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Review

Modulation of Life and Death by the Tumor Necrosis Factor Receptor-Associated Factors (TRAFs)

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The TNF receptor-associated factor (TRAF) family is a group of adapter proteins that link a wide variety of cell surface receptors. Including the TNF and IL-1 receptor superfamily to diverse signaling cascades, which lead to the activation of NF-κB and mitogen-activated protein kinases. In addition, TRAFs interact with a variety of proteins that regulate receptor-induced cell death or survival. Thus, TRAF-mediated signals may directly induce cell survival or interfere with the death receptor-induced apoptosis.

Keywords: TNF receptor superfamily, TRAF, Apoptosis, Cell survival

The tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamily comprise, respectively, a group of secreted or membrane-bound ligands and their receptors with diverse and widespread physiological functions, including apoptosis, osteoclastogenesis, and immune system regulation (Locksley *et al.*, 2001). The continually-expanding TNFR superfamily includes TNFR1 and 2, the lymphotoxin β receptor, CD27, CD30, CD40, Fas/CD95/Apo-1, OX40, 4-1BB, RANK/TRANCE-R, TRAMP/DR3, TRAIL receptors, and the low-affinity NGF receptor. A few TNFRs, including TNFR1 and Fas, use death domains in their intracellular regions to signal cell death, culminating in caspase activation. In contrast, the majority of TNFRs recruit a family of intracellular adapter molecules to promote cell survival by the activation of downstream protein kinase cascades, and ultimately, transcription factors in the NF-κB and AP-1 family. These transcription factors can then turn on numerous genes that are involved in proliferation, differentiation, and apoptosis (Baker *et al.*, 1996; Wallach *et al.*, 1999; Khaled *et al.*, 2001; Kim *et al.*, 2001).

A number of the intracellular molecules in these receptor-signaling pathways have been identified and characterized. Recently, a family of intracellular proteins were identified that appear capable of both negatively regulating cell death pathways and inducing the expression of genes that promote cell survival (Arch *et al.*, 1998). The first two name-giving members of this family were identified, due to their ability to bind to the cytoplasmic tail of TNFR2. They were consequently designated as the TNF receptor-associated factor (TRAF)1 and TRAF2 (Roth *et al.*, 1994). To date, six different proteins of this family were identified in mammals, three in *Drosophila melanogaster* (DTRAF1, DTRAF2, and DTRAF6), and one in *C. elegans* (Wajant *et al.*, 2001). Compelling evidence demonstrated that TRAFs serve as adapter proteins for a wide variety of cell surface receptors and govern diverse cellular responses. These culminate in cell survival, apoptosis, and differentiation. This review will focus on current knowledge regarding the structure and effector functions of this family of proteins.

Structure and function relationships of TRAF proteins

TRAF proteins are defined by a conserved C-terminal domain, the TRAF domain, which is further subdivided into a more divergent N-proximal (TRAF-N) and a highly conserved C-proximal (TRAF-C) sub-domain (Fig. 1). The TRAF domain is responsible for homo- and heterodimerization of the TRAF proteins, as well as for their direct and indirect interactions with cognate surface receptors. Structural differences between the TRAFs influence the range of receptors, heterodimerization partners, adapter molecules, and downstream signal transducers with which each TRAF interacts (Kaufman *et al.*, 1999). In addition to the TRAF domain, all of the TRAFs, except TRAF1, contain N-terminal RING finger domains. The deletion of the amino-terminal RING domain of TRAF proteins leads to a generation of dominant-negative TRAF mutants. This suggests that the RING domain of TRAF proteins is critical for downstream effector functions (Hsu *et al.*, 1996).

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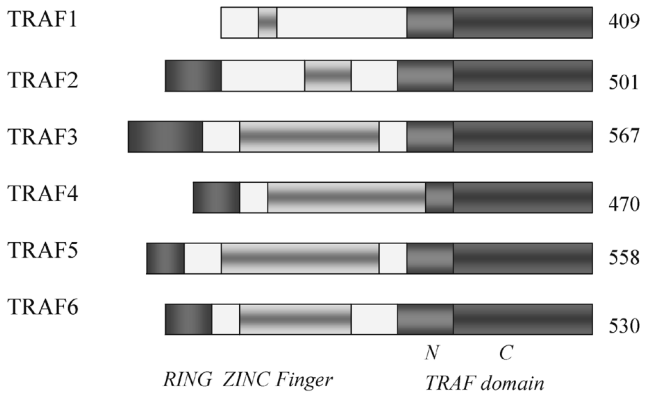

Fig. 1. Domain structure of the TRAF protein family.

Crystallographic studies of the TRAF domain of TRAF2 demonstrate that the TRAF-C forms an eight-stranded β-sandwich structure, whereas TRAF-N, a coiled-coil domain, forms a single α-helix. The TRAF domains of three TRAF2 molecules multimerize to form mushroom-like trimers with the TRAF-C domains as the cap and TRAF-N domains as the stalk (Park *et al.*, 1999). Further analyses of the TRAF domain with bound receptor peptides, derived from the cytoplasmic parts of TNFR2, showed that the receptor selectively interacts with each of the three TRAF-C subdomains (Ye *et al.*, 1999). There is no direct contact between the cytoplasmic tails of the receptors. These data show that the symmetrical ligand-mediated trimerization of receptors, and the self-association of TRAF molecules, lead to the formation of intracellular signaling complexes with a trimeric symmetry. Additional crystal structure studies of the TRAF domain of the human TRAF2, in complex with peptides from the TNFR family members CD40, CD30, OX40, 4-1 BB, and the EBV oncoprotein LMP1, revealed two conserved binding modes: a major TRAF2 binding consensus sequence, (P/S/A/T)x(Q/E)E, which could also be defined from biochemical studies (Lee *et al.*, 1996a), and a minor consensus motif, PxQxxD.

In contrast to the non-death domain receptor-TRAF2 interactions, the cytoplasmic death domain adapter molecule, TRADD (first identified as an interacting partner of TNFR1) binds TRAF2 through a comparably large protein-protein interface. Solution structural data indicate overlapping regions for the non-death domain receptor and TRADD binding to TRAF2, which has been confirmed by competition studies using peptides from the cytoplasmic part of CD40 and the N-terminus of TRADD (Tsao *et al.*, 2000). These results suggest that non-death domain receptors and TRADD compete for the TRAF2 homotrimer binding.

TRAF-interacting receptors and intracellular proteins

The TNF receptor superfamily can be subdivided into death domain-containing receptors and receptors lacking a death domain, both of which mediate signaling complexes that include TRAF proteins. Interestingly, non-death domain receptors directly bind one or more members of the TRAF protein family (Table 1). Although TRAF proteins cannot directly interact with death receptors, they are involved in death receptor signaling by indirect recruitment via additional adapter proteins, such as TRADD (Fig. 2). TARF6 also links the TRAF signaling transduction cascade to another intracellular signaling pathway that is triggered by IL-1 and IL-18 (Cao *et al.*, 1996; Means *et al.*, 2000). TRAF6 is indirectly recruited by the receptor signaling complex of IL-1 and IL-18 receptors by the adapter protein MyD88, and the kinase IL-1 receptor-associated kinase (IRAK). This leads to the activation of NF-κB.

Ligand-induced receptor oligomerization leads to the recruitment of the TRAF family and/or other intracellular proteins to the cytoplasmic domains of the receptor, which

Table 1. Interactions between TRAF protein family and cell surface receptors

Recepters	TRAFs
Non-death domain receptor	
TNFR2	TRAF1, TRAF2
CD30	TRAF1, TRAF2, TRAF3, TARF5
CD40	TRAF1, TRAF2, TRAF5, TARF6
OX40	TRAF1, TRAF2, TRAF3, TARF5
4-1BB	TRAF1, TRAF2, TRAF3
LT-βR	TRAF1, TRAF5
HVEM	TRAF1, TRAF2, TRAF3, TRAF5
TRANCE-R	TRAF1, TRAF2, TRAF3, TRAF5, TRAF6
CD27	TRAF2, TRAF3, TRAF5
Death domain receptor	
TNFR1	TRAF2 (indirectly via TRADD)
IL-1 & IL-18 receptor	
IL-1R	TRAF6 (indirectly via MyD88 & IRAK)
IL-18R	TRAF6 (indirectly via MyD88 & IRAK)

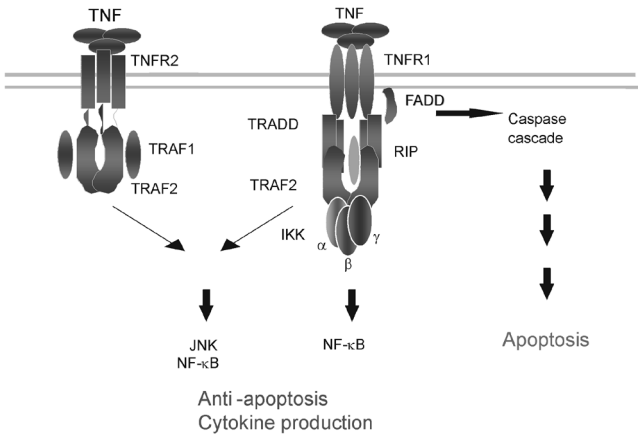

Fig. 2. Anti-apoptosis in TNFR-TRAF signaling pathways.

Table 2. TRAF-interacting intracellular adapter proteins and kinases

Adapter	TRAFs
TRADD	TRAF1, TRAF2
FADD	TRAF1, TRAF2
I-TRAF/TANK	TRAF1, TRAF2, TRAF3
TRIP	TRAF1, TRAF2
cLAP1	TRAF1, TRAF2
cLAP2	TRAF1, TRAF2
A20	TRAF1, TRAF2
FLIP/Caspaer	TRAF1, TRAF2
MIP-T3	TRAF3
T6BP	TRAF6
TTRAP	TRAF2, TRAF3, TRAF5, TRAF6
Kinases	TRAFs
RIP	TRAF1, TRAF2, TRAF3
NIK	TRAF1, TRAF2, TRAF3, TRAF5, TRAF6
IRAK	TRAF6
ASK1	TRAF1, TRAF2, TRAF3, TRAF5, TRAF6
TAK1	TRAF6
MEKK1	TRAF2, TRAF6
GCK	TRAF2
PKCζ	TRAF6
c-Src	TRAF1, TRAF3, TRAF6

results in the assembly of multiprotein signaling complexes. This leads to the activation of kinase cascades that stimulate NF-κB, AP-1, and Akt/PKB (Means *et al.*, 2000). Consequently, the association of TRAFs with a variety of kinases that are located proximal in these pathways was described (Table 2). In the case of RIP (Kellier *et al.*, 1998) and IRAK (kinases that are part of the TNF- and IL-1R complex, respectively), the essential role in TNF and IL-1 signaling could be confirmed in the respective knock-out mice. In contrast, the NF-κB-inducing kinase (NIK) (originally identified as the MAP3 kinase that interacts with TRAF2) was initially considered as essentially involved in TNF- and the IL-1-induced activation of NF-κB (Malinin *et al.*, 1997). However, alymphoplasia (aly) mice (a natural strain with a mutant NIK gene) remained competent in the TNF-induced NF-κB activation (Shinkura *et al.*, 1999). This argues against the physiological functions of NIK. MEKK1 associates with the RING-zinc finger domain of TRAF2 and TRAF6 upon TNF and IL-1 stimulation, respectively, in the *in vitro* experiments. Also, a dominant negative mutant of MEKK1 interferes with the JNK activation by these cytokines. However, unlike in the *in vitro* experiments, there was no effect in MEKKI-deficient embryonic cells on IL-1- and TNF-induced JNK activation. Two other MAP3Ks, ASK1 and TAK1, have been implicated in TRAF-dependent signaling pathways. TAK1 was identified as a TRAF6 interacting kinase that is involved in the IL-1-induced NF-κB activation (Wang *et al.*, 2001). ASK1, which was originally identified as an apoptosis-inducing kinase activated by TNF, was implicated in the TNF-induced JNK activation (Saito *et al.*, 1998). Thus, TRAF proteins may help to link the receptor-induced signals to the components of a functional MAP kinase cascade.

The signaling cascade that is triggered by the receptor recruitment of either TRAFs or the death domain that contains adapter proteins are modulated by proteins that can interfere with specific steps by modifying the composition of the multiprotein complexes and/or by blocking the protein-protein interactions and downstream effector functions. These proteins (Table 2) include A-20, c-IAPs (cellular inhibitor of apoptosis), TRIP (TRAF-interacting protein), I-TRAF/TANK (inhibitor of TRAF), and T6BP (TRAF6-binding protein). They may be an important link in the ability of the TRAF-dependent signal transduction to modulate cell survival (Rothe *et al.*, 1995; Rothe *et al.*, 1996; Cheng *et al.*, 1996; Lee *et al.*, 1997a). However, they are presently much less characterized in their functional role *in vivo*.

Anti-apoptosis in the TRAFs signaling pathways

TRAF1 TARF1 is a unique member of the TRAF family. It contains a single zinc finger and a TRAF domain. Its expression is restricted to the spleen, lung, and testis, in contrast to the more ubiquitous expression of other TRAFs. TRAF1 can recruit a number of distinct members of the TNFR superfamily, including TNFR2, CD30, CD27, 4-1BB, HVEM/ATAR, and TRANCE-R. Little is known about the biochemical function of TRAF1. The overexpression of TRAF1 in transgenic animals demonstrates the inhibitory role of TRAF1 on the antigen-induced apoptosis of CD8+ cells (Speiser *et al.*, 1997). However, the TRAF1 overexpression did not protect cells from TNF-induced apoptosis, suggesting that TRAF2 (to be discussed later) and TRAF1-mediated anti-apoptosis may be distinct. It is unclear how TRAF1 exerts its anti-apoptotic effect. Consistent with the *in vitro* overexpression studies in cell lines, the *in vivo* overexpression of TRAF1 does not affect NF-κB or JNK activation. Therefore, TRAF1 could achieve its anti-apoptotic effect either via cIAP recruitment, or through the use of some other, unknown pathway. Unlike TRAF1-mediated anti-apoptotic activity, the C-terminal cleavage product of TRAF1 that is cleaved by caspases mediates pro-apoptotic activity through sequestering TRAF2 from the TNFR1 complex (Jang *et al.*, 2001). Recently, activated T cells from TRAF1-deficient mice exhibited hypersensitivity to TNF, suggesting that TRAF1 is a negative regulator of TNF activity (Tsitsikov *et al.*, 2001). One possible mechanism of the TRAF1 inhibition of TNF signaling is that TRAF1 competes with TRAF2 for binding to TNFR2. Another possibility is that TRAF1 forms an inactive heterodimer by binding to TRAF2. Further work is needed to understand the precise biochemical basis of the inhibition of the TNFR2 signaling by TRAF1, and of the role of TRAF1 in the regulation of signaling by other TNFR family members.

TRAF2 TRAF2 was the first identified and well-characterized member of a larger TRAF family. Various transfection and overexpression experiments have suggested that TRAF2 is a crucial component of almost the entire TNFR superfamily signaling pathway. It initiates important downstream signaling events, such as the activation of the NF-κB family of transcription factors and activation of the MAP kinase cascade (Song *et al.*, 1997). NF-κB family members are able to interfere with apoptosis that is induced by a wide variety of stimuli, including exposure to ionizing radiation or chemotherapeutic agents, and cytokines of TNF family members (Baldwin *et al.*, 1996; Beg *et al.*, 1996; Wang *et al.*, 1996; Van Antwerp *et al.*, 1996).

The initial event in the TNF-induced activation of NF-κB is the ligand-induced formation of a multimeric TNFR1 complex (Fig. 2). Formation of the TNF/TNFR1 complex interacts with the death domain of the adaptor protein TRADD (Hsu *et al.*, 1995). Receptor-bound TRADD can then recruit TRAF2 and the RIP into the TNFR1 signaling complex. The role of TRAF2 in an *in vivo* setting was explored both by creating a gene-targeted deletion of the TRAF2 gene (Yeh *et al.*, 1997), and by overexpressing a dominant negative inhibitor of TRAF2 signaling (a truncated TRAF2 lacking the RING and zinc fingers) (Lee *et al.*, 1997b). These two models revealed mutually supportive findings on several points. First, even in the absence of functional TRAF2, TNF-induced NF-κB activation was nearly normal in the various cell types that were examined, including thymocytes, B cells, and fibroblast. Second, TNF-induced JNK activation was severely impaired under the same conditions. Third, the various cell types that were examined demonstrated an increased sensitivity to TNF-induced apoptosis in the absence of TRAF2. This supports the hypothesis that TRAF2 provides anti-apoptotic signals following TNF stimulation. Pre-treatment of the cells with cyclohiximide did not abrogate this disparity in sensitivity to TNF-induced apoptosis. This indicates that the relevant TRAF2-mediated anti-apoptotic pathway did not require new protein synthesis. Since the NF-κB-dependent anti-apoptosis (mentioned earlier) requires *de novo* gene expression, these findings support a model in which an NF-κB-independent effector pathway plays a critical role in TRAF2-mediated protection from cell death. This was confirmed in experiments that utilized mice that overexpressed both a dominant-negative TRAF2 and a regulation-insensitive IκB mutant, which served as a dominant negative inhibitor of NF-κB activity (Lee *et al.*, 1998). The thymocytes of these double-transgenic mice were 1000-fold more sensitive to TNF-mediated killing than those from normal mice, and more than 100 times more sensitive than those from mice that expressed either single dominant-negative inhibitor. Thus, TRAF2 and NF-κB mediate separate, but synergistic anti-apoptotic signals.

TRAF3 TRAF3 was described independently as a cytoplasmic factor that interacts with CD40, and LMP-1. TARF3 can have an inhibitory effect on NF-κB activation; it is also involved in the induction of cell death by the lymphotoxin-β receptor. Amino-terminal deletion mutants of TRAF3 that interfere with the recruitment of endogenous TRAF3 to this receptor have a dominant-negative effect on cell death that is induced by LT-β, but have no influence on TNF-triggered apoptosis (Force *et al.*, 1997). Null mutations of TRAF3 in mice demonstrate the role of TRAF3 in T-dependent immune responses (Xu *et al.*, 1996). All of the lymphoid organs TRAF3-/- mice were significantly smaller than those of the littermate controls. Although the number of lymphoid cells was only ~1% of those of the normal littermates, neither thymocytes nor bone marrow cells from TRAF3-/- mice showed changes in the relative percentages of lymphocyte subpopulations. Yet, T cells that were isolated from the TRAF3-deficient mice had intrinsic defects and were impaired in their ability to respond to antigen. Therefore, TRAF3 appears to be important in signaling cascades that promote the activation and survival of T cells.

TRAF4 TRAF4 (also named CART1) was isolated by differential screening of a cDNA library of lymph nodes that contained metastatic tumor cells. Interestingly, the highest sequence homology exists between *Drosophila* TRAF1 (DTRAF1) and mammalian TRAF4 (Wajant *et al.*, 2001). Moreover, the expression of TRAF4 can be followed throughout embryogenesis. It is predominantly found in undifferentiated cells, e.g., neuronal precursors or epithelial progenitor cells. Thus, it is possible that DTARF1 and mammalian TRAF4 represent prototypic archaic members of the TRAF family with conserved functions in differentiation from which the other TRAF family members were developed by divergent evolution. However, the analysis of TRAF4-deficient mice does not indicate an apparent role of TRAF4 in the differentiation. Unexpectedly, these mice exhibit tracheal malformation with resulting alterations in airflow to the lungs.

TRAF5 TRAF5 was identified by utilization of degenerate PCR primers that were homologous to a highly conserved region at the carboxy-terminal end of TRAF proteins, and independently in a yeast two-hybrid screen by use of the cytoplasmic tail of CD40 as bait (Ishida *et al.*, 1996; Nakano *et al.*, 1996). TRAF5 is highly expressed in the spleen, lung, and thymus. Targeted disruption of *Traf5* gene causes defects in the CD40- and CD27-mediated lymphocyte activation.

TRAF6 TRAF6 was isolated independently by the screening of an EST expression library, and by utilizing CD40 as bait for a yeast two-hybrid screen. Of the six TRAFs described to date, TRAF6 shows the least homology to the prototypical TRAF domain sequence. Furthermore, TRAF2, TRAF3, and TRAF5 bind to the membrane-distal domain in the cytoplasmic tail of CD40 and TRANCE-R; whereas, TRAF6 interacts with the membrane-proximal domain (Wong *et al.*, 1998). Another unique feature of TRAF6 is that, unlike

other TRAFs, it has also been implicated in IL-1 signaling, leading to the activation of NF-κB. Recently, it was reported that TRAF6-/- mice exhibit severe osteopetrosis and are defective in osteoclast formation due to defective signaling from TRANCE-R/RANK upon binding of TRANCE/RANKL/OPGL (Lomaga *et al*., 1999). Furthermore, TRAF6-/- mice are defective in normal B-cell differentiation, lymph node organogenesis, and IL-1 signaling. Thus, TRAF6 plays pivotal roles in immune and inflammatory systems and in bone remodeling *in vivo*. Interestingly, the involvement of TRAF6 in the activation of a distinct signaling pathway, the Akt/PKB pathway that is important for cell survival, has also been reported (Wong *et al*., 1999). In this regard, it has been shown that TRANCE induces the activation of Akt via c-Src and PI3-kinase, whereby the stimulation of TRANCE-R results in the formation of an endogenous ternary complex of TRANCE-R/c-Src/TRAF6.

Members of the TNFR superfamily are critically involved in a multitude of disease-relevant biological processes and are now being targeted for therapies against widespread human diseases. These include autoimmune disorders, osteoporosis, atherosclerosis, and cancer. The TRAF proteins serve as adapter proteins that link TNFR receptors to a wide variety of signaling cascades, which might be directly or indirectly involved in the pathophysiological processes. Further studies will be required to connect the TRAF signaling pathways to the processes, and to dissect their effector functions in the context of diverse envionmental stimuli.

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