The signaling adaptors and pathways activated by TNF superfamily

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Abstract

Members of the TNF receptor superfamily play pivotal roles in numerous biological events in metazoan organisms. Ligand-mediated trimerization by corresponding homo- or heterotrimeric ligands, the TNF family ligands, causes recruitment of several intracellular adaptors, which activate multiple signal transduction pathways. While recruitment of death domain (DD) containing adaptors such as Fas associated death domain (FADD) and TNFR associated DD (TRADD) can lead to the activation of a signal transduction pathway that induces apoptosis, recruitment of TRAF family proteins can lead to the activation of transcription factors such as NF-κB and JNK thereby promoting cell survival and differentiation as well as immune and inflammatory responses. Individual TNF receptors are expressed in different cell types and have a range of affinities for various intracellular adaptors, which provide tremendous signaling and biological specificities. In addition, numerous signaling modulators are involved in regulating activities of signal transduction pathways downstream of receptors in this superfamily. Most of the TNF receptor superfamily members as well as many of their signaling mediators, have been uncovered in the last two decades. However, much remains unknown about how individual signal transduction pathways are regulated upon activation by any particular TNF receptor, under physiological conditions.

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Keywords: Signal transduction; Adaptor molecule; Apoptosis; Cell survival

Contents

1. Introduction .................................................. 193
2. Signals for cell apoptosis .................................. 194
3. Signals for cell survival and inflammation ............... 196
3.1. TRAF family members ..................................... 196
3.2. Domains and structures of TRAF proteins ............. 197
3.3. Recruitment of TRAFs to signaling receptors ............ 198
3.3.1. Association of TRAF proteins with TNF receptors ........... 198
3.3.2. Association of TRAF proteins with non-TNF receptors ...... 199
3.3.3. Signaling complexes and membrane rafts ................ 199
3.4. TRAF-activated signal transduction pathways .......... 200
3.4.1. TRAF-mediated activation of NF-κB .................... 200
3.4.2. TRAF-mediated activation of JNK ..................... 201
3.5. Additional TRAF associated intracellular modulators ...... 202
4. Regulation of TNFR-mediated signal transduction pathways ........................................ 203
References ......................................................... 205

1. Introduction

Thus far, 29 TNF receptor family members have been identified in humans. Based upon their cytoplasmic sequences and signaling properties, these TNF receptors can be classified into three major groups [1]. The first group, including Fas, TNF-R1, DR3, TRAIL-R1, TRAIL-R2, and DR6, contains a death domain (DD) in the cytoplasmic tail. Activation of these death domain-containing receptors by their corresponding ligands can lead to recruitment of intracellular death domain containing adaptors such as Fas associated death domain (FADD) and TNFR associated...
2. Signals for cell apoptosis

The death domain containing receptors are a subset of the Type I transmembrane TNF-receptor superfamily proteins defined by the presence of a death domain in their cytoplasmic domains. This protein association domain is found in TNF-R1 and Fas as well as several others. While these receptors share some mechanisms of signal transduction, they are not identical. Fas, TRAIL-R1 and TRAIL-R2 interact with the FADD while TNF-R1 and DR3 interact with the adaptor TRADD [2,3]. These primary associations define and restrict the nature and complexity of subsequent intracellular signaling events.

Upon Fas-L binding, juxtaposition of the DD of Fas allows interaction with the DD-containing adaptor protein FADD. FADD is a 26kDa cytoplasmic protein that contains both a C-terminal DD and a homologous death effector domain (DED). Fas receptor ligation results in death-inducing signaling complex (DISC) assembly commencing within minutes of ligand binding and localizing initially FADD and then caspase-8 to the receptor complex [4–6]. Caspase-8 has two C-terminal DED that display homology to and can interact with the DED of FADD. Caspase-8 is produced as a zymogen, activated first by removal of a pro-domain, then by limited cleavage at an aspartate residue to separate the large active protease subunit of 20kDa from the smaller 10kDa subunit [7]. This mature caspase can process downstream pro-caspases as well as its own pro-caspase precursor. Caspase activation proceeds in a hierarchical manner until activation of the executor caspases, such as caspase-3, result in apoptosis. Interestingly, caspase-3 and caspase-8 are 10,000 and 100 times, respectively, more active as mature proteases than as zymogens, thus greatly limiting activity in their immature forms [8]. Using an FK506 binding fusion of caspase-8 to mimic an assembling DISC, inductive aggregation of caspase-8 allowed autoprocessing and initiation of caspase-mediated cell death, supporting an induced proximity model of caspase activation [9]. Type I apoptosis refers to the activation of this caspase cascade. The serial proteolytic cleavage of specific targets leads to the activation of effector caspases-3, caspase-6, and caspase-7. The effector caspases are responsible for proteolytic cleavage of various cellular substrates leading to the distinctive DNA fragmentation that marks apoptotic death.

Type II apoptosis relies on organelle dysfunction, of which regulation of mitochondrial dysfunction by Bcl-2 family proteins is the best characterized. The mitochondria serve a dual function both as an energy generating organelle and as a sink for pro-death molecules. The Bcl-2 family shares up to four Bcl-2 homology domains (BH1–4). Members such as Bcl-2 and Bcl-xL are anti-apoptotic and function through the sequestration of pro-apoptotic, BH3 domain containing proteins such as Bak [10]. During apoptosis, activated caspase-8 cleaves Bid releasing a potent pro-apoptotic truncated protein, tBid. tBid is targeted to the outer mitochondrial membrane [11]. Here, it induces the oligomerization of Bak, which is proposed to form an intramembranous pore complex allowing the release of cytochrome c [12]. Cytochrome c drives oligomerization of apoptotic protease activating factor-1 (Apaf-1) [13]. Apaf-1 contains a caspase activation and recruitment domain (CARD) that interacts with a homologous domain in caspase-9 completing, in the presence of ATP, the assembly of a functional apoptosome [14]. The apoptosome can cleave and activate procaspase-3 thereby completing the same cycle of activation as the initiator caspase-8. Thus, the death receptor regulation of cytochrome c release is a balance between activation of Bid and inhibition by Bcl-2 and related proteins.

In contrast to Fas, TNF-R1 only signals for cell death in certain circumstances (e.g. when protein synthesis is blocked). In most scenarios, TNF-R1 instead induces the transcription and activation of inflammatory genes. This suggests that TNF-R1 signaling provides a mechanism to suppress the apoptotic stimulus. Consistent with this, assembly of a signaling complex at TNF-R1 differs from Fas in that TNF-R1 primarily associates with TRADD. TRADD can in turn associate with the DD of FADD, thereby initiating the caspase-8 activation pathway. Alternatively, TRADD can recruit TRAF2, TRAF1 and receptor-interacting protein (RIP) to activate the NF-κB and JNK pathways, which protect cells from apoptosis and initiate inflammatory responses [15,16]. Animals with a targeted deletion for RIP are unable to activate NF-κB in response to TNF-R1 stimulation leading to TNFα-induced apoptosis [17]. This implicates RIP as the primary mediator of NF-κB activation. In contrast, TRAF2 deficiency results in an inability to activate...
the MAP kinase JNK in response to TNF stimulation [18]. This too results in an apoptotic response to TNF stimulation. In fact, recent evidence suggests that converting JNK activation from sustained activation to a transient response protects the cell from an apoptotic response. This raises two points of interest relevant to this discussion. First, the apoptotic outcome of TNF-R1 stimulation when either NF-κB or JNK activation is inhibited suggests the possibility that both pathways can contribute to an anti-apoptotic response rather than one being simply inflammatory [19]. Secondly, the observation that TRAF2 is primarily responsible for only JNK activation in TNF-R1 signaling, but both NF-κB and JNK activation in CD40 signal transduction, suggests that the TRAF2 binding proteins have a very important role in determining the outcome of incorporation of a TRAF2 signal.

Therefore, in an apparent dichotomy of activation, TNF-R1 assembling a signaling complex that activates both the caspase-8 apoptotic and the NF-κB and JNK anti-apoptotic pathways. This balance is regulated at numerous levels including strength of signal, regulation of receptor expression and anti-apoptotic gene induction (Fig. 1).

There is mounting evidence that cellular localization and expression of TNFRs plays a significant role in regulating whether death receptor ligation results in apoptosis or not. For instance, TRAIL binds to TRAIL-R1 in cells expressing this receptor and causes these cells to undergo apoptosis. TRAIL-R1 is broadly expressed on many tissues including the spleen and lung. Similarly, the TRAIL binding receptor TRAIL-R2 is highly expressed in the lung, heart, spleen and peripheral blood lymphocytes. TRAIL resistance in these organs confounds such broad expression of constitutively apoptotic death receptors. The presence of decoy receptors which express TRAIL binding ectodomains and either minimal cytoplasmic domains (DcR2) or GPI anchors (DcR1) exposes one mechanism by which apoptosis can be regulated in vivo [20].

The ability of death receptors to regulate the outcome of their ligation is an important aspect of their biology. At one
level, this is achieved by altering the level of expression and the presence or absence of decoy receptors. A recent study on TNFR mediated signaling suggests that strength of signal and complex stability are controlled by receptor ligand interactions. Therefore, whether a ligand is membrane bound or soluble would produce a profound effect on the outcome of receptor ligation [21]. However, as simple overexpression of death receptors leads to apoptosis, there must clearly be mechanisms to prevent ligand independent activation of death receptors. One such inhibitor is silentin of death domain (SODD). This protein was isolated by yeast two hybrid and was found to constitutively associate with TNF-R1 and DR3. SODD binds to the DD of TNF-R1 and DR3 at a site that overlaps and inhibits binding to TRADD [22]. TNF-R1 aggregation by ligand binding disrupts this association and allows uninhibited binding of TRADD. Other proteins are expected to serve analogous function for other death receptor families.

Although it has taken some time to describe the different players in DD receptor signal transduction, their rapid association at the membrane was recognized very quickly. In fact, no associated proteins can be detected bound to Fas in advance of Fas-L binding. However, stimulation with Fas-L induces clustering and the rapid recruitment of FADD and caspase-8 to the Fas DISC [4,23]. Interestingly, the DISC has been shown to assemble in the glycosphingolipid-rich microdomains in the cell membrane known as lipid rafts. This localization is dependent on an intact DD. Indeed, raft localization can be transferred from TNF-R1 to TNF-R2 by recombinantly grafting the TNF-R1 DD [24].

Clearly, as an initiator caspase, the expression and activation of caspase-8 critically regulates the outcome of DD containing receptor ligation. Accumulation of FADD and pro-caspase-8 at a receptor induces self-processing of caspase-8 resulting in cleavage of downstream effector caspases. Human caspase-10 is a closely related caspase for which there is no known murine homologue. The fact that mice with a targeted deletion of the gene for caspase-8 demonstrated perinatal lethality and resistance to TNF-R1, Fas and DR3 mediated apoptosis support the probable absence of a murine homologue for human caspase-10 [25]. It is also possible that there are functions unique to caspase-10 not apparent in the mouse [26]. In support of this possibility, patients with an inactivating mutation in the gene for caspase-10 presented with an autoimmune lymphoproliferative syndrome [27].

The initiator caspase-8 and caspase-10 have probably arisen by gene duplication as their genes co-localize on chromosome 2. A further such duplication probably generated the caspase-8 inhibitor FLICE-inhibitory protein (FLIP). FLIP is homologous to caspase-8 containing two DED and a caspase-like domain. However, the active site cysteine is substituted yielding a prototypically inactive protein. FLIP associates with FADD and caspase-8 and inhibits apoptosis by all known human death receptors [28]. Consistent with a protective effect, cells from animals with a targeted deletion of c-FLIP are very sensitive to death receptor stimulation. This could partly explain why simple overexpression of Fas, and the resulting titration of inhibitors such as FLIP and SODD, could result in apoptosis. The existence of a family of viral inhibitors related to c-FLIP supports the observation that this is an effective mechanism for inhibiting apoptosis.

RIP also serves as a target for regulation of the outcome of TNFR family member signaling. Cells sensitized to apoptosis by treatment with cyclohexamide showed a caspase-8-dependent cleavage of RIP protein at glutamic acid 324. This cleavage separates the N-terminal kinase domain (RIPn) from the C-terminal death domain (RIPc). This cleavage is performed specifically in vivo by caspase-8 and results in ablation of the RIP-mediated activation of NF-κB [29]. Indeed RIPc, as does a recombinantly produced RIP-DD protein, serves as a dominant negative inhibitor of NF-κB activation. Furthermore, while full length RIP inhibited the interaction between TRADD and FADD, binding of RIPc to TRADD enhanced TRADD DD interaction with FADD thereby augmenting caspase activation and apoptosis. RIP cleavage is also seen in TAIL and Fas stimulated cell lysates implying that RIP is a general molecular switch between apoptosis and activation in death receptor signaling.

Alternative regulation is achieved by targeting the activation of downstream caspase-3 and caspase-7 [30]. Originally described as baculoviral inhibitors of apoptosis (IAPs), c-IAP-1, c-IAP-2, and the X-linked X-IAP all specifically inactivate the effector caspases [31]. Structurally, each protein bears three baculovirus IAP repeats (BIR domains) and an N-terminal ring finger motif. This ring finger domain possesses a ubiquitin–protein ligase activity. X-IAP ubiquitination promotes proteosomal degradation of caspase-3 further enhancing the anti-apoptotic function of X-IAP in Fas induced apoptosis [32]. As argued by the existence of viral homologues of these IAP family members, they represent a very effective site for regulation of apoptosis.

3. Signals for cell survival and inflammation

3.1. TRAF family members

Tumor necrosis factor receptor associated factors (TRAFs) are a major group of intracellular adapters that bind directly or indirectly to many members of the TNF receptor and the IL-1/Toll-like receptor superfamly (Fig. 2) [3,33–55]. Till date, six mammalian TRAFs, TRAF1 through TRAF6, have been identified. TRAFs are evolutionarily conserved proteins with homologues found in Drosophila melanogaster (dTraf1, dTraf2 and dTraf3) and Caenorhabditis elegans (ceTRAF) [56]. In addition, TRAF domain homology has been found in proteins expressed by Dictyostelium discoideum and Arabidopsis thaliana [57,58]. Although TRAFs have no known enzymatic activities, they can induce the activation of several kinase cascades that ultimately lead to the activation of signal
transduction pathways such as NF-κB, JNK, ERK, p38 and PISK, which can regulate cellular processes ranging from cell proliferation and differentiation to apoptosis.

3.2. Domains and structures of TRAF proteins

All TRAFs are characterized by a highly conserved motif at the C-terminus, termed the TRAF domain. The TRAF domain mediates binding to the receptors, formation of homo- or heterodimers, and interaction with a number of intracellular proteins and signaling molecules. The TRAF domain is about 200 amino acids in length and is further divided into a TRAF-N and a TRAF-C domain [59,60]. The trimerization of TRAFs requires an intact TRAF domain [61]. TRAFs can self-associate as homo- or heterotrimers of TRAF1 and TRAF2 or TRAF3 and TRAF5 or TRAF6 and either TRAF2 or TRAF3 [59,60,62]. Furthermore, the TRAF-C domain has been shown to bind downstream
signaling molecules such as TANK and NIK and the TRAF-N domain has been shown to bind to anti-apoptotic molecules such as c-IAP1 and c-IAP2 [63–66].

The amino termini of all mammalian TRAFs, except TRAF1, have a RING finger motif, similar to those found in E3 ubiquitin ligases [67,68]. This is followed by five to seven zinc fingers [69]. Some reports indicate that TRAF6 may act as an E3 ubiquitin ligase leading to the activation of the NF-κB pathway [70,71]. Although it is unknown whether other TRAFs can act as E3 ubiquitin ligases, the RING finger is essential for NF-κB activation and the zinc finger domain is essential for JNK and NF-κB activation [46,72–74]. Interestingly, although both TRAF3 and TRAF5 have an isoleucine zipper domain between the zinc fingers and the TRAF-N domain, only the overexpression of TRAF3 activates NF-κB. So far, the function of this isoleucine zipper domain remains to be elucidated.

3.3. Recruitment of TRAFs to signaling receptors

3.3.1. Association of TRAF proteins with TNF receptors

In addition to indirect association of TRAF proteins with death domain containing TNF receptors through TRADD or RIP as mentioned earlier, many members of the TNFR superfamily bind to TRAFs directly through a cysteine-rich motif termed the TIM (Fig. 3). The amino acid compositions of the TIMs vary among different TNFRs. However, a major consensus sequence (P/SA/TxQ/E)E can be found in CD40, CD30, HVEM, OX40, p75NGFR and RANK that can bind to TRAF2, TRAF3 and TRAF5 [37,62,62,75–79]. TRAF6 appears to have a unique binding motif P-X-E-X-X-(aromatic/acid residue) that is present in RANK and CD40 [62,75].

Among the TIM-containing TNF receptors, CD40 and TRAF2-R2 have been investigated more extensively in terms of their association and functional relationships with TRAFs. Initially, TRAF3, TRAF5 and TRAF6 were identified by yeast-two-hybrid screen using the CD40 cytoplasmic tail [34,41,42]. Since TRAF1 can heterodimerize with TRAF3 and TRAF5 can heterodimerize with TRAF3, TRAF1 and TRAF5 can indirectly associate with CD40 [59,62]. Deletion analyses of the CD40 cytoplasmic tail has mapped the minimum TRAF6 binding site to the membrane proximal QEPEQENF motif and the TRAF2/TRAF3 binding site to the PVQET motif [62]. These in vitro observations have been confirmed by crystal structure analyses of the CD40 peptides containing PVQET and the TRAF domain of TRAF2 and TRAF3. Using a CD40 peptide VPQET, McWhirter et al. [80] demonstrated that every residue makes complementary contact with the TRAF domain of TRAF2, indicating the significance of the PVQET sequence. Using a longer CD40 peptide containing PVQET, Ni et al. showed that the CD40 fragment forms a hairpin loop when binding to TRAF3 [81]. Thus, mutational and structural analyses show overlapping but structurally distinct TRAF2 and TRAF3 interactions with CD40.

To investigate the biological functions of TRAF2, TRAF3 and TRAF6 in CD40 signaling, transgenic mice expressing CD40 mutants that abolish binding to TRAF2 and TRAF3 or TRAF6 were generated [82–84]. These studies confirmed the critical roles of these three TRAFs in immunoglobulin isotype switching and affinity maturation, B cell maturation and proliferation, and germinal center formation. However, the physiological roles of these individual TRAFs in these B cell functions remain controversial. Further study of the immune functions of TRAF2, TRAF3 and TRAF6 are hindered by the fact that mice deficient in each of these TRAFs die shortly after birth [18,85–87]. Nevertheless, TRAF2 knockout mice can be rescued by generating TRAF2/−/− and TRAF2/−/− or TRAF1−/− and TRAF2−/− double knockout mice. These double knockout mice have a normal primary IgM response but impaired secondary IgG responses [88]. This defect may be attributed to the absence of NF-κB activation upon stimulation of CD40, as observed in the TNF-R1−/−, TRAF2−/− splenocytes. Interestingly, TRAF6 deficient splenocytes also have impaired NF-κB activation upon CD40 stimulation, which suggests that both TRAF2 and TRAF6 are essential for this CD40 signaling pathway [86]. The role of TRAF3 in CD40 signaling is enigmatic. In vitro CD40 stimulation of the TRAF3 deficient B lymphocytes results in a normal proliferative response. However, reconstitution of mice with TRAF3−/− fetal liver cells reveals an essential role of TRAF3 in mounting a T-dependent immune response [85].

Many other non-death domain containing TNFRs including LTβR, XEDAR, 4-1BB, CD27, BCMA, AITR, TACI, and TROY have been shown to associate with TRAFs in yeast-two-hybrid screens and immunoprecipitation assays in overexpression conditions (Fig. 2). Recently, Xu and Shu showed by co-immunoprecipitation assay using transient transfected cells that the BAFF receptor interacts with TRAF3 but not the other TRAFs [89]. Some of these TRAFs, such as CD27 and 4-1BB, contain a TRAF2 binding motif similar to the PVQET motif found in CD40 [77]. At this moment, very little is known about the roles of TRAFs in the signaling pathways of many of these TNFRs because their potential functions have mostly been investigated using dominant negative mutants. Nevertheless, some of the in vivo functions of RANK, 4-1BB, XEDAR and CD27 have been examined and validated using mouse models. Since RANK signaling has been shown to be essential for osteoclast differentiation and activation, and the TRAF6−/− osteoclasts fail to differentiate, TRAF6 is probably a necessary mediator in the RANK signaling pathway [86,87]. Additionally, TRAF2 plays a critical role in 4-1BB signaling because IL-2 production is reduced upon CD3 and 4-1BB crosslinking on activated T cells from the TRAF2DN and TRAF2 knockout mice [90]. The role of TRAF6 in XEDAR signaling has also been supported by the lack of NF-κB activation after stimulation of a CD40-XEDAR chimeric receptor in TRAF6−/− MEFs [91]. Finally, co-stimulation of TRAF5 knockout T
shown to activate both the JNK and NF-

This MyD88–IRAK–TRAF6 signaling complex has been identified and have shown to bind to TRAF6 [94,95] .

The binding of TRAFs to the receptor may be dependent on the specific signaling complex formed by the receptor, and the amount of oligomerization of the receptor.

The receptor-TRAF signalsome seems to occur in membrane lipid rafts, which are regions of plasma membrane that are rich in sphingolipids and cholesterol. Lipid rafts are thought to serve as a platform for the formation of signalosomes upon receptor oligomerization [99]. In the case of CD40 signaling, it has been shown that upon CD40 engagement, TRAF2 and TRAF3 are recruited into lipid rafts [100]. In addition, the Src family kinase Lyn has been shown to translocate into lipid rafts and become phosphorylated [101]. In addition, targeting TRAF3 to the membrane fraction has allowed it to activate JNK [102]. The functional consequence of TRAF2 localization to the membrane may be dependent on the specific signaling complex formed by individual receptor in the TNFR family. In the case of CD30 signaling, translocation of TRAF2 to the membrane insoluble fraction appears to sensitize cells to TNF-induced apoptosis, possibly by depleting TRAF2 from the cytoplasm so that the anti-apoptotic complex (TRAF2:c-IAPs) cannot be formed [103]. However, TRAF2 has also been demonstrated to associate with TNF-R2 and caveolin-1, a structural component of caveolae, and this complex formation leads to NF-

3.3.2. Association of TRAF proteins with non-TNF receptors

TRAFs have been shown to associate with numerous receptors outside of the TNFR superfamily. These receptors include those in the interleukin-1 receptor/Toll-like receptor (IL-1R/TLR) family (IL-1, IL-18, TLR2 and TLR4), IL-17R, and the IL-1R1 receptor in the endoplasmic reticulum (ER). TRAF6 seems to be the only TRAF to member that associates with receptors in the IL-1/TLR family [60]. This interaction occurs through binding to the serine/threonine kinase IRAK. Upon ligand stimulation, an adaptor protein MyD88 is recruited to the IL-1R or TLR complex via a conserved cytoplasmic domain termed the Toll/IL-1R homology region (TIR) at the C-terminus. The death domain of the TIR at the N-terminus of MyD88 recruits IRAK to the receptor complex [93].

TRAF6 and IRAK-1 are also recruited to the IL-1R complex [94,95]. This MyD88–IRAK–TRAF6 signaling complex has been shown to activate both the JNK and NF-

TRAFs have also been shown to participate in the signaling pathways of IL-17R and IRE1. Using immunoprecipitation assays, IL-17R has been demonstrated to specifically bind to TRAF6, but not TRAF2. This observation is substantiated by the lack of IL-17-induced activation of NF-

Many receptors, both members and non-members of the TNFR superfamily, can deliver an extracellular signal into the cells through a signaling complex composed of TRAFs. The composition of these complexes and their specificity in activating downstream signals have yet to be elucidated. Are there pre-formed small complexes such as oligomerization of receptors greatly increases their avidity for TRAFs. Furthermore, since TRAFs can form homodimers or heterodimers, it is possible that TRAF trimers can be composed of various TRAFs to regulate the specificity of signaling. For example, in the CD40-TRAF complex, the TRAF trimer can consist of TRAF1, TRAF2 and TRAF6 or TRAF3, TRAF5 and TRAF6. The TRAF composition of the signaling complex may depend upon expression levels of these TRAFs in the cell, the affinities of each TRAF to its receptor, and the amount of oligomerization of the receptor. Furthermore, crystal structure analyses of CD40 peptides with the TRAF domain of TRAF2 reveal no direct contact between the cytoplasmic tails of the receptors, suggesting that individual receptor tails can bind distinct adaptor complexes [80]. This observation raises the possibility that each receptor tail can bind to one TRAF trimer. If a trimeric receptor complex can bind to three TRAF trimers, it would amplify the size as well as the strength of the signalsome.
MyD88–IRAK–TRAF6 or TRADD–TRAF2–RIP in the cytoplasm which are readily available to be recruited to the receptors upon ligand binding or are these individual signaling molecules just floating in the cytoplasm and waiting to be recruited to the activated receptor complex in the lipid raft? Perhaps scrutiny of these endogenous protein complexes through biochemical methods would shed some light on this subject.

3.4. TRAF-activated signal transduction pathways

Receptor-mediated multimerization of TRAFs in the signalosome has been found to activate multiple signaling pathways (Fig. 3). The best described of these are the JNK and NF-κB pathways which play a major role in the biological functions elicited by TNFR family members. In addition to these two pathways, reports have also documented TRAF-mediated activation of the ERK, p38, and PI3K pathways. However, many of the details pertaining to the activation of the ERK, p38, and PI3K pathways are rather unclear. Because of this, we will focus on the NF-κB and JNK pathways and will seek to describe the major points of interest regarding TRAF-mediated activation of these pathways.

3.4.1. TRAF-mediated activation of NF-κB

NF-κB is a homo- or heterodimeric transcription factor that binds to κB sites in the promoters of a large number of genes involved in cell survival, inflammation as well as innate and adaptive immune responses. The dimers are composed of the five Rel family members: NF-κB1 (p50) and its precursor p105, NF-κB2 (p52) and its precursor p100, RelA (p65), RelB, and c-Rel. NF-κB subunits are activated
Interestingly, low amounts of NF-κB activity following TNF or IL-1β plexes are still formed in MEKK3 as a promising candidate for mediating NF-κB downstream of both the TNF-R1 and the IL-1R [110]. Dis-

firmed and thus the role of TAK1 in TRAF-mediated NF-κB activation is currently unclear. Studies by a number of research groups show that while NIK does not likely play a role in TNFR-mediated activation of the type 1 NF-κB pathway, it is a crucial component of the type 2 NF-κB signaling pathway. The first hints of this arose from the fact that NF-κB transactivation activation is reduced in both α/β mice (which possess a mutated form of NIK) and NIK−/− mice following LTβR ligation [111,112]. Further studies showed that overexpression of NIK promotes p100 cleavage and introduction of the α/β mutation into NIK completely blocks processing of p100 [113,114]. In contrast to MEKK1, which activates both IKKα and IKKβ, overexpression of NIK specifically interacts with and phosphory-

lates IKKα [115-117]. In fact, NIK induced processing of p100 occurs in WT and IKKβ−/− cells, but not in IKKα−/− cells [115]. Thus, the type 2 NF-κB pathway seems to flow from the receptor to an uncharacterized TRAF, which then activates NIK. Activation of NIK then causes IKKα-mediated cleavage of p100 enabling p52-containing dimers to translocate to the nucleus and engage transactivation. As mentioned above, an alternative pathway for the acti-

vation of NF-κB has recently been described. The NF-κB2 precursor, p100, contains an IκB domain and thus dimers containing this precursor are sequestered in the cytosol. Re-

moval of the IκB domain by proteolytic cleavage allows dimers containing NF-κB2 to translocate to the nucleus and activate transcription. While it is known that p100 processing is a highly regulated event, the exact mechanisms through which TNFR family members activate this pathway were unclear until recently.

through two distinct pathways. RelA (p65), RelB, and c-Rel are all activated through the canonical or type 1 NF-κB pathway whereas NF-κB2 (p52) is activated by the alternative or type 2 NF-κB pathway (Fig. 3).

The type 1 NF-κB activation pathway dictates that upon exposure to TNF or other appropriate stimuli, IκB kinase (IKKs) are activated by mitogen-activated kinases (MAP3Ks) leading to phosphorylation and ubiquitination of the inhibitors of κB (IκB). Ubiquitination of IκB leads to their degradation thereby releasing NF-κB and allowing it to enter the nucleus and activate transcription of appropriate gene targets.

Extensive work has been carried out in an attempt to de-

termine the structural features of TRAFs that mediate the acti-

vation of the type 1 NF-κB pathway. Initial studies on TRAF2 and TRAF3 showed that the C-terminal TRAF do-

main is responsible for protein-protein interactions and the N-terminal region of TRAFs, containing a number of zinc (Zn) chelating domains, including a RING finger and a vari-

able number of Zn fingers, is necessary for TRAF mediated activation of downstream signaling pathways [34,46]. Fur-

ther analysis of this region showed that the RING finger of TRAF proteins is, in fact, the domain that is critical for NF-κB activation by TRAFs [72,74,108].

The importance of the RING finger in the signal activa-

tion is intriguing as a well documented function of RING finger domains is as an E3 ubiquitin ligase [109]. Recently, TRAF6 was shown to be able to function as an E3 ubiquitin ligase. Using purified proteins in vitro, Deng and cowork-

ers showed that TRAF5, in the presence of the E2 complex, Ubc13/Uev1A, synthesizes K63-linked polyubiquitin in a RING finger dependent manner [70]. Ubc13/Uev1A/TRAF6 causes ubiquitination of TRAF6 at the N-terminus, lead-

ing to activation of TGFβ-activating kinase (TAK1) [71]. TAK1, along with its regulators TAB1 and TAB2, are then able to directly phosphorylate IKKβ, thereby activating the type 1 NF-κB pathway. This biochemical data suggests that the TAK1 complex is an important link between TRAFs and the NF-κB pathway. However, the role of the TAK1/TAB complex in NF-κB activation has not been genetically con-

firmed and thus the role of TAK1 in TRAF-mediated NF-κB activation is currently unclear.

In addition to TAK1, a number of other MAP3Ks have been postulated to serve as TRAF-activated intermediaries in the activation of NF-κB. Genetic evidence points to MEKK3 as a promising candidate for mediating NF-κB activation downstream of both the TNF-R1 and the IL-1R [110]. Dis-

ruption of MEKK3 severely reduces NF-κB DNA-binding activity following TNF or IL-1β treatment of fibroblasts. Interestingly, low amounts of NF-κB DNA-binding com-

plexes are still formed in MEKK3−/− cells following TNF stimulation indicating that MEKK3 is not the sole activator of NF-κB. Thus, part of the reason for the confusion as to which MAP3K is responsible for mediating TRAF-induced activation of the type 1 NF-κB pathway likely stems from the fact that multiple MAP3Ks are involved in this process.

Further studies showed that overexpression of NIK promotes p100 cleavage and introduction of the α/β mutation into NIK completely blocks processing of p100 [113,114]. As mentioned above, an alternative pathway for the acti-

vation of NF-κB has recently been described. The NF-κB2 precursor, p100, contains an IκB domain and thus dimers containing this precursor are sequestered in the cytosol. Re-

moval of the IκB domain by proteolytic cleavage allows dimers containing NF-κB2 to translocate to the nucleus and activate transcription. While it is known that p100 processing is a highly regulated event, the exact mechanisms through which TNFR family members activate this pathway were unclear until recently.

Studies by a number of research groups show that while NIK does not likely play a role in TNFR-mediated activation of the type 1 NF-κB pathway, it is a crucial component of the type 2 NF-κB signaling pathway. The first hints of this arose from the fact that NF-κB transactivation activation is reduced in both α/β mice (which possess a mutated form of NIK) and NIK−/− mice following LTβR ligation [111,112]. Further studies showed that overexpression of NIK promotes p100 cleavage and introduction of the α/β mutation into NIK completely blocks processing of p100 [113,114]. In contrast to MEKK1, which activates both IKKα and IKKβ, overexpression of NIK specifically interacts with and phosphory-

lates IKKα [115-117]. In fact, NIK induced processing of p100 occurs in WT and IKKβ−/− cells, but not in IKKα−/− cells [115]. Thus, the type 2 NF-κB pathway seems to flow from the receptor to an uncharacterized TRAF, which then activates NIK. Activation of NIK then causes IKKα-mediated cleavage of p100 enabling p52-containing dimers to translocate to the nucleus and engage transactivation.

Currently, only a small subset of the TNFR superfamily members have been shown to activate the type 2 NF-κB pathway. These receptors include the LTβR, CD40 and the BAFF receptor 3 (BR3) [111-113,118-120]. The connec-

tion between these receptors and the components of the type 2 NF-κB pathway is strongly supported by the fact that mice deficient in CD40, LTβR, CD40L, LTα/LTβ, BAFF, NIK, IKKα, and p52 all exhibit similar phenotypes, having defects in secondary lymphoid tissue architecture [111,113,121-130]. While it is clear that these three recep-
tors all activate the type 2 NF-κB pathway, the adaptor molecule used by them to do so remains to be identified.

3.4.2. TRAF-mediated activation of JNK

Another signaling pathway activated by TNFR super-
family members culminates in the activation of the JNKs 1/2/3 (Fig. 3). Like all MAPKs, JNKs are sequentially ac-

tivated by a serine/threonine kinase cascade that proceeds from level 3 MAPK kinases (MAP3K) to level 2 MAPK ki-

nases (MAP2K), and finally to the activation of the level 1 MAPK kinases (MAPK). MAP3Ks are themselves activated by multiple mechanisms, including level 4 MAPK kinases (MAP4Ks) and TRAFs. Exactly how TRAFs activate MAP3Ks is still unclear but structural studies have shed some light on the regions of TRAFs that are required for activation of JNK. These studies show that the N-terminal Zn-binding domains are essential
Subcellular localization of different TRAF molecules also seems to be critical in determining whether JNK can be activated and to what extent. The different members of the TRAF family have been found to have different degrees of resistance to solubilization by detergent [102]. TRAFs 2, 4, 5 and 6 are relatively insoluble and all of these TRAFs also activate JNK when overexpressed. In contrast, TRAF3 is extremely soluble and is unable to activate JNK when overexpressed. Furthermore, artificial localization of TRAF3 to the plasma membrane using a myristilation tag allowed TRAF3 to activate JNK [102]. Thus, there appears to be an inverse correlation between solubility and ability to activate JNK.

Multiple MAP3Ks and MAP4Ks have been shown to act downstream of TRAFs to activate the JNK pathway (Fig. 3). Apoptosis signal-regulated kinase (ASK1) is activated following TNF treatment and is activated by overexpression of TRAFs 2, 5 and 6 [131–133]. The importance of ASK1 in TNF signaling is exemplified by the fact that overexpression of dominant negative forms of ASK1 results in not only inhibiting TNF and TRAF-induced JNK activation, but also TNF-induced apoptosis [131,132]. Recent genetic data supports a role for ASK1 in these processes as deletion of ASK1 results only in defective JNK activation but also apoptosis in response to TNF treatment [134]. Thus, ASK1 seems to be an important signal mediator of both pro- and anti-apoptotic signals elicited by some TNFR superfamily members.

However, a family of MAP4Ks has also been implicated in mediating the activation of JNK by members of the TNFR superfamily. These proteins include germlinal center kinase (GCK), germlinal center-like kinase (GLK) and germlinal center kinase related (GCKR). All of these proteins have been found to become activated in response to TNF and to elicit the activation of JNKs [135–139]. Both GCK and GCKR have been shown to directly interact with TRAF2 through the TRAF domain [140–142]. Both GCK and GCKR interact with and activate MEKK1, and GCK/GCKR-induced activation of JNK is dependent on MEKK1 and MKK4 (SEK1) [136,140–142].

While it appears that the GCK/GCKR pathway of JNK activation may be an important one, it may be so only in certain cell types. As this pathway uses MEKK1 as an intermediate, it can be functional only in cell types where MEKK1 is required for the activation of JNK by TNFR superfamily members. Genetic data from studies of MEKK1-deficient mice show that TNF- or IL-1β-mediated activation of JNK is disrupted only in embryonic stem (ES) cells, not in MEFs or macrophages [143]. Thus, the GCK/GCKR pathway appears to be essential only in very specific circumstances.

At this point, for numerous reasons, it is difficult to determine the relative importance of each of these kinases in the activation of JNK by TNFR superfamily members. First, many of the studies described above are based on data derived from overexpression studies, and there is limited genomic data to support many of these observations. Secondly, specific classes of receptors or even individual receptors may utilize different mechanisms to activate JNK. In addition, as TNFR superfamily members regulate many essential biological processes, it is possible that a great deal of redundancy exists between these pathways in order to ensure that TNFRs are able to transduce the signals required to elicit these vital processes. Lastly, cell type specificity may play a role in determining which pathway is utilized.

3.5. Additional TRAF associated intracellular modulators

The exact mechanisms through which TRAFs potentiate the signaling pathways described above are still unclear. To elucidate how TRAFs regulate signaling, several research groups have sought to identify intracellular TRAF-interacting proteins. In addition to the TRAF-associated receptors and MAP kinases mentioned above, many other TRAF-interacting molecules have been described (Fig. 2). These may function as modulators, regulating the activities of various TRAF-mediated signal transduction pathways. These include a number of TRAF-interacting proteins that either promote or repress activation of downstream signals. These molecules include the RING-finger containing proteins TRAF-interacting protein (TRIP), which associates with TRAF1 and TRAF2, the TRAF6-interacting proteins T6BP and TIF and the TRAF2 associating protein Pw1/Peg3 [144–147]. In addition, Act1 and TRAF2-binding protein (T2BP) interact with TRAF5 and TRAF2, respectively, and overexpression of either of these proteins leads to activation of both NF-κB and JNK [148,149].

Besides these apparent regulatory proteins, TRAFs also interact with a number of cellular structural proteins (Fig. 2). These proteins include p62 nucleoporin, a novel protein called MIP-T3, filamin and caveolin [104,150–152]. The function of these proteins is currently unknown but as cytoskeleton components play an integral role in signal complex formation, these proteins may have an important role in TRAF-mediated signaling. As mentioned previously, the anti-apoptotic proteins A20, c-IAP1 and c-IAP2 also interact with TRAFs and contribute to the anti-apoptotic effects elicited by these molecules [66,153].

However, the most extensively studied of the potential TRAF-associated modulators is TANK/TRAF, which interacts with all known TRAFs except TRAF4 [63,142,154]. The PXQXT motif in the middle of TANK was identified as the TRAF binding site and was shown to be able to compete with CD40 for TRAF binding [155]. Indeed, crystal structural studies demonstrated that TANK and CD40 bind to the same crevice in the TRAF domain of TRAF3 [155]. Functionally, TANK seems to have both positive and negative effects on TRAF-mediated NF-κB and JNK.
activation. When expressed at low levels, TANK potentiates the synergistic activation of NF-κB and JNK by TRAF2 [63]. However, when high levels of TANK are expressed, NF-κB activation by TRAF2, CD40 and TNF-R1 and TNF-R2 is actually inhibited [154]. The sequences located at the amino and the carboxyl-terminal regions of TANK appear to be responsible for the positive and negative effects, respectively. In addition, the amino-terminal portion of TANK, which is responsible for the positive effects on NF-κB activation, has been shown to interact with a number of serine/threonine kinases, such as TBK1, IKKα and IKKβ [156, 157]. Recent studies further identified an interaction between TANK and NEMO/IKKγ, suggesting that TANK may link TBK1 or IKKs to the IKK complexes for NF-κB activation [158].

As described above, a wide variety of proteins are capable of interacting with TRAFs. Some seem to be involved in determining the subcellular localization of TRAFs while others may regulate the activation of downstream signals. However, the exact functions of most of these proteins in TRAF-mediated signaling remain unclear.

One central question about TRAF signaling remains wholly unaddressed: how do TRAFs differentially activate distinct signaling pathways? This question is difficult to answer as the exact mechanism(s) by which TRAFs activate signaling are unknown. However, oligomerization of signaling complex components is surely a part of the activation procedure. As described above, the RING finger of TRAFs is required for NF-κB but dispensable for JNK activation. Thus, distinct but overlapping regions of TRAFs mediate activation of different signaling pathways. A single large signaling complex may form at the N-terminal region of TRAFs that mediates the activation of all signaling pathways. This complex would contain the IKK complex as well as MKKs, MAP3Ks, MAP4Ks, and all other molecules necessary for the activation of requisite signaling pathways. As all signaling components would be oligomerized (and thereby activated) at the same time, signaling specificity would occur through an unknown mechanism. A more appealing model for specific activation of individual pathways is that multiple signaling complexes may be able to interact with a single TRAF molecule at the same time. If this is in fact the case, a JNK-activating complex could associate with the Zn finger region of a TRAF at the same time that the NF-κB-activating IKK complex associates with the neighboring RING finger domain of the same TRAF. Likewise, other separate complexes may be utilized for activation of the p38, JNK or ERK pathways. TRAF-interacting proteins could regulate not only which signaling complex(es) associate with TRAFs, but also the activation state of these individual complexes. Binding of certain TRAF-interacting protein could either stabilize or destabilize the interaction of these complexes with TRAFs, thereby determining which signal is sent. They could also determine subcellular localization of TRAFs thereby potentially regulating the proximity of individual signaling complexes to TRAFs. If this is the case, it would provide a powerful level of regulation that can determine signal specificity in a cell-type specific manner.

4. Regulation of TNFR-mediated signal transduction pathways

The TNFR superfamily members, while all structurally related, mediate a broad spectrum of responses that range from an anti-apoptotic CD40 signal, to a proinflammatory TNF-R1 stimulus or apoptosis induced by Fas-L ligation. This range of responses are mainly a reflection of the variety of adaptor molecules that TNF receptors can interact with and is probably at its most varied in the case of TNF-R1 signaling. This DD containing receptor engages the FADD–caspase-8 complex as well as TRAF2 and RIP, resulting in activation of NF-κB and JNK. The outcome of TNF-R1 ligation is therefore a measure of the contribution of each pathway to the signal. This explains why Fas, which can recruit FADD but not TRAF2, is a very efficient mediator of apoptosis but cannot regulate inflammatory responses. TNF-R2 and CD40 in contrast, recruit TRAF2 but not FADD, leading to efficient activation of NF-κB and JNK, and are unable to elicit apoptosis.

Deficiencies in NF-κB activation, or inhibition of new protein translation render a cell exquisitely sensitive to TNF-R1 induced apoptosis [159, 160]. Expression of a dominant active mutant of IκBα produces a similarly sensitive response to TNF stimulation. The anti-apoptotic effect of NF-κB depends on the protective effect of the inflammatory genes induced [161]. As discussed above, NF-κB activation upregulates a panel of proteins, including A20, TRAF1, TRAF2, c-IAP-1, c-IAP-2, and XIAP [162, 163]. Although none of these proteins is singly able to inhibit apoptosis, their combined effects are to prevent cell death. They accomplish this by inhibiting the pro-apoptotic machinery and enhancing the cell survival response (Fig. 4). This panel of protein regulators describes a response shared with a subset of TNFR proteins that regulate inflammatory responses and enhance cell survival. As indicated above, blocking specific signal transduction pathways pharmacologically can prevent this cell survival program.

Additionally, degradation of specific proteins is increasingly being found to be a regulatory mechanism at the signaling level. Caspase-8 mediated RIP cleavage, to generate the pro-apoptotic RIPc fragment, is the result of specific cleavage just as is caspase activation. Similarly, caspase-3 can specifically cleave IκKα during TNF-induced apoptosis [164]. This cleavage is specific to TNF and results in elimination of IKKβ enzyme activity. The subsequent inability to activate NF-κB would inhibit the expression of anti-apoptotic NF-κB dependent genes, thus tilting the balance towards caspase-mediated apoptosis. Similarly, in direct opposition of one another, NF-κB activation up-regulates the Bak sequestering proteins Bcl-x and Bcl-2.

In contrast, caspase-8 cleaves Bid protein, supporting the
Fig. 4. Regulation of TNFR signal transduction. TRAF proteins play a central role in the outcome of TNFR regulation. Although many TRAF associating proteins have been described, many of their molecular mechanisms remain to be described. Anti-apoptotic or differentiative signals are indicated with green lines. Pro-apoptotic regulation is indicated with red.

generation of cytochrome c releasing Bak pores from the mitochondria. Again the outcome of TNF-R1 signaling is a measure of where the fulcrum is for that particular cell and the nature of the signal. Interestingly, NF-κB can also alter the magnitude of JNK activation. By inducing the X-IAP protein expression, NF-κB activation negatively modulates TNF-mediated JNK activation [165]. This regulation may convert the apoptogenic sustained JNK signal to a non-apoptotic transient signal [166].

Ubiquitination is another mechanism of degradation. c-IAP1 and c-IAP2 have been shown to contain ubiquitin protein ligase activity that is dependent on their RING domains. Just as the NF-κB mediated upregulation of c-IAP inhibits caspase-8 activation, pro-apoptotic signals result in the autoubiquitination of c-IAP proteins and their subsequent proteasome-mediated degradation [167]. Similarly, TRAF2 and TRAF6 ubiquitination has been shown to be key to the TRAF mediated activation of the NF-κB pathway although not necessarily degradation. The TRAF proteins recruited by TNF receptors also affect receptor signal regulation. Animals with a targeted deletion of the TRAF1 gene are hyper-responsive to TNF stimulation implying a negative regulatory role for TRAF1. The absence of Zinc fingers in TRAF1, a domain that has been shown to be critical to the functions of other TRAF proteins, supports a distinct role for TRAF1 in TRAF-mediated signaling [168].

The TNFR superfamily contains a large number of proteins that regulate a very broad array of developmental and differentiating processes. The excitement caused by the initial description of hemorrhagic necrosis by TNF pre-empted the discovery of a broader role for TNF and its family members in regulating inflammation. Indeed, a number of biologic TNF blocking therapies are being used now to inhibit the inflammation associated with Crohn’s disease and rheumatoid arthritis [169–171]. This approach will no doubt be applied to other receptor systems as the biology of TNFR family members is more carefully described. However, these therapies blocking receptor-ligand interactions can also inhibit beneficial as well as pathologic actions. By dissecting the signaling pathways associated with TNFR signaling, more specific sites of pharmacologic intervention will need to be described. Thus, the continued examination of TNFR signal transduction will provide us with the tools
for receptor or tissue specific interventions, allowing more targeted treatments that have fewer side effects.

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