunol.* 2007), Parkinson's (e.g., McCoy et al., *J. Neurosci.* 2006), Alzheimer's (e.g., McAlpine et al., *J. Neurosci*. 2008), multiple sclerosis, asthma, GVHD/GVT, Crohn's, sepsis, tuberculosis, and other inflammation and infection models. Using DN-TNFs, anti-TNF antibodies, and soluble TNFR decoy receptors, we are also exploring the roles of tmTNF and solTNF in dendritic cell-mediated activation of NK cells, to understand how each TNF ligand modulates the early innate immune response to infection.

One DN-TNF biologic, XPro^{TM}1595, has completed preclinical pharmacokinetic, pharmacology, and toxicology studies, and is now the subject of an approved IND in rheumatoid arthritis. This presentation will therefore address not only basic mechanistic studies of XPro1595 in in vitro and in animal models, but may potentially include potential clinical information.

With the availability of DN-TNFs, anti-TNF antibodies, and soluble TNFR decoys, the research tools now exist to explore the roles of solTNF and tmTNF in inflammation, autoimmunity, and immunity to infection. Our lab's efforts are focused on building on the existing genetic literature to further understand these two distinct TNF cytokines, and their very different (sometimes opposing) roles in inflammation and immunity.

MECHANISMS OF PATHOLOGICAL & THERAPEUTIC EFFECTSOF THE TNF FAMILY

TNF DRIVEN ACTIVATION OF INVARIANT NKT CELLS REGULATES COMBINED GUT AND JOINT INFLAMMATION

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Over the past years, it has become clear that TNF is a key player in the pathogenesis of spondyloarthritis, a devastating inflammatory rheumatic disorder which may lead to spinal ankylosis and other joint deformities. In addition, spondyloarthritis is often accompanied with extra-articular features such as uveitis, inflammatory bowel disease and psoriasis. However, the mechanisms by which TNF mediates pathology are only partially known. Particularly, the cellular targets sufficient to mediate the articular and extra-articular manifestations of spondyloarthritis remained to be defined, as well as the cellular constituents capable of modulating this TNF driven inflammation. Recently, we reported a peculiar role for TNF-R1 expressing mesenchymal cells in a mouse model of spondyloarthritis, characterized by enhanced TNF mRNA stability, resulting in Crohns´ like ileitis as well as peripheral arthritis (Armaka M., et al., J Exp Med 2008; 18;205(2):331-7). However, it remained unclear whether regulatory T cell subsets could modulate this inflammation. Here we report that invariant NKT (iNKT) cells, a glycolipid reactive regulatory T cell lineage, act as natural regulators of TNF driven inflammation. We unveil a novel mode of iNKT cell activation which occurs during chronic but not acute TNF exposure through generation of inflammatory dendritic cells. This in turn, leads to a spontaneous activation of iNKT cells by promoting CD1d mediated presentation of endogenous glycolipids. This mode of activation is capable of attenuating combined Crohn's like ileitis and joint inflammation occurring in TNFΔARE mice, and

thus may represent a natural counter regulatory mechanism to dampen TNF driven inflammation. Altogether, these observations provide new insights in the regulatory as well as the effector mechanisms of spondyloarthritis.

TNF PRIMING OF TYPE I IFN RESPONSES IN MONOCYTES AND MACROPHAGES

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TNF and type I interferons are key effectors of innate immune response. There is evidence in the literature that TNF has some antiviral activities and is able to induce type I IFN in certain cell types. The early activating effects of TNF on multiple cell types are well documented, but less is known about the effects of TNF on its major producers - macrophages, and little is known about delayed and chronic TNF responses.

We studied the temporal responses of primary macrophages to TNF and discovered that in myeloid cells TNF induces sustained expression of inflammatory genes, and delayed expression of IFN response genes such as STAT1 and IRF7 that enhance macrophage responses to cytokines and TLRs. IFN target genes were dependent on induction of an autocrine loop mediated by IFN-b, although TNF induced markedly lower levels of type I IFNs than did LPS. TNF induction of IFN-b production was dependent on IRF1, and downstream gene expression was mediated by synergy between low amounts of IFN-b and canonical TNF-induced signals. We demonstrated the physiological importance of TNF-induced expression of IFN-b and downstream genes in cell-based functional assays and in human in vivo systems. TNF treatment augmented macrophage production of type I interferons in response to subsequent TLR7/TLR8 and TLR9 ligands, showing priming function for TNF. Furthermore, neutralization of TNF strongly attenuated adenovirus-induced expression of IFN-b and IFN-dependent genes. Finally, we detected higher expression of type I interferons and downstream genes in synovial macrophages from patients with rheumatoid arthritis, an inflammatory condition that is driven by TNF. Thus, TNF activates an IRF1- and IFN-dependent autocrine loop that sustains inflammation and primes cells for strong responses to infectious pathogens if needed, while avoiding the high IFN production and potential toxicity associated with TLRs.

THE ANTI-TNF DRUG, INFLIXIMAB, HAS A PROINFLAMMATORY ACTION IN PATIENTS WITH TUMOR NECROSIS FACTOR RECEPTOR-ASSOCIATED PERIODIC SYNDROME (TRAPS)

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OBJECTIVE: Tumor necrosis factor receptor (TNFR)-associated periodic syndrome (TRAPS) is an autosomal-dominant autoinflammatory condition caused by mutations in the TNFRSF1A gene. Unlike other autoinflammatory diseases in which anti-TNF therapy is largely a successful treatment option, therapy with the anti-TNF drug infliximab is often ineffective in patients with TRAPS. Moreover, in certain cases, infliximab actually triggers severe episodes of inflammation. The aim of this study was to elucidate the mechanisms underlying such a reaction.
METHODS: Peripheral blood mononuclear cells (PBMCs) were obtained from patients with TRAPS. Both caspase 3 activity and NF-kappaB subunit activity were determined by enzyme-linked immunosorbent assay. Cytokine secretion was assessed, using a specific customized human multiplex bead immunoassay kit.
RESULTS: Unlike controls, cells from a family of 9 patients, all of whom carried the T50M mutation in TNFRSF1A, failed to respond to infliximab through proapoptotic induction of caspase 3 activity. Instead, we observed enhanced antiapoptotic c-Rel NF-kappaB subunit activity, accompanied by a significant increase in secretion of the proinflammatory cytokines interleukin-1beta (IL-1beta), IL-1 receptor, IL-6, IL-8, and IL-12.
CONCLUSION: Altered extracellular conformation of TNFRI, resulting from the T50M mutation in TNFRSF1A, results in failure of PBMCs to induce an apoptotic response to infliximab. We hypothesize that failure to shed infliximab-bound TNF/TNFRI from the cell surface of cells from patients with the T50M mutation triggers c-Rel activation, and that this leads to a marked increase in cytokine secretion and an increased proinflammatory response. In light of these findings, we strongly advise caution when prescribing infliximab as anti-TNF therapy to patients with TRAPS.

SCIENCE OF TNF BLOCKADE *IN VIVO*

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Pathogenic TNF is implicated in several autoimmune diseases, and therapies based on systemic blockade of TNF signaling are enjoying a remarkable clinical success with relatively rare side effects. However, without any doubt TNF possesses a number of non-redundant beneficial physiological functions as revealed in knockout mouse studies. Ablation of such functions may underlie unwanted complications, such as reactivation of infections. One of the possibilities why the anti-TNF therapy is working efficiently may be due to different threshold requirement for pathogenic and protective TNF signaling. Another possibility is that pathogenic TNF in affected organ may be easier accessible for blocking agents, as compared to TNF inside bactericidal granulomas where its signaling is needed for structural integrity. The third and the fourth possibilities are that pathogenic TNF is produced predominantly in distinct molecular form (soluble versus membrane-bound) or by distinct cellular sources. To address all these issues our lab is employing a growing panel of engineered mice and a growing panel of TNF inhibitors, including non-conventional ones. In particular, we are utilizing two types of novel humanized mice - a transgenic-knockout combination, and a human TNF gene "knockin" mouse. In both cases TNF gene remains under physiological control of its genomic regulatory sequences and demonstrates expected expression pattern. Although such mice are free of spontaneous diseases, pathogenic human TNF can be induced and can mediate deleterious functions in acute toxicity, or in model autoimmune diseases, such as experimental arthritis or autoimmune hepatitis. In each case the development of the disease can be completely or partially prevented by administration of various blockers of human TNF, and the therapeutic blockade protocols utilizing different blockers can be compared side-by-side and optimized. Additionally, in these mice human TNF is mediating protective functions in several infectious models, and the blockade modes allowing residual protective TNF signaling can be compared. Finally, inducible genetic TNF KO mice help to define residual TNF levels associated with distinct TNF functions, in particular, in organization of peripheral lymphoid tissues. Our overall goal is to find safer ways of TNF neutralization.

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TNF-INDUCIBLE INHIBITOR I-KAPPAB EPSILON MODULATES IL-2 EXPRESSION IN T CELLS THROUGH REGULATION OF C-REL: IMPLICATIONS FOR CHRONIC INFLAMMATORY DISEASE

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BACKGROUND: Inflammation can suppress T cell activation. Indeed, immune responses are restored in patients with active rheumatoid arthritis (RA) following anti-TNF therapy. To understand the mechanisms for this immune suppression, we developed an in vitro model and found that culture of mouse T cell hybridomas with TNF inhibited TCR-induced IL-2 mRNA and protein expression by up to 95%. This appeared to be due in part to inhibition of NFκB activation, as demonstrated by upregulation of IkappaB epsilon (IκBε) expression, and inhibition of the induction and nuclear translocation of c-Rel, when compared to control T cells.

OBJECTIVES: Since c-Rel is required for induction of IL-2 through its binding at the CD28 response region (CD28RR) in the proximal IL-2 promoter, and IL-2 is also crucial for peripheral T cell tolerance, we tested whether upregulation of IκBε by TNF was a key mechanism for regulation of IL-2 expression by modulatingc-Rel function.

Methods: CD28RR-driven luciferase expression was assayed in control and TNF-treated 11A2 cells co-transfected with c-Rel expression vector. Anti-CD3/CD28-induced proliferation was measured in splenic and lymph node T cells from IκBε wild-type (WT), heterozygote (HET) and homozygote knockout (KO) C57BL/6 animals. T cells were activated with anti-CD3, and NFκB and IκBε expression in nuclear and cytosolic fractions determined by blotting. IL-2 expression was determined by flow cytometry. Incidence and severity of collagen-induced arthritis (CIA) was monitored for 21d after onset and joints harvested for histological analysis.

RESULTS: Overexpression of c-Rel restored activation of pCD28RR in TNF-treated 11A2 T cells to levels observed in control T cells indicating that nuclear c-Rel is limiting in TNF stimulated T cells. Increased basal proliferative activity was observed in IκBε HET and KO cells. Anti-CD3-induced nuclear translocation of c-Rel was also more rapid in IκBε deficient T cells, and proliferation of KO T cells to low dose anti-CD3 was more vigorous. CD4+ T cell numbers expressing IL-2 was increased ∼1.6-fold in KO T cells compared to WT T cells. These differences were lost in the presence of CD28 co-stimulation. In a single study we found that the incidence of CIA was reduced to 35% for KO compared to 50-55% for WT, 36d post-immunisation. Unexpectedly, clinical scores were highest for HET animals, from disease onset. Whilst these parameters were comparable for WT and KO

mice at the outset, clinical scores appeared to increase for WT but decrease for KO animals. Importantly, histological analysis revealed ratios of mild moderate disease severity that were greatest for KO and least for HET.
CONCLUSION: IκBε is important for c-Rel-dependent regulation of IL-2, via the CD28RR of pIL-2. Deficiency of IκBε reduces TCR signalling thresholds to levels observed in the presence of costimulation. Preliminary data suggest that mice lacking IκBε develop less severe CIA, consistent with the homeostatic functions of IL-2. That IκBε heterozygotes appear to have more severe disease is intriguing and warrants further investigation.

TUMOR NECROSIS FACTOR-A P75 RECEPTOR IS REQUIRED IN POST-MI RECOVERY IN ADULT HEART

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BACKGROUND: Aging is a risk factor for coronary and peripheral artery disease. The role of TNF-α receptors (TNFR1/p55 and TNFR2/p75) in post-Acute Myocardial Infarction(post-AMI) recovery is not well understood. We hypothesized that signaling through TNFR2/p75 may be essential in post-AMI regeneration in adult heart.
Methodology:To test our hypothesis we evaluated post-AMI survival, cardiac function and angiogenesis in AMI model (LAD ligation) in age-matched young- and old-WT, -p75KO and -p55KOs.
RESULTS: In old-WTs there was significant 4-fold age-associated increase in post-AMI mortality. In young-p75KOs post-AMI mortality was approaching mortality in old-WTs (35% vs. 45%) whereas >1/2 of old-p75KOs (60%) died within 7 days post-AMI. No post-AMI mortality was observed in young-p55KOs and in old-p55KOs was lower than in young-WTs (10% vs. 15%). Compared to young-WT, between days 7 and 28 post-AMI functional recovery (% LV fractional shortening-FS) was 10% lower in old-WTs and young-p75KOs ($p<0.05$) and more than 20% lower in old-p75KOs ($p<0.001$). To evaluate ongoing MI injury, we immunostained hearts with cardiac troponin I (cTnI). Compared to non-infarcted tissue, cTnI expression was significantly increased in infarcted border-zone of young-p75Kos, old-WTs and old-p75Kos 7 days post-AMI and cTnI it was the highest in old-p75KOs. Compared to young-WTs there was significant decrease in capillary density (BS1-lectin staining) and the number of functional vessels (BS1-lectin perfusion) in old-WT and young-p75KOs in infarct/infarct border-zone. There was further 3-fold post-AMI decrease ($p<0.001$) in functional vessels in old-p75KOs

compared to old-WTs. To corroborate our findings to human condition peripheral blood(PB) endothelial progenitor cells(EPCs) from young volunteers and adult CAT-lab patients were evaluated for expression of angiogenic, pro-survival (VEGF, ANG-2, HIF-1alpha, ET-1) and stem cell-derived factors, receptors (SDF1, CXCR4 and GCSFR) by qRT-PCR. There was significant 2-5-fold ($p<0.02$) decreased in EPCs from adult patents. Transplantation of WT bone marrow(BM) mononuclear cells (MNC) into γ-irradiated p75KOs one month prior to HL surgery showed that resident old WT cells and BM-derived (GFP +) young WT cells proliferate (double Ki67/GFP +) in old p75KO ischemic tissue, whereas only BM-derived young p75 cells but not resident cells proliferate in old p75KO tissue. Conversely, WT/GFP cell from young mice survive (double TUNEL/GFP +) in old p75KO ischemic tissue, whereas all BM-derived young p75KO cells do not survive in old p75 ischemic tissue.
SUMMARY: Our data strongly suggests a critical role of signaling through TNFR2/p75 in post-AMI recovery in adult tissue through impairment of several ischemia-induced repair and regeneration processes.

TNF-ALPHA REGULATION OF CD38 EXPRESSION IN HUMAN AIRWAY SMOOTH MUSCLE: ROLE OF MAP KINASES AND NF-KAPPA B.

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The pleiotropic cytokine TNF-alpha has been implicated in airway inflammation and airway hyperresponsiveness (AHR), hallmarks features of asthma. Polymorphisms in the TNF gene cluster are associated with increased TNF-alpha production and risk of asthma. Our laboratory has demonstrated that in human airway smooth muscle (HASM) cells, TNF-alpha augments the expression of CD38, a type II transmembrane glycoprotein which synthesizes the calcium-mobilizing molecule cyclic ADP-ribose. Mice challenged intranasally with TNF-alpha develop AHR to inhaled methacholine. However, mice that are deficient in CD38 fail to develop AHR, indicating that CD38 expressed in the airways is required for cytokine-induced AHR. In HASM cells, TNF-alpha-induced CD38 expression is decreased in the presence of inhibitors of p38, JNK and ERK mitogen-activated protein kinases (MAPKs). The decreased CD38 expression by p38 and JNK MAPK inhibitors is associated with decreased activation of NF-kappa B, whereas the decrease by the ERK MAPK inhibitor is due to decreased stability of CD38 transcripts. TNF-alpha induced a 2-fold activation of a 3 kb cd38 promoter following its transfection in HASM cells. However, there was no activation of the promoter lacking the NF-kappa B site. These results demonstrate that TNF-alpha regulation

of CD38 expression in HASM cells is mediated transcriptionally through p38 and JNK MAPKs and NF-kappa B, and post-transcriptionally through the ERK MAPK. CD38 expression in the airways is required for TNF-alpha-induced AHR. Supported by NIH grant HL057498.

FUNCTIONAL CONSEQUENCES OF DISEASE-ASSOCIATED MUTATIONS IN TNFR1 ELUCIDATED BY TRANSCRIPTOME ANALYSIS

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Mutations in the TNFRSF1A (TNF receptor 1) gene cause the autosomal dominant, auto-inflammatory tumour necrosis factor receptor associated periodic syndrome (TRAPS). TRAPS is characterised by recurring attacks of fever, abdominal pain, arthralgia, myalgia, conjunctivitis, migratory skin lesions and systemic amyloidosis. The exact mechanism by which the periodic fevers are triggered is yet to be elucidated. Our aim was to identify genes and pathways, which are differentially modulated as a result of mutant receptor expression. We also investigated whether the alterations differed between different mutants, and whether or not signaling by the TNFR1 death domain was involved. Transcriptome analysis was used to determine the gene expression profile of HEK293 and SK-Hep-1 endothelial cells stably transfected with full-length constructs of wild type (WT) or TRAPS-associated mutant TNFR1. Compared to WT, cells expressing mutant TNFR1 showed down-regulation of genes that suppress immune responses and up-regulation of multiple genes involved in mediating inflammation. Both cell type-specific and mutant-specific profiles were identified. In endothelial cells, an inflammatory gene expression profile associated with a deregulation in the cytokine network and related signalling pathways was identified. Whilst in the HEK293 cells, a more general stress response expression profile was revealed. The expression of most genes was induced by a death domain-dependent mechanism since they were not induced by expression of TNFR1 mutants with an inactivated death domain. The differential expression profiles of WT vs. TRAPS-associated mutant TNFR1 transfected cells has identified a number of potentially key genes that may play a significant role in the pathogenesis of the disease and offer new insights into the alternative pathways activated by mutant TNFR1, providing candidates for drug targets.

THE ROLE OF TNF SIGNALING IN SPONTANEOUS COLITIS DEVELOPMENT IN MICE LACKING NEMO SPECIFICALLY IN INTESTINAL EPITHELIAL CELLS

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The intestinal epithelium plays an essential role in the maintenance of a healthy immune homeostasis in the gut. Previous results showed that NF-κB signaling in intestinal epithelial cells (IECs) is important for the maintenance of intestinal epithelial integrity. Specific inactivation of the gene encoding NF-κB Essential Modulator (NEMO/IKKγ) in mouse intestinal epithelium (NEMOIEC-KO mice) caused the development of severe spontaneous intestinal inflammation. NEMO deficiency in IECs resulted in complete inhibition of canonical NF-κB signaling, rendering these cells more vulnerable to TNF-mediated apoptosis. Moreover, NEMOIEC-KO mice that are also deficient for TNF receptor 1 did not develop spontaneous colonic inflammation, arguing that TNF signaling plays an essential role in disease development. It was therefore hypothesized that TNF-induced apoptosis of NEMO-deficient IECs might be a crucial event in disease induction by leading to the disruption of the epithelial barrier, thus allowing commensal bacteria to translocate to the lamina propria triggering an inflammatory response.

To address the cell specific function of TNF signaling in the development of colitis in NEMOIEC-KO mice we use genetically modified mice allowing the conditional manipulation of TNF signaling. To investigate the role of apoptotic TNFRI signaling in IECs we employ mice carrying conditional loxP-flanked alleles of FADD, an adapter molecule essential for death receptor-induced apoptosis and TRADD, an adapter essential for TNFRI signaling. FADDFL and TRADDFL mice were crossed with NEMOIEC-KO mice to generate mice lacking both NEMO and FADD or NEMO and TRADD in IECs. Unlike NEMOIEC-KO mice, double FADD/NEMOIEC-KO and TRADD/NEMOIEC-KO mice do not develop spontaneous colonic inflammation at young age. Therefore, TNFR1 mediated signaling and death receptor induced apoptosis in NEMO-deficient IECs is a crucial event for the development of intestinal inflammation in NEMOIEC-KO mice.

EFFECTS OF TNF-α ON RENAL HEMODYNAMICS AND EXCRETORY FUNCTION IN MICE

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TNF-α has been implicated in the pathogenesis of hypertension and renal injury. However, the direct effects of TNF-α on renal hemodynamic and excretory function are not yet clearly defined. In the present study, renal responses to intravenous infusion of TNF-α (0.3 ng/g/min) were examined in anesthetized mice. Renal blood flow (RBF) and glomerular filtration rate (GFR) were determined by PAH and Inulin clearances. The urine was collected from a cannula inserted into the bladder. Following the 60 min control clearance period, TNF-α infusion was initiated and 15 min were given for stabilization followed by another 60 min clearance period. TNF-α alone (n=7) caused decreases in RBF (7.9±0.3 to 6.4±0.3 mL/min/g) and GFR (1.0±0.1 to 0.6±0.1 mL/min/g) as well as increases in absolute (0.8±0.3 to 1.4±0.3 μmol/min/g) and fractional excretion of sodium (0.5±0.2 to1.5±0.4%) without affecting arterial pressure. TNF-α infusion also increased urinary 8-isoprostane excretion rate (8.1±1.1 to 11.1±1.3 pg/min/g). Pre-treatment with TNF-α blocker, etanercept (5 mg/kg, s.c; 24 & 3 hr before TNF-α infusion; n=6) abolished these responses. However, TNF-α induced an increase in RBF and caused attenuation of the reduction in GFR response in mice pre-treated with superoxide (O2-) scavenger, tempol (2 μg/min/g; n=6). TNF-α caused enhancement of the reduction in RBF response while natriuretic response remained intact in mice pre-treated with nitric oxide (NO) synthase inhibitor, L-NAME (0.1 μg/min/g; n=6). However, there was a marked reduction in sodium excretion in response to TNF-α in mice (n=6) pretreated with combined amiloride (17 μg/kg/min) and bendroflumethiazide (2.8 μg/kg/min) that would block distal tubular sodium transport. These data suggest that TNF-α induces renal vasoconstriction and hypofiltration via enhancing the activity of O2- and thus, reducing the bioactivity of NO. The natriuretic response to TNF-α is related to its direct inhibitory action on distal tubular sodium transport.

LIPOPOLYSACCHARIDE PLUS GALACTOSAMINE-INDUCED FATAL HEPATITIS MEDIATED BY SECRETED TNFα REQUIRES CASPASE-8 AND THE TWO BH3-ONLY PROTEINS BID AND BIM

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Apoptotic death of hepatocytes, a feature of many chronic and acute liver diseases, is often a consequence of over-activation of the immune system and caused by pro-inflammatory cytokines, such as TNFα. Injection of mice with lipopolysaccharide (LPS) plus the transcriptional inhibitor D(+)-galactosamine (GalN) results in rapid hepatocyte apoptosis. This is mediated by secreted TNFα signaling via TNF-R1, but the effector mechanisms are unclear. Our analysis of gene-targeted mice showed that caspase-8 is essential for this hepatocyte killing, as mice lacking caspase-8 specifically in hepatocytes were fully protected from LPS/GalN-induced fatal hepatitis. Loss of Bid, the pro-apoptotic BH3-only protein activated by caspase-8 and essential for Fas ligand-induced hepatocyte killing, resulted only in marginal reduction of liver damage and mortality. Remarkably, combined loss of Bid and another BH3-only protein, Bim, protected mice from fatal LPS/GalN-induced hepatitis. We provide evidence that the observed posttranslational activation of Bim is mediated by JNK, a kinase known to be activated downstream of TNF-R1 signaling. These observations identify caspase-8 and the BH3-only proteins Bid and Bim as potential therapeutic targets for treatment of liver diseases.

STRATEGIES TO INHIBIT THE TOXICITY OF SYSTEMIC TNF TREATMENT WITH RETENTION OF ITS ANTITUMOR EFFECT

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The application of the spectacular antitumor effect of TNF is still limited to local treatment schedules because of the unacceptable toxicity of systemic TNF administration. This toxicity is based on the strong pro-inflammatory nature of TNF. Indeed, in many cell types, TNF stimulates several signals towards transcription of numerous genes, encoding important molecules of inflammation, such as cytokines, adhesion molecules and enzymes. Al these signals result in acute inflammation and SIRS. Several lines of evidence from our group suggest that, in mice, the antitumor activities and induction of SIRS can be uncoupled. Indeed, mice can be protected against TNF toxicity with full retention of antitumor effects, by several strategies. These include inhibition of Matrix metalloproteinases (MMPs) and induction of HSP70. The mechanism of action of HSP70 appears to involve protection of the glucocorticoid receptor, which is clearly malfunctioning in TNF-treated mice. Moreover, the identification of mediators of the induction of SIRS by TNF is a priority, as it can lead to novel interventions. We have identified IL-17 as an essential mediator, which, surprisingly seems to be induced and expressed in a specific cell type of the gut, the Paneth cells. More new mediators of TNF toxicity have been described and will be presented on the conference.

RATIONAL DESIGN OF NEW MEANS FOR THERAPEUTIC MODULATION OF FUNCTION OF THE TNF FAMILY

THE STALK REGION OF THE TNF RECEPTORS HIGHLY DETERMINES SOLUBLE TNF RESPONSIVENESS

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Tnf receptor (tnfr)1 is similarly well activated by soluble tnf (stnf) and the membrane-bound form of tnf (memtnf), whereas tnfr2, is efficiently activated only by memtnf. To identify the molecular basis of differential stnf responsiveness, we used fibroblasts from tnfr1/tnfr2 double knockout mice stably expressing diverse tnfr-fas variants containing the cytoplasmic part of fas and assessed them in cytotoxicity assays.
We demonstrate that exchanging the first cysteine-rich domain (crd1) of tnfr1-fas with that of tnfr2 decreases stnf responsiveness. Chemical crosslinking experiments indicate a reduced ligand independent receptor oligomerisation. In contrast, exchange of the transmembrane region had only a mild effect on stnf responsiveness. We next exchanged the extracellular stalk region, highly different in length and o-glycosylation. Surprisingly, this resulted in a near to total exchange of the phenotype. Furthermore, the stalk region also controls ligand-independent receptor assembly as revealed from chemical crosslinking studies and confocal microscopy studies.
Together, we show that the tnfr responsiveness to stnf is determined by the stalk region as well as crd1 and suggest that stabilizing the pre-assembly of tnfr enables stnf to induce the formation of larger ligand/receptor clusters, a prerequisite for signal initiation.

THE EFFECT OF THERAPEUTIC ANTI-TNF AGENTS ON REVERSE SIGNALING VIA MEMBRANE TNF

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Tumor necrosis factor (TNF) is a pluripotent cytokine that exists as both soluble & type II transmembrane precursor proteins. Both forms of TNF demonstrate bioactivity on cells expressing TNF receptors, with some effects being distinct. In addition, a number of studies in immune cells have shown that engagement of the transmembrane form of TNF can transmit signals via a 'reverse signaling' mechanism into the ligand bearing cell. Inhibitors of TNF have demonstrated significant efficacy in the treatment of inflammatory diseases including rheumatoid arthritis (RA), psoriasis & Crohn's disease (CD). However, differences in the clinical profiles of currently marketed anti-TNF agents exist. For example, infliximab (an anti-TNF antibody) & etanercerpt (a TNFR-Fc fusion protein) have comparable efficacy in RA but infliximab has significant efficacy in treating CD & etanercept does not.
The different biophysical properties of the anti-TNF therapeutics have been proposed to account for differences in clinical efficacy. Etanercept dissociates more rapidly from membrane TNF than other marketed anti-TNF agents & consequently may be less efficient at inducing reverse signals through membrane TNF. Several reports have demonstrated that etanercept is less effective at inhibiting LPS-induced cytokine production by monocytes & less effective at inducing apoptosis of T cells expressing membrane TNF than other anti-TNF agents.
We have conducted experiments to address the ability of different anti-TNF agents to induce reverse signals via membrane TNF in these in vitro systems & will present our findings for discussion.

DETERMINANTS FOR PRODOMAIN INHIBITION OF THE TNF-α CONVERTING ENZYME (TACE) ZYMOGEN STATE

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The TNFα converting enzyme (TACE) is a zinc metalloproteinase that mediates shedding of multiple cell surface proteins. Regulation of TACE enzymatic activity is ultimately mediated via proteolytic removal of its inhibitory prodomain. Sequence determinants for TACE prodomain inhibition of the catalytic domain are yet to be identified. Surprisingly, although TACE and ADAM 10 (closest homolog) share only 23% sequence identity at their prodomains, the latter in isolation inhibits TACE with the same potency as TACE own prodomain. In contrast, the prodomain of

ADAM 9 inhibited TACE only weakly. Detailed analysis of ADAM prodomains revealed two short regions for which TACE and ADAM 10 depart dramatically from all other family members. We prepared TACE prodomain variants containing full or partial switches to ADAM 9 residues at those two regions and examined their functional properties. Variants containing ADAM 9 substitutions including amino acid residues 72-82 and 126-137 were fully inactive for TACE inhibition. A third variant comprising residues 114-125 was active, but at lower potency relative to wild type. All inactive variants appeared to be correctly folded. We conclude that TACE and ADAM 10 prodomains are functionally equivalent in a way that separates them from the rest of the ADAM family.

CREATION OF MUTANT LYMPHOTOXIN-ALPHAS WITH ENHANCED BIOACTIVITIES BY PHAGE DISPLAY TECHNIQUE

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Lymphotoxin-alpha (LT) has been considered as a promising new drug for cancer and immunotherapy because of a strong specific cytotoxicity to tumor cells and immune activating action. Serious drawbacks of LT as a clinical antitumor agent are the low stability and efficacy in vivo. Here, to overcome these problems, we attempted to create mutant LTs with lowered isoelectric point and enhanced bioactivities compared to wild type LT (wtLT). To create mutant LTs, a phage library displaying mutant LTs with randomized sequences in place of the all lysine residues was prepared. The library was subjected to several rounds of panning and we succeeded in obtaining bioactive lysine-deficient mutant LTs, Mut1, Mut2 and Mut3. By in vitro cytotoxic assay using HEp-2 cells, bioactivities of mutant LTs were found to be 30-, 7- and 6-fold higher than that of wtLT, respectively. The mutant LTs showed higher bioactivity against HT29.14S and MCF-7 cells as well. In addition, we showed that mutant LTs induced the strong and rapid activation of caspase compared to wtLT. These results indicated that mutant LTs would be promising anticancer agents. Now, we are examining the mechanism of strong bioactivity of mutant LTs.

PHARMACOLOGICAL INHIBITION OF Tpl2 KINASE BLOCKS TNFalpha PRODUCTION IN HUMAN MONOCYTES AND MAY REPRESENT A NOVEL TREATMENT FOR RHEUMATOID ARTHRITIS

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Tumor necrosis factor alpha (TNFalpha) is a pro-inflammatory cytokine that controls the initiation and progression of inflammatory diseases such as rheumatoid arthritis. Tpl2 is a MAPKKK in the MAPK (i.e. ERK) pathway, and the Tpl2-MEK-ERK signaling pathway is activated by the pro-inflammatory mediators TNFalpha, interleukin (IL)-1beta, and bacterial endotoxin (lipopolysaccharide (LPS)). Moreover, Tpl2 is required for TNFalpha expression. Thus, pharmacologic inhibition of Tpl2 should be a valid approach to therapeutic intervention in the pathogenesis of rheumatoid arthritis and other inflammatory diseases in humans. We have developed a series of highly selective and potent Tpl2 inhibitors, and we have used these inhibitors to demonstrate that the catalytic activity of Tpl2 is required for the LPS-induced activation of MEK and ERK in primary human monocytes. These inhibitors selectively target Tpl2 in these cells, and they block LPS- and IL-1beta-induced TNFalpha production in both primary human monocytes and human blood. In rheumatoid arthritis fibroblast-like synoviocytes these inhibitors block ERK activation, cyclooxygenase-2 expression, and the production of IL-6, IL-8, and prostaglandin E(2), and the matrix metalloproteinases MMP-1 and MMP-3. In LPS-challenged mice these inhibitors potently reduce serum TNFalpha levels. Importantly, in the murine model of collagen-induced arthritis, deletion of the Tpl2 gene causes a reduction in disease severity, further validating the role of Tpl2 in the pathogenesis of rheumatoid arthritis. Taken together, our results show that inhibition of Tpl2 in primary human cell types and in the murine LPS model can decrease the production of TNFalpha and other pro-inflammatory mediators during inflammatory events, and they further support the notion that Tpl2 is a potential therapeutic target for rheumatoid arthritis and other human inflammatory diseases.

THE DISCOVERY OF A SERIES OF NOVEL SMALL MOLECULE MACROCYCLIC TNF ANTAGONISTS

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We have developed an integrated platform for the synthesis and biochemical screening of macrocyclic molecules (EnsemblinsTM) that can interact with protein-protein drug discovery targets. Tumor necrosis factor alpha (TNF-α) has been linked to the pathogenesis of inflammatory disease, and agents that can prevent binding of TNF-α to its receptors have utility in the treatment of rheumatoid arthritis, Crohn's disease, psoriasis and ankylosing spondylitis. Currently marketed drugs are biologicals that target sequestration of TNF-α, and there are very few small molecules TNF-α antagonists at any stage of development. Ensemble Discovery has recently identified a series of selective and reversible small molecule macrocycles that competitively antagonise the activity of TNF-α on TNF receptors in both biochemical and cell-based assays, and show anti-inflammatory activity in vivo. These compounds are currently in pre-clinical development.

BIOLOGICAL ACTIVITY OF ECTODYSPLASIN A IS CONDITIONED BY ITS COLLAGEN AND HEPARAN SULFATE PROTEOGLYCAN-BINDING DOMAINS

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Mutations in the TNF family ligand EDA1 cause X-linked hypohidrotic ectodermal dysplasia (XLHED), a condition characterized by defective development of skin appendages. The EDA1 protein displays a proteolytic processing site responsible for its conversion to a soluble form, a collagen domain and a trimeric TNF homology domain (THD) that binds the receptor EDAR.

In frame deletions in the collagen domain reduced the thermal stability of EDA1. Removal of the collagen domain decreased its activity about 100-fold, as measured with natural and engineered EDA1-responsive cell lines. The collagen domain could be functionally replaced by multimerization domains or by cross-linking antibodies, suggesting that it functions as an oligomerization unit. Surprisingly, mature soluble EDA1 was poorly active when administered in newborn, EDA-deficient (Tabby) mice. This was not due to the collagen domain per se, but to a short stretch of basic amino acids located at the N-terminus of the collagen domain that confers EDA1 with proteoglycan-binding ability. In contrast to wild type EDA1, EDA1 with mutations in this basic sequence was a potent inducer of tail hair development in this

system,. Thus, the collagen domain activates EDA1 by multimerization, whereas the proteoglycan-binding domain may restrict the distribution of endogeneous EDA1 in vivo.

NOVEL MECHANISM OF ACTION FOR ANTI-LYMPHOTOXIN-ALPHA IN AUTOIMMUNE DISEASE: DEPLETION OF TH1 AND TH17 CELLS

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Aberrant autoimmune responses are often underpinned by dysregulated and uncontrolled CD4+ T helper (Th) cell subsets Th1 and Th17. We describe here a novel strategy to target Th1 and Th17 cells using a depleting monoclonal antibody directed to surface lymphotoxin (LT). The depleting anti-LTα mAb binds surface LTα1β2 but does not block LTβR signaling in vivo, and ameliorated disease in delayed-type hypersensitivity and collagen-induced arthritis (CIA) via Fcγ receptor mediated immunoablation. A mutation in the Fc tail, rendering the antibody incapable of FcγR binding and ADCC activity, abolished all in vivo effects. Anti-LTα was efficacious in established CIA whereas LTβR.Fc had no effect. Efficacy in CIA was associated with decreased IFN-γ and IL-17 antigen-specific T cell responses and rheumatoid-associated cytokines IL-6 and IL-1β within joints. These data indicate that depleting Th1 and Th17, with anti-LTα or other cell-specific antibodies, may be beneficial in the treatment of autoimmune disease.

THE DEVELOPMENT AND CHARACTERIZATION OF APRIL ANTAGONISTIC AGENTS

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APRIL (A Proliferation Inducing Ligand) is a TNF ligand originally characterized for its ability to promote proliferation both in vitro and in vivo. APRIL binds two receptors: TACI (Transmembrane Activator and CALM Interactor) and

BCMA (B Cell Maturation Antigen). In addition, APRIL also binds heparan sulphate proteoglycans (HSPG). APRIL is a type II transmembrane protein but is cleaved in the Golgi apparatus by a furin convertase and secreted as a soluble ligand. APRIL appears to be important in the induction or maintenance of several autoimmune diseases, including systemic lupus erythematosus (SLE), Rheumatoid Arthritis (RA), Sjögren's syndrome and multiple sclerosis. Furthermore, APRIL has been implicated as an important pro-survival factor in several B-cell malignancies, such as Chronic Lymphocyte Leukemia (CLL), Multiple Myeloma and Non Hodgkin Lymphoma. These effects appear to be in part mediated via APRIL interactions with BCMA and TACI, driving NF- B activation. A study using an in-vivo model for Multiple Myeloma and treatment with TACI-Fc to prevent survival signaling by both BAFF and APRIL, demonstrated a pivotal role for APRIL and BAFF in tumor proliferation. In addition, APRIL expression in patient `s serum appears to be a prognostic indicator and potentially relates to decreased survival rates of CLL and Diffuse Large B Cell Lymphoma. To study the role of APRIL in separation from BAFF, we developed novel APRIL antagonistic agents.

Fn14-TRAIL, A CHIMERIC INTERCELLULAR SIGNAL EXCHANGER, ATTENUATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Hallmarks of the pathogenesis of autoimmune encephalomyelitis include perivascular infiltration of inflammatory cells into the central nervous system, multifocal demyelination in the brain and spinal cord, and focal neuronal degeneration. Optimal treatment of this mechanistically complex disease entity will ultimately call for agents that target the spectrum of underlying pathogenic processes. In the present study, Fn14-TRAIL is introduced as a unique immunotherapeutic fusion protein that is designed to exchange and redirect intercellular signals within inflammatory cell networks, and in so doing, to impact multiple pathogenic events that yield a net anti-inflammatory effect. In this soluble protein, an Fn14 receptor component (capable of blocking the pro-inflammatory TWEAK ligand) is fused to a TRAIL ligand one (capable of inhibiting activated, pathogenic T cells). Sustained Fn14-TRAIL expression was obtained in vivo using a transposon-based eukaryotic expression vector. Fn14-TRAIL so expressed effectively prevented chronic

non-remitting paralytic disease in myelin oligodendrocyte glycoprotein (MOG)-challenged C57BL/6 mice. The suppression of disease in this model was reflected by decreases in clinical score, disease incidence, nervous tissue inflammation, and Th1, Th2 and Th17 cytokine responses. Significantly, Fn14-TRAIL's therapeutic efficacy could not be recapitulated simply by administering its component parts (Fn14 and TRAIL) as soluble agents, alone or in combination. Fn14-TRAIL's functional pleiotropism was manifest in its additional ability to attenuate the enhanced permeability of the blood-brain barrier that typically accompanies autoimmune encephalomyelitis.

NOVEL TRAIL VARIANTS FOR TARGETED CANCER THERAPY

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Targeting the apoptotic machinery of malignant cells is one attractive concept for the treatment of cancer, which is currently exploited for TRAIL at various stages of preclinical and clinical development. However, the presently published knowledge about TRAIL's clinical performance points to the need of a further optimization of TRAIL reagents.

In an attempt to improve TRAIL's tumor selective activity we generated new TRAIL fusion proteins comprising an antibody fragment (scFv) for targeting ErbB2. Further, a variant was designed, where TRAIL is expressed as a single polypeptide chain (scTRAIL). Apoptotic activity of scTRAIL is equivalent to a homotrimeric TRAIL molecule. We studied tumor targeting and apoptosis induction of scTRAIL based fusion proteins in comparison to conventional, homotrimeric TRAIL fusion proteins and nontargeted TRAIL. Interestingly, among the various TRAIL molecules, the tumor antigen targeted scTRAIL fusion protein showed highest apoptotic activity in vitro, with a predominant action via TRAILR2 signaling on Colo205 colon carcinoma cells. Pharmakokinetic studies revealed increased plasma half life of the targeted TRAIL fusion proteins compared to TRAIL. In vivo studies in a mouse tumor model with xenotransplanted Colo 205 cells confirm greater response to the tumor targeted ErbB2 specific scTRAIL fusion protein. The underlying mechanisms of this difference in apoptotic and anti tumoral activity will be discussed.

Together, in vitro and in vivo data are proof of concept of superior anti tumoral activity of targeted TRAIL molecules, likely via enforced apoptosis induction. Further,

we envisage that through targeting of TRAIL, potential side effects should be minimized. We propose that design concept of single chain TRAIL fusion proteins with tumor targeting capability represents a promising strategy to improve TRAIL's anti-tumoral action and to minimize potential unwanted actions on normal tissues.

DESIGNED RECEPTOR SPECIFIC RHTRAIL VARIANTS ENHANCE INDUCTION OF APOPTOSIS IN CANCER CELLS

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Cancer is one of the leading causes of death in the western society. In Europe every year 1.300.000 people are affected by cancer (new cases) and approximately 700.000 people die from the disease. Surgical resection, radiotherapy and chemotherapy remain the dominant weapons in the arsenal for the treatment of cancer, but apoptosis inducing therapies hold promise of selectivity targeting tumors while minimizing toxic side effects, as well as circumventing or overcoming acquired tumor resistance against conventional treatments[1].

A member of the TNF-ligand family, Tumor necrosis factor related apoptosis inducing-ligand (TRAIL, Apo2L) is attracting great interest as it selectively kills various types of cancer cells, and unlike other apoptosis inducing TNF-ligand family members, appears to be inactive against normal cells[2].

TRAIL is a promiscuous ligand as it binds to five different cognate receptors of the TNF-receptor family: the death receptor 4 (DR4, TRAIL-R1), death receptor 5 (DR5, TRAIL-R2) both containing a cytoplasmic death domain that transmits an apoptotic signal and to the decoy receptor 1 (DcR1, TRAIL-R3, TRIDD), decoy receptor 2 (DcR2, TRAIL-R4, TRUNDD) and the soluble secreted receptor OPG, that lack an intact death domain and therefore may act as antagonist receptors. The occurrence of DR4 or DR5 responsive tumor cells indicates that a DR4 or a DR5-receptor specific TRAIL variant will permit new and selective tumor therapies. Using the automatic design algorithm FOLD-X, we successfully generated DR5-selective TRAIL variants[3]. These variants do not induce apoptosis in DR4-responsive cell lines but show a large increase in biological activity in

DR5-responsive cancer cell lines. Even rhTRAIL wt insensitive ovarian cancer cell line could be brought into apoptosis. In addition, the design of a DR4 receptor selective TRAIL variant has been pioneered, which was a considerable challenge due to the lack of a crystal structure of the TRAIL-DR4 complex. Nevertheless our first results indicate that a single mutation a position 218 can result in a variant that induces apoptosis preferentially via DR4[4] resulting in a selective induction of apoptosis in EM-2 and ML-1 cell lines. Recently variants with enhanced receptor affinity have been created and we show that this leads to accelerated apoptosis induction of cancer cells whereas healthy cells remain unaffected. A model for the underlying mechanism of this enhanced apoptosis induction will be presented.

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APOMAB, A FULLY HUMAN AGONISTIC DR5 MONOCLONAL ANTIBODY INHIBITS TUMOUR GROWTH AND OSTEOLYSIS IN MURINE MODELS OF BREAST CANCER DEVELOPMENT AND PROGRESSION

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Apomab binds Apo2L/TRAIL death receptor DR5 and triggers apoptosis through activation of the extrinsic signaling pathway. In this study we assessed the cytotoxic effect of Apomab in vitro and evaluated its antitumour activity in murine models of breast cancer development and progression at both the orthotopic site and in bone. MDA-MB-231-TXSA breast cancer cells, tagged with a triple reporter gene construct were transplanted directly into the mammary fat pad or into the tibial marrow cavity of nude mice. Tumour progression with and without Apomab treatment was monitored in live animals and in real time using bioluminescence imaging, whereas the development of cancer-induced osteolysis was measured using high resolution micro-CT and histology. In vitro, Apomab treatment resulted in a dose-dependent increase in apoptosis in four of the nine well-established breast cancer cell lines

tested. This was associated with processing and activation of caspases 8, 10, 9 and 3, which was concomitant with activation of the Bcl2 family protein Bid and cleavage of the apoptosis target proteins PARP. In vivo, Apomab treatment resulted in complete regression of well-advanced tumours (1000 mm3) within the mammary fat pad and with no evidence of recurrence. Animals transplanted with cancer cells directly into their tibiae and left untreated, all developed large lesions that invaded the marrow cavity, eroded the cortical bone and tumour growth extended into the surrounding soft tissues. In contrast, Apomab treated mice showed remarkable inhibition of tumour growth within the marrow cavity and complete protection from breast cancer induced osteolysis. These results suggest that Apomab represents a potent immunotherapeutic agent with strong activity against the development and progression of breast cancer and highlights the need to clinically evaluate Apomab in patients with primary and metastatic disease.

ROLES OF THE TNF FAMILY IN NEURONAL DEVELOPMENT, FUNCTION & PATHOLOGY

CENTRAL AND SYSTEMIC ACTION OF CYTOKINES IN NEURODEGENERATION

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Immune responses have long been studied in central nervous system (CNS) diseases such as multiple sclerosis but mounting evidence suggests they have important roles in classical neurodegenerative diseases as well. Interestingly, many immune molecules have distinct functions in the CNS, which may or may not be identical to the ones the have in the systemic environment. The cytokine TGF-beta1, for example, is a potent modulator of autoimmune T cells in the periphery and was initially found to suppress the development of autoimmune encephalomyelitis (EAE), a model for aspects of MS in mice. We observed also beneficial, neuroprotective effects of TGF-beta1 in the brain but TGF-beta1 synthesis in glial cells promoted the accumulation of pathogenic T cells in the brain and aggravated EAE. Pharmacological inhibition of TGF-beta signaling ameliorated the paralytic disease and reduced the accumulation of T cells in the CNS. We concluded that the early production of TGF-beta1 in the CNS creates a permissive and dangerous environment for the initiation of autoimmune inflammation. To study the possible contribution of cytokines and other secreted signaling molecules in neurodegeneration more broadly we measured the levels of hundreds of these factors in plasma from patients with various CNS diseases. Using this approach we discovered that secreted signaling proteins in plasma, which we collectively call the

"communicome", change with Alzheimer's disease and can be used to classify disease or predict conversion in at-risk individuals. These systemic changes in cytokines and other factors may modulate immune responses in the CNS and potentially also provide information on pathophysiological processes occurring in the brain and other tissues.

In vivo AND in vitro VALIDATION OF TNF AS A KEY NEUROINFLAMMATORY MEDIATOR OF NEUROTOXICITY AND NEURODEGENERATION IN MODELS OF PARKINSON'S DISEASE

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BACKGROUND: Microglia activation and overproduction of inflammatory mediators in the CNS have been implicated in a number of neurodegenerative disorders including Parkinson's disease (PD). However, key molecular mediators of cytotoxicity that directly contribute to neurodegeneration have not been identified. A role for the pro-inflammatory cytokine Tumor Necrosis Factor (TNF) has been implicated in PD. Nigral midbrain dopaminergic (DA) neurons are extremely sensitive to TNF and the cerebrospinal fluid and post-mortem brains of patients with PD display elevated levels of TNF.

METHODS: Using novel engineered dominant negative TNF variants (DN-TNFs) and the decoy receptor etanercept, we found that TNF-dependent mechanisms are required for progressive *in vitro* and *in vivo* degeneration of the nigrostriatal pathway.

RESULTS: Specifically, inhibition of TNF signaling *in vitro* with anti-TNF biologics attenuated DA neuron loss even after delayed administration. *In vivo*, unilateral intrastriatal injections of 6-OHDA or intranigral chronic low dose LPS infusion resulted in a 65-70 % loss of ipsilateral nigral DA neurons; while co-administration of DN-TNFs on the lesion side reduced neuronal loss by half and attenuated the amphetamine-induced circling behavior indicative of striatal dopamine depletion. A single injection of a lentivirus encoding DN-TNF delivered intranigrally afforded similar neuroprotection.

Experiments are underway to identify targets and signaling pathways that transduce the neurotoxic effects of TNF as well as molecular regulators of microglial TNF production.

Conclusions: Timely inhibition of the TNF pathway may slow the progressive loss of dopaminergic neurons in PD [Funding by MJ Fox Foundation and NINDS 1R01NS049433-02].

THE CONTRIBUTION OF TNF TO SYNAPTIC PLASTICITY AND NERVOUS SYSTEM FUNCTION

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The cytokine TNFα, while primarily known for its role in inflammation, also serves important roles in nervous system function. Through activation of neuronal TNF-R1 receptors, TNFα causes the exocytosis of AMPA-type glutamate receptors and results in an increase of excitatory synaptic strength. Concurrently, TNFα application leads to an endocytosis of GABA-A receptors, and a decrease in inhibitory synaptic strength. In the brain, TNFα is released by glia and this release is increased when neural activity is reduced. The increase in glial TNFα release mediates homeostatic synaptic plasticity, where the nervous system compensates for a long-term reduction in neural activity levels by increasing the excitation and reducing the inhibition in the neural circuit. Blocking TNFα signaling prevents the activity-blockade-induced homeostatic plasticity, which is also absent in tissue from TNFα deficient mice. TNFα deficient mice are also lacking in one component of the *in vivo* visual cortical plasticity induced by monocular deprivation, a standard model of developmental plasticity. Specifically, TNFα is required for the strengthening of inputs from the open eye, following the closure of the other eye during a critical period of development. Further, TNFα deficient mice also lack the normal response to the chronic administration of anti-depressants, judged in two behavioral assays of anti-depressant function. Based on these results, we can use the TNFα -/- mice to explore the role of homeostatic plasticity and TNFα in the nervous system.

AXONAL SELF-DESTRUCTION AND NEURODEGENERATIVE DISEASE

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Naturally-occurring axonal pruning and neuronal cell death help sculpt neuronal connections during development, but their mechanistic basis remains poorly understood. We report that Amyloid Precursor Protein (APP) and Death Receptor 6 (DR6) activate a widespread caspase-dependent self-destruction program. DR6 is broadly expressed by developing neurons, and is required for normal cell body death and axonal pruning both *in vivo* and after trophic factor deprivation *in vitro*. Unlike

neuronal cell body apoptosis, which requires caspase-3, we show that axonal degeneration requires caspase-6, which is activated in a punctate pattern that parallels the pattern of axonal fragmentation. DR6 is activated locally by an inactive surface ligand(s) that is released in active form upon trophic factor deprivation, and we identify APP as a DR6 ligand. Trophic factor deprivation triggers shedding of surface APP in a beta-secretase (BACE)-dependent manner. Loss- and gain-of-function studies support a model in which a cleaved amino-terminal fragment of APP (N-APP) binds DR6 and triggers degeneration. Genetic support is provided by a common neuromuscular junction phenotype in mutant mice. Our results indicate that APP and DR6 are components of a neuronal self-destruction pathway, and suggest that an extracellular fragment of APP, acting via DR6 and caspase-6, contributes to Alzheimer's disease.

TNFalpha CHANGES DOWNSTREAM PATHWAYS OF NGF SIGNALLING FROM Erk1/2 TO Akt, WHICH BLOCKS NGF-DEPENDENT DIFFERENTIATION OF NEUROBLASTOMA CELLS

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Nerve growth factor (NGF) binds to its receptors, TrkA tyrosine kinase receptor and NTR, a member of the tumour necrosis factor alpha (TNFalpha) receptor family, thereby activating intracellular kinases, such as Erk1/2 and Akt. Neuroblastoma is a paediatric neuronal cancer, and overall cure rate is less than 40%. NGF can induce regression of neuroblastoma in early phases, but not in advanced phases, through TrkA and Erk1/2.

We have reported that NGF can induce TNFalpha synthesis in neuroblastoma cells, making a positive feedback loop of Akt activation via the NF-kB transcription factor. Akt activation in this cancer is known to be associated with poor outcomes of therapy. We found that the positive loop of Akt activation by TNFalpha changes downstream pathways of NGF signalling from an Erk1/2 branch to an Akt branch, changing the effects of NGF on neuroblastoma cells from differentiation to survival/proliferation. Thus, inhibition of TNFalpha signalling causes neuroblastoma cells to differentiate in response to NGF.

Therefore, NGF could contribute to poor outcome of neuroblastoma therapy in advanced phases by promoting Akt activation through TNFalpha synthesis. However, NGF can induce differentiation of neuroblastoma cells when TNFalpha signalling is inhibited.

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TNF RECEPTOR 2 SIGNALING: A TARGET FOR THERAPEUTIC INTERVENTIONS AGAINST NEURODEGENERATIVE DISEASES?

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Long term stimulation with TNF leads to a resistent phenotype against neurotoxic stimuli in neurons. We have investigated the signaling events underlying this effect. We can clearly show that long term protection is TNFR2 mediated and protein kinase B/Akt and NF-kappa B dependent. In addition, we can show the neuroprotective effect is not only effective in in vitro cell culture experiments but also in the living animal. Selective upregulation of TNFR2 by the blood-brain-barrier permeable statin Lovastatin was also shown to reduce neuronal lesions and improve learning behavior. Among the protective genes upregulated by TNF/TNFR2 via NFkappaB are calcium activated potassium channels which regulate the opening of calcium permeable glutamate receptors (NMDA receptor) key players in glutamate induced neuronal cell death.
Our results show that selective activation of TNFR2 can be crucial in the treatment of chronic and semi-chronic neurodegenerative diseases.

CASPASE 8 MEDIATES NEURON DEATH FOLLOWING in vivo AND in vitro EXCITOTOXIC 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, including epilepsia. We have recently shown that CNS neurons express components of the death receptor signaling cascades and the FADD-caspase 8 pathway is activated

and mediates neuron death after ischemic injury. Similarly, multiple DR signaling molecules including caspase 8 are activated in hippocampal neurons following kainate induced seizures. However, the involvement of caspase 8 to neuron fate after excitotoxic injury is not yet established.
To investigate the role of caspase 8 in neuron death after excitotoxic injury, we have used *in vitro* and *in vivo* models of kainate-induced damage, in mice deficient for caspase 8 specifically in CNS neurons (CamKIICrexCaspase8f/f)) and in neuronal cells (neuro2a) transfected with a dominant negative mutant for murine caspase 8 respectively. Here we show that mutant mice had significantly less severe kainic-acid induced seizures, decreased neuronal death and activation of caspase 3. Moreover, the absence of caspase 8 had no effect on NF-κB activation but led to a sustained increase in the levels of phosphorylated c-jun and JNK in the hippocampi of mutant mice. Similarly, neuronal cells deficient in caspase 8 were more resistant to kainate and NMDA-induced cell death. Overall our results support a functional role for caspase 8 in mediating excitotoxic death in neurons and identify potential new targets for the treatment of neurodegenerative diseases.

THE DEATH RECEPTOR ANTAGONIST C-FLIP IS NECESSARY FOR NEURONAL DIFFERENTIATION MEDIATED BY NEUROTROPHINS

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C-FLIP is an endogenous inhibitor of the signaling pathway triggered by the activation of death receptors. Beside its antiapoptotic role, c-FLIP also regulates cell proliferation. Here, we reveal a novel biological function for c-FLIP in neuronal differentiation. We show that c-FLIP is expressed in different regions of the mouse embryonic nervous system. The protein levels of c-FLIP drop in both spinal cord and cortex, but not in cerebellum, of the adult mice. Immunohistochemistry reveals that c-FLIP is expressed in embryonic neurons and decreases at later stages, whereas glial cells from adult mice exhibit increased immunoreactivity for c-FLIP. Specific lentiviral-based knockdown of c-FLIP in isolated motoneurons, SCGs and PC12 cells significantly reduces neurite outgrowth, even in the presence of the appropriate neurotrophin stimulus. Moreover, in PC12 cells, NGF-dependent activation

of ERKs and NF-kB are drastically reduced when endogenous c-FLIP is down-regulated. Finally, we demonstrate that c-FLIP interacts with TrkA, and not with p75NGFR, in an NGF-dependent manner. Altogether, we uncover an unexpected role of c-FLIP as a new critical player in the neurotrophin-induced MAPK/ERK-mediated neuronal differentiation process. Supported by MICINN, CIBERNED and Generalitat de Catalunya.

NEURONAL NF-κB (IKKβ) SUPPRESSES NEUROINFLAMMATION AND PROTECTS AGAINST NEUROLOGICAL DEFICITS IN DEMYELINATING CNS DISEASE

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In this study we applied conditional gene targeting in C57Bl6 mice to delete the inhibitor of NF-κB kinase β (IKKβ), a kinase essential for activation of the canonical NF-κB pathway, specifically in CNS neurons and immunized them with MOG_{35-55} peptide to induce experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis. Mice lacking neuronal IKKβ (nIKKβ-KO) showed similar disease initiation but subsequently developed a more severe, non-resolving disease compared to control mice. Histopathological analysis revealed enhanced inflammation during the early stages of disease and increased axonal damage during the chronic phase in nIKKβ-KO mice. Expression analysis of spinal cord from nIKKβ-KO mice in the early chronic phase of disease showed significantly reduced levels of neuroprotective molecules such as VEGF, FLIP and CSF-1R, and enhanced expression of immune mediators such as TNF, Th1- and Th17- related cytokines and chemokines that are known to be important for EAE pathogenesis compared to control mice. Our results show that NF-κB in CNS neurons plays a critical role in modulating the severity of autoimmune demyelinating disease, not only by enhancing neuroprotection but also by suppressing CNS immune responses, and emphasize the importance of neuroprotective strategies for the treatment of multiple sclerosis.

CELL DEATH MECHANISMS CONTROLLED BY THE TNF FAMILY

PROGRAMMED, ACCIDENTAL AND SECONDARY NECROSIS CONVERGE ON SIMILAR CELLULAR DISINTEGRATION FEATURES

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Programmed necrosis or necroptosis, accidental necrosis and secondary necrosis represent different modes of necrotic cell death. To study the subcellular events during TNF- and H2O2-induced necrosis, and anti-Fas-induced secondary necrosis, we performed high-resolution time-lapse microscopy. The cellular disintegration phase of the three types of necrosis is characterized by an identical sequence of subcellular events including lysosomal membrane permeabilization, mitochondrial hyper polarization, oxidative burst and plasma membrane permeabilization, although with different kinetics. H2O2-induced accidental necrosis starts immediately by lysosomal permeabilization. In contrast, during TNF-mediated programmed necrosis and secondary necrosis this is a late event preceded by a defined signaling phase. TNF-induced programmed necrosis depends on RIP1 kinase, mitochondrial complex I, and PLA2 activities, while H2O2-induced necrosis requires iron-dependent Fenton reactions. The common cellular and biochemical features associated with programmed, accidental or secondary necrosis lead to cellular leakage, suggesting a functional role in release of damage associated molecular patterns and intercellular communication during cell death.

RIBOFLAVIN KINASE COUPLES TNF RECEPTOR 1 TO NADPH OXIDASE AND APOPTOSIS

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Reactive oxygen species (ROS) produced by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase function as signaling molecules related to innate immunity and various cellular responses from cell growth to cell death. ROS are produced in response to plasma membrane receptor activation, however, the molecular mechanisms by which receptors like TNFR1 activate NADPH oxidase are poorly understood. We identified riboflavin kinase (RFK) as a novel death domain interacting protein that is recruited to TNFR1 upon TNFR1 ligation. RFK also binds to TRADD and, notably, to p22phox, the common subunit of NADPH oxidase enzymes Nox1-4. Strikingly, TNF induces proximity of TNFR1 with p22phox along with Nox1 and -2. In RFK-deficient cells, p22phox, Nox1 and -2 are excluded from recruitment to the TNFR1 signaling complex resulting in significantly reduced ROS production. In the absence of RFK, other ROS elicitors such as TLR agonists or angiotensin II also failed to induce ROS. This is expected because RFK phosphorylates riboflavin (vitamin B2) to flavin mononucleotide (FMN) that is converted to flavin adenine dinucleotide (FAD), an essential prosthetic group of NADPH oxidase. TNF-induced cytochrome c release and PARP cleavage are markedly reduced in RFK-deficient HeLa cells and in wild-type HeLa cells pretreated with NAC suggesting that RFK-dependent ROS production enhances caspase-mediated apoptosis. HeLa cells transfected with either siNox1 and siNox2 showed resistance to TNF-induced cell death and the combination of siNox1 and siNox2 almost completely blocked TNF-induced apoptosis, indicating functional redundancy of Nox1 and -2. Our data identify RFK as key adaptor protein physically and functionally coupling TNFR1 to NADPH oxidase.

ATIA, A MULTI-TASK PROTEIN THAT PROTECTS CELLS AGAINST TNF-INDUCED APOPTOSIS

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Tumor necrosis factor (TNF) is a proinflammatory cytokine that plays a critical role in diverse cellular events, including necrosis and apoptosis. Studies from many laboratories have demonstrated that the diverse TNF-mediated biological responses are achieved through activating multiple signaling pathways. Although

much information about TNF signaling has been obtained, many molecular aspects of TNF signaling remain unknown. Particularly, the decision of life and death in response to TNF is still poorly understood. To elucidate the mechanism of TNF-mediated survival effect, we identified a novel anti-apoptotic gene, ATIA (anti-TNF-induced apoptosis), which protects cells against TNF-induced apoptosis. We found that ATIA localizes on the cell plasma membrane and in mitochondria, and that TNF treatment increases its localization in mitochondria. We demonstrated that the different localization of ATIA allows it to have a complete different function and more importantly, the mitochondrial localization of ATIA is essential for its protective effect against TNF-induced apoptosis. The anti-apoptotic function of ATIA gene has been confirmed in vivo with our recently generated ATIA ko mice. We have also identified the interacting protein of ATIA in mitochondria and found that ATIA protects cells against TNF-induced apoptosis through regulating mitochondrial Redox pathway.

A RNA INTERFERENCE SCREEN IDENTIFIES NOVEL REGULATORS OF TNF-INDUCED PROGRAMMED NECROSIS

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Recent evidence indicates that TNF-like death cytokines can induce apoptotic and non-apoptotic forms of cell death. We have coined the term "programmed necrosis" to describe caspase-independent cell death induced by TNF-like cytokines. Besides an obligate requirement for the protein serine/threonine kinase RIP1 and the production of reactive oxygen species (ROS), relatively little is known about the molecular mechanisms that control TNF-induced programmed necrosis. In order to further illuminate the molecular pathway that governs programmed necrosis, we performed a targeted RNA interference (RNAi) screen. Our screen identified several kinases that specifically regulate TNF-induced programmed necrosis, but not apoptosis. Moreover, we have identified several viral gene products that interfere with TNF-induced programmed necrosis. Thus, a molecular circuit distinct from that of apoptosis controls caspase-independent non-apoptotic cell death and may serve as a host anti-viral defense mechanism. The molecular regulation of programmed necrosis and its physiological significance will be discussed.

COMPARTMENTALIZATION OF TNF-R1 SIGNALING: CASPASE-8 AND CASPASE-7 SEQUENTIALLY MEDIATE ACTIVATION OF ACID SPINGOMYELINASE IN INTERNALIZED TNF-R1 RECEPTOSOMES

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Our recent work revealed an important role of TNF-receptor-1 (TNF-R1) internalization in determining the biological outcome of TNF-stimulation (1-3). Internalized TNF-R1 forms TNF receptosomes and recruits TRADD, FADD and caspase-8 to transmit pro-apoptotic signals. TNF-induced apoptotic signaling further involves the generation of ceramide by acid sphingomyelinase (A-SMase) and subsequent activation of cathepsin D resulting in cleavage of the pro-apoptotic protein Bid (4). As all these signaling events occur in TNF receptosomes, we asked, whether A-SMase can be activated directly by caspase-8 within this compartment.
Confocal microscopy and immunomagnetically isolated TNF-receptosomes revealed co-localization of active caspase-8 with A-SMase and TNF-R1. The functional link between caspase-8 activity and A-SMase stimulation is demonstrated by the lack of TNF-induced A-SMase activation in caspase-8 deficient cells. However, caspase-8 failed to activate pro-A-SMase directly. We found that, instead, caspase-8 targets caspase-7 that binds to pro-A-SMase and directly cleaves the zymogen resulting in A-SMase activation. Our data suggest that activation of A-SMase requires sequential activation of caspase-8 and -7 within TNF-receptosomes.
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TNF RECEPTOR-1 (P55) AND DISC COMPONENTS TRANSLOCATE TO THE MITOCHONDRIA AND NUCLEUS IN TNF-TREATED HEPATOCYTES

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TNF can activate both inflammatory and apoptotic signaling in hepatocytes through the TNF receptor-1 (p55). The factors that determine which signaling pathway dominants are not clear but likely relate to the efficiency of death inducing signaling

complex (DISC) formation. The steps involved in DISC formation in hepatocytes are poorly understood. To characterize the formation for the DISC, we have assessed the appearance of DISC components within cultures of primary hepatocytes exposed to TNF alone or TNF with the sensitizing agent actinomycin D (Act D) using a combination of confocal and electron microscopy as well as cell fractionation, immunoprecipitation, and western blotting. These studies demonstrated rapid formation of a DISC involving TNF, TRADD, FADD, and Caspase 8 in whole cell lysates. The inclusion of Act D both accelerated and amplified the appearance of the DISC. Interestingly, the receptor along with DISC components also appeared within mitochondria within 30 minutes peaking at 60 minutes. Whereas TNFR1 consistently colocalized with TRADD in the cytosolic fraction, TNFR1 colocalization with TRADD was not observed in the mitochondria. However, TNFR1 in the mitochondria was associated with FADD and Caspase 8. The DISC comprised of TNFR1, TRADD, FADD, and caspase8 was also seen to form within the nucleus in TNF treated cells. Act D also enhanced the appearance of DISC components in both mitochondria and the nucleus. These observations suggest that TNF mediated signaling includes the translocation of TNFR-1 to intracellular organelles including the mitochondria and nucleus. Furthermore, the efficiency is amplified in the setting of Act D in cultured cells. How TNFR1 and the DISC within mitochondria and the nucleus participate in TNF mediated signaling or apoptosis remain under investigation.

FAS LIGAND SECRETION AND SELF-TOLERANCE IS REGULATED BY THE WISKOTT-ALDRICH SYNDROME PROTEIN

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The molecular mechanisms responsible for autoimmunity resulting from primary immunodeficiencies are unclear. The Wiskott-Aldrich Syndrome (WAS) is a primary immunodeficiency with a remarkably high incidence (40-70%) of autoimmunity. WAS is a X-linked disorder caused by mutations in the WASp (Wiskott-Aldrich Syndrome protein) gene. WASp is involved in regulation of actin polymerization

and enhances T cell activation and secretion of IL-2. In order to understand the molecular mechanisms responsible for WASp associated autoimmunity, WASp deficient mice were examined for autoimmune phenotypes. At ages above six months, we found that WASp deficient mice produce anti-DNA and antinuclear antibodies and develop proliferative glomerulonephritis with immune complex deposition. In examining immune regulatory pathways that may be disrupted in WASp deficiency, we found that activated CD4+ T cells of WASp deficient mice have reduced apoptosis after restimulation through the TCR due to a specific defect in Fas Ligand (FasL) secretion. Secreted and biologic FasL activity in supernatants of WASp deficient CD4+ T cells were reduced by at least 50% compared with genetically and age matched control T cells. However, FasL RNA and surface FasL levels were normal, indicating that synthesis and trafficking of FasL to the plasma membrane is not dependent on WASp. FasL can be secreted after metalloproteinase cleavage of membrane associated protein or secretion in exosomes derived from intracellular multivesicular bodies. Exosomal FasL is much more potent at inducing apoptosis than the soluble form. We found that that WASp deficient T cells secreted significantly less exosomal FasL from TCR restimulated CD4+ T cells compared to WT CD4+ T cells. This reduced secretion of exosomal FasL from WASp deficient CD4+ T cells results in a significant reduction of cell death on the Fas expressing indicator cell line, WEHI-279, in which apoptosis only occurs from FasL on exosomes. The effect of WASp deficiency on exosomal FasL secretion implicates WASp in FasL trafficking and regulation after TCR stimulation. These results provide a novel mechanism whereby a single gene deficiency can cause both immunodeficiency and predispose to autoimmunity.

TNFR1 HAS AN EARLY UBIQUITINATION-DEPENDENT, NF-κB-INDEPENDENT CELL DEATH CHECKPOINT

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The molecular mechanisms that determine whether TNFR1 triggers cell survival or cell death are not fully understood. Activation of NF-κB serves as a cell death checkpoint and its induction of pro-survival genes, or the lack thereof, is one mechanism that determines the outcome. Whether there are additional cell death checkpoints, which are not regulated by NF-κB, is not known. We recently discovered that ubiquitination of RIP1 determines whether survival or death is triggered by TNFR1. In the absence of ubiquitination, RIP1 rapidly associates with CASPASE-8 to induce death. However, if RIP1 undergoes ubiquitination, it forms a complex with NEMO and this serves to prevent RIP1 from engaging CASPASE-8. The loss of the ubiquitin acceptor site on RIP1, or the loss of NEMO, both

resulted in heightened sensitivity to TNF-induced cell death as a consequence of the rapid RIP1-CASPASE-8 complex formation. Therefore, ubiquitination of RIP1 and association with NEMO serves as an early cell death checkpoint to regulate RIP1-CASPASE-8 interaction, prior to the induction of NF-κB. Our data suggest a model whereby there are two sequential cell death checkpoints during TNFR1 signaling. The first checkpoint is transcription-independent, involving the ubiquitination of RIP1 and its sequestration from CASPASE-8 by NEMO. If this first checkpoint is intact, the RIP1-NEMO interaction then leads to the activation of IKK and the second cell death checkpoint mediated by NF-κB-dependent transcription of anti-death genes. The second checkpoint provides a genetically programmed pro-survival state that is long lasting. The first checkpoint can be disrupted physiologically by simultaneous activation of TNFR2, or pharmacologically by SMAC mimetics, which modulate the level of E3 ligases such as TRAF2 and CIAPs involved in the ubiquitination of RIP1.

TNFa AND Fasl CYTOTOXICITY IS REGULATED BY THE EXTRACELLULAR MATRIX PROTEIN CCN1 in vitro AND in vivo

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Cytokines of the TNF family regulate inflammation and immunity, and a subset of this family can also induce cell death in a context-dependent manner. Although TNFa is cytotoxic to certain tumor cell lines, it induces apoptosis in normal cells only when NFkB signaling is blocked. While FasL can induce cell death on its own, optimal response to FasL-induced apoptosis in adherent parenchymal cells must override the pro-survival effects of integrin-mediated cell adhesion. Here we show that CCN1 (CYR61), a matricellular protein upregulated at sites of inflammation and wound repair, synergizes with both TNFa and FasL to induce apoptosis by elevating cellular levels of reactive oxygen species (ROS). CCN1 can unmask the cytotoxic potential of TNFa without perturbation of NFkB signaling or de novo protein synthesis, leading to rapid apoptosis in the otherwise resistant primary human fibroblasts. CCN1 acts through direct interaction with integrins and heparan sulfate proteoglycans (HSPGs), triggering the generation of ROS through several cellular sources, including 5-lipoxygenase and the mitochondria. In the presence of TNFa, CCN1-induced ROS leads to an elevated and biphasic activation of JNK, which enables TNFa to trigger cell death. Likewise, CCN1 significantly enhances FasL-induced apoptosis through ROS accumulation, leading to hyperactivation of p38 and enhanced cytochrome c release. Mice with the genomic Ccn1 locus replaced with an apoptosis-defective Ccn1 allele are substantially resistant to TNFa-induced apoptosis in vivo, triggered by either direct injection of TNFa or by ConA-induced

TNFa synthesis. Similarly, these Ccn1 knockin mice are also severely blunted in Fas-mediated apoptosis. These results indicate that CCN1 acts as a physiologic regulator of the cytotoxicity of TNFa and FasL, providing the contextual cues from the extracellular matrix for cell death.

NON-CANONICAL NF-κB SIGNALING PATHWAY IS REQUIRED FOR TNFA AUTOCRINE AND NECROSIS IN L929 CELLS

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It is known that some caspase inhibitors (such as zVAD) alone are capable of inducing non-apoptotic/necrotic cell death in certain type of cells including L929 murine fibrosarcoma cells. In this study, we provide evidence that such cell death depends on TNFα autocrine which is controlled by the non-canonical NF-kB pathway. First, we found that zVAD-induced necrosis can be completely blocked by cycloheximine and actinomycin D, indicating that de novel protein synthesis is required for cell death. Second, it was found that TNF receptor 1 blocking antibody, TNFα neutralizing antibody and TNFα knockdown offered good protections against zVAD-induced cell death. Consistently, zVAD was found to promote TNFα autocrine, evidenced by an increased TNFα protein level and mRNA level in cells treated with zVAD. Next, we investigated the control mechanisms for TNFα autocrine and found that there is constitutive non-canonical NF-κB activation and zVAD is capable of promoting TNFα autocrine via further activation of NF-κB via the non-canonical signaling pathway. Finally, we identified the critical role of RIP1 in the non-canonical NF-κB signaling pathway. Taken together, we identified a novel mechanism responsible for zVAD-induced necrosis by promoting autocrine of TNFα via non-canonical NF-κB pathway.

THE FORM OF FAS LIGAND DETERMINES WHETHER RETINAL GANGLION CELLS SURVIVE OR DIE DURING GLAUCOMA

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Glaucoma is a leading cause of blindness worldwide and occurs when retinal ganglion cells (RGCs) die. However, the molecular mechanisms that cause RGC loss are largely unknown. The pro-apoptotic molecule FasL is expressed on ocular tissues, including retinal microglia and astrocytes, but whether this molecule contributes to retinal ganglion cell death during the development of glaucoma has not been addressed. In an experimental model of glaucoma, increased intraocular pressure results in elevated levels of TNF-α in the vitreous that activate microglia and coincide with RGC loss. However, TNF-α alone does not directly trigger RGC apoptosis. Here we show that RGCs are sensitive to FasL-mediated apoptosis and in a TNF-α model of optic neuropathy, RGC cell death is markedly reduced in both Fas deficient mice and FasL deficient mice. Moreover, FasL is a membrane-bound protein that can be cleaved to release a soluble protein with antagonist activity. We found that FasL is primarily expressed in the soluble form within the normal eye. To determine the function of membrane FasL in glaucoma, we constructed mFasL-only knock-in mice (ΔCS.1) by mutating the cleavage site in the FasL gene. ΔCS.1 mice only express mFasL and no sFasL and display accelerated loss of RGCs and more severe disease. Overall these data reveal that TNF-α triggers microglia and/or astrocytes to upregulate expression of membrane FasL, which in turn triggers apoptotic cell death in Fas positive RGCs. Therefore, during glaucoma or other optic neuropathies, genetic and/or other factors within the retinal microenvironment that increases the level of membrane FasL will accelerate loss of RGCs and promote disease severity. These data identify therapeutic targets for limiting loss of retinal ganglion cells associated with glaucoma and other optic neuropathies that will preserve vision and prevent blindness.

FATAL LYMPHOPROLIFERATIVE DISEASE IN FASL- AND TRAIL DOUBLE-DEFICIENT MICE DUE TO FAILURE OF MATURE LYMPHOCYTE ACTIVATION-INDUCED CELL DEATH

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To determine the relative roles of TRAIL and FasL in lymphocyte homeostasis and lymphomagenesis *in vivo* we generated mice defective in both molecules. FasL-mutant TRAIL-deficient B6.GT mice develop extreme splenomegaly and lymphadenopathy compared to age-matched FasL-mutant mice. Peripheral lymphoid tissues predominantly comprise CD3+CD4-CD8-B220+ double negative T cells (well known to exist in FasL- and Fas mutant mice and humans with ALPS), and increased numbers of conventional CD4 and CD8 T cells. In spleens, follicular B cells were also increased. Severe lymphoproliferative disease (LPD) resulted in premature death with B6.GT mice dying from 10-12 weeks of age and no mice surviving beyond 10 months. Despite TRAIL□s established role in tumor surveillance and control of tumor metastasis *in vivo*, and in contrast to reports of increased frequency of lymphoma in mice and humans bearing mutations in Fas and FasL, B6.GT mice died prematurely from uncontrolled polyclonal LPD with catastrophic hemorrhage, lymphocytosis, and thrombocytopenia – not from lymphoma or leukemia. Finally, the accumulating lymphocytes in B6.GT mice phenotypically resembled CD44hi CCR7- antigen-experienced lymphocytes that are normally deleted through activation-induced cell death (AICD). Consistent with this, B6.GT CD4 and CD8 T cell blasts are resistant to TCR-induced AICD, and to TRAIL-dependent apoptosis that occurs once TCR-stimulation is removed. Thus, TRAIL/TRAIL-Rs and FasL/Fas co-operatively control both the activation and execution phases of AICD. Nevertheless, the accumulation of lymphocytes results in autoimmunity, with high-titres of serum IgM and IgG autoantibodies, antibody deposition in the kidney, and frequent autoimmune skin lesions. Taken together these data demonstrate that FasL and TRAIL co-operatively control lymphocyte homeostasis *in vivo*.

DcR2 PROTECTS CANCER CELLS FROM TRAIL-INDUCED APOPTOSIS BY ACTIVATING Akt

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TRAIL triggers apoptosis upon engagement of its two death receptors, named DR4 (TRAIL-R1) and DR5 (TRAIL-R2) in many tumor cells. However some cancer cells are resistant to TRAIL-induced apoptosis by expressing the Decoy Receptors 2 (DcR2 or TRAIL-R4). We have previously shown that DcR2 inhibits TRAIL-induced cell death at the DISC level. TRAIL signaling can be also modulated by intra-cellular inhibitors such as survival kinases.
We demonstrate here that DcR2 can also regulates TRAIL-induced cell death through Akt activation. DcR2 protects cells from TRAIL toxicity and enhances cell proliferation in a Akt dependent manner. Akt inhibition, using either RNAi against PI3K or by overexpressing PTEN, restores TRAIL sensitivity of DcR2-expressing cells. Thus, despite DcR2 expression we show that pharmacological inhibitors of the PI3K/Akt pathway (e.g. Wortmannin and LY294002) sensitize DcR2-expressing cells to TRAIL-mediated apoptosis and reduce cell proliferation. Our findings indicate that DcR2 regulates TRAIL-induced cell death both at the membrane level but also downstream through Akt activation.

ANALYSIS OF KNOCKOUT/ KNOCKIN MICE THAT EXPRESS A MUTANT FasL LACKING THE INTRACELLULAR DOMAIN

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Fas ligand (FasL; CD178; CD95L) is a type II transmembrane protein belonging to the tumor necrosis factor family. Its binding to the Fas receptor (CD95; APO-1) triggers apoptosis in the receptor-bearing cell. Signaling through this pathway plays a pivotal role during the immune response and in immune system homeostasis. Similar to other TNF family members, the intracellular domain has been reported to transmit signals to the inside of the FasL-bearing cell (reverse signaling). Recently, we identified the proteases ADAM10 and SPPL2a as molecules important for the processing of FasL. Protease cleavage releases the intracellular domain, which then is able to translocate to the nucleus and to repress reporter gene activity. To study the physiological importance of FasL reverse signaling in vivo, we established knockout/knockin mice with a FasL deletion mutant that lacks the intracellular

portion (FasL☆Intra). Co-culture experiments confirmed that the truncated FasL protein is still capable of inducing apoptosis in Fas-sensitive cells. We are currently investigating signaling and proliferative capacity of B- and T- cells derived from homozygous FasL☆Intra mice to establish a role of FasL reverse signaling in co-stimulation. First results indicate an increased activation-induced proliferative response and increased Erk1/2 phosphorylation in lymphocytes expressing the truncated Fas Ligand, suggesting an inhibitory role for the intracellular Fas Ligand domain during lymphocyte activation.

XIAP LOSS CONVERTS FAS-INDUCED APOPTOSIS SIGNALING IN HEPATOCYTES FROM TYPE II INTO TYPE I

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Distinct cell types differ in the mechanisms by which the 'death receptor' Fas (APO-1/CD95) triggers their apoptosis. In lymphocytes (type I cells) activation of effector caspases by caspase-8 suffices for cell killing, whereas in hepatocytes (type II), amplification of the caspase cascade through caspase-8 mediated activation of the pro-apoptotic Bcl-2 family member Bid is essential. Our experiments revealed that gene targeting-mediated loss of the caspase inhibitor XIAP or treatment with Smac/Diablo mimetic IAP inhibitory drugs rendered hepatocytes independent of Bid for Fas-induced apoptosis signaling. These results show that XIAP is the critical discriminator between type I versus type II apoptosis signaling and suggest that IAP inhibitors should be used with caution in cancer patients with underlying liver conditions.

c-FLIP PROTEINS AT THE NEXUS POINT OF FATE-DETERMINING SIGNALS

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Frequently, a given death receptor (DR) signal must be redirected rapidly either to inhibit or to potentiate an apoptotic response. We have been especially interested in this kind of processes, requiring immediate, protein-synthesis-independent modifications of the regulatory molecules involved, including phosphorylation, ubiquitination, and proteolytic degradation (reviewed in Tran et al. Trends Biochem

Sci. 2004; 29: 601). The c-FLIP proteins, by their essential functions in the death-inducing signaling complex (DISC), are in a key position in directing DR responses. We have in previous studies shown that the stability of c-FLIP proteins is subject to isoform-specific regulation (Hietakangas et al. Mol Cell Biol. 2003; 23: 1278; Tran et a. Cell Death Differ 2003; 10: 1137; Poukkula et al. J. Biol. Chem. 2005, 280: 27345), but the underlying molecular mechanisms have not been known. We have now determined that serine 193 is a novel in vivo phosphorylation site of all c-FLIP proteins and observed that S193 phosphorylation selectively influences the stability of the short c-FLIP isoforms, c-FLIP-S and c-FLIP-R. In c-FLIP-L the phosphorylation seems to have a different role, not related to the stability. The phosphorylation of this residue is operated by PKC-alpha and beta. S193 mutations do not affect the ability of c-FLIP to bind to the DISC, although S193 phosphorylation is increased by death receptor stimulation. Instead, S193 phosphorylation affects the intracellular level of c-FLIP-S, which in turn determines the sensitivity to death receptor-mediated apoptosis. These results reveal that the differential stability of c-FLIP proteins is regulated in an isoform-specific manner via PKC-mediated phosphorylation. These results are discussed in the context of the different immediate regulatory modalities of DR responses (Holmström et al. EMBO J. 2000, 19: 5418; Tran et al. J. Biol. Chem. 2001; 276: 16484; Meinander et al. J. Immunol. 2007, 178: 3944).

Posters
ROLES OF THE TNF FAMILY IN TISSUE HOMEOSTASIS AND NORMAL DEVELOPMENT

CHEMOKINES AS TARGETS OF EDA - A ROLE IN HAIR FOLLICLE DEVELOPMENT?

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Hypohidrotic ectodermal dysplasia (HED) is characterized by sparse hair, and defects in the number and shape of teeth and several exocrine glands (like sweat and lachrymal glands. HED is caused by mutations in genes of the ectodysplasin (Eda) pathway including the TNF ligand Eda, the Eda receptor (Edar), or the adapter protein Edaradd.

Eda is involved in ectordermal organ development and is crucial for the initiation of hair follicle (HF) development in mouse. The ectodysplasin A1 isoform and its receptor Edar activate the NF-kappaB pathway during development.

In order to identify the direct target genes of Eda we have performed a microarray analysis on genes differentially expressed upon 90 min or 4 h stimulation of

embryonic skin with recombinant Eda during at the time of initiation of hair follicle development (embryonic stage E14 in mouse). We found 6 chemokines upregulated after 1,5 and/or 4 h induction by Eda. These findings were verified by using qPCR. The expression pattern of all 6 chemokines was analyzed by in situ hybridization in developing skin appendages of wild type and Eda deficient mice. This experiment allowed us to narrow down the potentially physiologically important Eda targets to 2 chemokines: Cxcl10 and Cxcl11. These chemokines were specifically expressed in nascent hair follicles, as was their receptor Cxcr3. The relevance of these chemokines in hair development was tested by in vitro skin culture and by analysis of Cxcr3 deficient mice.

CASPASE-8 DEFICIENCY IN EPIDERMAL KERATINOCYTES TRIGGERS AN INFLAMMATORY SKIN DISEASE

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Expression of enzymatically inactive caspase-8, or deletion of *caspase-8* from basal epidermal keratinocytes, triggers chronic skin inflammation in mice. This inflammation is substantially decreased on TNF null background. However, unlike similar inflammation resulting from arrest of NF-κB activation in the epidermal cells, the disease resulting from caspase-8 deficiency is not prevented but only slowed down by TNF deficiency, and does not depend on dermal macrophage function. It also does not depend on interleukin-1 or expression of the Toll-like receptor adapter proteins MyD88 or TRIF. Temporal and spatial analyses of the alterations in gene expression that result from caspase-8 deficiency reveal that the changes are initiated before birth, around the time that cornification develops, and occur mainly in the suprabasal layer. Our findings suggest that caspase-8 serves to restrain, and TNF – to potentiate, the response of the epidermal keratinocytes to endogenous activators of inflammation, presumably generated in association with the conrnification process.

ROLES OF THE TNF FAMILY IN CANCER

SUPPRESSION OF TUMOR GROWTH AND INTERRUPTION OF TUMOR ANGIOGENESIS BY SELECTIVE INHIBITION OF EITHER TNFR1/P55 OR TNFR2/75 IN MURINE MODEL OF LUNG CARCINOMA

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BACKGROUND: Tumor necrosis factor-α(TNF), a pro-inflammatory cytokine, binds two distinct receptors TNFR1/p55 and TNFR2/p75. TNF is continuously produced in all tumors and tumor microenvironment. TNF has been implicated in many tumor-associated processes such as tumor growth and survival, differentiation, invasion, metastases and secretion of cytokines, pro-angiogenic factors, and metalloproteinases. We hypothesized that inhibition of signaling via one or the other but not both of TNF receptors may have deleterious effect on tumor growth.
METHODOLOGY: To test our hypothesis we injected WT, p55KO and p75KO mice with $5x10^5$ mouse lung carcinoma line (LLC) mixed with 50 μl of Matrigel into the flank. Tumors were measured weekly using electronic calipers. Bone marrow (BM)-derived endothelial progenitor cells (EPC) were tracked into the tumor using BM/GFP transplantation model in combination with BS1-lectin perfusion and staining. Tumors bisected form all genotypes 14 days post-inoculation were collected and processed for immunostaining for CD31, TUNEL, TNF and VEGF expression and imaged using confocal microscopy. Alterations in angiogenic and apoptotic signaling pathways in whole tumor tissue were analyzed using gene array analysis. Stably transfected LLC cells using combination of shRNA plasmids for TNFR2/p75 receptor were performed to confirm our findings in-vivo.
RESULTS: Compared to day 1, tumors in WTs became ∼343 % and ∼ 933% larger between days 14 and 21, respectively, whereas at the same period, tumor growth was inhibited in both p75KOs (by 46% and 51% on days 14 and 21, respectively, $p<0.05$) and p55KOs (by 42% and 38% on days 14 and 21, respectively, $p<0.05$). TNF levels in tumor tissue were statistically significant within genotype when comparing expression in normal skin vs tumor tissue in WT 10-fold ($p<0.001$), in p75KO 8.7-fold ($p<0.001$) and p55KO 6-fold ($p<0.03$). Compared to WTs, CD31 immunostaining revealed significant 80% ($p<0.001$) and 60% ($p<0.02$) decreases in capillary density in tumor tissue of p75KO and p55KO, respectively. TUNEL

staining showed ~50% increases in TUNEL (+) cells in tumors of both p75KO and p55KO. VEGF expression was decreased by more than 50% ($p<0.001$) in p75KO and 40% ($p<0.02$). Compared to WT and p55KO mice there was ~50% ($p<0.05$) decrease in the incorporation of BM-derived EPCs into the functional capillary network in p75KOs. Gene array analysis showed that absence of either receptor had significant inhibitory effect (>2-fold) on several genes of angiogenic, anti-apoptotic and pro-survival pathways that include, but not limited to, VEGF A and B, HIF1-alpha, MAPK14(p38), HGF, Il1b, Il12a, Pgf, NFkB, Faim, Api5, Bnip3, Cxcl1, 2 and 10, and several other genes. As a proof of principle and because anti-tumor effects were more pronounced in p75KOs, we stably transfected LLC's with 1, 2 or 4 TNFR2/p75 shRNA plasmid combinations and found 70%, 74% and 90% inhibition in TNFR2/p75 expression, respectively. Percent apoptotic (PI staining - sub-G0/G1 population) LLCs treated with 80 ng/ml TNF revealed direct correlation with percent inhibition of TNFR2/p75 achieved by shRNA transfection. qRT-PCR of shRNAs-transfected TNF-treated LLCs harvested after 24hrs showed statistically significant decreases in VEGFA, HGF, PLGF, CXCR4, and MMP2 expression levels.

SUMMARY: Our results indicate that absence of the signaling through either TNFR2/p75 or TNFR1/p55 in the tumor microenvironment only inhibits tumor growth by ~50%, by negatively regulating tumor angiogenesis. Significantly higher deficiency in tumor angiogenesis in p75KOs vs p55KOs demonstrate inability of host p75KO EC's to survive in "hostile" tumor microenvironment (high TNF level) as well as impede incorporation of BM-derived p75KO EPC's into tumor vasculature, suggesting that inhibition of tumor angiogenesis is a primary mechanism of tumor growth inhibition in p75KOs. Up-regulation of LLC apoptosis and down regulation of pro-angiogenic factors in TNFR2/p75 shRNA-transfected and TNF-treated LLC's further supports the role played by TNFR2/p75 in tumor growth and angiogenesis. Indeed, our in vivo and in vitro findings suggest that inhibition of TNFR2/p75 or TNFR1/p55 in tumor tissue in vivo may deliver "double hit" by affecting survival of both tumor and endothelial cells, while continues high levels of TNF in tumor tissue will have self-destructive effect.

INHIBITION OF TNFa/NIK/NF-kB TRANSDUCTION PATHWAY AS A POSSIBLE TARGET FOR PROSTATE CANCER TREATMENT

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AIMS: TNFa induces cell death, but also cell proliferation by activation of NF-kB, which may also be activated by IL-1a. The aim of this study was investigate components of NIK transduction pathway in normal prostate (NP), benign prostatic hyperplasia (BPH) and prostatic carcinoma (PC).
Methods: Immunohistochemical and Western blot analyses were performed in 20 samples of NP, 35 samples of BPH, and 93 samples of PC (21 low, 51 medium and 21high Gleason grades).
RESULTS: In NP, cytoplasm of epithelial cells immunostained intensely to TNFa (80% of samples), TNFRI (80%), TRAF-2 (60), NIK (100%), Ikka/B (80%), IkBa (60%) and p-IkB (40%); weakly to NF-kB-p50 (60%); and negative to NF-kB-p65. In PC cytoplasm of epithelial cells immunostained was intensely to TNFa (100% samples low, 88.8% medium and 100% high Gleason grades), TNFRI (80.9%), NIK (100%), Ikka/B (increasing with Gleason), IkBa (increasing with malignancy) and p-IkB (increasing with malignancy), p50 (increasing with malignancy) and p65 (increasing with Gleason) and weakly to TRAF-2 (19%) Nuclear inmmunostaining was only observed for NF-kB (p50 and p65), only in PC and increasing with Gleason grade.
CONCLUSIONS: NF-KB (p50 and p65) enhances cell proliferation. Since other study (carried out in the same patients) revealed that immunoexpression of IL-1a or TNFa, increase in PC, inhibition of these cytokines might be a possible target for PC treatment, because such inhibition could decrease the activity of all transduction pathway members that activate several transcription factors as NF-kB, Elk-1 or ATF-2.
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ERK and P38 PROMOTE SURVIVAL IN VITRO BY TNF-ALPHA STIMULATION IN PROSTATE CANCER

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AIMS: TNFα and IL-1a are two pro-inflammatory cytokines involved in prostate cancer, linked to its ability to activate intracellular signalling pathways that involve mitogen-activated protein kinases (MAPKs), such as ERK or p38, by binding to its receptors. The inmunoexpression of TNFa and IL-1 and its receptors are increased in prostate cancer in comparison with normal prostates. The aim of this study was to evaluate the effect of these cytokines in the apoptotic index of the androgen-dependent prostatic cancer cell line LNCaP, when ERK and p38 are inhibited.
METHODS: LNCaP cells were exposed to different concentrations of TNFa or IL-1α and treated with ERK (PD98059) and P38 (SB203580) inhibitors. Then, its apoptotic indexes were measured by flow cytometry (iodate propidium incorporation).
RESULTS: TNFa induces apoptosis in LNCaP when we increased TNFa concentration (10, 20, 50, 75, 100 ng/ml), but no differences were found when LNCaP were treated with IL1a. After preincubation with SB203580 and PD98059, TNFa treatment we found a significative increasing of apoptosis.
CONCLUSIONS: When LNCaP cell cultures were treated with p38 and ERK inhibitors, increase the frequency of apoptosis indicating that p38 and ERK could exert an important role in prostatic tumour promotion by TNFa stimulation. Hence, down-regulation of p38 and ERK activity by specific pharmacological inhibitors may represent a strategy to clinical improve efficacy of TNFa in androgen-dependent prostate cancer.
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FUNCTION AND SIGNALING OF TRAIL IN PRIMARY HUMAN LEUKEMIA CELLS

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TRAIL is a promising potential future cytostatic drug that induces apoptosis in a variety of human cancer cells, but not in normal cells. In addition, our group recently described that TRAIL mediates tumor cell survival and proliferation in certain tumor cells. So far, little is known about TRAIL signaling in primary human tumor cells.
To study TRAIL signaling in primary tumor cells, we amplified fresh tumor cells from children with acute leukemia in immuno-supprimized NOD/SCID mice and performed subsequent in vitro experiments. TRAIL-induced apoptosis was observed in 8/14 patients (57%), whereas 6/14 samples showed apoptosis resistance or alternative TRAIL effects (43%). Four samples even showed increased survival or proliferation after stimulation. According to published data, inhibition of NF-κB activation by biochemical inhibitors increased apoptosis sensitivity in TRAIL-sensitive samples, but was unable to overcome complete apoptosis resistance towards TRAIL. As determined by FACs analysis, surface expression of TRAIL receptors was not detectable in all samples. In contrast, significant cytoplasmic expression of the TRAIL-receptors was found by immunohistochemistry. Moreover, TRAIL sensitive patients showed an upregulation of TRAIL-R2 (DR5) after tunicamycin treatment.
To further investigate TRAIL signaling pathways in primary leukemia, we recombinantly expressed different TRAIL variants with and without tags and a polymerizing isoleucine zipper domain which we can now use for further in vitro and in vivo studies on the function of TRAIL.
Taken together, these data will allow a better understanding of TRAIL signaling mechanisms in primary human tumor cells.

STUDIES ON THE MECHANISM OF ACTION AND RESISTANCE TO TNFALPHA IN SOFT TISSUE SARCOMAS

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BACKGROUNG: Isolated limb perfusion (ILP) with TNFalpha and melphalan is an innovative approach to treat unresectable soft tissue extremities sarcoma (STS). The well-studied mechanisms of TNF action are an increase in the endothelium permeability for melphalan (DNA alkylant), and a selective destruction of tumor vessels.

However, the direct apoptotic effect of TNF on sarcoma cells remains controversial. Recently we demonstrated that histological response to ILP may be influenced by p53 status (Muret at al., 2008). This is in agreement with several reports indicating a relationship between sensitivity to TNF and p53 activity. The aim of the present study was to further investigate the effect of TNF on sarcoma cells and better understand the role of p53 in controlling the outcome of ILP.
METHODS: Studies were carried on 12 human STS cell lines. P53 gene was sequenced; expression of TNF receptor was examined by flow cytometry. Cytotoxic effects of TNF and melphalan were assessed with MTT and by TUNEL performed on sarcoma tumor slices cultured ex vivo with TNF and melphalan.
RESULTS: All cell lines were resistant to TNF. However, after TNF treatment of tumor slices, we observed apoptosis of both endothelial and sarcoma cells. Sequencing of p53 and chromosomal analysis revealed that all studied cell lines carry either p53 mutation/deletion or Mdm2 amplification. TNFR I was expressed by 6 cell lines. CONCLUSIONS: The results of ex vivo studies suggest, that during ILP, TNF may directly kill sarcoma cells. The resistance of sarcomas to TNF and relation to p53 function will be discussed.

LEUKOCYTE-DERIVED TNF-α PROMOTES TUMOR GROWTH IN A SPONTANEOUS MODEL OF MAMMARY CARCINOGENESIS

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Solid tumors comprise tumor cells and surrounding stromal cells, mostly of hematopoietic origin. Cancer cells and infiltrating leukocytes communicate through a complex network of pro-inflammatory molecules, mostly unknown. Critical is the role of the transcription factor NF-kB and of the inflammatory mediator TNF–α, which, through a multifaceted interaction, eventually promote cancer development and progression, at least in some tumor types.
We have investigated the role of TNF–α in HER-2/neuT (NeuT) transgenic mouse model of mammary carcinogenesis, which, because of the expression of the mutated

rat neu oncogene under the control of the MMTV promoter, spontaneously develops mammary carcinomas during life time.
Bone-marrow transplantation (BMT) experiments from TNF–α KO mice into NeuT recipients significantly delay the onset and reduce the number of affected mammary glands, indicating that the relevant source of TNF–α fostering tumor promotion is of BM origin. Whole mount analysis of mammary glands confirms the less severe tumor phenotype of mice transplanted with BM from TNF–α KO mice. BMT experiments performed at different time points during tumor progression (8, 15, 20 weeks of age) indicate that TNF–α is critical during early steps of mammary tumorigenesis but still active also at later time points when carcinomas in situ and invasive carcinomas are already present. This finding differs from other models such as skin carcinogenesis in which the role of TNF–α is relevant mainly for tumor initiation/promotion.
To clearly identify which leukocyte population is the relevant source of TNF–α, BMT experiments have been performed with donor mice deficient for TNF–α in selected cellular compartments, such as monocytes, T cells and B cells. Preliminary data indicate that TNF–α produced by macrophages and T cells is critical for tumor development and progression, whereas B cell-derived TNF–α has no role.
From these experiments we expect to uncover the role of TNF–α in the various phases of mammary transformation and progression and to identify the best time window to neutralize its activity using specific monoclonal antibody.

ROLE OF B CELL IN TWO-STAGE SKIN CARCINOGENESIS

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Tnf-/- and Tnfr1-/- mice are highly resistant to two-stage chemical carcinogenesis (DMBA/TPA) of the skin developing 90% fewer tumours than wild-type mice, but the mechanism of this action is not fully understood. More recently, in a genetic model of skin cancer, B cells were found to be important effector cells of cancer-related inflammation. As both Tnf-/- and Tnfr1-/- mice have defects in B cell activity (lack of follicular dendritic cell network, no germinal centre formation and sustained IgG response) our hypothesis was that this B cell defect could contribute to the resistance of these mice to chemical carcinogens. First we found that B cells had a

tumour-promoting role in DMBA/TPA skin carcinogenesis. RAG2-/- mice, which lack mature B and T cells, were resistant to skin carcinogenesis and adoptive transfer of B cells from wild type mice restored tumour development. B cell deficient mice were also resistant to DMBA/TPA carcinogenesis. We then adoptively transferred B cells from wild type mice to Tnf-/- mice and tumour development was significantly increased ($p<0.05$). The RAG2-/- mice were still resistant to skin carcinogenesis when we adoptively transferred B cells from Tnf-/- mice. Therefore we suggest that the TNF produced by B cells plays an important role in their tumour promoting phenotype, and our data support the hypothesis that TNF is a crucial cytokine in cancer and inflammation.>

CO-EXPRESSION OF CD40L AND CD70 BY MELANOMA CELLS STIMULATES ANTI-TUMOUR IMMUNE RESPONSE

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The lack of costimulatory molecules on tumour cells results in inefficient stimulation of tumour-reactive T lymphocytes. The costimulatory signal can be provided by members of the TNFR super family, including CD27, CD40, 0x40 and 4-1BB, which are expressed on the T-cell membrane. In several murine tumour models, we have shown that stably transfected tumour cells, expressing CD40L or CD70 were able to enhance an anti-tumour response and favour a long-lasting immune memory. However in the MHC Class I-negative B16F10 murine melanoma model, the anti-tumour responses induced with CD40L or CD70 expressions remain slight. To enhance it, we associated an allogenic molecule H-2 K^d. B16F10 tumour growth slows significantly when CD40L and CD70 are co-expressed by tumour cells and the association with H-2 K^d molecule enhances this effect. Growth kinetics of mock and CD40L-CD70-H-2 K^d-expressing B16F10 tumours in immunocompetent versus nu/nu and beige mice suggested that $CD8^+$ T lymphocytes and NK cells are involved in this anti-tumour immunity. A delay in mock tumour growth is observed when CD40L-CD70-H-2 K^d-expressing B16F10 cells and mock tumour cells are injected simultaneously and contra-laterally. It is also shown that *in vivo* immunisation of immunocompetent mice with CD40L-CD70-H-2 K^d B16F10 tumour cells improved the generation of cytotoxic lymphocytes against the wild-type melanoma cells.

These observations suggest that new immunotherapeutic protocols, using semi-allogenic fibroblasts expressing costimulatory molecules and tumour-associated antigens or semi-allogeneic CD40L-CD70-expressing tumour cells, can be conceivable. Consequently, we tried to find drugs, which could enhance expression

of costimulatory and MHC class I complexes on melanoma cells and we have shown that Rho GTPases inhibitors could be good candidates. Indeed, *in vitro* treatments with statins, isoprenyl transferase inhibitors, C3 exotoxin and small interfering RNA against RhoA are able to stimulate membrane expression of costimulatory and MHC class I molecules.

NOVEL ASPECTS OF SIGNALING ACTIVATION BY THE TNF FAMILY

ESSENTIAL ROLE OF TNF-INDUCIBLE ZINC FINGER PROTEIN A20 ON THE DEATH RECEPTOR-MEDIATED APOPTOTIC CELL DEATH THROUGH THE BLUNTING JNK SIGNALLING

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Nuclear factor-kappaB (NF-kB)-dependent gene expression and apoptosis play crucial roles in numerous cellular process, and defects in their regulation may contribute to a variety of disease including cancer. It is well established that inhibition of NF-kB activation increases susceptibility to death receptor-mediated cell death, concurrent with caspases and prolonged c-Jun N-terminal kinase (JNK) activation, and reactive oxygen species (ROS) accumulation. However, the molecular mechanisms are unclear. Here we show that Cys2/Cys2 zinc finger protein, A20/TNFAIP3 has an essential role to block the tumor necrosis factor (TNF)-induced apoptotic cell death through the repressing the prolonged JNK activation. The ectopic expression of A20 in NF-kB deficient cells including IKKb-/-, p65-/- and TRAF2,5-/- fibroblast inhibits TNF-induced prolonged JNK activation as well as apoptotic cell death without any change of expression level of NF-kB-inducible inhibitor JNK signaling pathway. Co-immunoprecipitation and mutational analysis revealed that A20 directly interacts with the conserved N-terminal half of MAP3 kinase in a TNF dependent manner. More over, this stimuli-dependent interaction of A20 with MAP3 kinase might selectively suppress the prolonged JNK activation and apoptosis through ubiquitination pathway upon TNF ligation. Our findings uncover a key role for A20 in the antiapoptotic response of NF-kB activation. This work was supported by the Korea Science & Engineering Foundation through the Infection Signaling Network Research Center (R13-2007-020-01002-0) at Chungnam National University, and a grant from the National R&D Program for Cancer Control Ministry of Health & Welfare, Repubic of Korea (No: 0720560).

ADP-RIBOSYLATION OF HUMAN TNF-ALPHA BY ADP-RIBOSYLTRANSFERASE-1 (ART1)

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Mono-ADP-ribosyltransferases (ARTs) are GPI-anchored ectoenzymes that covalently modify cell surface or soluble target proteins by transferring ADP-ribose from NAD+ to arginine residues. These enzymes are thought to play a role in inflammation and immunity.
ART1 has been shown to ADP-ribosylate different membrane-bound and secreted proteins, including the antimicrobial peptide defensin alpha 1 (DEFA1/HNP-1). ART1 can be released from the cell membrane by cleavage of its GPI-anchor by GPI-specific phospholipases, such as the bacterial enzyme, Bacillus cereus phospholipase C (PI-PLC). GPI-anchored proteins can also be released by an endogenous secretory enzyme that is abundant in human plasma (GPLD1).
We identified ART1 transcripts in human peripheral blood leukocytes, as well as in heart and skeletal muscle, by RT-PCR analysis. Soluble ART1, released from the surface of transfected cells by PI-PLC, was found to ADP-ribosylate recombinant human TNFa in vitro. Furthermore, co-transfection of HEK293 cells with ART1 and human TNFa resulted in modification of TNFa at at least 2 distinct sites, i.e. one within the domain shed from the cell surface by the action of the metalloproteinase TNFa Converting Enzyme (TACE), and one on the stalk that remains connected with the cell membrane after cleavage by TACE. Binding assays indicate that ADP-ribosylation reduces binding of TNFa to its receptor (TNFRII).

TRANSCRIPTIONAL NETWORKS AND SYSTEMS BIOLOGY

PKC- AKTS TOGETHER DURING TNF SIGNALLING

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Protein kinase C (PKC) is a family of serine/threonine kinases that play important role in cell proliferation and cell death regulation. There are three classes of PKC isozymes: conventional (α, β, β and γ), novel (δ, ε, η, θ and μ) and atypical (ζ and λ/ι). PKCε acts upstream of Akt to protect breast cancer MCF-7 cells from cell death induced by members of the tumor necrosis factor-α (TNF) family, including TNF and TNF-Related Apoptosis-Inducing Ligand (TRAIL). We have shown that over-expression of PKCε in MCF-7 cells (MCF-7/PKCε) primarily affected the intrinsic or mitochondrial cell death pathway. This included an increase in anti-apoptotic Bcl-2 and a decrease in pro-apoptotic Bid compared to vector-transfected control cells (MCF-7/Neo). It also attenuated translocation of Bax from the cytosol to mitochondria. Activation of Akt by PKCε was associated with an increase in human homolog of Mdm 2 (Hdm2) and a decrease in p53. This was accompanied by a decrease in Bid mRNA.

Since TNF causes activation of the transcription factor,
nuclear factor kappa B (NF-κB) leading to the induction of a number of anti-apoptotic proteins, we examined if PKCε upregulates Bcl-2 via the NF-κB pathway. Treatment of MDA-MB-231 cells that overexpress PKCε or PKCε-overexpressing MCF-7 cells with the NF-κB inhibitor enhanced cell death by TNF but did not alter Bcl-2 level. Knockdown of NF-κB subunits p65 or p52 also did not decrease the levels of Bcl2. Knockdown of PKCε decreased phosphorylation/activation of cAMP-response element-binding protein (CREB) and a modest decrease in Bcl-2. Our results suggest that PKCε acts in concert with several signaling pathways to exert its antiapoptotic function.

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A PTP4A3 PEPTIDE PIMAP39 MODULATES TNF-alpha LEVELS AND ENDOTOXIC SHOCK

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LPS stimulation of primary macrophages initiates intracellular signaling pathways leading to activation of MAPK and its subsequent influence in cytokine production. We recently identified a LITAF-STAT6(B) complex that regulated TNF-alpha. We also know that p38 MAPK activates LITAF gene expression and PTP4A3 inhibits LITAF promoter activity in response to LPS stimulation. However, the LPS-induced cascade in the p38/LITAF/TNF signaling pathway remains unclear. Here, we identified PTP4A3, a protein tyrosine phosphotase, as a novel negative regulator of LPS-induced LITAF/TNF-alpha production. PTP4A3 elicits its negative role by dephosphorylating p38alpha MAPK in response to LPS stimulation of primary macrophages. PTP4A3 expression is up-regulated in primary macrophages and undergoes tyrosine phosphorylation in a Src family kinase-dependent manner. Further structure-function analysis revealed that a unique short peptide (PIMAP39) derived from PTP4A3 is capable of mimicking the functionality of full-length PTP4A3 to selectively dephosphorylate p38alpha and indirectly suppress LPS-induced LITAF-STAT6B complex when it is translocated from the cytoplasmic region to the nucleus of cells. Treatment of mice with PIMAP39 significantly attenuates the severity of adverse host responses to LPS stimulation, and in some cases provides complete resistance to a lethal dose of LPS due to suppression of TNF-alpha production. All together, these results reveal a previously unrecognized role for PTP4A3 pathway in response to LPS.

CROSS-TALK OF APOPTOSIS SIGNAL-REGULATING KINASE 1 (ASK1) AND HIF-1ALPHA PROTEIN AS ESSENTIAL PATHWAY FOR LIGAND-INDUCED TOLL-LIKE RECEPTOR 4-MEDIATED EXPRESSION OF PRO-INFLAMMATORY CYTOKINES

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Sepsis is the leading cause of death in intensive care units, which reflects detrimental host response to infection where lipopolysaccharide (LPS) shared by Gram-negative bacteria acts as a potent activator of immune cells via Toll-like receptor 4 (TLR4). It is expressed in different cell types including myeloid cells, the key effectors of innate immune reactions. TLR4 downstream signalling leads to the expression of pro-inflammatory cytokines – TNF–α, IL-6 and IL-12. By now many aspects of TLR4 downstream signalling leading to TNF–α and ILs' production remain unclear. In this study we have found that LPS-induced TLR4 signalling triggers cross-talk of ASK1 downstream pathway and hypoxia inducible factor 1 alpha (HIF-1α) in THP-1 human myeloid cells. Both pathways are activated via redox-dependent mechanism associated with tyrosine kinase/phospholipase C-1γ-mediated activation of protein kinase C α/β that activates NADPH oxidase and therefore production of ROS that activate both HIF-1α and ASK1. ASK1 contributes to stabilisation of HIF-1α protein via activation of p38 MAP kinase that directly phosphorylates HIF-1α. Knockdown of HIF-1α in THP-1 cells with siRNA suggested that this protein supports TLR4-dependent production of pro-inflammatory cytokines – TNF–α and IL-6 by protecting the cells against depletion of ATP and therefore against death. On the other hand, the activity of ASK1 was observed to be controlled by PI 3 kinase pathway that protects the cells against pro-apoptotic action of ASK1. However, according to our recent experiments, ASK1 is involved in TLR4-mediated expression of pro-inflammatory cytokines not only through its cross-talk with HIF-1α protein, influencing other pathways as well.

EXPRESSION OF TNFALPHA-ACTIVATED NFKAPPAB-DEPENDENT GENES IS AFFECTED BY HYPERTHERMIA AND ACTIVE HSF1

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NFkappaB is a family of transcription factors that regulate numerous genes important for pathogen- or cytokine-induced inflammation, immune response and cell proliferation. NFκB also activates several genes that promote cell survival, which contributes to aggressive tumor growth and resistance to chemotherapy and radiation in cancer treatment. HSF1 is the primary transcription factor responsible for cellular response to different forms of stress (e.g., a heat shock), which upon stress-induced activation binds regulatory DNA elements, termed heat shock elements (HSE), present in promoters of heat shock proteins (HSPs) genes, and activates their expression. In general, HSPs function as molecular chaperones in regulation of cellular homeostasis and promoting survival. HSPs over-expression is frequently found in many types of cancer, and is usually associated with poor prognosis. On the other hand, however, hyperthermia is an adjuvant treatment used to sensitize cancer cells to radio- and chemotherapy, possibly affecting pathways that promote cell survival. Here we aimed to address possible mechanisms by which hyperthermia and HSF1-dependent signaling interfered with NFkappaB-dependent pathways. The U2OS osteosarcoma human cell line was used as an experimental model. The heat shock response was induced by mean of hyperthermia (incubation at 43oC for one hour). Alternatively, cells were transfected with mutated constitutively active HSF1 with deletions in regulatory domain (HSF1deltaRD) to activate HSF1-dependent signaling in the absence of the heat shock. Cells were incubated with TNFalpha cytokine to activate the NFkappaB pathway, and then expression of NFkappaB-regulated genes was assessed by RT-PCR. The activation of the NFkappaB signaling pathway was monitored by mean of degradation of IkappaBalpha inhibitor and appearance of active DNA-binding NFkappaB forms in nuclear extracts.

We have observed that TNFalpha-induced activation of NFkappaB was perturbed in cells subjected to hyperthermia, and four hours recovery in physiological temperature was necessary to allow full activation of NFkappaB. On the other hand, NFkappaB remained to be fully activatable by TNFalpha treatment in cells containing constitutively active mutated HSF1 at normal temperature. Interestingly, however, expression of several TNFalpha-activated and NFkappaB-dependent genes, including genes encoding TNFalpha and IL-6, was down-regulated in the presence of active HSF1. Our findings clearly indicates functional interference among hyperthermia, HSF1- and NFkappaB-dependent signaling pathways.

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ROLES OF THE TNF FAMILY IN INFECTIOUS DISEASES AND INTERRELATIONSHIP OF THE TNF FAMILY AND PPR-SIGNALING

PHOSPHATASE PTP1B NEGATIVELY REGULATES MYD88- AND TRIF-DEPENDENT PROINFLAMMATORY CYTOKINE AND TYPE I INTERFERON PRODUCTION IN TLR-TRIGGERED MACROPHAGES

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Toll-like receptors (TLRs) are primary sensors to detect conserved patterns on microorganisms, thus acting as the important components of innate immunity against invading pathogens. Protein tyrosine phosphatase-1B (PTP1B) has been shown to be a critical negative regulator of insulin pathway and other cellular signaling, however, whether and how PTP1B regulates TLR-triggered innate response remain to be investigated. We report here that PTP1B can markedly decrease TNF, IL-6 and type I IFN production by macrophages stimulated with LPS, CpG ODN, or Poly I:C. Accordingly, knockdown of endogenous PTP1B expression increases production of TNF, IL-6 and type I IFN in macrophages stimulated with TLR ligands. Phosphatase activity-disrupted mutant PTP1B cannot inhibit TLR-triggered production of proinflammatory cytokines and type I IFN, indicating PTP1B exerts its suppressive activity in phosphatase-dependent manner. PTP1B inhibits TLR ligands-induced activation of MAPKs, NF-KB, and IRF3, furthermore, cotransfection of PTP1B inhibits both MyD88- and TRIF-induced transcription of TNF and type I IFN reporter genes in a dose-dependent manner. In addition, PTP1B inhibits LPS-induced Tyk2 and STAT1 activation. Therefore, we demonstrate that phosphatase PTP1B is a physiological negative regulator of TLR signaling via suppression of both MyD88- and TRIF-dependent production of proinflammatory cytokine and type I IFN in macrophages. Our results provide new mechanistic explanation for negative regulation TLR response and suggest PTP1B as a potential target for the intervention of the inflammatory diseases.

SHP-2 PHOSPHATASE DIFFERENTLY REGULATES MYD88- AND TRIF-DEPENDENT SIGNAL TRANSDUCTION IN LPS RESPONSE

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Toll-like receptors (TLRs) activate expression of proinflammatory cytokine and type I interferon through MyD88- and TRIF-dependent pathways. By now, multiple regulators have been found to control its activation to a proper level, leading to elimination of invading pathogens while avoiding excessive inflammation. We previously reported that SHP-2, a widely expressed cytoplasmic tyrosine phosphatase, negatively regulated TRIF-dependent signaling. In this report, we demonstrate that SHP-2 enhances LPS-induced TNF-α in addition to its inhibitory effect on LPS-induced IFN-β production. Although SHP-2 inhibits ERK, JNK, and p38 MAPK activation in the late stage of TLR4 signaling, it increases LPS-induced IKK-α and NF-κB activation. While inhibits TRIF-induced TNF-α reporter gene expression, SHP-2 enhances MyD88-activated NF-κB and TNF-α reporter gene expression, demonstrating that SHP-2 increases TLR4-mediated TNF-α production by enhancing MyD88-depedent NF-κB activation. These results demonstrate that SHP-2 can differently regulate MyD88- and TRIF-dependent signal transduction.

CHARACTERISTICS AND FUNCTION OF ECTROMELIA GENE EVM008

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Poxvirus-encoded TNF receptor homologs block the activity of the pro-inflammatory cytokine TNF-alpha.The archetypal TNF receptor homolog T2 is encoded by Myxoma virus and is required for pathogenicity in rabbits. TNF receptor homologs have been found in other poxviruses, such as Cowpox, Vaccinia, Variola and Ectromelia viruses (EV, mouse pox). Cowpox virus encodes four viral TNFRs, known as cytokine response modifiers (Crm) B, C, D and E. CrmB and CrmD exhibit binding properties that are practically identical to T2. T2 and all four Crm proteins contain a N-terminal TNF receptor homology domain that is necessary and sufficient to bind and sequester TNF-alpha. The C-terminus of T2, CrmB, and CrmD contains no homology to cellular TNF receptors or other cellular proteins. This region functions as a chemokine-binding site, named smallpox virus-encoded chemokine receptor (SECRET) domain. Other SECRET

domain-containing proteins (SCP) were identified in certain strains of Cowpox, Vaccinia and Ectromelia viruses.
We identified an additional function encoded in the ectromelia gene EVM008, a SCP homolog. EVM008 interacts with all mouse FcgRs. In a genome-wide screen for secreted poxviral proteins, EVM008 was found to specifically bind mouse monocytes. This was a surprising finding given EVM008's presumed function of binding to chemokines. Immunoprecipitation and mass spectrometry with EVM008 indentified mouse FcgRs, CD64, CD32b, CD16 and CD16-2. These interactions were confirmed with recombinant proteins and cell lines engineered to express all different FcgRs. Surface plasmon resonance analysis revealed that EVM008 has low nonomolar to picomolar affinity for FcgRs. Functionally, EVM008 protein was able to compete with murine IgG2a for binding to mouse FcgR, CD32b.These results suggest that the C-terminal region contains host modulatory function in addition to its SECRET domain, which is to inhibit antibody-induced host immune response.

IMPORTANCE OF TRAF1 SIGNALING DOWNSTREAM OF 4-1BB IN MEDIATING CD8 T CELL SURVIVAL

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During an acute immune response, CD8 T cells undergo rapid expansion followed by a contraction phase during which the majority of activated T cells die, leaving a few survivors to persist as memory T cells. The regulation of T cell survival is critical at each stage of this response. We have shown that tumor necrosis factor receptor (TNFR) associated factor (TRAF) 1, a protein recruited to several costimulatory TNFR family members, is required for maximal CD8 T cell responses to influenza virus in mice. In vitro, TRAF1-deficient antigen-activated T cells accumulated higher levels of the pro-apoptotic BH3-only family member Bim. However, when the memory CD8 T cells were allowed to compete for survival signals in the absence of antigen in vivo, the TRAF1-deficient T cells showed decreased recovery compared to TRAF1-sufficient T cells. This defect in T cell recovery in vivo was alleviated by introduction of siRNA to down modulate Bim in TRAF1-deficient memory T cells. 4-1BB, a TNFR family member that recruits TRAF1, has been implicated in prolonging the survival of activated and memory CD8 T cells; however, the role of TRAF1 in 4-1BB signaling has not been elucidated. We demonstrate that TRAF1 plays a critical role in survival signaling downstream of 4-1BB during CD8 T cell expansion in response to viral infection in vivo. Further analysis reveals that TRAF1-deficient T cells show increased levels of Bim following 4-1BB signaling. TRAF1-deficient CD8 T cells fail to activate ERK in response to 4-1BB ligation and inhibition of ERK signaling downstream of 4-1BB in wild-type cells leads to

increased Bim levels. Thus, TRAF1 has a pro-survival effect in CD8 T cells via the 4-1BB-mediated ERK-dependent Bim downmodulation.
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THE ROLE OF 4-1BBL IN RESCUING FUNCTIONALLY IMPAIRED HIV-SPECIFIC CD8 T CELLS

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During chronic infection, HIV-specific CD8 T cells exhibit progressive signs of functional impairment, attributed to persistent antigenic stimulation, up-regulation of the inhibitory receptor PD-1, and declining T cell help. Strategies that directly improve CD8 T cell function offer the potential of restoring immune control of HIV. 4-1BBL is emerging as an important stimulator of antiviral CD8 T cell responses. Regardless of the PD-1 status of the donors, we have reported that 4-1BBL, when combined with CD80 or CD70, expands a population of Ag-specific CD8 T cells expressing multiple markers of effector function, from the functionally impaired starting population. In contrast, CD70 in combination with CD80 was insufficient for these effects. The unique contribution of 4-1BBL correlated with down-regulation of the proapoptotic molecule Bim in activated CD8 T cells. Decreasing the level of TRAF1 in T cells using siRNA resulted in increased levels of Bim in the 4-1BBL-stimulated CD8s. Thus, costimulation via 4-1BBL leads to TRAF1-dependent Bim down-modulation in T cells, resulting in the rescue of otherwise functionally impaired antigen-specific T cells in vitro. Work is in progress to investigate the role of the TRAF1-Bim-axis in the functional impairment of T cells in chronic progressors of HIV infected patients as compared to elite controllers directly ex vivo. TRAF1 has been shown to provide a crucial survival signal for memory T cells in mice via Bim modulation. We are currently investigating TRAF1 and Bim expression in virus-specific CD8 memory T cell populations from healthy and HIV infected subjects. These studies will provide further validation of TRAF1 expression as an important mediator of T cell survival and a potential correlate of CD8 responsiveness (This work is supported by grant number 74492 of the Canadian Institutes for Health research and by a scholarship from the Ontario HIV treatment network (to C. W)).

THE ROLE OF GITR IN CD8 T CELL RESPONSES TO INFLUENZA VIRUS

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Glucocorticoid-induced TNF receptor family-related gene (GITR) is a costimulatory member of the TNFR superfamily. GITR is present on naïve T cells and gets further upregulated upon T cell activation. Little is known about the role of GITR during an immune response to infection. To examine GITR's role on CD8 T cells during a viral infection, wildtype and GITR-/- TCR transgenic OT-I CD8 T cells were transferred to B6 recipients in ratios of 1:1. The next day mice were infected with a recombinant influenza virus that carries the OVA epitope recognized by OT-I, Influenza A/X31-OVA. At the peak of both the primary and secondary response to virus 2-4 fold fewer OT-I GITR-/- T cells were detected as compared to OT-I T cells in spleen, lung, mediastinal lymph node and bone marrow. The decreased numbers of OT-I GITR-/- cells were not due to differences in kinetics, homing or proliferation. Rather GITR appeared to be important for the survival of CD8 T cells. Systemic administration of agonisitic anti-GITR antibodies also enhanced influenza-specific CD8 T cell expansion. OT-I GITR-/- cells failed to respond to anti-GITR, indicating that the effect of antibody was intrinsic to the T cells. These results show that GITR is an important survival molecule on CD8 T cells during both primary and secondary antigen-specific expansion to infection.

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THE ROLE OF TRAF1 IN REGULATING TRAF2 STABILITY DOWNSTREAM OF 4-1BB SIGNALING

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The TNFR family member 4-1BB plays a critical role in the survival of CD8 memory T cells which are crucial for the development of a more efficient immune response upon re-exposure to a pathogen. 4-1BB recruits TRAF1 and TRAF2 to activate NF-kB and MAP kinase signaling pathways. Results from our lab have

shown that TRAF1 is critical in signaling downstream of 4-1BB for ERK dependent downregulation of the pro-apoptotic molecule Bim and prolonged memory T cell survival. It is unknown how TRAF1 regulates this signaling and here we show that TRAF1 is crucial for stabilizing TRAF2 downstream of 4-1BB signaling. In the absence of TRAF1, TRAF2 is rapidly degraded via the proteasome. A yeast two hybrid screen was performed to identify novel proteins involved in TRAF1's function. Among the interacting proteins identified were PSMC3, a regulatory subunit of the 26S proteasome. Preliminary co-immunoprecipitation experiments confirm that TRAF1 and PSMC3 interact together in mammalian cells. The interaction of PSMC3 with other proteins has been shown to specifically protect them from degradation. These results suggest that TRAF1 may have a critical role in dictating the magnitude and duration of the signaling downstream of 4-1BB by stabilizing TRAF2.

MOLECULAR CHARACTERIZATION OF CRMD, THE TNF AND CHEMOKINE DECOY RECEPTOR OF POXVIRUS

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CrmD is a soluble TNF decoy receptor encoded by Ectromelia virus, the causative agent of mousepox, and other members of Poxviridae viral family. CrmD is composed of two structurally and functionally independent domains, the N-terminal domain (NTD) that shares the classical structural features of the extracellular domain of TNF receptors composed of four cystein rich domains (CRDs), and the C-terminal domain (CTD) with no sequence similarity to any known cellular protein. We have shown the CTD is actually a chemokine-binding domain that can interact with a small set of chemokines with high affinity disrupting the leukocyte recruitment to sites of infection triggered by these chemokines. Thus, the CTD of CrmD named SECRET (smallpox virus encoded chemokine receptor) is a novel domain for chemokine binding.

CrmD is a crucial virulence factor for Ectromelia virus infection since CrmD-disrupted viruses revealed a loss of lethality of up to 6 logs compared to wild type viruses in mousepox susceptible mice. To study the role of each CrmD domains we have created different forms of CrmD that cannot bind TNF but still interact with chemokines. We have generated a collection of mutants in the NTD of CrmD and have identified two mutants disrupted for TNF binding but with the same affinity for chemokines as the wild type form of CrmD.

Up to date, despite the importance of TNF as a proinflammatory factor, there are few reports describing molecularly the binding of a member of TNF ligand superfamily (TNFLSF) to a TNF receptor. Therefore, these mutants can provide important

insights of how the TNF-CrmD interaction is established and also provide clues of how TNF interacts with cellular receptors.
We have also produced truncated forms of CrmD expressing only the SECRET domain that mantain high affinity for chemokines but are unable to bind TNF. These short versions of CrmD together to the mutant forms will help us to investigate in vivo the molecular mechanism used by CrmD to block both TNF and chemokines.

NOVEL ASPECTS OF IMMUNE REGULATION BY THE TNF FAMILY

STRUCTURAL AND FUNCTIONAL ANALYSIS OF DEATH RECEPTOR-6

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Death receptor-6 is a death domain containing receptor of the tumor necrosis factor-receptor superfamily that is apparently involved in the regulation of proliferation and differentiation of T- and B-lymphocytes. Cystein-rich domains of DR6 as well as its death domain and the C-terminal CARD-like region are highly conserved in vertebrates from zebrafish to humans. Overexpression of DR6 in some cell lines leads to apoptosis, to activation of NF-κB and stress kinases of JNK/SAPK family. DR6-knockout mice have expanded T-cell populations, increased secretion of Th2 cytokines in response to mitogens, and grafted DR6-/- HSC induce more severe graft-versus-host disease. Also DR6-/- B-cells show increased proliferation in response to stimulation. DR6 ligand has not been, in contrast to majority of other TNFR family members, yet identified.
We found that DR6 is an extensively posttranslationally modified protein including S-palmitoylation and both N- and O-glycosylation. Six N-glycosylation and one S-palmitoylation sites were precisely mapped to appropriate asparagines and cysteine respectively. The juxtaposed linker region (between cystein-rich domains and the transmembrane part), which also contains Ser/Thr/Pro-rich region with clustered putative O-glycosylation sites, is required for the plasma membrane localization of DR6. N-glycosylation, but interestingly not S-palmitoylation, apparently plays a role in targeting of DR6 into detergent-resistant glycosphingolipid-enriched microdomains.
We have also prepared a set of agonistic monoclonal antibodies to DR6 and used them for the analysis of DR6 signaling both in DR6-infected cell lines and in peripheral blood leukocytes, considering mainly the activation of NF-κB and JNK/AP1 pathways.

CHARACTERIZATION OF DR6 SIGNALING. CELL SPECIFICITY IN APOPTOSIS INDUCTION AND NF-κB ACTIVATION

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DR6 has been one of the last DRs to be identified. However 10 years after, its ligand is still unknown and its functional characterization is very weak. It was determined that this receptor induces apoptosis (though its induction depends on the cell line), and activates NF-κB and JNK. KO mice studies have shown that DR6, like other TNFRs, plays a role in the immune system, being important in the regulation of Th cells differentiation. This function is not related to the activation of the apoptotic pathway, but to JNK activation. Recently it has been described that DR6 is shed from the membrane by MMP14, which is often overexpressed on tumor cells. The extracellular region of DR6 released can modulate the response of the dendritic cells against the tumor, thus favoring the immune escape of the tumor.

We have studied the functionality of DR6 in two cell lines, one (HEK293) which is non transformed, while the other (HeLa) is a tumor cell line. The ectopic expression of DR6 induces JNK activation in both cell lines, but it is able to induce apoptosis and NF-κB activation only in HeLa cells. We have previously observed that ectopically overexpressed DR6 is released from the membrane of HEK293 cells. To check whether the shedding is responsible for the lack of NF-κB activation, we generated a DR6 deletion mutant in which the region comprising from the last CRD to the transmembrane domain is missing. This receptor mutant is functional and is not shed. However, is still unable to induce apoptosis and NF-κB activation, ruling out that shedding of the receptor is a mechanism to regulate these two signaling pathways in HEK293 cells.

It is conceivable that the different behavior of HEK293 and HeLa cells as for DR6-mediated apoptosis and NF-κB activation is due to the fact that these two pathways are dependent on intermediary proteins which are defective or not expressed in HEK293

ESSENTIAL ROLE OF TNFRSF25/TL1A IN THE DEVELOPMENT OF ALLERGIC LUNG INFLAMMATION AND INFLAMMATORY BOWEL DISEASE

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We identify the TNFRSF25/TNFSF15 pair as critical trigger for allergic lung inflammation, a cardinal feature of asthma. TNFRSF25 (TNFR25) signals are required to exert Th2 effector function in Th2 polarized CD4 cells and to costimulate IL-13 production by glycosphingolipid activated NKT cells. In vivo antibody blockade of TNFSF15 (TL1A), the ligand for TNFR25, inhibits production of Th2 cytokines including IL-13 and lung inflammation even when administered days after airway antigen exposure. Similarly, blockade of TNFR25 by a dominant negative transgene, DN TNFR25, confers resistance to lung inflammation in mice. Allergic lung inflammation-resistant NKT deficient mice become susceptible upon adoptive transfer of w.t. NKT cells, but not after transfer of DN TNFR25 transgenic NKT cells. The TNFR25/TL1A pair appears to provide the earliest signal for Th2 cytokine production in the lung and therefore may be a valuable drug target in attempts to attenuate lung inflammation in asthmatics.
In studies in inflammatory bowel disease using the SCID/RAG model for disease induction, antagonistic anti TL1A administered in vivo completely blocks the pathogenicity of adoptively transferred CD45RBhigh cells. TNFR25 signals serve as costimuli for CD4 effector cells and as inhibitors for the suppressive activity of T regulatory cells. Blockade of TL1A thus has a dual effect on the molecular and cellular pathway of disease pathogenesis.

APRIL (TNFSF13), A SIGNIFICANT IMMUNE MODULATOR IN A MODEL OF RHEUMATOID ARTHRITIS, IN ALLERGIC LUNG INFLAMMATION AND IN ORAL/MUCOSAL IMMUNITY

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APRIL (TNFSF13) and BAFF (TNFSF13B) share receptors on B and T cells. Although BAFF has been implicated in autoimmune arthritis and other autoimmune diseases, the role of APRIL has been less well defined. Here we show that in newly generated APRIL-/- mice the incidence of autoimmune rheumatoid arthritis induced by chicken type II collagen is significantly reduced. In the absence of

APRIL the Th2/Th1 antibody ratio is increased, IL-13 production enhanced and IL-17 production diminished while T cell proliferation is increased. APRIL therefore supports Th1 and IL-17 responses while suppressing production of IL-13 and other Th2 cytokines. Blockade of APRIL may be a valuable adjunct in the treatment of rheumatoid arthritis.
The suppression of Th2 responses and of IL-13 production by April suggests that Th2 type diseases such as asthma may be increased in APRIL-/- mice. Indeed,lung inflammation in the ovalbumin model of asthma was significantly exaggerated in APRIL-/- mice in comparison to wild type litter mate controls.
APRIL also is known to regulate IgA antibody levels. We demonstrate that April deficiency has strong effects antigen specific isotype levels following oral immunization.

CONTROL OF LYMPHOCYTE FUNCTION AND REPERTOIRE BY THE TNF FAMILY

ROLES OF NIK IN THYMIC EPITHELIAL CELLS FOR THE ESTABLISHMENT OF CENTRAL TOLERANCE

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Thymic epithelial cells in the medulla (mTECs) expressing wide varieties of tissue-restricted self-antigens (TRAs) play pivotal roles in establishing self-tolerance through critical dialogue with developing thymocytes. We have previously demonstrated unique actions of Nik together with Ikk-alpha for the establishment of central tolerance by studies using a strain of mouse bearing a natural mutation of the Nik gene (aly mice) and Ikka-deficient mice, respectively; grafting embryonic thymi from both strains onto athymic nude mice manifest organ-specific autoimmunity in the recipient mice. Although reduced expression of TRA genes together with structural abnormalities of mTECs including the lack of Aire-positive cells are common features of both strains, exact roles of these NF-kB activating factors in establishing central tolerance still remain elusive. In the present study, we demonstrate that Nik-mutant aly mice have the defect in the production of regulatory T-cells, which is due to the lack of CD80-high mTECs with the use of thymus graft experiment. Interestingly, levels of CD80 expression from CD11c-positive thymic DCs from aly mice were also reduced, although CD80 expression levels from splenic DCs were retained, suggesting that reduced CD80 expression from thymic DCs in aly mice is organ-specific. The results suggest that Nik plays important roles for organizing the thymic microenvironment, thereby contributing to the prevention of autoimmunity. Because lymphotoxin-beta receptor (Ltbr)/Cd40-double

deficient mice showed less severe thymic phenotypes compared with those from Nik-mutant mice, Nik-dependent thymic organogenesis appears to be mediated through receptors beyond LTbR and CD40.

LYMHOTOXIN-B-RECEPTOR SIGNALING IS REQUIRED TO LICENSE DC FOR CROSS-PRIMING

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Early during the initiation of an immune response, dendritic cells (DC) interact closely with CD4+ T cells, during which time signals are delivered from the activated antigen (Ag)-specific T cell to the DC, many in the form of tumour necrosis factor (TNF) superfamily ligand-receptor interactions. These signals are critical for the maturation, function and survival of DC, and thereby dictate the capacity of DC to prime a robust CD4+ T cell response. Among these instructive cues, activated CD4+ T cell expressed CD40L interaction with DC-expressed CD40 is required to license DC for cross-presentation of exogenously acquired Ag for priming help-dependent CD8+ T cell responses.

We have previously demonstrated a requirement for Ag-specific CD4+ T cell-expressed lymphotoxin (LT)ab for DC stimulatory function and optimal CD4+ T cell priming, suggesting the involvement of an additional TNF superfamily receptor-ligand pair, LTab-LTbR in licensing DC for cross-priming CD8+ T cell responses. Confounding this conclusion is the expression of LTbR on lymphoid stromal cells. Using a LTbR signaling inhibitor, bone marrow chimeras and adoptive transfers, we selectively removed expression of LTab or LTbR from Ag-specific CD4+ T cells or DC, respectively, to identify a DC-intrinsic requirement for LTbR signaling for maturation, licensing and T cell priming.

Specifically, absence of the LTab-LTbR licensing signal results in delayed CD8+ T cell proliferation, defective CD25 upregulation, and failure of the memory CD8+ T cell pool to persist and expand. DC denied the LTbR licensing signal were impaired in CD86 upregulation, and CD86-/- DC recapitulated the stimulatory defect observed in the absence of DC-intrinsic LTbR signaling. We conclude that DC-intrinsic LTbR signaling via Ag-specific CD4+ T cell-derived LTab is required to license DC for cross-priming of CD8+ T cell activation and proliferation and for programming CD8+ T cells for persistent memory.

LYMPHOID TISSUE INDUCER CELLS CONTROL CD8A-DENDRITIC CELL HOMEOSTASIS VIA THE LYMPHOTOXIN-BETA RECEPTOR PATHWAY

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Antigen-presenting CD8alpha- dendritic cells (DC) proliferate within lymphoid tissues under control of the Lymphotoxin-beta receptor (LTbetaR), however the cellular networks providing the trophic signals remain unknown. We show that LTbeta-deficient T or B lymphocytes or RAG-/- mice exhibited a normal profile of splenic DC subsets. However, RAG mice lacking the cytokine receptor-gammac (RAGgammac-/-) exhibited a specific decrease in the CD8alpha- DC subsets, phenocopying LT-deficient mice, implicating their participation in a common pathway controlling DC homeostasis. Signaling by gammac, but not LTbetaR, was required for integrity of the interleukin (IL)-7 receptor alpha (IL-7Ralpha)-expressing LTi cell population in the spleen. In contrast, enforced LTbetaR signaling with an agonist antibody in RAGgammac-/- mice restored the proliferative capacity of CD8alpha- DC subsets, indicating gammac-deficiency impacts LTi cells expressing LTalphabeta. LTbetaR-signaling further induced clustering of CD4+ DC around the spleen central arteriole, wherein LTi cells reside in intimate contact with DC, revealing a cellular network controlling DC homeostasis. Blockade of IL-7Ralpha signaling in RAG mice impacts DC-LTi clustering which specifically affects CD8alpha- DC subsets, similarly to LTbetaR-deficient RAG mice. Together, using both genetic and pharmacological approaches, we demonstrate the CD4+IL7R+CD3-CD11c- lymphoid tissue inducer (LTi) cells control the homeostasis of CD8alpha- DC subsets through the LTbetaR pathway.

ROLE OF TNFRI AS A COSTIMULATORY MOLECULE DURING THE EARLY PHASE OF TCR STIMULATION IN CD3 T LYMPHOCYTES

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Several members of the TNFR superfamily deliver costimulatory signals to T cells and this has reshaped the classical "two-signal" (TCR plus CD28) model of T cell activation. In this study the contribution of TNFRI to the triggering of T cell immune responses was examined. TNFRI deficient (TNFRI KO) CD3+-enriched T cells showed a significant delay in cell cycle kinetics, compared to wild-type (WT) cells. This delay was not associated with alterations in the cell surface expression of the TCR complex, apoptosis or activation markers (CD25, CD69) and could not be compensated by CD28 costimulation. Interestingly, the upregulation of CD25 expression was also delayed in TNFRI KO CD3 T cells after TCR stimulation and regulation of IL-2 receptor expression may be one mechanism by which TNFRI contributes to early T cell proliferation. A comparison of the activation of downstream MAPK, NF-κB and NFAT signaling pathways following TCR stimulation did not reveal any differences between the two cell types. Similarly, the expression kinetics of other co-stimulatory molecules (OX-40, ICOS, TNFRII) was similar, except for a delay in OX-40 upregulation in TNFRI KO cells. To investigate whether our in vitro findings could have in vivo consequences, we challenged TNFRI KO and WT mice with oxazolone, which was used to generate an acute contact hypersensitivity T cell reaction. Ear swelling in oxazolone-sensitized mice was significantly reduced in TNFRI KO compared to WT groups indicating that TNFRI KO T cells are less effective in priming a robust and persistent allergenic reaction. Overall our results suggest that the TNFRI acts as a positive costimulator of T cell responses in vitro and in vivo and we are currently working to define the mechanism involved and its consequences for T cell differentiation.

LYMPHOTOXIN–β RECEPTOR SIGNALING SUPPORTS A UNIQUE STROMAL CELL NICHE THAT SUPPORTS IGA CLASS SWITCH RECOMBINATION IN THE INTESTINAL LAMINA PROPRIA

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The intestinal Lamina Propria is an important site for the production of IgA that protects against mucosal infections and controls the growth of commensal bacteria. The Lymphotoxin (LT) pathway has been shown to be critical for IgA production in mucosal tissues, however its precise contribution to this process remains obscure. We have confirmed that Lymphotoxin-beta receptor (LTβR) signals are required in the non-hematopoietic compartment for IgA production, and here we have shown that these signals are not required in radio-resistant gut epithelial cells. By applying different stromal cell markers, we have identified a unique $BP3^+$ stromal cell niche within the small intestinal lamina propria that absolutely requires LTβR-derived signals. This stromal cell niche is the site of CCL2 production as well as a nexus for RORγt-expressing LTi-like cells, AID^+ B cells and other IgA-promoting cytokines such as TNFα and iNOS. In addition, we have observed a strong dependence on CCR2 for the presence of LTi-like cells in the intestinal lamina propria. As $Rorc(gt)^{gfp/gfp}$ mice that lack LTi-like cells exhibit a decrease in frequency of IgA^+ B cells, we hypothesize that the CCL2/CCR2 axis is a means for recruiting LTi-like cells to this niche to maintain a microenvironment that promotes IgA class switch recombination.

FUNCTIONAL CHARACTERIZATION OF CHIMERICAL RECEPTORS RANK-TNFR2 AND ANALYSIS OF THEIR INTERACTION WITH ADAPTOR PROTEINS TRAF

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TNFR2, one of the two known receptors of TNF, is only able to interact with the transmembrane form of the ligand. With the aim of studying its signalling independently from TNFR1, we have generated chimerical receptors in which the extracellular region of TNFR2 has been replaced with the extracellular region of RANK (RANKec-TNFR2). We have also generated several chimerical receptors with some deletions in their intracellular region, so that one or both TRAF2 interactions sites of TNFR2 are deleted. All chimerical and mutants receptors have been cloned in the tagged expression vector pCMV1-Flag.
The chimerical receptors RANK-TNFR2 are completely functional as compared to wild type TNFR2 as they are unable to trigger apoptosis by themselves when ectopically overexpressed. However, they activate NF-κB and JNK and are able to interact with the adaptor proteins TRAF1, TRAF2 and TRAF3. Through an in vitro assay with fusion proteins containing the intracellular region of TNFR2 fused to GST, we show that whereas the interaction with TRAF2 and TRAF3 appears to be direct, the interaction with TRAF1 is indirect and dependent on the previous interaction of TRAF3 with the receptor. The interaction of TRAF1 and TRAF3 with the receptor appears to occur through any of the two TRAF2 binding sites of TNFR2. We also show that overexpression of TRAF1 and TRAF3 originates an inhibitory effect over the ability of RANKec-TNFR2 to activate both NF-κB and JNK, provided that the chimerical receptors include at least one of the two TRAF2 binding sites. As for NF-κB, we show that the inhibitory effect of TRAF1 is stronger than that of TRAF3.

TNF RECEPTOR ASSOCIATED FACTOR 1 (TRAF1) MODIFIES CRYSTALLINE SILICA INDUCED LUNG INJURY

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The TNF receptor associated factor (TRAF) family of proteins link specific members of the TNF receptor family with secondary signal transduction proteins. Activity of the TNF-α receptors are modified by recruitment of TRAF1 and TRAF2. TRAF1 is unique in its restriction to specific cells of lung, testis and spleen. It is markedly induced by TNF in lung epithelial cells and macrophages, and it is

suggested to be a negative regulator of TNF cytotoxicity. We recently demonstrated increased intratracheal TNF-α induced lung and liver injury in transgenic TRAF1 null mice, marked by elevated chemokine production in lung tissue and BAL as well as marked release of liver enzymes with gross hepatic cell death. In order to determine what role TRAF1 might play in a model of TNF-α mediated chronic lung injury, C57BL/6 TRAF1-/- and WT controls were treated with crystalline silica (intratracheal, 200-400μg in 50μl saline) and harvested 28 days later. In response to silica, in comparison to WT, chemokine mRNA levels were suppressed in the TRAF1-/- mice at 28 days. TUNEL assay and pro-surfactant protein B IFC demonstrated large numbers of TUNEL positive nuclei, including SP-B positive cells. The total number of cells isolated in bronchoalveolar lavage was decreased, and although the percentage of lavaged cells that were neutrophils was increased, the number of PMNs in BALF did not differ. The number of lavagable alveolar macrophages, however, was significantly reduced in the TRAF1 null mice. Though TRAF1 appeared protective in an exogenous TNF-α induced injury, it may contribute to lung injury in the setting of silicosis. We also propose that enhanced apoptosis in the absence of TRAF1, both in leukocytes and parenchymal cells limits the inflammatory response.

TRAF1 REGULATES TWEAK AND LTAB2-INDUCED NFKB SIGNALLING

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The Tumor Necrosis Factor (TNF) Receptor Associated Factor (TRAF) proteins are important mediators of TNF receptor signaling. While TRAF2 has been implicated in the activation of the classical and alternative NFκB signaling pathway by TNF receptors, the function of TRAF1 is rather unknown. TRAF1 is regulated by NFκB and is typically not expressed in non-stimulated cells. To investigate the function of TRAF1 in TNF receptor signaling, we therefore ectopically expressed TRAF1 in cancer cell lines. To rule out that TRAF1-related effects are caused by non-physiological TRAF1 expression levels, we selected for clones having TRAF1 expression levels that were comparable to those induced in the corresponding parental cell lines by TNF. Using these clones, we found that TRAF1 inhibits LTαβ2- and TWEAK-induced translocation of TRAF2 into a Triton X 100 insoluble compartment. This correlated with enhanced activation of the classical NFκB pathway by the LTαβ2 and TWEAK. In contrast, TNFR1, which does not induce TRAF2 depletion, signals activation of the canonical NFκB pathway with comparable efficiencies in the presence and absence of TRAF1. We conclude that the ratio of TRAF1/TRAF2 is a crucial factor, which determines the capability of TRAF2-degrading TNF receptors to stimulate the classical NFκB pathway.

MECHANISMS OF PATHOLOGICAL & THERAPEUTIC EFFECTS OF THE TNF FAMILY

TRAIL INDUCED CELL DEATH IN COLON CARCINOMA CELLS IN VIVO AND IN VITRO: SENSITISATION BY ONCOGENES AND BY OTHER THERAPEUTICS

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Most data on the therapeutic potential and expression of TRAIL in colorectal cancer has come from *in vitro* studies using tumour cell lines. To gain a clearer understanding about the susceptibility of patient tumours to TRAIL, we derived primary human cancer epithelial cells (1). Increased apoptosis was observed in both primary PAP60 and MIH55 after treatment with SuperKiller TRAIL. Treating patient tumour xenograft/ SCID mouse models with Killer TRAIL *in vivo* for 5 consecutive days suppressed tumour growth, although less efficiently compared to *in vitro* experiments.The previous *in vivo* finding showed that evading apoptosis induced by anticancer drugs characterizes many types of cancers. This results in the need for combination therapy. We have previously shown that quercetin induces the degradation of Ras oncoproteins (2). We have provided evidence that quercetin enhanced TRAIL induced apoptosis by causing the redistribution of DR4 and DR5 into lipid rafts (3). Based on these results, this study provides a challenging approach to enhance the efficiency of TRAIL-based therapies (4). Oncogene-specific sensitization to TRAIL induced apoptosis has been previously shown by our lab (5) and by others. Here, we will present data by using oncogene specific models, that mutant oncoproteins can sensitise cells to TRAIL induced cell death, in specific synergy with targeted therapeutics.

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RELATION OF PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINES WITH MEDIATORS OF BONE REMODELATING SYSTEM IN PSORIATIC ARTHRITIS

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We determined pro-inflammatory (IL-1, IL-6, TNF-α) and anti-inflammatory (IL-4, IL-13) cytokines in serum and synovial articular fluid at 27(15=m,12=f) patients with psoriatic arthritis(PA) (CASPER CRITERIA).The age ranged between 27-50 years. Normal subjects were 20 volunteers aged 30years with no forms of joint infectious,neoplastic or autoimmune diseases. The cytokines and mediators of bone remodelation (osteoprotegerin-OPG-, RANKL/RANK) were determined by the immunoenzimatique ELISA sandwich technique.
The serum levels were:IL-1(between 2.67 and 6.99 pg/ml;mean range of 4.6±2.45 pg/ml;),IL-6 (between 6.5 and 15.98 pg/ml; mean range of 11.25±4.75 pg/ml) and TNF-α between 2.05 and 4.95 pg/ml;mean range 3.5±1.45 pg/ml.The level of pro-inflammatory cytokines in the articular synovial fluid was: IL-1 between 2.34 and 7.26 pg/ml (mean range 4.8±2.46 pg/ml), IL-6 between 11.1 and 31.7 pg/ml (mean range 21.4±10.3 pg/ml) and TNF-α between 5.75 and 15.25 pg/ml (mean range 10.5±4.75 pg/ml).The seric level of IL-4 was undetectable in majority of patients (n=20);values between 0.2 pg/ml and1.4 pg/ml, and in the articular synovial fluid values between 0.20 and 0.50 pg/ml were detected. The seric level of IL-13 was 2.67 and 4.99 pg/ml (mean range 3.22±1.45 pg/ml), and in the synovial articular fluid values of 2.07 and 14.8 pg/ml (mean range 5.8±0.45 pg/ml).The PA study group had seric values of sRANKL,marker of bone resorbtion, ranged between 36.97 and 66.12 pg/ml (mean range 51.98±10.74 pg/ml).The values in the synovial articular fluid were between 50.23 and 68.35 pg/ml(mean range 60.05±2.98 pg/ml).Patients with PA had seric values of OPG, marker of osteosynthesis, between 28.65 and 35.79 pg/ml(mean range 31.69±1.39 pg/ml); in the synovial articular fluid the values of OPG were between 219.89 and 249.46 ph/ml (mean range 235.12±8.79 pg/ml).The values of sRANKL were in a good positive correlation (rC = 0.57, RC2= 0.3292 in respect to TNF-α) with the seric values of pro-inflammatory cytokines. While TNF-α was not regularly detected in PA patients but in cases with major articular involvement. The seric values of OPG were in a good negative correlation (rC= 0.57, RC2= 0.3294 in respect to TNF-α) with the seric value of pro-inflammatory cytokines.Our study

showed that seric levels of OPG were in a strong negative relation (r= -0.87, R2= 0.7718) to that of sRANKL in PA patients.The low seric levels of anti-inflammatory cytokines (Il-4, Il-13) in PA suggested a deficit of activation/production though there were indication of a local synovial production of Il-13.
The values of RANKL in the synovial articular fluid higher than in blood is a good marker of PA in respect to bone activity as well as a criteria to select patients which would evolve to severe bone erosions and would benefit from a specific therapy in time.

THE REGULATION OF THE AMOUNT OF EXTRA CELLULAR DNA FIBRES BY THE TNF SUPER FAMILY

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It has been demonstrated that collagen fibres play an important role in the process of the cell assembling in order to form tissues, this mechanism is due to the adhesive characteristic of those fibres from witch has been explained their involvement in the platelets aggregation after a vascular breach. However, it is known that DNA fibres are strongly anionic witch makes them able to adhere to the surrounding cells after their liberation, this data allows us to hypothesise that nucleic acids are also involved in the cell assembling and in the platelets aggregation, therefore the aim of this work is to give a hence insight into the physiological consequences induced by the release of nucleic acids after apoptosis.

The experiences stated in this paper demonstrate that DNA has several similarities with collagen including:
1. The existence as long and firm fibres in an aqueous medium.
2. Abundance in the interstitial fluids.
3. Adhesive characteristic.
4. Platelets aggregation in the bloodstream.
Further, this article proves that DNA fibres participate in the pigmentation of tissues by adsorbing different pigments coming from the metabolism of haemoglobin; witch might provide a better understanding into the pigmentation of the skin and the hair.
The insubordination of cells to apoptosis, that is described in the common definition of cancer, leads to the decrease of the extra cellular DNA fibres amount that are essential for the cell assembling, in this case, the cancerous cells will be free to migrate and proliferate throughout the human body. This concept suggests that DNA fibres are of big importance in the retaining of the cancerous cells into one single place so that they might be submitted to apoptosis. On the other hand, as TNF family has been reported as inflammatory, pro-coagulant and anti-cancerous it would be logical to state that TNF enhances the amount of DNA fibres by activating

apoptosis and therefore it is necessary to study the metabolism of TNF family and their interactions with different molecules in order to bring new medical approaches regarding the disorders related to the increase or the decrease of extra cellular DNA fibres, for instance, the swelling of inflammatory regions is due to the accumulation of DNA fibres caused by the exaggeration of necrosis witch is why anti-TNF drugs are beneficial in the treatments of inflammatory disorders such as polyarthritis.

INDUCTION OF PRIMARY SCLEROSING CHOLANGITIS-LIKE DISEASE BY DR5-MEDIATED APOPTOSIS

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It is still controversial whether tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or anti-TRAIL receptor (DR5 or DR4) monoclonal antibodies (mAbs) induce toxicity in vivo. Here, we show that agonistic anti-DR5 monoclonal antibody (mAb) treatment triggered cholangiocyte apoptosis and subsequently induced cholangitis and cholestatic liver injury in C57BL/6 (B6) mice, but not BALB/c mice. Cholangiocyte isolate from B6 mice were sensitive to TRAIL/DR5-mediated killing, however hepatocytes from each strain and BALB/c cholangiocyte were resistant in vitro. Notably, anti-DR5 mAb-induced cholangitis exhibited the typical "onion-skin" histological appearance reminiscent of human primary sclerosing cholangitis (PSC). Human cholangiocytes constitutively expressed DR5, and intriguingly TRAIL expression and apoptosis were significantly elevated in cholangiocytes of human PSC and primary biliary cirrhosis (PBC) patients. Thus TRAIL/DR5-mediated apoptosis may substantially contribute to chronic cholestatic disease, particularly PSC.
Collaborators: Kenichi Ikejima, Shunhei Yamashina, Kyoko Okumura, Tomonori Aoyama, and Nobuhiro Sato (Dept. of Gastroenterology, Juntendo Univ), Kenichi Harada, Hiroko Ikeda, and Yasuni Nakanuma (Human Pathology, Kanazawa Univ.)

LUPUS SERUM INDUCES SKIN INFLAMMATION THROUGH TNFR1 SIGNALING PATHWAY

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by high levels of autoantibody and multi-organ tissue damage including kidney and skin. Skin manifestations are frequent in patients with SLE and because of limited understanding of the involved pathogenic mechanisms there is no specific treatment. Skin injury is also frequent in the lupus-prone MRL/lpr mice. We investigated whether lupus serum containing high levels of immunoglobulin can cause skin injury. We found that serum from patients and mice with lupus induced skin inflammation following intradermal injection in normal mice. Depletion of immunoglobulin G in lupus serum abrogated the ability of lupus serum to induce skin inflammation.

Immunohistochemistry demonstrated that tumor necrosis factor receptor (TNFR) 1 but not TNFR2 was expressed in the skin lesions caused by injected lupus serum. The expression of NF-kB, iNOS and MCP-1 were also markedly increased in the induced skin lesions. The severity and incidence of lupus serum-induced skin inflammation were significantly decreased in TNF-a-deficient mice. Skin inflammation induced by lupus serum was abrogated in TNFR1-deficient mice. In contrast TNFR2 or IL-1R-deficient mice developed skin lesions comparable to those observed in normal mice injected with the same lupus sera. Our studies demonstrate that lupus serum causes skin injury and TNFR1 exerts a crucial role in the pathogenesis of lupus serum-induced skin injury by engaging TNFR1 but not TNFR2 or IL-1R. Therefore, disruption of TNFR1-mediated signaling process may be of therapeutic value in patients suffering of skin in SLE.

CD8 T CELL-MEDIATED IMMUNE RESPONSES AGAINST CROSS-PRESENTING LIVER SINUSOIDAL ENDOTHELIAL CELLS LEADS TO HEPATITIS

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Virus-specific CD8 T cell responses are considered to be targeted directly against virus-infected hepatocytes leading to development of hepatitis. However, hepatocytes are separated from circulating leukocytes by liver sinusoidal endothelial cells (LSEC) that are capable to cross-present antigen to CD8 T cells. After hepatocellular infection with recombinant OVA-expressing adenovirus and adoptive transfer of OVA-specific CTLs we observed that the OVA-specific CTL response caused strong ALT elevation and leukocyte accumulation. Importantly, using a tie2-H2-Kb transgenic mouse, where antigen is solely presented in the context of H2-Kb on LSEC but not on hepatocytes, we also detected liver damage after infection with AdOVA and adoptive transfer of OVA-specific CTLs. In contrast, presentation of antigen only on hepatocytes in CRP-H2-Kb transgenic mice did not lead to comparable ALT elevation. Antigen-recognition on LSEC was rapidly followed by elimination of this cell population in vitro and in vivo, as documented by ultrastructural analysis. Sinusoidal platelet accumulation was observed, but sinusoidal thrombosis was not responsible for liver damage, but rather expression of the T cell effector cytokine TNFa. Although liver damage was mediated by TNFa that was released from effector CD8 T cells stimulated by LSEC cross-presenting antigens from virus-infected hepatocytes, cross-presentation of circulating antigens by LSEC or application of TNFa alone did not suffice to induce liver damage. Importantly, viral infection rendered hepatocytes sensitive to the pro-apoptotic effect of TNFa through upregulation of the pro-apoptotic molecule Bim. Taken together, we provide evidence that cross-presentation of antigens from virus-infected hepatocytes initiates CD8 T cell-mediated hepatitis, which is caused by T cell-derived TNFa and requires licensing of hepatocytes by virus-infection.

FUNCTIONAL CONSEQUENCES OF MUTATIONS IN TNFRSF1A, COMPARISON OF IN VITRO CELLULAR EVENTS TO FINDINGS FROM TISSUE SAMPLES ISOLATED FROM PATIENTS WITH TUMOUR NECROSIS FACTOR RECEPTOR-ASSOCIATED PERIODIC SYNDROME (TRAPS)

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Mutations in TNFRSF1A are associated with a rare autosomal dominant condition known as TRAPS, and have been identified in families worldwide. TRAPS is associated with recurrent fevers and abdominal pain, +/- signs and symptoms of centrifugal rashes, myalgias, arthritis, periorbital oedema, erythema and amyloidosis. Onset is usually in infancy but may present in middle age. Corticosteroids and anti-TNF therapy have variable efficacy. Our study focuses on TNFR1 expression and trafficking associated with TRAPS mutations by expressing recombinant full length wild type (WT) and mutant forms in mammalian cell lines (HEK 293T) and investigating associated intracellular events. Our findings are compared to results from patients' samples.

WT and mutant TNFR1 constructs were cloned and expressed in HEK 293T cells under doxycycline-induced regulation. Expression levels were monitored by Western blotting and FACS analysis. Receptor levels were measured by a combination of immunoprecipitation, Western blotting and FACS. Downstream signalling of TNFR1 was investigated by studying NF-kB activation, apoptosis and cytokine release.

An antibody against the splice mutant variant (C158delinsYERSSPEAKPSPHPRG) has been developed and tested in functional assays to distinguish between WT and mutated receptor. We will present data correlating in vitro findings to results from patients' samples.

TNFR1 mutations are associated with increased NF-kB activation and impaired receptor shedding in some patients. Additional mutant forms in this study enable us to look at a variety of mutations to gain further insights into the pathophysiology of TRAPS. Further studies will investigate intracellular signalling downstream of mutant TNFR1 in patients and healthy controls.

SIGNALING PATHWAYS OF MUTANT TUMOUR NECROSIS FACTOR RECEPTOR 1(TNFR1)

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Tumour Necrosis Factor Receptor Associated Periodic Syndrome (TRAPS) is an autoinflammatory disease which is associated with autosomal dominant mutations in the gene encoding TNFRSF1A. To explore the mechanisms underlying inflammation associated with mutant TNFR1, we investigated associated signaling pathways in cells carrying mutant forms of TNFR1 in comparison with wild type. SK-HEP1 cells were transformed with either wild type (WT) or different mutant of TNFR1(C33Y, R92Q or T50M). Perturbation of phosphorylation was detected in cells carrying mutant receptor: AKT phosphorylation at Thr308 and serine473, were enhanced in cells carrying the C33Y mutation, relative to WT and other mutant types. Phosphorylation of P38, C-raf and Gsk were reduced in C33Y transfected cells.

EFFECT OF INFLIXIMAB TREATMENT ON CIRCULATING MONOCYTES IN RHEUMATOID ARTHRITIS PATIENTS: DIFFERENCES BETWEEN EARLY AND LATE DISEASE

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TNF plays a key role in the pathogenesis of RA and TNF blockade using infliximab (IFX) has profound therapeutic effects, which differ between early and late disease. However, the molecular mechanisms of action of IFX remain unclear, particularly regarding differential effects at different stages of RA. This study investigated the effects of IFX on levels of circulating leucocytes in RA patients, with both early and late disease.

The two RA cohorts comprised late RA patients (failed 2 DMARDs, n=16), and a DMARD naïve early RA cohort (randomised to IFX (n=12) or steroid (n=16) infusions with concurrent methotrexate therapy). Cells were stained with phenotypic markers, including CD3, CD4, CD14, CD16, CD25 and FoxP3, and samples were analysed at baseline and week 2, using 4 colour flow cytometry with inclusion of counting beads.

The absolute number of CD14low monocytes was significantly higher at baseline in late compared with early RA ($p = 0.0001$), whereas increased CD16+ granulocyte ($p < 0.0001$) and CD4+CD25+FoxP3+ T reg ($p = 0.04$) numbers were observed in early RA. Both CD14high and CD14low monocyte counts were depleted by approx 30% at week 2 in IFX-treated late RA patients, but not in early RA patients, irrespective of therapy.>
Significant differences in circulating leucocyte counts were observed between early and late RA. Furthermore, IFX-induced monocyte depletion, at week 2, was only observed in late disease. These data suggest that mechanisms of action of IFX may differ in RA patients at different stages of disease and may be relevant to different responses observed at these disease stages.

APRIL EXPRESSION AND TARGETTING IN AUTOIMMUNE DISEASES

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Rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and multiple sclerosis (MS) are autoimmune diseases in which B cells and auto-antibodies have been implicated. This explains the recent clinical test of a soluble form of the transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI, TNFRSF-13b) (TACI-Ig) in these pathologies. TACI-Ig is a decoy for the B-cell activation factor (BAFF, TNFSF-13b) and a proliferation inducing ligand (APRIL, TNFSF-13). Own to this antagonism activity, TACI-Ig treatment is inducing a severe depletion of mature B cells (BAFF antagonism) as well as blocking the generation of new Ig switch and plasma-cell survival (BAFF and APRIL antagonism). One may think that APRIL without BAFF antagonism may be more selectively targeting pathological auto-antibody producing plasma cells. To test this hypothesis, we have first studied APRIL expression in lesions from RA, SLE and MS patients. APRIL expression is upregulated in all these lesions compared to healthy tissues due to the ongoing inflammatory reaction and the local retention of secreted APRIL by tissue heparan sulfate proteoglycan (HSPG). This creates similar survival niches for plasma cells that we recently identified in the mucosa. The recent generation of an antagonist monoclonal antibody against murine APRIL allowed us to further test whether APRIL targeting may help ameliorating RA and SLE. Preliminary experiments indicate that indeed APRIL targeting reduce significantly the incidence of these two diseases in animal models, and experiments are ongoing to characterize the pathological events that are affected by APRIL targeting.
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TNFα SHEDDING AND EPIDERMAL INFLAMMATION IS CONTROLLED BY JUN/AP-1

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Inducible, epidermal deletion of JunB and c-Jun in adult mice causes a Psoriasis-like disease. This phenotype is likely mediated by the pro-inflammatory cytokine TNFα, although the molecular mechanism is still unknown. We show that epidermal Jun proteins control TNFα shedding by direct transcriptional regulation of TIMP-3, a specific inhibitor of the TNFα converting enzyme (TACE). Upon epidermal JunB/c-Jun deletion, TIMP-3 is downregulated and TACE activity is increased leading to massive keratinocyte-mediated TNFα shedding. As a result, a prominent TNFα–dependent cytokine cascade is initiated in the epidermis inducing severe skin inflammation and perinatal death from cachexia in newborn pups. Importantly, this phenotype can be fully rescued in a TNFR1 null background or by epidermal re-expression of TIMP-3. These findings reveal how Jun/AP-1 proteins control skin inflammation through a TIMP-3/TACE/TNFα pathway. This discovery lends support to established TNFα therapies and provides new targets for the treatment of TNFα-driven skin pathologies.

DISTINCT CONTRIBUTIONS OF T CELL-DERIVED AND MACROPHAGE-DERIVED TNF IN PATHOGENESIS OF COLLAGEN INDUCED ARTHRITIS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

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Anti-TNF therapy is highly efficient against autoimmune diseases, such as rheumatoid arthritis (RA), autoimmune psoriasis and Crohn's disease. In contrast, TNF blockade in multiple sclerosis (MS) may result in disease exacerbation, suggesting opposing roles of TNF in autoimmunity. Consistent with this, several cases were reported with CNS demyelination episodes in RA patients on anti-TNF therapy.
To further dissect the role of TNF in RA and MS, we studied collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) in a panel of mice with cell-type specific TNF ablation (Grivennikov et al. Immunity, 2005 Jan;22 (1):93–104). Macrophages and neutrophils appeared to be the main producers of pathogenic TNF in CIA. In contrast, TNF from the same cell types was protective in EAE. Moreover, TNF gene deletion in macrophages and neutrophils resulted in increased numbers of autoreactive CD4 T cells during disease development in both models, indicating that TNF from macrophages may control early expansion of autoimmune T cells. Distinct roles were also found for T cell-derived TNF in both CIA and EAE. TNF from this source was dispensable for development of CIA, but played deleterious role during EAE by contributing to CNS damage. Our findings demonstrate that the pathogenic or protective effects of TNF in autoimmunity are dependent on the cellular source and further highlight the need for refining anti-TNF treatment in RA to avoid detrimental adverse effects.

HSP70 DEPENDENT ZINC PROTECTION AGAINST TNF-INDUCED LETHAL INFLAMMATION ALLOWS FOR A SAFER ANTITUMOR THERAPY

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Tumor Necrosis Factor (TNF) is a very potent antitumoral molecule. However the severe inflammation induced by its systemic injection prevents the use in anti-tumor therapy. Here we report that addition of ZnSO4 to the drinking water of mice can protect these mice from the inflammatory effects of TNF (TNF induced hypothermia, serum IL6 and nitric oxide levels, TNF-induced bowel cell death and lethality). We could also show that ZnSO4 uptake leads to induction of Heat Shock Protein 70 (HSP70) in several organs especially in the gastrointestinal tract. The protective effect of ZnSO4 was completely absent in mice deficient in the major HSP70-inducible gene, hsp70.1, whereas transgenic mice constitutively expressing the human HSP70. A gene, under control of a ß-actin promoter, were also protected against TNF, indicating that an increase in HSP70 is necessary and sufficient to confer protection. The therapeutic potential of the protection induced by ZnSO4 was tested in a TNF/IFN -based antitumor therapy. Hsp70.1 wild-type mice treated with ZnSO4 were significantly protected against TNF-induced lethality while the antitumor effect was fully intact. In contrast, the Hsp70.1 deficient mice succumbed to the severe inflammation induced by TNF. In conclusion, these data show that zinc can protect against the detrimental effects of TNF in a HSP70 dependent way and that protection by zinc could be helpful in developing a safer anticancer therapy with TNF/IFN.

RESISTANCE OF SPRET/EI MICE TO TNF-INDUCED LETHAL SHOCK

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Tumor necrosis factor (tnf) is a cytokine with a potent antitumor activity, but has also been identified as a central mediator in lethal shock. Injection of tnf induces a systemic inflammatory response syndrome leading to hypotension, liver failure and finally death. Consequently, the use of tnf as antitumor drug is limited to local treatments. tnf has also been shown to be centrally involved in the development of arthritis, ibd and ms.

We found that spret/ei, an inbred mouse strain derived from m.spretus, and (bxs)f1 mice are extremely resistant to tnf-induced lethal shock. All tnf-induced metabolic changes (il6, no, alt) occur at much reduced levels in both spret/ei and (bxs)f1 mice. (bxs)f1 mice are also protected against a tnf/ifng antitumor therapy, while tumor regression still occurs.
In order to identify loci conferring resistance to tnf-induced lethality, we performed a backcross between (bxs) f1 and c57bl/6. We found loci on proximal chr2 and distal chr6, and a sensitivity locus on chr11. Any discussion of candidate genes is still speculative. However, the traf2 gene, coding for traf2, involved in activation of nf-kb and the jnk pathway, is located on proximal chr2. And an even more prominent candidate gene could be tnfrsf1a, coding for tnfr1, located on distal chr6. An in vitro study using mefs and primary macrophages showed that tnfr1 is still functional in spret/ei. m.spretus-derived cells were even more sensitive than c57bl/6 cells to tnf/actd induced cell death. Furthermore, spret/ei and (bxs)f1 were not protected against tnf/galn-induced lethal hepatitis, which strictly depends on tnfr1. currently we are performing a genetic analysis of the tnfrsf1a gene. Twelve sequence variations were found in the cdna between spret/ei and c57bl/6. Six of these are unique for spret/ei. In the 5′ promotor region, we found 22 variations in spret/ei vs c57bl/6. These may be responsible for differential binding of transcription factors and hence different levels of receptor expression in spret/ei.

ABLATION OF c-FLIP AUGMENTS CD95- AND GALACTOSAMINE/LPS-INDUCED LIVER INJURY THROUGH ACTIVATION OF c-JUN N-TERMINAL KINASE (JNK)

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Apoptosis receptor signaling through CD95 (Fas/Apo-1) or CD120a (TNF-R1) causes significant hepatocellular injury and was shown to be relevant to a variety of liver diseases. Upon ligand binding, an intracellular signaling complex termed death-inducing signaling complex (DISC) forms and mediates autolytic caspase 8 activation. The caspase 8 homologue FLICE-inhibitory protein (FLIP) is a regulatory molecule that prevents recruitment of pro-caspase 8 to the DISC and its activation. To evaluate the importance of FLIP in CD95 and TNF-R1 signaling in hepatocytes, mice conditionally lacking c-FLIP (flip -/-)were generated using the cre/loxP system under control of the albumin promoter. To induce acute liver injury, wildtype (wt) and flip -/- mice were injected with PBS, agonistic CD95 antibody (Jo-2; 0.15 microg/g) or galactosamine (gal)/LPS. Flip -/- mice exhibited significantly higher degrees of liver injury measured by serum transaminases (ALT: 2237 vs 259; flip -/- vs wt; $p< 0.01$) and TUNEL positive, apoptotic cells in liver histology at 4 hours from Jo-2 or Gal/LPS compared to wt mice. In parallel caspase

activation at 4 hours was found to be increased in flip -/- mice (caspase-8: 1.3-fold, -3: 1.3-fold, -9: 2.5.fold) and the survival decreased by 50% following 48 hours of treatment with Jo-2. To identify a mechanism that drives cellular injury in flip -/- mice, we examined the proapoptotic kinase c-Jun N-terminal kinase (JNK) following treatment. In wt mice injection with Jo-2 caused a predominant phosphorylation of the p46 isoform of the JNK protein compared to PBS treated mice. In flip -/- animals we observed a 2-fold increase of p46 phosphorylation and a 8-fold increase of the p54 isoform following Jo-2 injection. Gal/LPS caused an even stronger increase of both p46 and p54 phosphorylation when compared to Jo-2 treated flip -/- mice or wt mice. In summary we were able to demonstrate that loss of c-FLIP augments early liver injury following CD95 or TNF-R1 activation through an increased, rapid activation of JNK.

RATIONAL DESIGN OF NEW MEANS FOR THERAPEUTIC MODULATION OF FUNCTION OF THE TNF

MODULATORY EFFECT OF SILIBININ ON THE CYTOKINE PRODUCTION AND FUNGICIDAL ACTIVITY OF HUMAN MONOCYTES INFECTED IN VITRO WITH PARACOCCIDIOIDES BRASILIENSIS

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Introduction and Objectives: Silibinin is the major active component of silymarin (Silybum marianum), a polyphenolic plant flavonoid that has anti-inflammatory, cytoprotective and anticarcinogenic effects. The modulatory effect of silibinin on monocyte function against P.brasiliensis (Pb18) has not yet been demonstrated. The objective of the present study was to evaluate the effect of this flavonoid on tumor necrosis factor-alpha (TNF-alpha) and interleukin-10 (IL-10) production and on fungicidal activity of human monocytes challenged in vitro with Pb18. Methods and Results: Peripheral blood monocytes from 15 healthy individuals were pre-incubated in the absence or presence of different concentrations of silibinin (5 and 50 uM) for 60 min at 37oC. The cells were challenged with Pb18 in a ratio of 50 monocytes per one fungus for 4h and 18h and cytokines were determined in supernatant of cultures by enzyme immunoassay (ELISA). Fungicidal activity of monocytes against Pb18 was assessed after pre-incubation of these cells with or without interferon-gamma (IFN-gamma) for 18h, culture with or without silibinin and challenge with Pb18 for 4h. The co-cultures were plated onto BHI-agar for viable fungi recovery. The pre-treatment of monocytes with silibinin induced an

inhibitory effect on TNF-alpha and IL-10 production, but not affected fungicidal activity of monocytes against Pb18. Conclusions: This inhibitory effect of silibinin on TNF-alpha production, a cytokine involved in the pathogenesis of paracoccidioidomycosis, could contribute to the establishment of new adjuvant therapeutic alternatives for this mycosis.

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APOPTOSIS-INDUCING ACTIVITY OF SOLUBLE TRAIL EXPRESSED IN MESENCHYMAL STEM CELLS ON HUMAN CANCER CELLS

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TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, was originally reported to induce apoptosis in various tumour cells lines, but not in normal cells, both in vitro and in vivo. TRAIL-induced apoptosis is mediated by the transmembrane receptors death receptor 4 (DR4) (also known as TRAIL-R1) and DR5 (TRAIL-R2). TRAIL can also bind decoy receptor 1 (DcR1; TRAIL-R3) and DcR2 (TRAIL-R4) and OPG (Osteoprotegerin). Due to lack of a functional death domain, these decoy receptors are unable to elicit the activation of the downstream apoptotic signaling pathway. The limitations of TRAIL as anti-tumour agent are determined by apoptosis resistance and delivery problems. Therefore, we have designed expression constructs that contain full-length (membrane-bound) and soluble (aa114-281) forms of TRAIL. The soluble TRAIL (sTRAIL) construct contains an exogenous signal peptide, as well as a Furin cleavage site (Furin CS) and a Leucine Zipper (ILZ) motif to force trimerisation. These cassettes were then cloned into adenoviral vectors that were used to transduce bone marrow-derived Mesenchymal Stem Cells (MSCs). MSCs are particularly attractive cells for the targeted delivery and local production of therapeutic proteins in tumours. They can be easily isolated and expanded in vitro, have the ability to accumulate at the site of cancer and can be genetically modified with viral vectors. Western blots, following adenoviral transduction, showed TRAIL expression from our constructs. The culture supernatants of the MSCs expressing TRAIL were transferred to various cancer cell lines: Colo205, A2780, Panc-1, Colo357 and HCT116. Subsequent, apoptosis measurements showed that sTRAIL, can trigger apoptosis in TRAIL sensitive cell lines (Colo205, A2780, HCT116, Colo357) whereas almost no apoptosis could be detected in Panc-1 cells. Supernatants from cells expressing full-length membrane-bound TRAIL could not transfer the apoptosis-inducing activity. Finally, we demonstrated the in vivo therapeutic potential of TRAIL-secreting MSCs in a

murine xenograft tumour model of Colo205 cells. Injection of MSCs loaded with the adenoviral vectors expressing sTRAIL led to tumour regression in this model. In conclusion, the use of MSCs as delivery vehicle for TRAIL holds promise and warrants further investigations.

ANALYSIS OF LIGAND RECEPTOR INTERACTION WITH NEW SINGLE CHAIN TNF DERIVATIVES

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Bioactive TNF forms a homotrimer in which the binding sites for the receptors are located at the clefts between the individual TNF monomers. One TNF molecule can thereby bind three receptor molecules. Based on covalently stabilized TNF (single chain TNF; scTNF; Krippner-Heidenreich et al., J. Immunol. 180, 2008, 8176) we have constructed TNF molecules effectively binding only one or two receptor molecules. This was obtained by introduction of mutations into individual receptor binding sites of the scTNF molecule.
In equilibrium binding studies performed at 0°C we compared these "mono-", "di-" and "trivalent" TNF derivatives regarding their binding affinities. At this temperature membrane diffusion of receptors is largely prevented and therefore information regarding their oligomerization status should be obtained. For example, the binding affinities of mono-, di- and trivalent scTNF molecules should be comparable, provided the receptors exist as monomers on the cell membrane. In our experiments we found significantly reduced affinities with the monovalent or divalent scTNF molecules, clearly demonstrating initial binding to more than a single receptor at once, thereby suggesting the existence of preformed receptor multimers e.g. via PLAD interaction. Comparing TNFR1 and TNFR2 interesting differences were found: TNFR2 reacts more sensitive towards the loss of individual binding sites in the ligand, a possible hint for differences in the preassembly of the receptors.

DOMINANT NEGATIVE TNF PROTECTS FROM MYCOBACTERIUM BOVIS BCG AND ENDOTOXIN-INDUCED LIVER INJURY WITHOUT COMPROMISING HOST IMMUNITY TO MYCOBACTERIUM TUBERCULOSIS AND BCG INFECTIONS

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Inhibition of TNF by TNF receptor fusion proteins such as etanercept or anti-TNF antibodies such as infliximab and adalimumab that inhibit both soluble TNF (solTNF) and transmembrane TNF (tmTNF), has proven to be a highly efficacious treatment for human pathologies such as rheumatoid arthritis, Crohn's disease, and ulcerative colitis. However, the clinical use of TNF inhibitors has been linked to serious adverse events such as an increased risk for infections including tuberculosis, either newly acquired or by reactivation of latent infection. The growing literature establishing the importance of tmTNF in immunity has generated support for the hypothesis that selective inhibition of solTNF while sparing tmTNF may be anti-inflammatory yet maintain host response to pathogens. This hypothesis was tested using a novel dominant-negative (DN) strategy of selective TNF neutralization, consisting of blocking solTNF while sparing tmTNF, in mouse models of Mycobacterium tuberculosis and Mycobacterium bovis BCG infections and in acute liver inflammation. XENP1595, a DN-TNF biologic, was compared to etanercept, a TNFR2-IgG1 Fc fusion protein inhibiting both murine solTNF and tmTNF. XENP1595 protected mice from acute liver inflammation induced by endotoxin challenge in BCG-infected mice, but in contrast to etanercept, did not compromise host control of M. tuberculosis and BCG infections in terms of bacterial burden, granuloma formation, and innate immune responses. This study showing that a selective inhibitor of solTNF did not suppress host immunity to M. tuberculosis and M. bovis BCG infections, yet protected mice from BCG/LPS-induced liver injury suggests that the risks associated with the first generation of nonselective TNF inhibitors might be reduced by use of a DN-TNF biologic that spares the protective effects of tmTNF. A reduction in the infection risks associated with current anti-TNF drugs may also allow the safer use of novel anti-TNF therapies in other inflammatory diseases.

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THE TUMOR NECROSIS FACTOR-A CONVERTING ENZYME (TACE) IS REGULATED BY PHYSIOLOGICAL IONIC STRENGTH

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The tumor necrosis factor-α converting enzyme (TACE) cleaves membrane-bound precursor TNFα to generate its soluble, secreted form. Thus, TACE plays a critical role in the control of inflammatory responses and TACE inhibitors have been shown to block septic shock and other TNFα-driven immune reactions in animal models. Over a decade after the isolation and identification of TACE, we have only an incipient understanding of regulatory events affecting its activity, both at the molecular and cellular levels. We previously reported that surprisingly, relatively low concentrations of NaCl inhibit TACE. Here we show that this effect is also observed with other alkaline metals within a similar concentration range (IC50 values within 50–100 mM for LiCl, NaCl, RbCl and CsCl). NaCl did not seem to affect TACE protein conformation or thermodynamic stability, as determined by circular dichroism and fluorescence spectroscopy. We also show that low NaCl concentrations induce TACE activation in vitro. Unexpectedly, at concentrations above the physiological range, TACE catalytic activity is dramatically augmented by Li+, Na+ and Rb+ ions, while Cs+ fails to produce this increase in activity. We do not know the basis of this stimulatory effect, but the site involved seems to restrict access to relative large ions such as Cs+. Taken together, our results suggest that at physiological ionic strength, TACE may sit at a delicate balance where small monovalent ions may either induce or repress its activation and proteolytic activity. Finally, it may be the case that TACE activity is regulated to a significant extent in vivo by electrostatic interactions with co-factors yet to be identified.

ENHANCED ANTI-TUMOR EFFICACY OF A DR5-SPECIFIC TRAIL VARIANT OVER RHTRAIL IN A BIOLUMINESCENT OVARIAN CANCER XENOGRAFT MODEL

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Recombinant human (rh) trail is clinically evaluated as novel anticancer drug. rhtrail-dr5 (e195r/d269h), a rhtrail variant that specifically binds to pro-apoptotic

dr5 receptor, has recently been developed. We investigated whether rhtrail-dr5 is more efficient than rhtrail in combination with cisplatin in dr5 expressing human a2780 ovarian cancer cells. Cisplatin strongly enhanced dr5 surface expression. both rhtrail and rhtrail-dr5 in combination with cisplatin induced high levels of caspase 3 activation, apoptosis and cell kill with rhtrail-dr5 being most potent. Small interfering rna against dr5 completely inhibited apoptosis induction by these combinations. Anti-tumor efficacy of rhtrail-dr5 versus rhtrail was determined in an intraperitoneal growing bioluminescent a2780 xenograft model. Intraperitoneal administration of rhtrail-dr5 delayed a2780 tumor progression, reflected in a mean light reduction of 68.3% ($p = 0.015$), while rhtrail or rhtrail-dr5 plus cisplatin resulted in 85% ($p = 0.003$) and 97% ($p = 0.002$) reduction, as compared to a2780 tumor progression in vehicle treated animals. combination of rhtrail-dr5 with cisplatin was more effective than cisplatin alone ($p = 0.027$). in conclusion, our results show that rhtrail-dr5 was superior over rhtrail also in combination with cisplatin against dr5 expressing ovarian cancer.

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TARGET CELL-RESTRICTED STIMULATION OF THE CD95 (APO-1/FAS) DEATH RECEPTOR WITH VARIOUS BISPECIFIC CD20XCD95 ANTIBODIES

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It is well established that agonistic antibodies directed to the cd95 (apo-1/fas) death receptor induce apoptosis of cd95-positive and sensitive cell lines. However, in vivo the therapeutic use of such monoclonal cd95 antibodies is hampered due to the expression of cd95 on normal cells such as hepatocytes and the application of agonistic antibodies in mice led to fatal hepatic failure (ogasawara et al., 1993). For in vivo application it seems to be necessary to confine cd95-mediated apoptosis to predefined target cells. This "target cell restriction" can be achieved by using bispecific antibodies with dual specificities: one directed to cd95 and the other to a target antigen on a tumor cell (jung et al., 2001; herrmann et al., 2008).
Here we chose the cd20 molecule as a target antigen which is a well established tumor antigen for b-cell lymphomas. we could show that chemically hybridized bispecific fab2 fragments consisting of the apo-1 antibody and different cd20

antibodies were able to induce in vitro an effective killing of the fas-sensitive b-lymphoblastoid cell line skw6.4 and to a lower extend of the less fas-sensitive b-lymphoblastoid cell lines jy, bjab and c1r. Fas-sensitive cells of the cd20-negative t-cell line jurkat were not killed by the bispecific fab2 fragments. This proves the target cell restriction and the necessity for the presence of the target antigen. First in vivo experiments using a lymphoma scid mouse model to evaluate the therapeutic activity of cd20xapo-1 antibodies showed a prolonged survival of cd20xapo-1 treated mice.

SENSITIZATION OF PROSTATE CANCER CELLS TO TRAIL-MEDIATED APOPTOSIS BY THE TUMOR-SUPPRESSING MATAIRESINOL LIGNAN

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells causing minimal toxicity to normal tissues, which makes TRAIL a promising anti-cancer agent. However, not all cancers are sensitive to TRAIL-mediated apoptosis and combinatorial treatments have been sought after. Here we observed that among tumor suppressing plant lignans matairesinol (MAT) was particularly effective as a death receptor-sensitizing agent. Therefore, MAT was tested for its efficacy to sensitize hormone-dependent prostate cancer cells. It was observed to sensitize the LNCaP cells to TRAIL-induced apoptosis both in the presence and absence of androgen. The sensitization involved increased Bid cleavage and mitochondrial depolarization. Androgen-dependent LNCaP prostate cancer cells have constitutive activation of the phosphatidylinositol 3-kinase (PI-3 K)/Akt survival pathway, which makes them resistant to TRAIL-mediated apoptosis. Treatment with MAT decreased Akt activity. In addition, MAT-sensitized LNCaP cells could be rescued from TRAIL-induced apoptosis by ectopic expression of a constitutively active form of Akt. The LNCaP cells displayed distinct mechanisms of TRAIL-resistance depending of the presence of androgen. Due to high frequency of excessive PI-3 K/Akt signaling in cancer, targeting this survival pathway with MAT could benefit the treatment of tumors, including prostate cancer.

RECEPTOR ACTIVATION BY SOLUBLE OX40 LIGAND AND 41BB LIGAND IS ENHANCED BY OLIGOMERIZATION AND CELL SURFACE IMMOBILIZATION

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The ability of many members of the tumor necrosis factor (TNF) ligand family to activate their corresponding receptors depends on oligomerization status and trimer stability of the ligand. Here we examined the effect of oligomerization and trimer stability on receptor activation by the immunostimulatory ligands OX40L and 41BBL. Recombinant soluble human OX40L and 41BBL assemble into trimers, but are practically inactive despite binding to their corresponding receptors OX40 and 41BB, respectively.
Oligomerization of soluble Flag-tagged variants of OX40L and 41BBL trimers by crosslinking with anti-Flag antibody increased the receptor activation by the ligands. The relevance of trimer stability was investigated by introduction of the tenascin-C (TNC) trimerization domain, which led to strongly enhanced signaling by anti-Flag antibody oligomerized Flag-TNC-OX40L and Flag-TNC-41BBL. Oligomerization by introduction of an immunoglobulin Fc-domain activated receptor signaling by hexameric Flag-OX40L and Flag-TNC-41BBL. To activate selectively receptor signaling on target cells we used antibody fragment (scFv)-ligand fusion proteins recognizing the tumor stroma antigen fibroblast activation protein (FAP). ScFv-OX40L and scFv-41BBL showed a cell surface antigen-dependent increase in receptor activation by the ligand domain of the molecule

RELEVANCE OF OLIGOMERIZATION AND CELL SURFACE IMMOBILIZATION FOR THE ACTIVITY OF SOLUBLE VARIANTS OF CD27L AND GITRL

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Trimer stability and oligomerization status can be crucial for the ability of TNF ligand family members to activate their cognate receptors. However, detailed information regarding the requirements for the immunostimulatory-acting CD27L (CD70) and glucocorticoid-induced TNF-related ligand (GITRL) is still lacking. To investigate this topic we used trimeric Flag-tagged, hexameric Fc and tenascin-C stabilized forms of both TNF ligands. Soluble Flag-tagged CD27L trimers and hexameric Fc-Flag-CD27L were inactive, even after oligomerization with anti-Flag antibodies. In contrast, hexameric Fc-Flag-GITRL induced receptor signaling. The stabilized Flag-TNC-trimers of both CD27L and GITRL were highly active after oligomerization. Based on these results, we generated antibody fragment (scFv)-ligand fusion proteins and looked for selective activation of receptor signaling on targeted cells. The scFv-GITRL fusion protein recognizing fibroblast activating protein (FAP) was active on FAP-positive and to a lesser extent also on FAP-negative cells. In contrast, the anti-FAP variant of CD27L lacked activity towards both cell types. Taken together, hexamerization and/or oligomerization increased the activity of soluble GITRL. CD27L activity required trimer stabilization and oligomerization. In addition, target-restricted cell surface immobilization can trigger selective ligand activity.

TRAIL FUSED TO SURFACTANT PROTEIN-D AND COLLECTIN-11 AS MODEL FOR TNF-SF COLLECTIN FUSION PROTEINS

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Ligands of the TNF superfamily require homotrimerization of their extracellular receptor-binding domain (RBD) for biological acitivity. Here we describe a novel construction principle based on different trimerization/scaffold domains that can be fused to the C-Terminus of the ligands. As scaffold we employed domains

derived from human collectins surfactant protein-D (SPD) or collectin-11. Thus the designed fusion proteins are fully human except an interdomain positioned linker element. Several TNF related apoptosis inducing ligand (TRAIL)-collectin variants were engineered and characterized and the linker element or type of scaffold both were found to affect expression and biological activity. A clear ranking with respect to technical feasibility of the engineered proteins could be derived from the data. Finally, a TRAIL-SPD based fusion protein variant was profiled in more detail. The purified protein was found to induce apoptosis in several established human cancer cell lines but not on primary human hepatocytes. An initial pharmacokinetic analysis revealed a terminal half life of >6 hours, making it an attractive candidate for further development. The construction principle can be applied to other TNF-SF ligands such as CD95L/FasL, LIGHT and APRIL and represents a promising strategy for future therapeutic applications.

MUTANT TNF ELICITS MUCOSAL AND SYSTEMIC IMMUNITY AGAINST HIV-1 AND INFLUENZA VIRUS

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The development of safe and effective mucosal vaccine adjuvants is critical for the improvement of mucosal vaccines. Therefore, it is necessary to design new mucosal adjuvants, which can effectively induce both systemic and mucosal immunity. Application of cytokines for mucosal adjuvant has currently attracted because cytokines play important roles in biological host-defense system. Recently, using phage display techniques, we have created mutant TNF (mTNF-K90R), which has a stronger bioactivity than wild-type TNF. In this study, we assessed the potential of mTNF-K90R as a mucosal adjuvant for the development of mucosal vaccines against HIV and influenza virus. Mice were intranasally immunized with OVA or recombinant HIV-1 Env gp120 or influenza virus HA protein alone or together with mTNF-K90R. Characterization of immune responses following mTNF-K90R revealed elevated OVAspecific IgG1 Abs, a Th2-type Ab subclass. Consistent with this, mTNF-K90R was found to enhance OVA-specific Th2-type cytokine production in splenocytes from immunized mice. Intranasal coadministration of gp120 or HA with mTNF-K90R induced higher levels of systemic IgG Abs responses than those of antigen (Ag) alone. Further, Ag-specific IgA responses in the nasal and

vaginal mucosa were significantly induced in the group receiving mTNF-K90R plus Ag. These results suggested that mTNF-K90R could be a potential mucosal adjuvant for enhancing both systemic and mucosal immune responses.

DEVELOPMENT OF A NOVEL METHOD USING PHAGE DISPLAY, GENE SHUFFLING AND IMPROVED RECEPTOR SELECTIVITY TO PRODUCE MUTANT TNFS

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TNF is a major cytokine that regulates many biological processes, such as host defense and tumor cell death, via the receptors TNFR1 and TNFR2. Moreover, TNF levels correlate with the severity of inflammatory diseases such as rheumatoid arthritis. TNF is therefore a drug target for autoimmune disease therapy. Functional analysis of each receptor will provide valuable information that will aid the development of drugs with increased efficacy and safety. To this end, we constructed libraries of mutant TNFs in which 6 amino acids in the receptor binding site were randomly substituted, using phage display techniques, and isolated TNF receptor-selective mutants. However, the generation of a phage library with a large repertoire (>one hundred million) was impeded by the limitation of transformation efficiency of Escherichia coli. Consequently, it proved difficult to construct a high quality library of mutants with 7 or more amino acid substitutions. To overcome this problem, we performed panning against a specific receptor using two different libraries, each with a different set of 6 amino acid substitutions. We then constructed a randomized high quality library with 12 amino acid substitutions by integrating the libraries using gene shuffling technology. Our new approach has enabled identification of TNF receptor-selective mutants with greater efficiency and selectivity than was possible from existing libraries. We believe that this new approach will provide a valuable contribution to the elucidation of the regulatory functions of biomolecules for therapeutic purposes.

ANTI-INFLAMMATORY EFFECTS OF A NOVEL TNFR1-SELECTIVE ANTAGONISTIC TNF MUTANT ON ESTABLISHED MURINE COLLAGEN-INDUCED ARTHRITIS

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Blocking the binding of TNF to TNF receptor subtype 1 (TNFR1) is believed to be a promising strategy for the treatment of rheumatoid arthritis (RA). However, because of the unavailability of any TNFR1-selective antagonist, the therapeutic potency of this strategy still remains to be confirmed. To this end, we recently developed a novel TNFR1-selective antagonistic TNF mutant (R1antTNF) and reported that this agent efficiently inhibits TNF/TNFR1-mediated signaling in vitro. Here, the anti-inflammatory effects of R1antTNF on a murine collagen-induced arthritis (CIA) model have been investigated. To improve in vivo stability, polyethylene glycol-modified R1antTNF (PEG-R1antTNF) was prepared. Using site-specific PEGylation technology, the in vivo half-life of R1antTNF was improved without loss of antagonistic activity. In the CIA model, the clinical score of the mice given PEG-R1antTNF was improved equally or even more effectively than with Etanercept treatment, which is clinically applied for TNF-blockade. Recently, there has been much concern over the increased susceptibility to pathogenic infection caused by TNF blockade. Therefore, we assessed whether PEG-R1antTNF and Etanercept had similar deleterious effects in adenoviral infection model. The data suggest that PEG-R1antTNF has less negative effects on viral clearance than Etanercept. We conclude that selective inhibition of TNF/TNFR1-mediated signaling could be effective in treating RA and that PEG-R1antTNF could serve as a novel anti-inflammatory drug for this purpose.

RECEPTOR INTERACTION OF TUMOR NECROSIS FACTOR (TNF) BASED ON 3D STRUCTURAL ANALYSIS OF A FULLY ACTIVE TNFR1-SELECTIVE TNF MUTANT

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We previously reported the successful isolation of a fully active TNFR1-selective TNF mutant (clone R1-6) using the phage display technique. Because TNFR1 selectivity of R1-6 might provide important information for structure-based drug discovery, we examined the structural basis of the TNFR1 selectivity of R1-6 by X-ray crystallography. The R1-6 loop structure near mutational residues 31 and 32 was different from that in wild-type TNF, which is thought to be due to an R32G mutation from a sterically bulky arginine residue to a flexible glycine residue. Docking simulation using the TNFR structures to examine TNFR1 selectivity suggested that R31A and R32G mutations strongly influence electrostatic interactions with TNFR2, and that an L29K mutation contributes to the binding of R1-6 to TNFR1. This phage display technique can be used to efficiently construct functional mutants for analysis of the TNF structure-function relationship, which might facilitate in silico drug design based on receptor selectivity.

THE DEVELOPMENT OF FULLY ACTIVE RECEPTOR-SELECTIVE TUMOR NECROSIS FACTOR (TNF) MUTANTS

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Tumor necrosis factor (TNF) is an important cytokine that regulates various biologic processes through TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). The contribution of each receptor to these processes, however, is unclear. Here, we used phage display techniques to generate receptor-selective TNF mutants that activate only one TNFR. Six amino acids in the receptor binding interface (near TNF residues 30, 80, and 140) were randomly mutated and two phage libraries comprising over 5 million TNF mutants were constructed. From these libraries, we successfully isolated 4 TNFR2-selective candidates and 16 TNFR1-selective candidates. The TNFR1-selective candidates were highly mutated near residue 30, whereas TNFR2-selective candidates were highly mutated near residue 140 and conserved near residue 30. These findings indicate that the residues essential for binding to TNFR1 and TNFR2 differed and provide important information about TNFR-selectivity. Purified clone R1-6 remained fully bioactive and had full affinity for TNFR1 without activating TNFR2. Additional studies using R1-6 will further elucidate the biology of the TNF/TNFR system and facilitate receptor-selective drug discovery.

THE ROLE OF HEPARAN SULFATE PROTEOGYCANS IN APRIL SIGNALLING

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APRIL (A PRoliferation Inducing Ligand or TALL-2 and TRDL-1) is a member of the tumor necrosis factor (TNF) superfamily and binds two TNF receptors: TACI (Transmembrane activator and calcium modulator and cyclophilin ligand interactor) and BCMA (B-cell maturation antigen). APRIL has tumorigenic properties, but is also important for the maintenance of humoral immune responses. Recently, APRIL was also shown to bind heparan sulfate proteoglycans (HSPGs). Using point mutants of APRIL, that lack either HSPG-binding capacity or the ability to bind TACI and BCMA, we have shown that HSPG-binding is crucial for both IgA production and B cell proliferation. We found that the HSPG interaction provides a platform for APRIL cross-linking and oligomerization, and thus effective presentation to the receptors, but did not appear to mediate direct signaling. Intriguingly, APRIL also stimulates survival of several cell lines derived from solid tumors that lack both BCMA and TACI, suggesting there is either a third APRIL receptor, or that HSPGs directly signal or function as co-receptors in this context. To study this we have generated stable cell lines that express either WT-APRIL or the non-HSPG binding form, which we plan to inject into nude mice and study tumor outgrowth. TACI has also been shown to interact with HSPGs, which is potentially interesting in light of the high levels of syndecans produced by several B cell malignancies and the TACI-high signature of several multiple myelomas. To study this interaction we have also generated a non-HSPG binding form. Such interactions potentially open up an exciting new arm of TNF receptor-ligand biology.

ENHANCEMENT OF ANTITUMOR PROPERTIES OF RHTRAIL BY AFFINITY INCREASE TOWARD ITS DEATH RECEPTORS

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Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily and has been shown to induce apoptosis in cancer cells but not normal cells. TRAIL was discovered by virtue of its structural homology to members of the TNF gene superfamily. It is a promiscuous ligand as it binds to five different cognate receptors of the TNF-receptor family: the death receptor 4 (DR4, TRAIL-R1), death receptor 5 (DR5, TRAIL-R2) containing a cytoplasmic death domain through which TRAIL can transmit an apoptotic signal and to the decoy receptor 1 (DcR1, TRAIL-R3, TRIDD), decoy receptor 2 (DcR2, TRAIL-R4, TRUNDD) and the soluble secreted receptor OPG, that may act as antagonist receptors, lacking an intact death domain. TNF-related apoptosis-inducing ligand (TRAIL) receptors (DR4 and DR5) are therefore promising targets for cancer therapy.

The design of rhTRAIL variants either with improved affinity or selectivity toward one or both death-inducing receptors is thought to enhance the therapeutical potential of TRAIL. Here we demonstrate that a single amino acid mutation at the position of glycine 131 to lysine or arginine in wild-type rhTRAIL significantly improved the affinity of rhTRAIL toward its death receptors, with the highest affinity increase observed for the DR4 receptor. These variants were able to induce higher *in vitro* levels of apoptosis in cancer cells responsive to only DR4 or to both death receptors and could therefore increase the potential use of rhTRAIL as an anticancer therapeutic agent.

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IMPROVED TUMOR CELL KILLING BY TRAIL REQUIRES SELECTIVE AND HIGH AFFINITY RECEPTOR ACTIVATION

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Apoptosis can be activated by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in a wide range of tumor cells, but not in non-transformed cells. TRAIL interaction with receptors DR4 or DR5 induces apoptosis, whereas DcR1, DcR2 and osteoprotegerin are decoy receptors for TRAIL. TRAIL variants (single mutant, D269H, and double mutant D296H/E195R) engineered to selectively activate DR5 but not DR4 enhanced pro-caspase-8 processing and apoptosis in tumor cells, compared to wild-type TRAIL. D269H/E195R showed a 17 fold faster kinetics of receptor activation than wild-type TRAIL. The robust receptor activation was not due to altered 3D ligand structures or aggregation but to high affinity and selectivity towards DR5, which prevented ligand-induced receptor heteromerization and neutralization by decoys. These findings have important implications for the design of anticancer therapeutic agents and cytokine engineering.

COMPUTATIONAL DESIGN OF RECEPTOR SELECTIVE TRAIL VARIANTS

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a potential anticancer drug that selectively induces apoptosis in a variety of cancer cells by interacting with death receptors DR4 and DR5. TRAIL can also bind to decoy receptors (DcR1, DcR2, and osteoprotegerin receptor) that cannot induce apoptosis. Different tumor types respond either to DR4 or to DR5 activation and chemotherapeutic drugs can increase the expression of DR4 or DR5 in cancer cells. Thus, DR4 or DR5 receptor-specific TRAIL variants have enormous potential as new tumor-selective therapies. Therefore, we designed receptor specific TRAIL variants that bind selectively to either DR4 (2) or DR5 (1) by using the computational protein design algorithm FoldX. Various in vitro receptor binding assays and cell based biological activity assays demonstrated that the designed TRAIL variants selectively bound to and induced apoptosis via either DR4 or DR5. DR5 selective TRAIL variants induced apoptosis more potently than wild-type TRAIL. Moreover, it was shown that the kinetics of apoptosis induction were increased by an order of magnitude (Szegezdi et al., submitted). This increase in kinetics could however not solely be explained by the ~3-fold improvement in association rate constant for DR5 binding. Computer simulation of TRAIL-receptor interaction revealed that prevention of ligand-induced receptor heteromerization combined with the increased affinity towards DR5 produced these enhanced receptor-activation kinetics.

In conclusion, computational protein design was effective in the development of receptor-specific TRAIL variants. Moreover, the designed receptor selectivity also enhanced the kinetics of receptor activation. We believe that this method could be generally applicable to design faster acting and more potent variants of other promiscuous cytokines as well.

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EFFICACY OF ANTI-TNF-A ANTIBODIES IN INTESTINAL CELL LINES SYSTEM

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Inflammatory bowel disease is a group of disorders characterized by inflammation of the gastrointestinal tract. One of the most important cytokines in the pathogenesis of the disease is tumor necrosis factor alpha (TNF-α) - an agent which stimulates the cascade of pro-inflammatory signaling in various cell types of the intestinal wall. Recently, antibodies targeted against this cytokine have been successfully implemented in IBD therapy, namely infliximab and adalimumab. The aim of this project was to evaluate the efficiency of those drugs in blocking the TNF-α-mediated pro-inflammatory responses. As a model system we used cultures of the epithelial, myofibroblastic, and lymphocytes/ macrophage cell lines. For monitoring the changes of mRNA levels we used RT-PCR.
Phosphorylation of signal transduction proteins was determined by Western Blot. For testing the specificity of the transcription factors we used EMSA. To investigate the interaction of the drugs with the cells we performed (immuno) fluorescent microscopy. We observed the cytokine specific responses in cell lines by means of phosphorylation of STAT, p38 MAPK, CREB proteins, increased binding to the NF-kB specific probe and increased production of pro-inflammatory cytokines such as TNF-α and IL-8. Both drugs prevented TNF-α specific increase of TNF-α and IL-8 mRNA in epithelial cells, but only adalimumab showed a complete inhibitory effect on TNF-α -induced TNF-α and IL-8 mRNA production in all three cell lines tested. We observed that fluorescently labeled infliximab, but not adalimumab, accumulated at the surface of myofibroblasts, which can explain its limited inhibitory activity in those cells. We can conclude that anti- TNF-α antibodies could be successfully tested in cell line based system.

ROLES OF THE TNF FAMILY IN NEURONAL DEVELOPMENT, FUNCTION & PATHOLOGY

TUMOR NECROSIS FACTOR RELATED APOPTOSIS INDUCING LIGAND-RELATED SIGNAL TRANSDUCTION IN NEURODEGENERATIVE PROCESSES

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Tumor necrosis factor related apoptosis inducing ligand (TRAIL) has been shown to significantly contribute to amyloid β dependent neurotoxicity.
Recently, an array of genes modulating TRAIL cytotoxicity has been identified, providing evidence for a role of pathways such as Wnt and JNK in cell susceptibility to proapoptotic cytokines. Here, the significance of involvement of the Wnt signalling in TRAIL-mediated neutoxicity has been investigated. Western blot analysis of Wnt in human neuronal-like cell lines showed significantly decreased Wnt expression when these were treated with TRAIL in vitro. In addition, both phosphorylation of GSK-3β and degradation of cytoplasmic β catenin were increased, as well as hyperphosphorylation of the τ protein, bringing about the picture of neuronal damage. As a further demonstration of the interactions of TRAIL with the Wnt pathway, the addition of specific GSK-3β inhibitors to neuronal cultures resulted in rescue of a significant percent of cells from TRAIL-induced apoptosis. The rescue was total when caspase-8 inhibitors were used in combination with GSK-3β inhibitors. Thus, it appears that in addition to trigger the caspase signalling, TRAIL also interferes with the Wnt pathway, redundantly concurring to neurotoxicity. These data suggest that the Wnt pathway substantially contributes to the TRAIL-related neurotoxicity and indicate the TRAIL system as a candidate target for pharmacological treatment of Alzheimer's disease and related disorders.

APOPTOSIS REGULATION IN NEUROSECRETORY CELLS OF HYPOTHALAMUS OF AGED TNF-KNOCKOUT MICE

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Tumor necrosis factor (tnf) takes part in regulation of many processes, including carcinogenesis and apoptosis. However we did not find many investigations which devoted of features of apoptosis regulation in tnf-knockout (tnf-/-) mice. These mice have not serious morphological anomalies in their development, but perhaps, they have disorder in many biological processes, such as inflammation, programmed cell death etc. So, the aim of our investigation was to study possible pathways of apoptosis regulation without TNF in hypothalamic neurosecretory cells in young and old mice. We studied magnocellular supraoptic and paraventricular nuclei of hypothalamus. We detected the amount of apoptotic proteins (caspase-8, -9, Bax, Bcl-2, Mcl-1), neuropeptide vasopressin (immunocytochemical method) and apoptosis level (luminescent microscopy in ethidium bromide stained slices) in tnf-knockout young and old mice comparing wild type C57Bl/6 (WT) mice. We shown the age-related activation of apoptosis in magnocellular neurosecretory hypothalamic nuclei in tnf-knockout mice and in WT ones, so, we can conclude that the apoptosis activation in aging does not depend on presence of gene tnf. We obtained the increased level of caspase-8 in aged tnf-/- mice and no changes in amount of caspase-9 in the same ones. So, this apoptosis is caspase-8-mediated. Besides, misbalance of members of Bcl-2-protein family synthesis (the increase of proapoptotic (Bax) and the decrease of antiapoptotic (Mcl-1, Bcl-2) proteins) play important role in developing of apoptosis in neurosecretory system in aging.

CELL DEATH MECHANISMS CONTROLLED BY THE TNF FAMILY

ASSOCIATION OF FASL AND CAVEOLIN-1 IN LIPID RAFTS IS ESSENTIAL FOR THE ONSET OF FASL-MEDIATED CELL DEATH

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Fas-ligand belongs to the TNF family of proteins and is expressed by activated T lymphocytes, immune-privileged cells and some type of tumor cells. It can induce

apoptosis through death receptor Fas/CD95 or by the reverse signaling pathway. The important condition for inducing Fas-ligand - dependent cell death is its localization to rafts, cholesterol- and sphingolipid enriched micro domains of plasma membrane, involved in regulation of different signaling complexes. Here we show that the molecular basis for Fas-ligand translocation to rafts, and subsequent induction of Fas - mediated cell death is the association of Fas-ligand and caveolin-1, the main protein component of rafts, which is also a suppressor of cell tumor transformation. Formation of Fas-ligand - caveolin-1 complex needs dephosphorylation of both molecules and intact N-terminal sequence of Fas-ligand bearing putative caveolin-1-binding motif. Dephosphorylation of Fas-ligand and caveolin-1 can be due to a decreased expression and activity of p59Fyn-kinase in dying cells. We predict that caveolin-1-binding motifs found in sequences of other members of TNF family is responsible for their translocation to rafts and activation of ligand-dependent functions. Thus, calveolin-1 plays an important role in activation and regulation Fas-ligand - dependent cell death.

TNF ALPHA INDUCE SHEDDING OF CD30 AND CD45 AND LDH RELEASE FROM K-562 CELLS

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TNF-alpha can induce cell death I different hematological cell line which effects mostly depending on cell membrane TNF super-family receptor expression. Since determination of LDH release from cultured cells in-vitro reflects early membrane alterations, we compared LDH release with changes in cell membrane molecule expression during cultures of K-562 cells treated in vitro with TNF-alpha in dose and time-dependent manner. Before and after treatments K-562 cells were analyzed by Flow cytometry (apoptosis and cell membrane molecule expression), by LDH release assay and by estimation proteins in supernatant and cell surface by Western blot techiques. The significant increase in LDH release was associated with decrease cell membrane molecule expression including CD45 and CD30 as highly expressed and CD45RA and CD38, as low expressed after TNF-alpha treatment. However, percentage of decrease of all examined molecules is not uniform, and appears to depend on the respective level of pre treated values and molecule type. In addition, presence of these molecules was confirmed in supernatants using western blot analyses. These results indicated the complexity of events on cell membrane, including association between LDH release and shading of membrane molecules following TNF-alpha mediated processes.

DIFFERENTIAL ROLE OF FLIP ISOFORMS IN TNF-INDUCED CELL LIFE AND DEATH RESPONSES

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Tumor necrosis factor-alpha (TNF) is a major mediator of apoptosis, immunity and inflammation. Aberrant TNF responses can lead to human diseases such as cancer, osteoporosis, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease. In leukemia cells, TNF can drive divergent responses by switching the character of the TF-1 cells (erythroleukemia cell line) from a TNF-responsive proliferative phenotype (life) into an apoptotic death response. The caspase-8/FLICE-inhibitory protein (FLIP) generally acts as an inhibitor of death receptor-mediated apoptosis by blocking caspase pathways initiated by receptor activation. The short FLIP isoforms, FLIPS and FLIPR, have been shown to block death receptor-induced apoptosis, whereas the role of FLIPL is still unclear. Here we investigate the expression of FLIP isoforms using real-time PCR and western analysis; we show that life/death switching is due in part to an enhanced FLIP basal expression in the proliferative phenotype versus the apoptotic one. Results also show that in TF-1 cells the specific siRNA silencing of the FLIPL isoform, or a combination of both FLIPL and FLIPS isoforms, is enough to switch the proliferative phenotype of these cells into an apoptotic phenotype. However, specific FLIPS siRNA silencing does not drive these cells into apoptosis. These data show the importance of the enhanced FLIP basal levels in leukaemia cells and demonstrate the predominant role of FLIPL in TNF-induced life/death switching pointing this isoform as a good potential therapeutic target for the treatment of erythroleukemia.

DEATH WITHOUT LIGANDS: NOVEL APPROACH IN INDUCTION OF TNF RECEPTOR SUPERFAMILY MEDIATED CELL DEATH

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Tumor necrosis factor alpha (TNF-α) is a cytokine capable of inducing hemorrhagic necrosis and sometimes even full regression of tumors in vivo. Unfortunately, systematic administration of TNF-α is not possible because of its severe adverse effects. TNF-α was, as a prototipe molecule, included in the family of structurally related soluble or membrane bound biologically active ligands, member of which is also

TRAIL. Action of ligands starts with binding to their receptors, which initiates clustering of receptors, being the first step in signal transduction.
In order to achieve clustering of the receptors without ligands, we introduced histidine tags (HIS-10) into the extracellular part of the TNF-R1 molecule. HIS-10 tag has also been introduced into the truncated TRAIL-R1 molecule (in which the extracellular part of the TRAIL-R1 molecule was deleted) to prove that clustering of receptors is sufficient for inducing the downstream cascade and the extracellular parts of TRAIL-R1 receptors are not required for signal transduction. HIS-10 tags in the extracelullar parts of TNF-R1 and TRAIL-R1 serve for association via Zn2+ ions alone or Zn2+ ions together with biologically compatible chelating molecule, TETA (1,4,8,11-Tetraazaciclotetradecane-1,4,8,11-acetic acid). This leads to clustering of receptors and consequently to activation of the downstream cascade which results in cell death. This approach would be applicable for the other members of the TNF receptor superfamily as well as for other situations where signaling depends on protein clustering.

INVOLVEMENT OF ENDOCYTOSIS IN TRAIL-R1/DR4 AND TRAIL-R2/DR5 TRAFFICKING AND SIGNALING

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TRAIL (TNFα Related Apoptosis Inducing Ligand) is as other ligands of the TNFα family expressed mainly on surface of hematopoietic cells (T lymphocytes, monocytes, dendritic and NK cells, neutrophils). Binding to its pro-apoptotic receptors TRAIL-R1/DR4 or TRAIL-R2/DR5 triggers apoptosis preferentially of transformed cells and plays important role in the immune surveillance. Receptor clustering and their intracellular associations with adaptor protein FADD and pro-caspase-8 represent essential first steps of TRAIL-induced apoptotic signaling. Also ligand-triggered internalization of death-receptors such as TNFR1 or Fas/CD95 is apparently crucial for the induction of receptor-mediated apoptosis, but for TRAIL receptors the published data are not as clear. Thus our project is aimed on clarifying significance of endocytosis and death receptor trafficking for TRAIL-induced cell death. In order to distinguish the involvement of clathrin-dependent and -independent endocytosis, we performed set of experiments with biochemical inhibitors like dansylcadaverine, methyl-β-cyclodextrin, 5-(N-ethyl-N-isopropyl)amiloride and chlorpromazine on TRAIL-sensitive colon cancer cell lines, and we compared this approach with siRNA-mediated downregulation of the gene expression of the selected members of the endocytic machinery (AP2, clathrin heavy chain, caveolin-1 or cdc42). We analyzed rate of TRAIL receptors

endocytosis, efficacy of DISC formation and kinetics of TRAIL-induced apoptosis. The relevant results pointing to a role of the internalization of TRAIL-death receptors will be shown and discussed.

GENERATION OF LMP1-TR1/2 FUSION PROTEINS AND RECOMBINANT TRAIL

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Usually, TNF-related apoptosis-inducing ligand (TRAIL) induces apoptosis in tumor cells, whereas normal cells are resistant. Therefore, TRAIL is considered as a potent future cytotoxic drug for the treatment of cancer. However, we described in 2003 that, instead of inducing apoptosis, TRAIL mediates cell survival and even proliferation in certain tumor cell lines. Previous experiments showed that TRAIL-induced proliferation was mediated by the same two TRAIL receptors which also signal TRAIL-induced apoptosis: TRAIL-receptor 1 and 2.
In order to generate tumor cell lines with constitutively active TRAIL signaling, we constructed fusion proteins consisting of the HA-tagged transmembrane domain of the EBV oncoprotein LMP1, fused to the intracellular signaling domains of TRAIL receptor 1 or 2. The LMP1 part mediates spontaneous oligomerization of the chimeras within the membrane and thus, constitutive activity of the fused TR signaling domains. In TRAIL-resistant HEK293T cells, LMP1-TR1 and LMP1-TR2 activated NFκB, while in TRAIL-sensitive SHEP cells both fusion proteins induced apoptosis. Thus, the two constitutive active proteins were able to mimic TRAIL stimulation. We will use these tools to monitor the influence of constitutive TRAIL signaling on tumor cell lines both in vitro and in vivo.
Additionally, we produced recombinant TRAIL suitable for stimulation of cells as well as for immune precipitations with and without a polymerizing isoleucine zipper domain. This protein will allow us to biochemically analyze DISC assembly in different cell lines and especially primary leukemic cells, which are amplified in NOD/SCID-mice prior to use.

ENHANCED CASPASE-8 ACTIVATION WITHIN THE DISC PRIMES OVER THE MITOCHONDRIAL PATHWAY TO SENSITIZE DCR2 EXPRESSING CELLS TO TRAIL INDUCED CELL DEATH AFTER CHEMOTHERAPY

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Combining TRAIL with various cytotoxic agents has been previously reported as a powerful way to induce apoptosis in TRAIL resistant cell lines. Yet the molecular mechanisms underlying sensitization to TRAIL-induced cell death remain poorly understood.
The present study focuses on DcR2 (TRAIL-R4) expressing cells which are resistant to TRAIL-mediated apoptosis by inhibition of caspase-8 processing within the DISC. Using three different groups of chemotherapeutic agents at pharmacological relevant conditions, we show that DcR2 expressing cell lines can be sensitized to TRAIL. Importantly, we demonstrate that the synergistic effect is neither impaired by Bcl-2 or Bcl-XL overexpression nor Bax deficiency, but severely inhibited by c-FLIP-L overexpression. We also provide evidence that chemotherapeutic agents promote caspase-8 recruitment and processing within the TRAIL DISC. This set of results indicates that the control of caspase-8 activation at the DISC level primes over the mitochondrial apoptotic pathway to sensitize DcR2 expressing cells to TRAIL induced cell death upon chemotherapy.

INHIBITION OF CASPASES ENHANCES THE CYTOTOXIC EFFECT OF TNF IN HUMAN MYELOMONOCYTIC U937 CELL LINE

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The eukaryotic cells treated with tumor necrosis factor (TNF) initiate programmed cell death, apoptosis, which is mainly mediated by caspase cascade activation. Therefore inhibition of caspases seems to be a perfect level of control in the process of apoptosis. In our research we worked with the human myelomonocytic U937 cell

line which is resistant to TNF, while undergoing apoptosis induced by the cytokine in presence of translation or transcription inhibitors. Interestingly, the U937 cells stimulated with a combination of TNF and the broad-spectrum caspase inhibitor zVAD.fmk became sensitive to TNF, revealing a caspase-independent mechanism of cell death. Moreover, the phenotype of dying cells suggested necrotic-like mechanism of cell death. Similar results were obtained after co-stimulation with TNF and caspase-8 specific inhibitor zIETD.fmk. Further experiments with TNF and caspase-3 specific inhibitor zDEVD.fmk revealed that the lethality of U937 cells was not enhanced which rather excluded caspase-3 engagement in the phenomenon. Flow cytometry analysis indicated that in cells treated with combination of TNF and zVAD.fmk the level of reactive oxygen species (ROS) increased significantly. In addition, it was found that the cytotoxic effect of TNF and zVAD.fmk was prevented by preincubation with antioxidant butylated hydroxyanizole (BHA) what confirmed the role of ROS in the mechanism of death. Further experiments were aiming at identifying possible regulatory mechanisms of TNF/zVAD induced cell death of U937 cells. It was found that p38 kinase phosphorylation but not activation of JNK kinase or mobilization of NF-κB transcription factor can be involved in modulation of cell death induced by TNF in the presence of caspase inhibition. Summarizing, the U937 cells when properly manipulated, seem to be a suitable model for studies on interplay between TNF-mediated apoptosis and necrosis as well as can be used for the analysis of regulatory mechanisms of different modes of TNF-induced cell death.

ANCHORAGE DEPENDENCY OF TNF AND CD40 LIGAND-INDUCED MAPK SIGNALLING

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The central role of the TNF receptor family members CD40 and TNFR1 and their ligands in immune regulation, disease pathogenesis and cancer therapy necessitates the in-depth understanding of their signalling circuits in a wide range of tissue types. Tpl2/Cot is a serine/threonine kinase that plays an important regulatory role in TNF and CD40 ligand-induced MAPK signal activation by operating upstream of MEK, the ERK kinase. Here, we show that CD40 ligand and TNF-mediated Tpl2 signal transduction in fibroblasts and carcinoma cells depends on cell adhesion. We have found that in suspended cells stimulated with TNF, the activity of Tpl2 and the phosphorylation of MEK and ERK are impaired. Overexpression of the Tpl2 kinase potently engaged MEK/ERK signal transduction in adherent fibroblasts but not in cells in suspension. MEK and ERK phosphorylation were also diminished in CD40 ligand-stimulated cells removed from their matrix whereas NF-κB activation

remained largely intact. Concomitantly, non-adherent carcinoma cells vigorously underwent apoptosis when stimulated with CD40 ligand, in line with the pro-survival effects of ERK signaling in these cells. Our findings reveal a novel crosstalk between TNF family receptor and anchorage induced signal transduction which may provide valuable insight into the complex biology of TNF receptor family members.

LIFEGUARD DIRECTLY INTERACTS WITH NEUROBLASTOMA-DERIVED SULFHYDRYL OXIDASE

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Lifeguard (LFG) is a member of a new class of death receptors inhibitors termed Fas-Apoptotic Inhibitory Molecules (FAIM). Indeed, LFG was first identified for its ability to protect Hela cells from Fas-mediated cell death but not from Tumor Necrosis Factor Receptor 1 (TNF-R1). Later, it was shown that the overexpression of LFG prevents from Fas-mediated neuronal death. In the same way, the downregulation of LFG (by RNAi) leads to sensitization to Fas-triggered apoptosis in both cerebellar granule cells and cortical neurons. In order to understand how LFG acts in Fas-mediated cell death, we carried out two hybrid approach using LFG as a bait. In this regard, we observed the direct interaction between the N-terminal part of LFG and a protein named Neuroblastoma-Derived Sulfhydryl Oxidase (SoxN), which was confirmed by co-immunoprecipitation. SoxN, a member of the sulfhydryl oxidase/Quiescin6 family, contains two main domains: a protein disulfide-isomerase-type thioredoxin and an ERV1 domain. Downregulation of SoxN expression protects to Interferon-gamma (IFN-γ)-induced apoptosis of neuroblastoma cells, positioning SoxN as an attractive target protein modulating the protective effects of LFG against Fas-mediated neuronal death.
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MEMBRANE FAS LIGAND CLEARS BACTERIA AND SOLUBLE FAS LIGAND PROTECTS HOST TISSUE DURING S. AUREUS INDUCED ENDOPHTHALMITIS

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PURPOSE: Endophthalmitis is an infection of the posterior segment of the eye that often results in retinal tissue damage as a direct consequence of the bacterial toxins, as well as, an indirect consequence of the host inflammatory response. We previously demonstrated that Fas ligand (FasL) is necessary for clearance of bacteria from the posterior of the eye. However, FasL is a membrane-bound protein that can be cleaved from the cell surface to release a soluble molecule with antagonistic activity and the function of the two forms in endophthalmitis is unknown. We hypothesize that membrane FasL rapidly turns on inflammation to clear the bacteria, while soluble FasL turns off inflammation to prevent non-specific retinal damage. To test this we constructed a novel mutant knock-in mouse that expresses only membrane FasL and is unable to produce soluble FasL (ΔCS.1 mice). We predict these mice will display an increased ability to clear large bacterial loads, but in the absence of soluble FasL prolonged inflammation will increase non-specific retinal damage.
METHODS: ΔCS.1 knock-in mice were created by mutating the FasL metalloproteinase cleavage site, preventing formation of soluble FasL. B6/129-WT and B6/129-ΔCS.1 mice received intravitreal injections of either 500 or 5000 CFU *S. aureus* (RN6390). Clinical examinations were performed at 24-96 hours post injection. H&E retinal sections were used to assess retinal damage. Neutrophil infiltration was assessed quantitatively using a myeloperoxidase (MPO) assay.
RESULTS: All WT mice successfully cleared a low dose (500 CFU) *S. aureus* infection with little to no retinal damage, but could not clear a high dose (5000 CFU) *S.aureus* infection, resulting in complete retinal destruction in 80% of the mice. By contrast, 100% of the ΔCS.1 mice successfully cleared a low dose *S.aureus* infection but displayed greater retinal damage that coincided with prolonged neutrophil infiltration. As predicted by our hypothesis, ΔCS.1 mice were capable of clearing a high dose *S. aureus* infection in 80% of the mice.
CONCLUSIONS: The membrane form of FasL is critical for triggering innate immunity that clears bacteria, while soluble FasL is necessary for terminating inflammation and limiting non-specific tissue damage.

TRAIL RESISTANCE IN NON-TRANSFORMED CELLS IS PROVIDED BY MULTIPLE FACTORS

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Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor family of death ligands. TRAIL is able to bind to two death inducing receptors, DR4 and DR5, and to two decoy receptors, DcR1 and DcR2. Through binding to DR4 and DR5, TRAIL can induce apoptosis selectively in tumour cells. Normal, untransformed cells are resistant to TRAIL, however, very little is known about the mechanism of their resistance. Approximately 50–60% of tumour types are also resistant to TRAIL. To sensitise these tumours to TRAIL without causing toxicity to normal cells, the mechanism that protects non-transformed cells from TRAIL should be identified.

As a typical non-transformed cell type, primary human dermal fibroblasts were used. We have shown that expression of the decoy receptors is not the reason for resistance. Inhibition of protein synthesis could sensitise the cells to crosslinked TRAIL-induced apoptosis, indicating that there is a block in the intracellular signalling pathway. Cell fusion experiments of fibroblasts with the TRAIL-sensitive colon carcinoma cell, Colo205 confirmed that presence of anti-apoptotic proteins protects fibroblasts from TRAIL-induced apoptosis.

Examination of the TRAIL-induced signal transduction pathway revealed that multiple factors contribute to TRAIL resistance.

DOWNREGULATION OF C-FLIP PROMOTES CASPASE-DEPENDENT JNK ACTIVATION AND REACTIVE OXYGEN SPECIES ACCUMULATION IN TUMOR CELLS

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NF-κB inhibits cell death through suppression of the caspase cascade, the c-Jun N-terminal kinase (JNK) pathway, and reactive oxygen species (ROS) accumulation. To suppress this antiapoptotic function of NF-κB might be a promising strategy to increase susceptibility of tumor cells to stress-induced cell death. We have recently shown that tumor necrosis factor (TNF)α induces caspase-dependent and -independent JNK activation and ROS accumulation in c-Flip-/- murine embryonic fibroblasts (MEFs). To apply this observation to tumor therapy, we knocked down c-FLIP by RNA interference in various tumor cells. Consistent with the results using c-Flip-/- MEFs, we found that TNFα stimulation induced caspase-dependent prolonged JNK activation and ROS accumulation, followed by apoptotic and necrotic cell death in various tumor cells. Furthermore, TNFα and Fas induced the cleavage of mitogen-activated protein kinase/ERK kinase kinase (MEKK)1, resulting in generation of a constitutive active form of MEKK1 leading to JNK activation in c-FLIP knockdown cells. Given that ROS accumulation and necrotic cell death enhance inflammation followed by compensatory proliferation of tumor cells, selective suppression of caspase-dependent ROS accumulation will be an alternative strategy to protect cells from ROS-dependent DNA damage and compensatory tumor progression.

SEVERAL MECHANISMS FOR THE REGULATION OF HUMAN T CELL BLAST ACTIVATION BY FASL AND APO2L/TRAIL

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The Fas-FasL pathway plays an important role in the homeostasis of mature lymphocytes, defects causing autoimmune lymphoproliferative syndromes (ALPS), with Apo2L/TRAIL and their receptors playing an apparently more limited role.

Human T cell blasts are not sensitive to FasL or Apo2L/TRAIL-induced apoptosis unless they get re-activated, but both ligands inhibit their growth in the absence of cell death induction due to a cell cycle arrest in S-G2/M. The induction of the negative cell cycle regulator p21WAF-1 by FasL or Apo2L/TRAIL in both CD4+ and CD8+ T cell blasts seems to be the main regulatory mechanism. p21 levels are controlled through a mechanism dependent on caspase activation and on H202 generation. These results suggest that the etiology of ALP syndromes could be related not only with defects in apoptosis induction, but also in cell cycle regulation. In addition, we have shown that the induction of Bim expression during the process of human T cell blast generation is strictly dependent on FasL/Fas-mediated signalling. The main pathway by which Fas signalling regulates the levels of Bim expression in human T cell blasts is the death-domain- and caspase-independent generation of discrete levels of H2O2, which results in the net increase of Foxo3a levels. These results connect the two main pathways described until the moment for the control of T cell responses: death receptor-mediated activation-induced cell death and apoptosis by cytokine deprivation, which is dependent on Bim levels.

TNFALPHA-INDUCED ACTIVATION OF NFKAPPAB PROTECTS AGAINST UV-INDUCED APOPTOSIS SPECIFICALLY IN P53-PROFICIENT CELLS

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Signaling pathways that depend on p53 or NFkappaB transcription factors are essential components of cellular responses to stress. In general, p53 is involved in either activation of cell cycle arrest or induction of apoptosis, while NFkappaB exerts mostly anti-apoptotic functions; both regulatory pathways apparently interfere with each other. Here we aimed to analyze effects of NFkappaB activation on DNA damage-induced apoptosis, either p53-dependent or p53-independent, in a set of human cell lines. Four cell lines, HCT116 and RKO colon carcinoma, NCI-H1299 lung carcinoma and HL60 myeloblastoma, each of them in two congenic variants either containing or lacking transcriptionally competent p53, were used. Cells were incubated with TNFalpha cytokine to activate NFkappaB and then treated with ultraviolet or ionizing radiation to induce apoptosis, which was assessed by measurement of the sub-G1 cell fraction. We observed that treatment with TNFalpha resulted in a significant reduction in the frequency of apoptotic cells in UV-irradiated p53-proficient lines (with exception of UV-resistant NCI-H1299 cells). This anti-apoptotic effect was lost when cells were pretreated with

parthenolide, an inhibitor of NFkappaB activation. In marked contrast, TNFalpha-pretreatment of p53-deficient lines resulted in an increased frequency of apoptotic cells after UV irradiation (with exception of HL60 cells). Such anti- and pro-apoptotic influence of TNFalpha was less obvious in cells treated with ionizing radiation. The data clearly indicates functional interference of both signaling pathways upon the damage-induced apoptotic response, yet the observed effects are both cell type- and stimulus-specific.
This work was supported by the Ministry of Science and Higher Education, grants 3T11A01929 and PBZ-MNiI-2/1/2005.

INFLUENCE OF OPTINEURIN ON ADENOVIRUS E3-14.7K MEDIATED TNF-RESISTANCE

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Recently we have shown, that adenovirus E3-14.7K (14.7K) expressing cells are protected from TNF-mediated apoptosis by inhibition of TNF-Receptor-1 (TNFR1) internalization. In the presence of 14.7K the recruitment of essential adaptorproteins involved in both apoptosis signalling (TRADD, FADD, and caspase-8) and clathrin-mediated endocytosis (rab5 and dynamin2) to the activated TNFR1 is blocked. The 14.7K interacting protein optineurin seems to link 14.7K not only to the apoptotic pathway but also to the membrane trafficking network via its additional interacting partners rab8 and huntingtin. Therefore we investigated wether the interaction of 14.7K with optineurin is essential to prevent TNF-mediated apoptosis.
We generated several mutants of 14.7K and characterized their ability to protect cells from apoptosis and to interact with optineurin. Finally 14.7K expressing cells were treated with optineurin targeting siRNA to examine the importance of optineurin for 14.7K mediated inhibition of apoptosis.
The 14.7K mutants defined three groups: (i) ones, which interacted with optineurin and protect cells from cell death, (ii) others that interacted with optineurin but failed to protect, and those (iii) that were incapable for interaction and inhibition of apoptosis. Interestingly the suppression of optineurin by siRNA transfection did not revert the TNF-resistant phenotype.
Collectively, these data suggest that optineurin might not be essentially involved in 14.7K mediated inhibition of TNFR1 induced apoptosis.
Additional work is under progress to address the effect of optineurin on TNFR1-internalization.

CD137-INDUCED CELL DEATH IN PERIPHERAL BLOOD MONONUCLEAR CELLS

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CD137 Ligand and its receptor are members of the TNF and TNF receptor superfamilies, respectively, and are potent immunoregulatory proteins. Via bidirectional signal transduction, the CD137/CD137 Ligand system can co-stimulate T-cell activation and proliferation and interestingly, we have found that reverse signaling via the CD137 Ligand can inhibit cell proliferation and induce apoptosis in peripheral blood mononuclear cells (PBMCs).
Our studies have shown that CD137 selectively induces cell death in the T-cell subpopulation of the PBMCs. Our investigation into the mechanism underlying this T-cell death program shows that it is not TNF dependent despite the large secretion of TNF by the CD137 treated PBMC mileu nor is it Fas dependent. There is evidence that the cell death induced is via the intrinsic pathway due to mitochondrial depolarisation of the cells as well as the concomitant production of reactive oxygen species (ROS). Scavenging ROS with hydrogen peroxide and superoxide anion inhibitors rescues the cell from death suggesting that CD137-induced apoptosis is regulated by intracellular ROS in T-cells.
The balance between leukocyte proliferation and cell death is critical in immune homeostasis. Further investigation into CD137-mediated cell death is expected to increase our understanding of the regulation of immune responses and contribute to the development of new approaches for the treatment of autoimmune diseases and cancer.

REGULATION OF CELL DEATH BY c-FLIP PHOSPHORYLATION

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c-FLIP proteins modulate death receptor responses by docking to the activated death receptor complex and preventing the formation of active caspase-8 homodimers. The splice variants have characteristic structural features, with distinct roles in death receptor signaling. We have shown earlier that c-FLIP(S) and c-FLIP(L) are ubiquitylated and subsequently degraded by the proteasome. However, c-FLIP(S)

has a dramatically shorter half-life than c-FLIP(L), suggesting that different mechanisms regulate their turnover. We have previously demonstrated that efficient ubiquitylation and subsequent degradation of c-FLIP(S) requires the presence of a C-terminal splicing tail. Here we have mapped a phosphorylation site that affects the ubiquitylation of c-FLIP and identified a signaling pathway that mediates this post-translational modification. In conclusion, our study reveals that c-FLIP phosphorylation is an important determinant of death receptor signaling and cellular survival.

MATHEMATICAL MODELING OF APOPTOSIS AS A FUNCTION OF C-FLIP TURNOVER AND AT RANDOM PARAMETER DISTRIBUTIONS

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The expression levels of the caspase-8 inhibitory c-FLIP proteins play an important role in regulating death receptor-mediated apoptosis, and experimental data as well as mathematical models have indicated that the concentration of c-FLIP is a key factor in determining the sensitivity of cells to signals coming through this pathway. Experimental studies have shown that c-FLIP levels can be rapidly modified by changes in the stability of c-FLIP. The present work applies mathematical modeling to study how c-FLIP stability regulates apoptotic signalling. Experimental studies of apoptosis often report the proportion of cells in a cell population, which undergo apoptosis after a given time in specified conditions. However, such results are not appropriate to use for verification or tuning of mathematical models that describe the behavior of a single cell. To address this problem, variations between individual cells are taken into account by characterizing protein concentrations and reaction parameters as random variables. The behavior of a population of cells is studied by Monte-Carlo simulation and these stochastic simulations are demonstrated to provide predictions in qualitative agreement with experimental findings. The simulation results indicate that the stability of c-FLIP has a strong influence on sensitivity to extrinsic apoptotic signalling, and that destabilization of c-FLIP is followed by apoptosis induced in response to death receptor ligation, thereby being in agreement with experimental findings.

RIP1, A JACK-OF-ALL-TRADES

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RIP1 is a crucial adaptor kinase on the crossroad of a cell's decision to live or die as it can differentially signal to cell death, autophagy, and activation of NF-κB and MAPKs. RIP1 consists of three domains: a kinase domain, an intermediate domain and a death domain. Studies in Jurkat cells have shown that RIP1 kinase activity is necessary to initiate death receptor-induced necrosis (necroptosis). In this study, we examined the contribution of the different RIP1 domains to necroptosis. Therefore, L929 cells were retrovirally transduced with three domain deletion mutants of RIP1 (delta KD, delta ID, delta DD). We demonstrate that RIP1 deltaID performs dominant negative effects on death receptor-induced necrosis in the presence of caspase-inhibitors whereas it induces a shift to apoptosis in the absence of caspase-inhibitors. In addition, this shift can be inhibited by necrostatin-1, a specific inhibitor of the RIP1 kinase activity. These results suggest that RIP1 can act at the decision point between necrotic and apoptotic signaling and that the intermediate domain might have a regulatory role in this decision. Furthermore, to study the physiological relevance of RIP1 in necroptosis, we are currently generating a conditional kinase-dead RIP1 knock-in mouse.

Subject Index

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