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Review

Tumor necrosis factor signaling

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Abstract

A single mouse click on the topic tumor necrosis factor (TNF) in PubMed reveals about 50 000 articles providing one or the other information about this pleiotropic cytokine or its relatives. This demonstrates the enormous scientific and clinical interest in elucidating the biology of a molecule (or rather a large family of molecules), which began now almost 30 years ago with the description of a cytokine able to exert antitumoral effects in mouse models. Although our understanding of the multiple functions of TNF *in vivo* and of the respective underlying mechanisms at a cellular and molecular level has made enormous progress since then, new aspects are steadily uncovered and it appears that still much needs to be learned before we can conclude that we have a full comprehension of TNF biology. This review shortly covers some general aspects of this fascinating molecule and then concentrates on the molecular mechanisms of TNF signal transduction. In particular, the multiple facets of crosstalk between the various signalling pathways engaged by TNF will be addressed.
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Keywords: apoptosis; IKK; immunity; JNK; necrosis; NF-κB; TNF; TRAF2

Abbreviations: ASK1, apoptosis signal-regulated kinase-1; aSMase, acidic sphingomyelinase; BIR, baculovirus IAP repeat; DD, death domain; DED, death effector domain; DISC, death-inducing signalling complex; FADD, Fas-associated death domain protein; FAN, factor associated with neutral sphingomyelinase activation; GSK, germinal center kinase; IAP, inhibitor of apoptosis protein; IKK, inhibitor of NF-κB kinase; IL, interleukin; I-κB, inhibitor of kappa B; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LTa, lymphotoxin-a; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; MEKK, mitogen-activated protein kinase/Erk kinase kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor kappa B; PARP, poly(ADP-ribose) polymerase; nSMase, neutral sphingomyelinase; PI3K, phosphoinositide-3OH kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLAD, pre-ligand binding assembly domain; PTEN, phosphatase and tensin homologue deleted on chromosome 10; RHD, rel homology domain; RING, really interesting new gene; RIP, receptor-interacting protein; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; TACE, TNF alpha-converting enzyme; SODD, silencer of death domain protein; TANK, TRAF-associated NF-kappaB activator; TNF, tumor necrosis factor; TRADD, TNF receptor-associated death domain protein; TRAF, TNF receptor-associated factor; Trx, thioredoxin

General aspects of TNF biology

The principle of an antitumoral response of the immune system *in vivo* has been recognized already about 100 years ago by the physician William B Coley. About 30 years ago, a soluble cytokine termed tumor necrosis factor (TNF) has been identified that is produced upon activation by the immune system, able to exert significant cytotoxicity on many tumor cell lines and to cause tumor necrosis in certain animal model systems. In 1984, the cDNA of TNF was cloned, the structural and functional homology to lymphotoxin (LT) α was realized, and several years later, two membrane receptors, each capable of binding both cytokines, were identified. Subsequently, it was recognized that TNF is the prototypic member of a large cytokine family, the TNF ligand family.

TNF is primarily produced as a type II transmembrane protein arranged in stable homotrimers (Figure 1).[1,2] From this membrane-integrated form the soluble homotrimeric cytokine (sTNF) is released via proteolytic cleavage by the metalloprotease TNF alpha converting enzyme (TACE).[3] The soluble 51 kDa trimeric sTNF tends to dissociate at concentrations below the nanomolar range, thereby losing its bioactivity. The 17 kDa TNF protomers are composed of two antiparallel β-pleated sheets with antiparallel β-strands, forming a 'jelly roll' β-structure, typical for the TNF ligand family, but also found in viral capsid proteins.[4]

The members of the TNF ligand family exert their biological functions via interaction with their cognate membrane receptors, comprising the TNF receptor (TNF-R) family.[5] The members of the TNF-R family contain one to six cysteine-rich repeats in their extracellular domain, typically each with three cysteine bridges.[6] Two receptors, TNF-R1 (TNF receptor type 1; CD120a; p55/60) and TNF-R2 (TNF receptor type 2; CD120b; p75/80) bind membrane-integrated TNF (memTNF) as well as soluble TNF (sTNF), but also the secreted homotrimeric molecule lymphotoxin-α (LTα). The functional role of LTα in man is largely undefined and will not be discussed in this review. TNF-R1 and TNF-R2 each contain four cysteine-rich repeats in their extracellular domains and form elongated shapes, which interact with the lateral grooves of the trimeric ligand formed between each two of its three protomers.[6,7] Ligand-dependent trimerization of

the receptors was long considered as the key event for signal initiation. However, initial receptor activation now appears more complicated, because the distal cysteine-rich domains of TNF-R1 and TNF-R2 mediate homophilic interaction of receptor molecules in the absence of ligand. These preligand binding assembly domains (PLAD)[8] may therefore keep receptors in a silent, homomultimerized status and antagonize spontaneous autoactivation, the latter being frequently observed upon overexpression. Accordingly, ligand binding to the preformed TNF-R complex either induces an activating conformational change of an *a priori* signal competent receptor complex or it allows the formation of higher-order receptor complexes, which acquire signal competence.

TNF-R1 is constitutively expressed in most tissues, whereas expression of TNF-R2 is highly regulated and is typically found in cells of the immune system. In the vast majority of cells, TNF-R1 appears to be the key mediator of TNF signalling, whereas in the lymphoid system TNF-R2 seems to play a major role. Generally, the importance of TNF-R2 is likely to be underestimated, because this receptor can only be fully activated by memTNF, but not sTNF.[9] The cause for this difference is not fully understood yet, but the different stabilities, that is half-lifes, of the individual ligand/receptor complexes may contribute to this.[9,10] The extracellular domains of both receptors can be proteolytically cleaved, yielding soluble receptor fragments with potential neutralizing capacity.[11] Owing the lack of cooperativity in ligand binding, however, the affinities of soluble receptors are low compared to their membrane-integrated forms. TNF neutralizing agents for clinical use that were constructed on the basis of the soluble receptors have therefore been engineered as dimeric IgG fusion proteins.[12] Like TNF, TNF-R2 is cleaved by TACE.[13] The processing enzyme(s) responsible for TNF-R1 cleavage is still undefined, but TNF-R1 cleavage is obviously an important step in the regulation of cellular TNF responsiveness, as cleavage-resistant TNF-R1 mutations are linked with dominantly inherited autoinflammatory syndromes (TNF-R1-associated periodic syndromes; TRAPS).[14]

The intracellular domains of TNF-R1 and TNF-R2 that do not possess any enzymatic activity define them as representatives of the two main subgroups of the TNF-R family, the death domain-containing receptors and the TRAF-interacting receptors, respectively. TNF-R1 contains a protein–protein interaction domain, called death domain (DD).[15] The DD can recruit other DD-containing proteins and couples the death receptors to caspase activation and apoptosis.[16] In addition, as described in detail below, TNF-R1 is also a potent activator of gene expression via indirect recruitment of members of the TNF receptor-associated factor (TRAF) family. TNF-R2 directly recruits TRAF2, induces gene expression and intensively crosstalks with TNF-R1.

TNF is mainly produced by macrophages, but also by a broad variety of other tissues including lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissue. Large amounts of sTNF are released in response to lipopolysaccharide and other bacterial products. In concert with other cytokines, TNF is considered to be a key player in the development of septic shock.[17] Whereas high concentrations of TNF induce shock-like symptoms, the prolonged exposure to low concentrations of TNF can result in a wasting syndrome, that is, cachexia. This can be found for example in tumor patients. Indeed, the biological mediator of cachexia, originally described in an animal model of trypanosoma infection and thus called cachectin, has been later unravelled as TNF.[18]

TNF exerts an extreme spectrum of bioactivities and most cells show at least some TNF responsiveness. In general, TNF may be considered to represent a major proinflammatory mediator, with an optional capacity to induce apoptosis. In (patho)physiological situations, TNF shows a remarkable functional duality, being strongly engaged both in tissue regeneration/expansion and destruction. One important example is the role of TNF in neurodegeneration. CNS-specific overexpression of TNF in transgenic mice revealed infiltrating $CD4^+$ and $CD8^+$ T cells, astrocytosis, microgliosis and demyelination.[19] Although from these transgenic animal models, TNF and TNF-R signalling has been implicated as important for the onset of demyelinating disease, TNF must also be recognized as a reactive cytokine that is upregulated in response to traumatic and excitotoxic injury of the brain, thus potentially exerting protective functions. Of interest, TNF receptors can have counteracting functions, at least in neuronal tissues, as recently demonstrated in a murine model of retinal ischemia, where TNF-R1 apparently aggravated tissue destruction, whereas TNF-R2 was protective via activation of PKB/Akt.[20] In a different transgenic model, a targeted AU repeat deletion of 69 bp in the 3′untranslated region of the TNF gene resulted in an enhanced stability of TNF mRNA and elevated protein level in fibroblasts, but not in a significant change in TNF responses to LPS challenge. These animals develop clinical signs of arthritis and colitis.[21] However, when this modified TNF transgene is crossed into TNF-R2-deficient mice, a near to normal phenotype is observed, pointing to a significant contribution of this receptor for the development of chronic inflammatory diseases.[21] A further example for the two-edged role of TNF *in vivo* is liver regeneration after partial hepatectomy. In TNF-R1-deficient animals, hepatocyte DNA synthesis is severely impaired, indicating that TNF signalling through TNF-R1 is involved in liver regeneration.[22] In contrast, in models of acute hepatotoxicity TNF acting via TNF-R1 appears as a key player in liver destruction.[23] Owing to its strong proinflammatory and immunostimulatory activities, TNF is, in general, an important mediator of progression of many autoimmune diseases. Important examples are rheumatoid arthritis and inflammatory bowel disease (Crohn's disease), where significant clinical improvement can be achieved when patients are treated with TNF neutralizing agents.[24,25] Thus, the question as to whether TNF contributes to or protects from tissue damage in acute or chronic diseases is probably wrongly posed. The accumulating data rather suggest a very differential TNF action and indicate that tissue type, precise cellular context and TNF-R composition, timing and duration of TNF action are important parameters determining the net effect of TNF action *in vivo*.

The developmental role of TNF and its receptors has also been addressed by gene targeting approaches. TNF, LTα and LTβ have been inactivated either alone or in combinations and the respective receptors (TNF-R1, TNF-R2, LTβR) were targeted as well. Knockout mice lacking LTβR, the receptor for

a heterotrimeric ligand consisting of LTα and LTβ, are devoid of all lymph nodes, Peyer's patches and gut-associated lymphatic tissue, providing clear evidence for the essential role of this receptor in secondary lymphoid tissue development. Interestingly, TNF$-/-$ and TNF-R1$-/-$ mice share some features of the LTβR$-/-$ phenotype, but also reveal unique characteristics, thus defining both redundant and nonredundant functions for each of these molecules in lymph node formation.[26,27]

Further, in full accordance with the important role of TNF as a mediator of the innate immune system, impaired defense against certain intracellular pathogens is observed in TNF-R1- and TNF-deficient animals, whereas parameters of the adaptive immune system like $CD8^+$ T-cell cytotoxicity, mixed lymphocyte responses, T-cell-independent B-cell response and most parameters of T-cell-dependent B-cell response remain grossly normal.[28] TNF- or TNF-R1-deficient mice show enhanced sensitivity when challenged with, for example, *Mycobacterium tuberculosis*,[29] *Lysteria monocytogenes*[30] or *Leishmannia major*.[31] In mycobacterial infections, especially the formation of granuloma is TNF dependent.[32] TNF-R1- and TNF-deficient mice, but not TNF-R2 knockout animals, die from a fulminant necrotizing encephalitis when orally infected with a low-virulent strain of *Toxoplasma gondii*.[33] However, in other infection models, TNF knockout mice show delayed pathological reactions when challenged with pathogens. This has been observed for example in rabies virus infection,[34] the acute phase of infection by *Yersinia enterocolitica*,[35] and a model of cerebral malaria.[36] Independent from its role in host defense, TNF might also play a role in downregulating the immune system after a successful response.[37] Together, these examples clearly show that the specific role of TNF in infection is highly dependent on the type of the pathogen, the general context and stage of the infection. Very recent data from patients with inflammatory bowel disease, who have been treated Qwith the TNF-R2–IgG fusion protein *etanercept* to antagonize TNF activity, support a crucial role of TNF in defense against intracellular pathogens: during treatment an exacerbation of *Mycobacterium tuberculosis* enteritis was observed.[38]

The first described and thus the name giving action of TNF was its antitumoral activity in mouse tumor models.[39] As a result of TNFs strong cytotoxic activity on some tumor cells *in vitro*,[40] TNF was initially considered as a widely applicable, direct tumoricidal reagent. However, meanwhile it is evident that TNF-mediated tumor rejection *in vivo* is dependent on a functional immune response and most likely independent of TNF's capability to induce directly apoptosis in tumor target cells.[41] Moreover, systemic TNF application in humans is limited by severe side effects, ranging from influenza-like symptoms to the development of life-threatening symptoms of shock.[17] Nevertheless, more recent data show that TNF can be successfully applied as a tumor therapeutic under conditions that prevent systemic TNF action. Thus, high concentrations of TNF in combination with the chemotherapeutic drug melphalan, applied under isolated limb perfusion conditions, yielded superior response rates and limb salvage in metastatic sarcoma.[42] The underlying mechanism involves destruction of the tumor vasculature leading to a necrotic destruction of the tumor.[43] For a successful application of TNF in other tumor entities future developments need to accommodate these results, aiming at genetically engineered TNF-based constructs that display site-specific action.

TNF-induced activation of NF-κB

Nuclear factor kappa B (NF-κB) comprises a group of dimeric transcription factors consisting of various members of the NF-κB/Rel family. NF-κB proteins are involved in the transcriptional activation of a huge number of inflammatory-related genes in response to cytokines, for example, TNF and IL-1, bacterial products and some forms of physical 'stress', for example, UV radiation or reactive oxygen species.[44] In the recent years, it became also evident that NF-κB induces a variety of antiapoptotic factors, which is of importance for the regulation of TNF-R1-mediated triggering of the apoptotic machinery of the cell (see below). In mammalian cells, five members of the NF-κB/Rel family are known: NF-κB1/p50, which is constitutively processed from its precursor p105 by proteolysis, NF-κB2/p52, which is inducibly processed from its precursor p100, c-Rel, RelA/p65 and RelB.[45] The NF-κB/Rel proteins share the conserved Rel homology domain (RHD), which mediates dimerization, DNA binding, nuclear localization and interaction with members of the I-κB protein family – the inhibitory counterparts of the NF-κB/Rel proteins.[45] The I-κB family is characterized by six or seven ankyrin repeats and includes I-κBα, I-κBβ, I-κBγ, I-κBε, Bcl-3 and the precursors of NF-κB1 and NF-κB2, p105 and p100, respectively, that contain seven carboxy-terminal ankyrin repeats in addition to their amino-terminal RHD. I-κBα, I-κBβ and I-κBε also contain an amino-terminal regulatory domain that allows stimulus-induced degradation of these proteins.[46] When functions of NF-κB or I-κB are described in the following paragraphs, in most cases, we refer to data obtained with p65/p50 heterodimers and the I-κBα isoform.

In an uninduced state, cellular I-κB proteins interact with NF-κB dimers to mask their nuclear location sequence, thereby retaining the ternary complex of NF-κB and I-κB in the cytoplasm. TNF, like a variety of other inducers, can stimulate proteolytic degradation of I-κB by the proteasome, thus liberating NF-κB and allowing nuclear translocation.[46] For full activation, NF-κB must be further modified by phosphorylation of its subunits. Several kinases including mitogen activated protein kinases (MAPK) and protein kinase C (PKC) isoforms have been implicated in this secondary modification of NF-κB activity (see below). The level at which the various I-κB degradation-inducing signalling pathways converge is the activation of a multicomponent protein kinase complex, the I-κB kinase (IKK) complex. The activated IKK complex is able to phosphorylate the regulatory domain of I-κB and this marks it for recognition by an SKP1-Cullin-Fbox-type E3 ubiquitin–protein ligase complex. The IKK complex is believed to comprise a heteromer of two related I-κB kinases, called IKK1 and IKK2 (IKKα and IKKβ),[47–49] the regulatory protein NEMO (Fip-3, IKKγ, IKKAP)[50–54] and a homodimer of the heat shock protein-90 (Hsp90), as well as two or three molecules of the Hsp90-asscociated cdc37 protein.[55] NEMO-deficient cells are completely impaired in NF-κB activation by all inducers investigated so far[56–58] suggesting that this

noncatalytic component has an essential role in the IKK complex. Analyses of IKK1- and IKK2-deficient mouse embryonic fibroblasts revealed that both I-κB kinases are nonredundant in their function and contribute differently to TNF-induced NF-κB activation (Table 1). IKK2-deficient mice are strongly impaired in TNF-induced I-κB phosphorylation, but nevertheless show residual DNA binding of NF-κB, significant production of NF-κB-driven genes and almost unchanged p65 phosphorylation after TNF stimulation.[59,60] In contrast, TNF-induced I-κB phosphorylation and DNA binding of NF-κB were found to be unaffected in IKK1 deficient mouse embryonic fibroblasts in two studies,[61,62] but another study reported significant reduction in TNF-induced NF-κB binding and inhibition of NF-κB target genes.[63] There is also evidence that IKK1, but not IKK2, has a role in a second pathway leading to the activation of NF-κB2 by promoting its processing from the p100 precursor. Interestingly, this pathway is independent of I-κB degradation,[64,65] However, there is yet no evidence that this pathway plays a role in TNF signalling. In good agreement with an essential role of the IKK complex in TNF-induced NF-κB activation is that all effects of this response are completely abrogated in mouse embryonic fibroblasts (MEFs) of IKK1–IKK2 double-deficient mice.[66] In particular, this indicates that the recently identified IKK-related kinases TBK/T2K/NAK[67–69] and IKKε[70] cannot substitute for IKK1 and IKK2 in TNF-signalling.

The initial event in TNF-induced activation of the IKK complex is ligand-induced reorganization of preassembled TNF-R1 complexes (Figure 1). The intrinsic property of the death domain of TNF-R1 to self-aggregate and therefore to signal independent of ligand is masked in preassembled TNF-R1 complexes by binding of the silencer of death domain (SODD) protein.[71] After ligand binding, SODD dissociates from TNF-R1 complexes and the death domain-containing adaptor protein TRADD is recruited to the death domain of TNF-R1 by homophilic interactions of the death domains.[72] TNF-R1-bound TRADD then serves as an assembly platform for binding of TNF receptor-associated factor (TRAF) 2 and the death domain-containing serine–threonine kinase RIP (receptor-interacting kinase).[73] TRAF2 is a member of the phylogenetically conserved TRAF protein family.[74] The characteristic feature of the TRAF proteins is a carboxy-terminal homology domain of about 180 aa, the TRAF domain, which mediates a wide range of protein–protein interactions including binding to MAP3 kinases, various regulators, and non-death domain-containing members of the TNF receptor

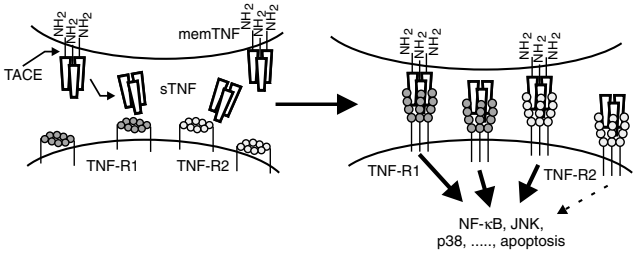

Figure 1 Membrane-bound TNF (memTNF) and soluble TNF (sTNF) derived thereof, both bind to two members of the TNF-receptor superfamily, TNF-R1 and TNF-R2. While memTNF activates both TNF receptors, sTNF predominantly stimulates TNF-R1 and has limited signalling capacities on TNF-R2

family.[74] With the exception of TRAF1, all TRAF proteins have an amino-terminal RING finger, which is followed by five or seven evenly spaced zinc fingers.[74] The association of TRAF2 to TNF-R1-bound TRADD is mediated by the interaction of its carboxy-terminal TRAF domain with the amino-terminal death domain of TRADD.[75] In contrast, RIP is recruited to the DD of TNF-R1-bound TRADD via its carboxy-terminal death domain.[73] RIP is also able to interact with TRAF2 via its amino-terminal kinase domain and its central intermediate domain.[73] However, studies with TRAF2- and RIP- deficient mouse embryonic fibroblasts have shown that both molecules can be independently recruited into the TNF-R1 signalling complex.[76] Moreover, these studies showed that TRAF2 is sufficient to recruit the IKK complex into the TNF-R1 signalling complex whereas RIP is necessary for the activation of the IKKs.[76] Although RIP is able to interact with NEMO[51,77] in the yeast two-hybrid system, studies with RIP-deficient cells showed that this interaction is dispensable for recruitment of the IKK complex to TNF-R1.[76] Thus, a minimal model of a TNF-induced NF-κB activation comprises TRAF2, acting as a receptor proximal adaptor (via TRADD binding) that recruits the IKK complex to the TNF-R1 signalling complex, thereby enabling RIP to activate the kinases of the IKK complex (Figure 2). However, there are several lines of evidence that the interplay of TRAF2, RIP and the IKK complex is more complicated. In RIP-deficient cells, similar amounts of IKK1 and IKK2 are found in the TNF-R1 signalling complex compared to wt cells, but the amount of coprecipitated NEMO is significantly reduced.[78] These data, together with the finding that increasing amounts of TRAF2 interfere with the interaction of IKK1 and NEMO,[78] suggest that TRAF2 binding weakens the coherence of the IKK complex. Remarkably, RIP is able to compensate the TRAF2 inhibitory effect on the IKK1/2–NEMO interaction[78] and possibly stabilizes the IKK complex after TRAF2-mediated recruitment to the TNF-R1 signalling complex. A puzzling detail in this regard is the observation that in RIP-deficient cells a significantly increased amount of TRAF2 and TRADD is recruited to TNF-R1 after TNF treatment[76,78] without an effect on the recruitment of IKK1/2.[78] Overexpression studies[73] and reconstitution experiments in RIP-deficient cells[76,79] suggest that the kinase activity of RIP is dispensable for IKK activation. It is conceivable that RIP activates the IKKs indirectly via mitogen-activated protein kinase kinase kinase MEKK3, as RIP can interact with MEKK3, and MEKK3-deficient mouse embryonic fibroblasts show strongly reduced NF-κB activation in response to TNF and IL1.[80] However, marginal TNF-induced NF-κB activation has been reported for MEKK3$-/-$ mouse embryonic fibroblasts pointing to the possibility that other kinases can substitute to some extent for MEKK3.[80] Indeed, a possible candidate is MEKK1, which is able to interact with TRAF2 as well as with RIP after TNF stimulation in human HEK293 cells and is activated by TNF in a RIP-dependent manner in the human T-cell line Jurkat.[81,82] Inconsistent with a role of MEKK1 in TNF-induced NF-κB activation are findings showing that this response is normal in MEKK1-deficient embryonal stem cells as well as in fibroblasts and macrophages derived from MEKK1-deficient mice.[83,84] However, it cannot be ruled out that this discrepancy is based on cell- or

Table 1 Phenotype of knockout mice and/or cells deficient in TNF signalling-related molecules

Knockout	Phenotype	Reference
A20	Development of severe inflammation and cachexia High sensitivity against TNF and LPS Prolonged TNF-induced activation of NF-κB and JNK but no effect on IL1-induced NF-κB activation	121
aSMase	Partly reduced TNF-induced cell death	306
ASK1	Normal overall appearance Strongly reduced TNF-induced apoptosis, but no changes in TNF-induced caspase-8 activation Rapid (30') TNF-induced activation of JNK and p38 is normal but prolonged activation is reduced Fas-induced activation of JNK and p38 is reduced	144
Caspase-8	Impaired TNF-induced cell death in caspase-8 deficient Jurkat cells Embryonic lethal around 12.5 days postcoitum Impaired apoptosis-induction by TNF-R1-, Fas and DR3	199,191
Cathepsin B	Partly reduced TNF-induced cell death in hepatocytes	314
GSK-3	Embryonic lethality around 13.5–14.5 days postcoitum caused by TNF-induced liver degeneration Reduced TNF- and IL1-dependent NF-κB activation Increased TNF sensitivity	114
FADD	Embryonic lethal around 10–12 days postcoitum Strongly reduced TNF-induced apoptosis Impaired TNF-induced activation of acidic SMase	193,194,198
FAN	Normal overall appearance No TNF-dependent activation of neutrale SMase but normal activation ERKs	170
FLIP	Embryonic lethal around 10.5–11.5 days postcoitum Impaired cardiac development MEFs highly sensitive towards TNF-induced apoptosis	327
JunD	MEFs show increased TNF-induced cell death	328
IKKα	Abnormalities in limb, skeleton and skin development Reduced TNF-induced NF-κB activation in MEFs	61–63,66,92
IKKβ	Embryonic lethal around 13–14 days postcoitum (liver degeneration) Rescued by crossing with TNF-R1 -/- Strongly reduced TNF and IL1-induced NF-κB activation in MEFs Increased TNF sensitivity in thymocytes	59,66,329,330
NEMO/IKKγ	Impaired TNF-induced NF-κB activation in a IKKγ-deficient B-cell line Embryonic lethality around 13–14 days postcoitum (liver degeneration) Skin lesions in heterozygous female mice Impaired TNF-induced NF-κB activation in MEFs	50,56,57, 58
IKK1+IKK2	Impaired TNF-induced NF-κB activation in MEFs	66
MKK3	Normal overall appearance TNF- but not IL1-induced p38 activation was reduced TNF-induced JNK activation was unchanged	163
MKK4	Moderate reduction in TNF-induced JNK activation	139
MKK7	Strong reduction in TNF-induced JNK activation	139
MKK4 + MKK7	p38 activation by TNF is largely unaffected	139
MEKK1	Normal TNF- and IL1-induced activation of JNK and NF-κB in fibroblasts and macrophages	84
MEKK3	Embryonic lethal around 10.5 –11 days postcoitum Impaired NF-κB activation by TNF and IL1	80
NIK	Normal TNF signalling	331

Table 1 *(continued)*

Knockout	Phenotype	Reference
PARP	Normal overall appearance Increased TNF sensitivity Reduced TNF-dependent NF-κB activation	106,332
PKC-zeta	Normal overall appearance, but reduced number of Peyer's patches Strongly reduced activation of NF-κB (DNA binding and reporter gene) by TNF and IL1 but normal IKK activation in some cell types Enhanced TNF sensitivity of MEFs but no liver apoptosis	86
RIP	Early (3 days) postnatal lethality Massive thymic cell death at 18 days postcoitum MEFs highly sensitive against TNF-induced apoptosis Impaired TNF-dependent NF-κB activation but normal NK activation	162
RelA	Embryonic lethal around 15 days postcoitum Impaired TNF-induced NF-κB activation in MEFs MEFs show increased sensitivity against TNF-induced apoptosis	333,334
T2K	Embryonic lethal around 15 days postcoitum Rescued by crossing with TNF-R1-/- TNF sensitivity is unchanged in MEFs Impaired TNF-induced upregulation of NF-κB-dependent genes despite normal NF-κB translocation	68
TRAF1	Enhanced TNF-R2 signalling	335
TRAF2	Impaired TNF-induced JNK activation	138
TRAF5	No phenotype in TNF signalling	336
TRAF2+TRAF5	Impaired TNF-induced NF-κB activation	337

species-specific distinct roles of various MAP3K in TNF signalling.

Although TRAF2 has been mainly recognized as a physical link between the TNF-R1 signalling complex and the IKK complex it seems possible that this molecule has additional functions in NF-κB activation. Indeed, the TRAF2-related TRAF6 molecule is able to interact with PKCζ, an atypical protein kinase C,[85] which has also been implicated in TNF-induced NF-κB activation.[86] Analyses of cells derived from PKCζ-deficient mice point to a cell type-specific and potentially multi-functional role in TNF-mediated NF-κB activation (Table 1). While in lung tissue of PKCζ-deficient mice IKK activation by TNF is impaired, embryonal fibroblasts from the same animals show IKK activation similar to WT cells.[86] Nevertheless, DNA binding of NF-κB and transcription of an NF-κB-driven luciferase reporter gene have been found to be significantly reduced also in this cell type. A possible explanation for these observations could be the capability of PKCζ to phosphorylate p65 in its RHD, which is responsible for DNA binding.[86,87] In this regard, PKCζ resembles the catalytic subunit of protein kinase A (cPKA), which can be released from an I-κB/NF–κB/cPKA complex in response to LPS and which is involved in NF-κB activation by phosphorylation of RHD serine 276.[88] In this case, phosphorylation of serine 276 is necessary to allow recruitment of the transcriptional coactivator CPB/p300. Interaction of CPB/p300 and p65 is mediated by two motifs within p65, one within the RHD comprising phosphorylated serine 276 and one unphosphorylated site in the C-terminal region of p65, which is only accessible upon phosphorylation of serine 276.[88] The role of PKCζ-dependent phosphorylation of p65 would then be clearly distinct from the action of some other kinases, which stimulate NF-κB activity by phosphorylation of p65 in its carboxy-terminal transactivation domain without affecting DNA binding (see below). The cell type-restricted NF-κB deficiency in knockout cells could be because of the functional redundancy of PKCζ and the closely related atypical PKCλ, which display different expression patterns. Indeed, PKCζ as well as PKCλ interact with the IKK complex after TNF stimulation[89] and both kinases indirectly interact with RIP via the adaptor protein p62.[90] The complex role of PKCζ exemplifies that activation of the IKK complex, degradation of I-κB, nuclear translocation of p65 and DNA binding may not suffice for efficient transcription of NF-κB responsive genes. To obtain full NF-κB function, additional stimulation of the transactivation potential of p65 by phosphorylation at serines 529 and 536 appears to be required. This can be accomplished by constitutively active kinases like casein kinase II, which is able to phosphorylate serine 529 of p65 after its release from I-κB,[91] or by the serine–threonine kinase Akt upon TNF stimulation via an IKK1-dependent pathway.[92] TNF can stimulate Akt in a cell type-specific manner via the phosphoinositide-3OH kinase (PI3K) pathway.[93–101] In agreement with a role of the PI3K/Akt pathway in TNF-induced NF-κB activation, it has been found that the dual specificity phosphatase PTEN, which dephosphorylates and inactivates phosphatidylinositol 3-phosphate, inhibits TNF-induced transcription of NF-κB-driven genes.[102,103] However, although these studies partly used the same experimental models, there are considerable discrepancies regarding the steps in

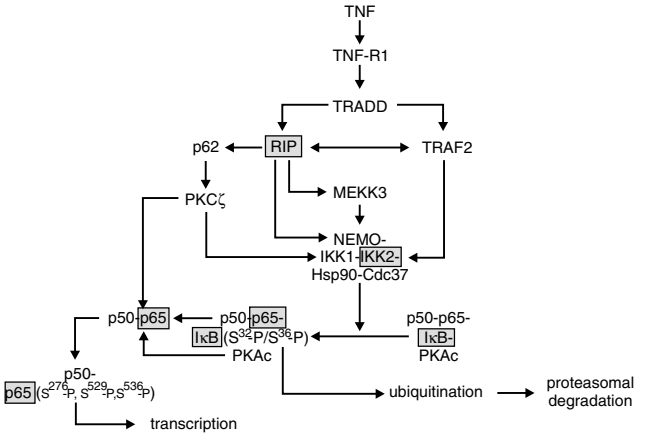

Figure 2 Scheme of TNF-R1-induced NF-κB activation. Components of the NF-κB pathway that can be cleaved by caspases during apoptosis are shown in gray boxes

NF-κB activation that are affected by PTEN-dependent inhibition of Akt. Two recent studies using IKK1- and IKK2-deficient mouse embryonic fibroblasts reported different results concerning the role of IKK1 and IKK2 in Akt-mediated transactivation of the p65 subunit of NF-κB.[92,104] In both studies, IKK2 was necessary for Akt-dependent p65 transactivation, whereby in one study myristoylated and therefore constitutively active Akt (Myr-Akt) and in the other study TNF was used to induce transactivation of p65.[92,104] However, in the study of Sizemore *et al*.[104] TNF-induced transactivation was in addition dependent on IKK1 and the Akt pathway, whereas overexpressed Myr-Akt was able to transactivate p65 in IKK1-deficent cells.[104] In agreement with an important role of IKK1 in TNF-induced transactivation of p65, another report using IKK1-deficient mouse embryonic fibroblasts shows that TNF-induced upregulation of the endogenous NF-κB target genes I-κBα, IL6 and M-CSF is impaired in these cells.[63]

Another level of complexity in TNF-induced NF-κB activation became apparent from the analyses of mice deficient for poly(ADP-ribose) polymerase 1 (PARP-1; see Table 1), a nuclear DNA repair enzyme activated by DNA strand breaks.[105] Unexpectedly, PARP-1$-/-$ mice were highly resistant to LPS-induced endotoxic shock because of an impaired NF-κB response towards the inflammatory mediators LPS and TNF.[106,107] While degradation of I-κB and translocation of NF-κB were normal in PARP-1-deficient cells, DNA-binding and transcriptional activation were found to be severely reduced.[106,107] Subsequent *in vitro* studies have then shown that PARP-1 interacts with both p65 and p50 by two independent domains.[108–110] However, there are contradictory data concerning the importance of the PARP-1 enzymatic activity for NF-κB activation.[108,109,111–113] TNF is able to activate PARP-1 via the production of ROS, which in turn causes PARP-inducing DNA damage. However, it is an open question whether TNF-induced PARP-1 activation via ROS is a prerequisite for its role in TNF-induced NF-κB activation or whether PARP-1 exerts its NF-κB-supporting capability independent of prior activation. Interestingly, TNF-induced PARP-1 activation by the production of ROS and subsequent DNA damage has also been implicated in the regulation of the balance between apoptosis and necrosis (see below). It will be interesting to see whether the roles of PARP-1 in NF-κB activation and in cell death induction are related in some way.

Remarkably, mouse embryonic fibroblasts from mice deficient for GSK3β or TBK/T2K/NAK, two kinases previously not thought to be involved in TNF signalling, also exert reduced NF-κB-dependent transcription, but normal nuclear translocation and DNA binding in response to TNF, suggesting a role of these kinases in p65 transactivation, also.[68,114] Kinase-inactive mutants of TBK/T2K/NAK failed to interfere with TNF-induced NF-κB activation, thus the role of TBK/T2K/NAK in TNF-dependent NF-κB activation might be rather of structural nature than implying its enzymatic capabilities.[67,69] Nevertheless, a more complex, maybe multifunctional, role of TBK/T2K/NAK in NF-κB activation is possible as this kinase is able to phosphorylate IKK2[69] and its kinase activity is necessary for NF-κB activation by overexpression of TANK.[67] However, the molecular mechanisms underlying the effects of GSK3ß and TBK/T2K/NAK on TNF-induced NF-κB activation are not defined yet.

While the pathways leading from TNF/TNF-R1 interaction to activation of the IKK complex and NF-κB are comparably well understood, the mechanisms involved in the termination of the TNF-induced NF-κB response are rather unclear. There is evidence that TNF-selective, but also rather globally acting feedback mechanisms are utilized to terminate TNF-induced NF-κB activation. Complexes of soluble TNF and TNF-R1 are rapidly internalized[115,116] opening the possibility that degradation in secondary lysosomes contributes to termination of TNF responses. By contrast, TNF-induced internalization seems to be required for efficient stimulation of some (JNK, aSMase, apoptosis), whereas other signalling pathways (nSMase, ERK, NF-κB) initiated by TNF-R1 are independent of internalization.[117]

NF-κB-dependent upregulation of NF-κB inhibitory proteins is another powerful mechanism involved in feedback inhibition of the NF-κB pathway. In particular, I-κBα and A20 have been identified as NF-κB inducible genes that are required for the postinduction repression of TNF-induced NF-κB activation.[118–120] As I-κBα targets free p65/p50 subunits, it inhibits NF-κB activation by a variety of stimuli. In contrast, analyses of mouse embryonic fibroblasts of A20-deficient fibroblasts point to a more selective role. Mouse embryonic fibroblasts deficient for A20 show persistent activation of the IKK complex and prolonged DNA binding of NF-κB in response to TNF, whereas termination of the IL1-induced NF-κB activation remains unaffected.[121] In contrast to the TNF-selective effects observed in A20-deficient cells, biochemical and transient overexpression studies point to a more general regulatory role of A20 affecting various MAP3K associated with NF-κB activation.[122–124] A20 can interact with TRAF2[120] as well as NEMO.[77] In agreement with the IKK inhibitory role of A20 and its recruitment to the TNF-R1–IKK signalling complex it has been found that the capacity of the IKK complex, precipitated from whole cell lysates, to phosphorylate I-κB is greater than that of the TNF-R1-associated IKK complex.[77] Interestingly, A20 promotes the phosphorylation of IKK1/2 in the context of the TNF-R1–IKK signalling complex.[77]

IKK2 undergoes progressive autophosphorylation at multiple serine residues in its carboxy-terminal end, thereby decreasing its kinase activity.[125] It is therefore tempting to speculate that A20 stabilizes or promotes this autoinhibitory state of IKK2.

Stimulation of TNF-R1 can lead to a strong activation of the apoptotic pathway, in particular when protein synthesis is globally reduced or when the NF-κB pathway is compromised. Therefore, the fact that the NF-κB pathway targets, among others, a variety of antiapoptotic genes is of special interest. Indeed, there is growing evidence that the NF-κB and the apoptotic pathway are tightly connected. This aspect of TNF signalling is therefore discussed below in the context of TNF-induced apoptosis.

TNF-induced activation of JNK and p38-MAPK

TNF regularly induces the activation of kinases of the stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) group.[126] The JNK isoforms are distantly related to mitogen-activated protein kinases (ERKs) and, like ERKs, are activated by dual phosphorylation on tyrosine and threonine residues. Upon activation, JNK kinases translocate into the nucleus and enhance the transcriptional activity of transcription factors, for example, c-Jun and ATF2, by phosphorylation of their amino-terminal activation domains.[127] c-Jun belongs to a group of basic region-leucine zipper proteins that dimerize to form transcription factors commonly designated as activator protein-1 (AP-1).[127] However, JNK kinases have also functions not related to c-Jun phosphorylation. The AP-1 proteins have an important role in a variety of cellular processes including proliferation, differentiation and induction, as well as prevention of apoptosis.[127] Although TNF-induced JNK activation and c-Jun phosphorylation have been implicated in upregulation of collagenases,[128] the chemoattractant MCP-1,[129] E-selectin[130] and in the regenerative response to liver injury,[131,132] the importance of JNK activation for TNF-mediated cellular responses is otherwise poorly understood.

TNF-induced activation of the JNK pathway occurs through a nonapoptotic TRAF2-dependent pathway (Figure 3).[133–138] It is evident from knockout mice and mice overexpressing a dominant-negative form of TRAF2 that this adaptor is necessary for coupling the JNK pathway to TNF-R1.[137,138] Analyses of MKK7- and MKK4- deficient mouse embryonic fibroblasts suggest that MKK7 is essentially involved in TNF-induced JNK activation, whereas MKK4 contributes to optimal TNF-mediated JNK activation, but is not sufficient to evoke this response alone.[139] The distinct roles of MKK7 and MKK4 in TNF-induced JNK activation most likely reflect the different, but complementary substrate specificities of these kinases. MKK7 preferentially phosphorylates threonine 180 of JNK,[140] whereas MKK4 mainly phosphorylates tyrosine182.[139] In this regard, it has been shown that MKK7 is able to activate WT JNK, but to a lesser degree also mutated JNK harboring a Tyr182Phe substitution, whereas MKK4 is only able to activate wt JNK.[139] Therefore, TNF-induced activation of JNK seems not to depend completely on dual JNK phosphorylation. Thus, phosphorylation of threonine 180 by MKK7 could be sufficient to activate JNK in response to TNF and MKK4 could enhance TNF–MKK7-mediated JNK activation by phosphorylation of tyrosine 182. Remarkably, TNF activates MKK7 but not MKK4,[141] suggesting that the basal activity of MKK4 is sufficient to allow maximal activation of JNK in response to TNF. While knockout mice have clearly proven the pivotal roles of TRAF2 and MKK7 for TNF-R1-mediated JNK activation, the nature and function of the MAP3K, which must fill the gap between TRAF2 and MKK7, has not been satisfactorily identified yet. Based on their ability to interact with TRAF2, the JNK-inducing MAP3 kinases MEKK1 and ASK1 potentially have a role in TNF-induced JNK activation,[81,142,143] but studies in mice deficient for these kinases could not support an essential role in TNF-induced JNK activation.[84,144] However, as discussed in detail later, a role of ASK1 in prolonged TNF-induced JNK activation, which occurs under apoptotic conditions, is supported by the ASK1 knockout mice. It is also possible that a role of ASK1 and/or MEKK1 in TNF-induced JNK activation is masked by redundancies. Indeed, biochemical data indicate that several parallel pathways link TNF-R1/TRAF2 to MKK7 and JNK.

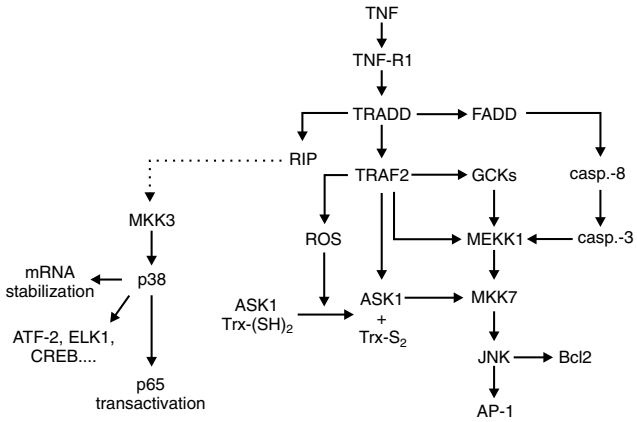

Figure 3 Scheme of TNF-R1-induced activation of p38–MAPK and JNK. JNK activation by TNF can occur by caspase-dependent and/or caspase-independent pathways. The latter is based on 'reactive oxygen species'-dependent and/or GCK-dependent activation of the JNK cascade module, whereby both are regulated by TRAF2. TNF-R1-mediated, caspase-dependent activation of JNK takes place as a consequence of apoptosis induction and is therefore dependent on the TRADD–FADD–caspase-8 axis. The poorly defined connection between RIP and MKK3 is indicated by a dotted line

There is evidence that TRAF2 mediates TNF-induced JNK activation through interaction with members of the germinal center kinase (GSK) family (Figure 3). The GCK family comprises a group of serine–threonine kinases homologous to the yeast Ste20p kinase that can be subdivided into two groups according to the structure of their carboxy-terminal regulatory domain. The carboxy-terminal regulatory domain of group I GCKs contains two or more PEST motifs, binding sites for SH3-domains and a highly conserved stretch of about 350 aa comprising a leucine-rich amino-terminal part and a so-called C-terminal region.[145] Group I GCKs act as proximal activators of MAPK pathways by phosphorylation of MAP3Ks.[145] Group II GCKs are homologous in their amino-terminal kinase domain to group I GCKs but differ drastically

from these molecules in the architecture of the carboxy-terminal regulatory domain. In particular, GCKs of the group II do not stimulate any of the currently known MAPK pathways.[145] Several group I GCKs (germinal center kinase (GCK),[146] GCK-like kinase (GLK),[147] GCK-related (GCKR),[148] HPK/GCK-like kinase (HGK),[149] NIK-like embryo-specific kinase (NESK),[150] TRAF2 and Nck-interacting kinase (TNIK)[151]) have been implicated in TNF-mediated JNK activation because of the following findings: they become activated by TNF (NESK, HGK, GCKR, GLK), interact with TRAF2 (TNIK, GCK, GCKR[152]) and dominant-negative mutants of these kinases interfere with TNF-induced JNK activation (NESK, HGK, GCKR). The group I GCKs seem to channel stimulation of TNF-R1 to the JNK cascade by concomitant interaction with TRAF2 and MEKK1 and phosphorylation-dependent activation of the latter.[146,153] GCK-induced MEKK1 activation correlated with the enhancement of the oligomerization of MEKK1,[153] a process, which on its own is sufficient to drive the activation of MEKK1-dependent signalling pathways.[81,153] The suggested role of group I GCKs in TNF-mediated JNK activation is mainly based on correlation (TNF/TRAF2 activates GCKs – GCKs activate JNK) or transient overexpression experiments with dominant-negative mutants, which are difficult to interpret in terms of causal relations, considering that related molecules may compete for common endogenous upstream or downstream signalling components. Additional studies, especially analyses of knockout mice, will therefore be necessary to figure out the relative (and maybe cell-type specific) contribution of the various GCKs to TNF-induced JNK activation.

A second GCK-independent pathway used by TNF-R1 to activate JNK is based on the production of reactive oxygen species (ROS) and activation of apoptosis signal-regulating kinase-1 (ASK1), a member of the MAP3K family (Figure 3).[154] Stimulation of TNF-R1 can result in the TRAF2-dependent increase of ROS of mitochondrial origin by yet poorly understood mechanisms.[155] Stimulation of TNF-R1 induces interaction of ASK1 with TRAF2 and leads to activation of ASK1 by antioxidant-sensitive mechanisms.[156,157] In agreement with the latter, thioredoxin (Trx) has been identified as an ASK1 interacting protein.[157] Trx contains a redox-active center composed of two cysteine residues and can exist in an oxidized form containing a disulfide-bridge in the active center (Trx-S_2) or in a reduced form with two free SH-groups (Trx-$(SH)_2$). Trx can be oxidized by various ROS and thereby protects to some extent from the cytotoxic effects of TNF,[158] which is partly based on the production of these molecules. Under reducing conditions, Trx exists in its Trx-$(SH)_2$ form and is able to bind and inhibit ASK1.[157] The generation of ROS leads to the oxidation of Trx-$(SH)_2$ to Trx-S_2, which is no longer able to interact with ASK1.[157] In addition, it has been shown that TNF-induced ASK1 activation and TRAF2–ASK1 interaction require prior dissociation of Trx-ASK1 complexes.[159] Thus, a model obtrudes in which TNF-induced generation of ROS leads to the oxidation of Trx, release of ASK1 from inhibitory Trx-ASK1 complexes and subsequent formation of JNK-inducing TRAF2–ASK1 complexes.[159] Remarkably, TRAF2-dependent activation of ASK1 correlates with oligomerization of the kinase.[156] Thus, both TRAF2-GCK-mediated and TRAF2–ASK1-mediated activation of JNK seem to involve oligomerization-dependent activation of MAP3Ks. TRAF2 has therefore a dual role in TNF-induced ASK1 activation, firstly as an inducer of Trx-ASK1 dissociation by ROS induction and secondly, as an activating oligomerization scaffold for ASK1. In agreement with the existence of parallel TRAF2-dependent, JNK-activating pathways (TRAF2–GCKs–MEKK1 *versus* TRAF2–ROS–ASK1), it has been found that Trx-$(SH)_2$ completely blocks TRAF2–ASK1 interaction, but has only a partial inhibitory effect on TRAF2-mediated JNK activation.[159]

TNF not only robustly activates the JNK-inducing MAP kinase cascade, but also the p38-MAPK signalling cascade. Indeed, many aspects of TNF-induced JNK activation hold also true for TNF-induced activation of the p38–MAPK cascade (Figure 3). Both, JNK and p38, are transiently activated by TNF, but show prolonged activation under apoptotic conditions. Moreover, TRAF2, ASK1 and MEKK1, which have been implicated in TNF-induced JNK activation, are also strong inducers of the p38–MAPK pathway.[146,154,160] Nevertheless, there is clear evidence that upstream and downstream of the MAP3 kinase level differences exist between JNK and p38 activation. For example, the various GCKs, which have been implicated in TNF-induced activation of JNK, are unable to stimulate the p38–MAPK cascade.[147,149–151,161] In addition, it has been shown that a deletion mutant of RIP, lacking its intermediate domain, interferes with TRAF2-mediated activation of p38–MAPK, but failed to inhibit TRAF2-induced JNK activation.[146] Thus, TNF may signal p38–MAPK activation via an axis comprising TRAF2 and RIP, whereas JNK activation occurs via TRAF2–GCKs–MEKK1/ASK1. In agreement with the latter, TRAF2 knockout mice are impaired in TNF-induced JNK activation,[138] whereas RIP-deficient mice appear normal in this regard.[162] Unfortunately, studies concerning activation of p38–MAPK in mice deficient for TRAF2 and/or RIP have not been published yet.

Mouse embryonic fibroblasts of MKK3-deficient mice show a strong reduction in TNF-induced activation of p38–MAPK, but no effect on TNF-induced JNK activation (Table 1).[163] In agreement with the important role of the p38-MAPK signalling pathway as a mediator of inflammatory processes, TNF-induced production of IL1 and IL6 is almost completely blocked in MKK3$-/-$ mouse embryonic fibroblasts. However, the exact mode of action of p38–MAPK in TNF-induced upregulation of IL1 and IL6 is not clear yet. p38–MAPK may act by activation of various transcription factors including ATF2, CHOP, CREB and ELK1[164] but it could also enhance TNF-induced production of IL1 and IL6 by increasing the stability of the respective mRNAs via activation of MAP kinase-activated protein kinase 2.[165,166] Remarkably, p38–MAPK has also been discussed as a mediator of NF-κB transactivation.[167,168] To which extent the various effector mechanisms of p38–MAPK are of importance in TNF-induced production of inflammatory cytokines remains to be seen.

TRADD-independent signalling pathways

A number of proteins have been described to interact with TNF-R1 outside its death domain, some of them with

undefined impact on TNF signals. The WD-repeat protein FAN (factor associated with neutral sphingomyelinase activation) has been identified by its binding capacity to the membrane-proximal region of TNF-R1.[169] FAN interacts with one of its five WD-repeats with a nine AA stretch of TNF-R1 located directly in the membrane-proximal region of the DD and appears involved in the activation of the neutral sphingomyelinase (nSMase).[169,170] Defects in cutaneous barrier repair have been found in mice with a genetic deletion of FAN.[170] The role of nSMase-derived ceramide is largely undefined, especially as at least in some cells nSMase becomes only activated very transiently during only the first 3 min of TNF stimulation.[171] A proapoptotic function of nSMase has been described in one report using a dominant-negative form of FAN,[172] whereas ERK1/2 and phospholipase A2 activation are not linked to FAN.[173] Other proteins binding TNF-R1 upstream the DD include a regulatory component of the 26S proteasome, called TRAP2, 55.11 or p97, that binds to aa residues 234–308 of TNF-R1, and which might be involved in TNF-mediated regulation of proteasomal functions.[174–176] In addition, the mitochondrially localized Hsp75, also called TRAP1, has been found to bind TNF-R1 membrane proximal of the DD.[177,178]

TNF-induced activation of the classical MAP kinases, that is the ERKs, is, in most cells, absent or only moderate when compared to TNF activation of the stress-activated protein kinases[179] (see above) or compared to activation of ERKs via mitogenic receptor tyrosine kinase (RTK) pathways.[180] Rather, a negative feedback of TNF on ERK activation triggered by RTK signals has been observed in several cell lines.[181] TNF-R2 can also induce JNK, but not ERKs.[182] A protein containing a domain with low DD homology termed MADD or Rab3-GAP binds to the DD of TNF-R1 and is able to activate MAP kinase pathways.[183,184] Various splice variants of MADD exist which are also termed DENN.[185] Further, the adapter protein Grb2 binds to a PLAP motif of TNF-R1, thereby potentially linking this receptor via SOS to Ras, c-Raf and the ERKs.[186] This signal, however, appears not sufficient to efficiently activate the MAP kinases, as FAN/nSMase-derived ceramide acting on the ceramide-activated protein kinase (CAP-K;[187]) is necessary to fully activate c-Raf.[186] Consistent with these data, survival of osteoclasts by TNF is mediated by Akt and ERKs and can be blocked by inhibitors of the ERK-activating kinase MEK-1, but also by a peptide interfering with FAN/PLAP domain interaction.[188]

Molecular mechanisms of TNF-induced cell death

Like other death receptors, TNF-R1 is able to signal cell death via its cytoplasmic death domain in a variety of cell lines. However, *in vivo* TNF-induced apoptosis seems to have only a minor role compared to its overwhelming function in the regulation of inflammatory processes. Indeed, the high systemic toxicity of TNF is caused by cellular mediators like NO and not related to its apoptosis-inducing capability.[189] Remarkably, mice deficient in p65 or other components involved in TNF-induced NF-κB activation are embryonally lethal or die early after birth because of massive TNF-dependent liver failure (Table 1). Thus, the death-inducing capability of TNF is masked *in vivo* by concomitant activation of NF-κB. Although other death receptors are also able to activate the NF-κB pathway, they show prominent apoptotic functions *in vivo*. Moreover, in some experimental situations Fas-mediated NF-κB activation becomes apparent only when parallel apoptosis induction is blocked.[190] Thus, while in TNF-R1 signalling NF-κB activation dominates over apoptosis-induction, in Fas or TRAIL-R1/2 signalling apoptosis-induction is dominant over NF-κB activation. As outlined below in detail, the apoptotic and the NF-κB pathway are inhibitory to each other. Thus, there is a situation that both pathways are connected by self-amplifying inhibitory circuits that may be responsible for the predominant *in vivo* specification of TNF-R1 as an NF-κB-inducing receptor and of Fas as a death-inducing receptor.

There is genetic evidence from knock-out mice and mutagenized cell lines that all death receptors investigated so far critically depend on the death domain-containing adaptor protein FADD and caspase-8 and -10 to induce cell death (Table 1).[191–199] In the case of Fas and the TRAIL death receptors, a death-inducing signalling complex (DISC) that contains FADD and caspase-8/10 has been defined by immunoprecipitation of the endogenous molecules. Studies with deletion mutants of Fas, FADD and caspase-8 show that activated Fas recruits FADD by homophilic interaction of the DD of these molecules.[200] Receptor-bound FADD in turn interacts with caspase-8/10 via the death effector domains contained in the amino-terminal parts of both molecules.[201,202] There is evidence that the Fas-DISC is a supramolecular complex of several trimeric or higher-order Fas complexes.[203] Thus, FADD-mediated recruitment brings several caspase-8/10 molecules in close proximity and thereby facilitates autoproteolytic activation of these proteases. In contrast to Fas and TRAIL death receptors, TNF-R1 is indirectly linked to FADD, namely by TRADD which is also responsible for bridging TNF-R1 to TRAF2 and the IKK complex.[75] However, while several groups were able to immunoprecipitate the TNF-R1-IKK signalling complex from TNF-treated cells (see above), a comparable demonstration of the TNF-R1-DISC was not successful yet. The lack of an immunoprecipitable TNF-R1-DISC could reflect a comparably low stability of the complex, but could also indicate that the efficient formation of such a complex requires special, yet poorly understood circumstances. In agreement with the existence of mechanisms that selectively regulate the formation and/or activity of the TNF-R1-DISC, several groups have observed that depletion of TRAF2, which is a major part of the TNF-R1 signalling complex, but does not or only modestly interact with Fas, sensitises cells for the apoptotic action of TNF,[204–210] whereas Fas- and TRAIL-mediated cell death remain unaffected.[205,210] A TNF-R1 selective apoptosis-regulating process could be the TRAF2-mediated recruitment of the antiapoptotic cIAP1 and cIAP2 proteins to TNF-R1.[210,211] cIAP1 and the closely related cIAP2 protein have been originally identified as molecules present in the TNF-R2 signalling complex.[212] Both are typical members of the inhibitor of apoptosis protein family,[213] which bind and inhibit caspase-3 and -7 via their amino-terminal BIR (baculovirus IAP repeat) domains,[214] a structural feature common to all

IAP family members.[213] In agreement with an antiapoptotic role of TRAF2-mediated recruitment of cIAP1 and cIAP2, it has been found that concerted overexpression of TRAF1, TRAF2, cIAP1 and cIAP2 efficiently interferes with TNF-R1-induced apoptosis and activation of caspase-8.[215] So, it seems that in the TNF-R1 signalling complex TRAF2-bound cIAP1/2 molecules are able to block activation of caspase-8, which is independently recruited into the TNF-R1 signalling complex via the TRADD–FADD axis and which is otherwise no substrate for these IAP proteins.[214] Depletion of cytoplasmic, therefore TNF-R1 accessible, TRAF2 can be induced by stimulation of non-death domain-containing members of the TNF receptor family able to recruit TRAF2 and to induce its subsequent proteasomal degradation.[207,210] To destine TRAF2 for proteasomal degradation the molecule has to be ubiquitinated.[207,209] Indeed, additional to their caspase inhibitory BIR domains the carboxy-terminal RING domain of cIAP1 and cIAP2 can act as E3 ubiquitin ligase[216] involved in the proteasomal degradation of caspase-3 and –7,[217] and cIAP1 and cIAP2 themselves.[216] Although TRAF2 mediates recruitment of cAP1 and cIAP2 into the TNF-R2 signalling complex with comparable efficiencies,[210] only cIAP1 seems to ubiquitinate TRAF2.[207] In agreement with the hypotheses that TRAF2 depletion interferes with the formation of the caspase-8 inhibiting TNF-R1-TRAF/IAP complex mentioned above, it has been demonstrated that TNF-R2 stimulation enhances TNF-R1-induced caspase-8 activation.[208,210] Hence, TNF-R2-dependent enhancement of TNF-R1-induced cell death appears to be based on two tightly linked mechanisms: First, on competition of the two TNF-Rs for binding of TRAF2 and the TRAF2-associated anti–apoptotic cIAP1 and cIAP2 proteins, and second, on the cIAP1-initiated degradation of TRAF2, which in turn enhances receptor competition for the remaining TRAF2, cIAP1 and cIAP2 molecules. Accordingly, cIAP1 would have an anti-apoptotic function upon recruitment into the TNF-R1 signalling complex, but would switch to a net proapoptotic function upon recruitment into the TNF-R2 signalling complex.

Downstream of caspase-8 processing TNF-R1-induced apoptosis occurs in principle via the same routes as described for other death receptors like Fas and TRAIL-R1/2 (see the parallel review articles). In brief, in type I cells active caspase-8 alone is sufficient to robustly induce caspase-3 activity and the execution phase of apoptosis (Figure 4). Thus, release of cytochrome c from mitochondria and subsequent formation of the caspase-3-inducing apoptosome complex are dispensable for the apoptotic process in this type of cells (see the parallel review articles). However, in type II cells caspase-8-mediated activation of caspase-3 is inefficient and the apoptotic process therefore depends on a mitochondria-dependent amplification loop (Figure 4). Small quantities of active caspase-8 produced in type II cells are sufficient to activate proteolytically the BH3 domain-containing Bcl2 family member Bid.[218,219] The truncated carboxy-terminal fragment of Bid (tBid) generated this way translocates to the mitochondria and promotes release of cytochrome *c*[218,219] and Smac/Diablo[220,221] in a Bax/Bak-dependent manner.[222] The release of cytochrome *c* from mitochondria into the cytosol allows the ATP-dependent formation of a caspase-3 activating 'apoptosome', consisting of cytochrome *c* itself, Apaf-1 and caspase-9.[223] Complementary to the action of the caspase-3-inducing apoptosome, Smac/Diablo binds and antagonizes the caspase inhibitors xIAP, cIAP1 and cIAP2.[220,221] Activated caspase-3 is able to activate caspase-8[224–226] thereby creating a positive feedback loop.[225,226] to the pivotal role of the mitochondrial cytochrome c release in type II cells, these type of cells can be experimentally distinguished from type I cells by overexpression of Bcl-2. While in type I cells apoptosis induction by death receptors is not sensitive towards Bcl-2 overexpression, this molecule protects type II cells from the apoptotic effects of death receptors.[227] Since the amount of active caspase-8 seems to be critical to determine type I or type II responsiveness and TNF-R1-mediated caspase-8 processing is less efficient compared to Fas, it seems conceivable that the same cells could behave as a type I cell or a type II cell, depending on the death receptor subtype, that is Fas or TNF-R1, respectively.

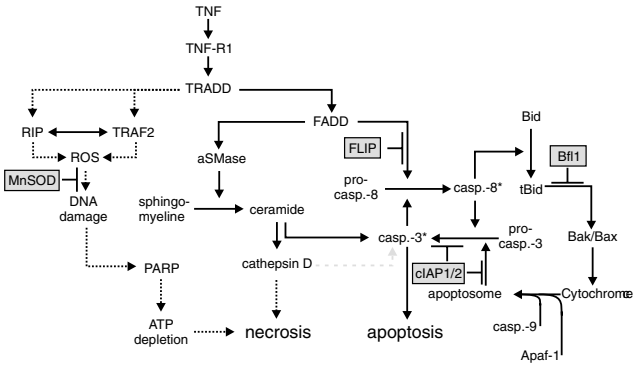

Figure 4 TNF-R1 stimulates a death-inducing network. Necrosis-related relationships are shown by dotted lines, apoptosis-related relationships are depicted with solid lines. NF-κB-inducible inhibitors of the cell death network are highlighted with gray boxes. Note that the active caspase-3 (casp.-3*) cleaves and inhibits PARP, thereby blocking the necrotic response. Cathepsin D-mediated activation of caspase-3 occurs most likely indirectly by Bid cleavage and is therefore shown by a gray dashed line. The role of RIP in the production of ROS has not been experimentally addressed yet. Thus, this relation is deduced from the receptor-proximal position of RIP and the finding that RIP is essentially involved in death receptor-induced necrosis

cIAP1, cIAP2 and TRAF1 have been identified as NF-κB target genes.[215,228,229] Thus, ablation of the NF-κB pathway may interfere with the action of the caspase-8 inhibitory TNF-R1-TRAF/IAP complex and therefore sensitises for the apoptotic action of TNF. In particular, this is in good accordance with the increased TNF sensitivity observed in various knock-out mice where TNF-induced NF-κB activation is compromised (Table 1). Remarkably, the NF-κB pathway targets also several other antiapoptotic factors, including cFLIP,[230,231] IEX-1L,[232] Bfl-1/A1,[233–235] XIAP,[236] Bcl-Xl [234,237,238] and the TRAIL decoy receptor TRAIL-R3.[239] All or part of these genes are most likely involved in or responsible for the variety of global, non-TNF-R1 selective anti-apoptotic effects of NF-κB. Upregulation of NF-κB, dependent anti-apoptotic genes seems not to be the only way by which TNF counteracts induction of apoptosis by itself or other death inducers. In accordance with the known anti-apoptotic properties of the PI3K/Akt pathway, caused by phosphorylation and

inactivation of proteins involved in apoptosis,[240–242] there is growing evidence that the stimulation of this pathway by TNF not only contributes to TNF-dependent NF-κB activation, but also independently mediates some additional antiapoptotic signals of TNF.[98,100]

While the NF-κB pathway negatively regulates the apoptotic program, ongoing apoptosis in turn interferes with the activation of NF-κB. This is because of the caspase-mediated cleavage of several of the components utilized by this pathway including RIP, [243,244] TRAF1, [245] I-κB, [246] IKK2, [247] HPK1, [248] NIK,[249] Akt[252] as well as p50 [250] and p65 [250,251] themselves. Noteworthy, in most cases caspase-mediated cleavage results in release of fragments that can act in a dominant-negative fashion towards their noncleaved counterparts. Thus, caspaseaction not only reduces the amount of signalling intermediates necessary to transduce a NF-κB response, but also creates novel peptides that actively interfere with NF-κB activation. While IKK2, [247] p65, [250,251] I-κB [246], Akt [252] and HPK1 [248] are mainly cleaved by caspase-3-related caspases during the effector stage of apoptosis, NIK, [249] RIP [243,244] and TRAF1 [245] are cleaved by caspase-8 during the initiator phase of death receptor-induced apoptosis. Thus, caspase-8-generated cleavage products derived from RIP, TRAF1 and NIK may have a special role in TNF-R1-induced apoptosis by blocking the concomitantly induced anti-apoptotic NF-κB response. As death receptors are in principle able to activate both the apoptotic and the NF-κB pathway, the regulatory network of these pathways allows a highly flexible cellular behavior in response to stimulation of death receptors, especially TNF-R1. Aside from NF-κB activation, stimulation of c-Jun N-terminal kinase is a second cellular response to TNF in common to all cell types, which could play a role in apoptosisinduction by TNF-R1. As outlined in more detail in the following, there is evidence for an apoptosis-related crosstalk of the JNK pathway with both the NF-κB, and the apoptotic pathway itself.

Although c-Jun N-terminal kinase is robustly activated by TNF via TNF-R1 in almost every cell line investigated, the role of JNK for TNF function, especially TNF-mediated apoptosis, is still poorly understood. An essential role of the JNK signalling pathway in excitotoxic stress-induced neuronal apoptosis [253,254] and UV-stimulated apoptosis [255] has been clearly demonstrated, in particular in studies with mouse embryonic fibroblasts derived from JNK-deficient mice. The proapoptotic action of JNKs seem mainly dependent on their capability to phosphorylate c-Jun, a component of the heterodimeric transcription factor AP-1, [255] but can also be related to phosphorylation and inhibition of Bcl-2.[256–258] Nevertheless, the JNK pathway can also have an anti-apoptotic function, for example during neuronal development [259] or in thymocytes.[260] The ambivalent function of the JNK pathway in different apoptotic scenarios is also reflected in its role in TNF-induced apoptosis. While mouse embryonic fibroblasts of JNK1−/−-and JNK2−/−-deficient mice show increased sensitivity against TNF-induced apoptosis, [261] mouse embryonic fibroblasts of mice deficient for ASK1, an MAP3K implicated in TNF-R1-mediated JNK activation, are significantly protected against TNF-induced cell death.[144] Moreover, studies in other cell types using inhibitors of the JNK pathway also revealed a proapoptotic function of the JNK pathway in TNF-induced cell death.[262–268] The different roles of JNK in TNF-induced apoptosis could be partly related to celltype- specific effects, but may also mirror that TNF engages JNK by more than one pathway. Indeed, especially under apoptotic conditions, TNF activates JNK, but also p38, with biphasic kinetics.[264] The first transient phase of JNK activation is caspase-independent [269,270] and almost completely inhibited in MKK7- deficient mouse embryonic fibroblasts.[139] In contrast, the second phase of TNF-induced JNK activation correlates with apoptosis-induction and is blocked by caspase inhibitors. Remarkably, mouse embryonic fibroblasts of mice deficient of ASK1 show reduced sensitivity against TNF-induced apoptosis and a reduction in apoptosis-related delayed JNK activation, but are undisturbed with respect to transient rapid TNF-induced JNK.[144] Thus, this delayed prolonged type of JNK activation by TNF may rather reflect a common response to the activation of the apoptotic pathway, as described elsewhere, [271,272] than being a genuine, direct TNF-mediated effect. Nevertheless, caspase-mediated activation of JNK could trigger a self-amplifying apoptotic loop, for example, by upregulation of death ligands. In agreement with this idea, a kinase-dead mutant of ASK1 weakens TNF-induced apoptosis.[143,154,273] In addition, it has been recently shown that ASK1 can trigger a caspase-dependent, [274] but also a caspase-independent [275] pathway leading to cell death. In this regard, NF-κB activation inhibits prolonged TNF-induced JNK activation [267,268,276] and this has been attributed to the upregulation of JNK inhibitory proteins, XIAP1 [267] in one study and GADD45β [276] in another study. Both molecules are identified and characterized under conditions, where TNF-induced NF-κB activation is compromised in the absence of caspase inhibitors. Therefore, it is possible that XIAP1 and GADD45ß are not directly involved in the regulation of JNK activity, but interfere with prolonged TNF-induced and ASK1-mediated JNK activation by blocking apoptosis and caspase activation as described above. A role of ASK1 in the delayed phase of TNF-induced JNK activation and apoptosis induction is also in agreement with the finding that ASK1 is activated by reactive oxygen species (ROS).[156] Indeed, the generation of ROS can have a dual role in TNF signalling. On one hand, it can promote NF-κB activation, which is redox-sensitive [277] but on the other hand, ROS have also been implicated as mediators of TNF-induced apoptosis.[278–283] Remarkably, manganous superoxide dismutase (MnSOD), which acts as a scavenger of potentially toxic superoxide radicals, is an NF-κB-dependent target gene of TNF.[282,283] This emphasizes again that NF-κB activation and apoptosis signalling by TNF are tightly connected by a regulatory network (Figure 4). ROS-generating compounds can themselves induce caspase-activation and apoptosis via the mitochondrial pathway, in the context of TNF signalling ROS production seems to act as an amplification mechanism.

Noteworthy, production of ROS has not only a role in apoptosis but also in necrosis. This form of cell death is largely independent of caspases, morphologically quite different from apoptosis and *in vivo* associated with inflammation.[284] Necrosis can be induced by death receptors including TNF-R1 by a RIP-dependent pathway.[285] In contrast to its role in the NF-κB pathway, the role of RIP in induction of necron's is

dependent on the kinase activity of the molecule.[285] As FADD, but not caspase-8, is essentially involved in death receptor-induced necrosis, FADD seems to be the bifurcation point of death receptor-mediated apoptosis and necrosis.[285] There is evidence that death receptor-induced apoptosis and necrosis do not simply act as parallel linear death pathways. Under apoptotic conditions caspase-mediated cleavage of RIP may block the necrotic pathway. Consequently, inhibition of the FADD-caspase axis of cell death by use of caspase inhibitors or FADD deletion mutants lacking their DED leads to sensitiation to death receptor-induced necrosis[286–289]. A pivotal role in mediating the necrotic response has been attributed to the production of ROS.[288,290,291] How RIP is involved in the production of ROS and how this is related to the capability of TRAF2 to induce ROS[155] is still an open question. TNF triggers oxidative stress (see also JNK) and mitochondria-dependent production of ROS.[278,292–294] As a consequence of ROS production, DNA is damaged leading to the activation of PARP-1. Excessive action of PARP-1 consumes high amounts of NAD^+ and leads to ATP depletion, creating thereby conditions that favor necrosis instead of apoptosis.[295–298] Remarkably, PARP-1 is cleaved by caspases into an amino-terminal fragment retaining DNA-binding capabilities and a carboxy-terminal fragment without catalytic activity.[299] In particular, the amino-terminal PARP-1 fragment acts as a dominant-negative variant for uncleaved PARP-1.[299] As ATP is required for apoptosis, PARP-1 cleavage acts as a switch between apoptotic and necrotic cell death.[300] The fact that Fas is a more robust activator of caspase-8 compared to TNF-R1 may explain why in some cells that undergo apoptosis in response to Fas stimulation, TNF-R1 triggers a necrotic form of cell death.[300,301] Nevertheless, inhibition of the apoptotic pathway can unmask the capability of Fas to trigger the necrotic response.[288,290]

Several studies have implicated acidic SMase and ceramide release in TNF-induced cell death.[171,302–304] As cells deficient for acidic SMase are still TNF sensitive,[305] TNF-induced ceramide production seems to have no general, obligatory role in TNF-induced cell death. Nevertheless, the demonstration of reduced TNFsensitivity of aSMase−/− mouse embryonic fibroblasts[306] and several other studies point to aSMase and ceramide production as celltype specific modulatory factors of TNF-induced cell death.[307] TNF-R1-induced activation of aSMase occurs by a FADD-dependent, but caspase-8 independent pathway.[308,309] In so far the pathway leading to aSMase activation is identical to the receptor-proximal necrotic pathway it remains to be verified to which extent the aSMAse pathway is related to the necrotic response. Ceramide release by aSMase can contribute to the activation of caspases and can, in addition, lead to the activation of the lysosomal compartment, in particular cathepsin D, which specifically binds ceramide.[310] Although lysosomal enzymes have been implicated in necrosis (for review see [284]) they can also activate caspases and induce apoptosis,[311] most likely via Bid cleavage.[312] The potential relevance of cathepsins for TNF-induced cell death[313–315] is most obvious for cathepsin B, as mice deficient for this protease are resistant to TNF-mediated hepatocyte apoptosis and liver injury.[316]

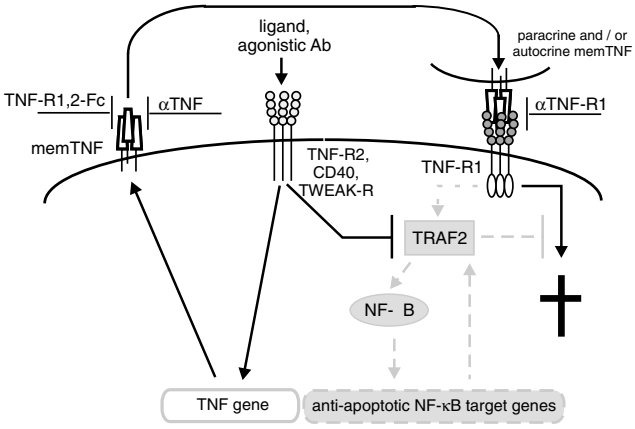

Figure 5 Model of non-death domain-containing receptor-induced apoptosis. Non-death domain-containing receptors trigger two events that cooperatively induce apoptosis: (1) Inhibition of TRAF2-mediated NF-κB activation and deviation of TRAF2-containing antiapoptotic complexes (gray) and (ii) induction of endogenous membrane-TNF that stimulates in addition the death receptor TNF-R1

Mechanisms of TNF-R2-induced apoptosis

Early studies with soluble TNF gave inconsistent results with respect to an apoptosis-inducing capability of the nondeath domain-containing receptor TNF-R2. [317,318,319] However, the use of agonistic TNF-R2-specific antibodies clearly showed that exclusive triggering of this receptor is in some cells sufficient to induce cell death.[320–322] Analyses with TNF and TNF receptor-specific neutralizing antibodies showed that stimulation of TNF-R2 does not directly engage the apoptotic program, but relies on the induction of endogenous, membrane-bound TNF, which subsequently activates TNF-R1 (Figure 5).[323,324] Remarkably, the action of the endogenously produced membrane-bound TNF on TNF-R1 is drastically enhanced by the existing stimulation of TNF-R2. Thus, apoptosis induction by the non-death domain-containing receptor TNF-R2 is not only dependent on the production of endogenous TNF and expression of TNF-R1, but also by the TNF-R2-dependent process described above leading to depletion of TRAF2 and TRAF2-associated protective factors. Similarly, apoptosis induction by the other members of the TNF receptor family lacking a death domain has also been attributed to the induction of endogenous TNF and concomitant depletion of TRAF2.[323–326]

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