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COMMENTARY

Apoptotic, Non-apoptotic, and Anti-apoptotic Pathways of Tumor Necrosis Factor Signalling

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ABSTRACT. Early events in the signalling of tumor necrosis factor-receptor 1 (TNF-R1), which is the main TNF receptor on most cell types, have been clarified recently. A multimolecular signal transducing complex from which several pathways originate rapidly forms upon TNF-induced aggregation of the receptor. Although fully capable of transducing apoptotic signals, which depend on the adapter Fas-associated death domain protein (FADD) and on the subsequent recruitment/activation of the apoptotic proteases, TNF-R1 usually does not kill cells; this is due to the induction of a complex cytoprotective response that requires TNF-receptor associated factor 2 (TRAF2), a signal transducer that couples TNF-R1 to both nuclear factor κB (NFκB)-dependent and NFκB-independent transcriptional events implicated in induction of genes protecting from TNF cytotoxicity. Although absolutely required for cytoprotection, TNF-receptor associated factor 2 is not sufficient to protect cells from TNF, thus suggesting that it may act in concert with additional TNF-R1 complex components. In this commentary, we will discuss some critical aspects of TNF-R1 signal transduction that are not fully understood: Why do cells not die before the protective protein synthesis has occurred? What are the mechanisms implicated in the termination of each TNF-R1-elicited response? Are there regulatory mechanisms capable of influencing the composition of the TNF-R1 complex and, consequently, the propagation of specific signals? BIOCHEM PHARMACOL **56**;8:915–920, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. tumor necrosis factor; tumor necrosis factor receptors; NFκB/Rel family; TRAF family

TNFα† is a cytokine that is produced by activated macrophages as well as by several other cell types, including lymphocytes, fibroblasts, and hepatocytes [1–3]. TNFα is produced as an integral transmembrane precursor (mTNFα) from which the soluble form (sTNFα) is proteolytically cleaved and released. The effects of TNFα are mediated by two distinct cell surface receptors that are expressed simultaneously on almost all cell types: p55TNF-R1 and p75TNF-R2. While the former is constitutively expressed at a rather low level, the expression of the latter is inducibly controlled by a number of extracellular stimuli acting at both the transcriptional and the post-transcriptional level [2]. Although p75TNF-R2 can independently activate some cellular responses (including activation of NFκB and SAPKs/cJun N-terminal kinases; see below), p55TNF-R1 seems to be entirely responsible for TNF signalling in most cell types. The identification of TRAF2 (see below) as a signalling molecule used by both receptors has provided the molecular framework to understand the partially overlapping responses elicited by TNF-R1 and TNF-R2.

Crystallographic studies indicate that activation of TNF-Rs occurs through ligand-mediated oligomerization [4]; indeed, TNFα has a spontaneous tendency to trimerize, and its only function may be to cross-link the receptors loaded on the cell membrane, thus leading to the juxtaposition of their cytoplasmic tails. The cytoplasmic regions of both p55TNF-R1 and p75TNF-R2 lack recognizable enzymatic domains; therefore, their ability to transduce signals depends on the interaction with specific transducers (Fig. 1), whose identification and molecular cloning in the last 3 years has aided in the understanding of the proximal events in TNF-R1 signalling [5, 6]. TRADD has been identified by two-hybrid screening in yeast as a TNF-R1 associated protein that contacts the receptor in a ligand-dependent manner [7]. The TNF-R1 region that interacts with TRADD is a COOH-terminal domain to which the cytotoxic function of the receptor is mapped [8]. TRADD is thought to act as an adapter, whose function is to recruit two downstream transducers, FADD and TRAF2 [9]. While FADD [10, 11] directly interacts with the apoptotic proteases [12, 13], thus triggering cell death, TRAF2 [14] is

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†*Abbreviations:* CHX, cycloheximide; EF, embryonic fibroblasts; FADD, Fas-associated death domain protein; FLICE, FADD-like interleukin 1β converting enzyme; IκB, inhibitor of NFκB; I-TRAF, inhibitor of TRAF; JNK, cJun N-terminal kinase; NFκB, nuclear factor κB; NIK, NF-κB inducing kinase; RIP, receptor interacting protein; SAPK, stress-activated protein kinase; TNFα, tumor necrosis factor-α; TNF-R, TNF-receptor; TRADD, TNF-receptor 1 associated death domain containing protein; and TRAF2, TNF-receptor associated factor 2.

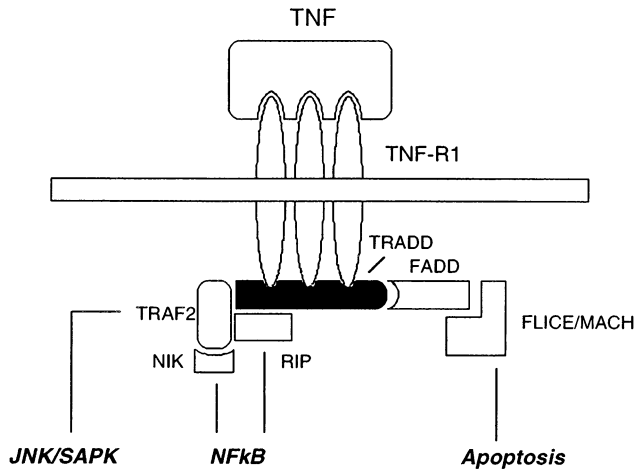

FIG. 1. Schematic representation of p55TNF-R1 and its signal transducers. Upon ligand-induced trimerization, the adapter TRADD and the downstream signal transducers FADD, TRAF2, and RIP are rapidly recruited to TNF-R1. The involvement of each downstream protein in the transduction of specific signals is shown.

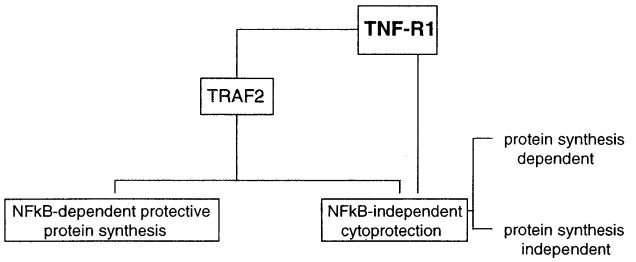

FIG. 2. The cytoprotective response. Shown are NFκB-dependent and NFκB-independent cytoprotective pathways that are implicated in the induction and maintenance of cellular resistance to TNF. The existence of TRAF2-independent cytoprotective pathways is suggested by the inability of overexpressed TRAF2 (which activates JNK/SAPK, AP1, and NFκB) to protect cells from a challenge with TNF + CHX (see text).

required for the activation of two distinct pathways. The first one, which requires the recruitment of the recently identified protein-kinase NIK [15], leads to the phosphorylation of the NFκB inhibitory subunit (IκBα) at two N-terminal serines (Ser 32/36); phospholylated IκBα is then degraded, thus liberating NFκB, which enters the nucleus and activates transcription [9, 16]. The other pathway, which is independent of NIK [17, 18], is devoted to the activation of the JNKs/SAPKs [19–21], which, in turn, stimulate transcription by phosphorylating and activating a number of transcription factors, including c-Jun, ATF2 and TCF/Elk. Therefore, while TNF-R1-induced apoptosis requires FADD, stimulation of gene expression mainly occurs through TRAF2-dependent pathways. p75TNF-R2 does not recruit TRADD but directly interacts with TRAF2, which links this receptor to both NFκB and JNK/SAPK activation pathways [16, 21], thus explaining the partially overlapping signalling properties of the two TNF receptors.

The first (and most important) biological role of TNF-stimulated gene expression is to protect cells from TNF cytotoxicity; indeed, most TNF-treated cells are resistant to apoptosis unless TNF treatment is co-delivered with protein/RNA synthesis inhibitors (such as cycloheximide and actinomycin D). Evidence for a TRAF2 role in turning on protecting genes (Fig. 2) originally came from experiments with a TRAF2 mutant that acts in a dominant negative fashion (TRAF2ΔN); while most normal and neoplastic cells are resistant to TNF cytotoxicity, those expressing TRAF2ΔN are doomed to death upon TNF-R1 cross-linking [19, 20]. This suggests that TNF-R1 sends two different signals: the first activates the cell death pathway; the second protects from it. As a general rule, the second signal overcomes the first, and the cell will survive. One critical downstream mediator of TRAF2-dependent cytoprotection is transcription factor NFκB, as shown by TNFα sensitivity of both cells lacking the main NFκB subunit (p65) [22] and of cells expressing a phosphorylation-defective IκBα mutant (IκBα Ser 32/36>Ala) that irreversibly sequesters NFκB, thereby acting as a "super-repressor" of NFκB function [19, 23, 24]. The extent to which different cells are sensitized to TNF cytotoxicity by knocking out NFκB function may vary. However, in our hands, sensitization to TNFα-induced apoptosis by TRAF2ΔN, which is supposed to act as a rather general inhibitor of TRAF2-elicited cellular responses, is usually much higher than that obtained by selectively blocking NFκB activation, in both continuously cultured tumor cells and highly differentiated primary cells [25]. This would suggest that TRAF2 transmits cytoprotective signals through alternative pathways, some of them being independent of transcription factor NFκB (Fig. 2). The identification of these pathways and the characterization of their mode of regulation will be a relevant challenge. Moreover, it must be considered that although TRAF2 is *required* for the induction of a cellular response protecting from TNF cytotoxicity (as shown by experiments with TRAF2ΔN), overexpression of TRAF2 (which leads to the activation of NFκB, JNK/SAPK, and AP1, thus mimicking several non-cytotoxic TNF-induced signals) is not *sufficient* to protect cells from apoptosis induced by TNF + CHX [25]. This points to a critical role of additional TNF-R1 complex components and suggests that the integration of multiple signals must occur to protect cells from TNF.

The very recent availability of TRAF2-deficient mice generated by gene targeting [26] has allowed a better definition of the role of TRAF2 in TNF-R1 signalling (Fig. 3). In contrast to their wild-type counterpart, several TRAF2-/- lineages showed high sensitivity to TNF-dependent apoptosis, thus confirming that TRAF2 is an absolutely required component of the cytoprotective apparatus and that its absence results in the abrogation of the cytoprotective response. Consistent with the described TRAF2 requirement for JNK/SAPK activation [19–21], EF obtained from TRAF2-deficient mice were unable to up-regulate JNK/SAPK activity upon TNF treatment; con-

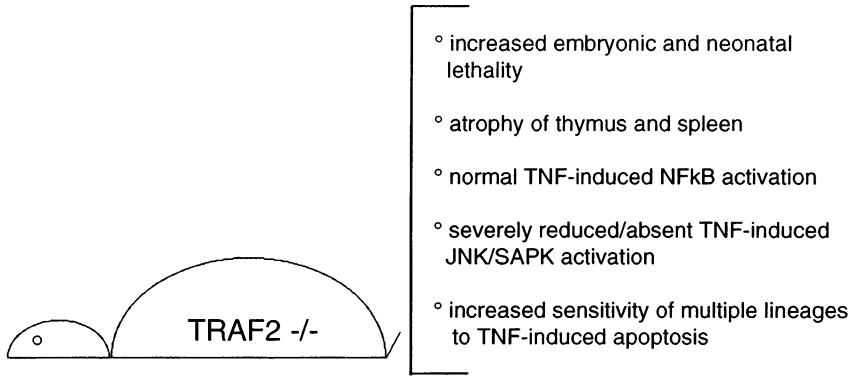

FIG. 3. Phenotype of TRAF2 deficient mice. Knockout mice lacking TRAF2 display increased embryonic and neonatal lethality, atrophy of thymus and spleen, and depletion of B cell precursors. Moreover, several cell lineages were sensitive to TNF-induced apoptosis, thus indicating the absence of the cytoprotective response. TRAF2 deficiency is also associated with a severe reduction in JNK/SAPK activation and with a conserved NFκB activation.

versely, these cells were surprisingly capable of NFκB activation in response to TNF-R1 engagement, thus indicating the existence of TRAF2-independent NFκB-activation pathways. One of these pathways may require the protein-kinase RIP [27], an additional TNF-R1 complex component [28] whose participation in NFκB signalling has been suggested both by studies with dominant negative mutants [28] and by the absent TNF-induced NFκB activation of a RIP-deficient Jurkat cell line [29].

This simple model *makes sense*. Indeed, a very high number of conditions have been shown to determine a local or systemic increase in TNFα levels in mammals [1]. Therefore, a vigorous and prevailing protective mechanism is required to avoid unwanted death of cells exposed to TNFα. However, many relevant and unresolved questions need to be addressed. First of all, what happens in the time-lag between TNF-R1 engagement and the translation of protective genes, i.e. why doesn't the cell die before protective gene products have been synthesized? Indeed, although NFκB is activated within minutes following exposure to TNF, transcription, translation, and accumulation of protective gene products may require some hours; during this period, the death pathway must be kept on stand-by status to avoid the unwanted propagation of death signals. This suggests the existence of a cytoplasmic mechanism regulating either FADD recruitment to TRADD or the subsequent activation of proteases; in the absence of this (hypothetical) rapidly acting (presumably transcription-independent) mechanism, the cell would be killed rapidly by TNFα in spite of the induction of the transcription-dependent cytoprotective response. Indeed, we already know that the activation of the FADD>proteases apoptotic pathway by a strictly related receptor, Fas/Apo1/CD95, occurs in tens of seconds [30], being that FADD and the first protease of the pathway (FLICE/MACH) are recruited almost instantly to the engaged receptor. We can also hypothesize that this protective mechanism must be switched off a few hours after TNF-R1 engagement; at that time, a cell that is destined to survive (i.e. a cell wherein TRAF2-dependent protective genes have been induced) will remain viable and will also be able to express (in a cell-type dependent fashion) other TNFα-inducible genes, such as those encoding cytokines and adhesion molecules. On the other hand, if protective proteins have not been synthesized, the cell will die. As regards the nature of the signals that keep the death machinery blocked before the synthesis of protective proteins, we have only a few clues. The most important comes from studies on Fas signalling and is the observation that FADD exists in unstimulated cells as both unphosphorylated and serine-phosphorylated forms, with only the latter recruited to cross-linked Fas [30]. Therefore, one possible mechanism implicated in the regulation of FADD recruitment to receptors is its phosphorylation by yet unknown protein-kinases. Importantly, since FADD is serine-phosphorylated in unstimulated cells, the activity of its kinase must be either constitutive or (more likely) responsive to extracellular signals independent of Fas/TNF-R1 aggregation, which may dictate cellular sensitivity to FADD-dependent apoptotic pathways. However, no reports have been published to date that investigate the role of FADD phosphorylation in FADD recruitment to the TNF-R1/TRADD complex. An alternative possibility is that post-translational modifications of TRADD may differentially affect its ability to interact with FADD or TRAF2, thus regulating the composition of TNF-R1 complexes. The intriguing possibility that TRAF2 may also be involved in this transcription-independent protective mechanism has been suggested by the behavior of TRAF2-deficient EF cells [26]. Indeed, differently from most cell types, wild-type EF cells are resistant to TNF + CHX treatment, thus indicating that resistance to TNF cytotoxicity in these cells is transcription/translation independent. Because TRAF2-/- EF are killed by TNF + CHX, it is highly likely that TRAF2 directly blocks apoptotic effectors or recruits protective proteins. A similar protein synthesis-independent protective effect of TRAF2 was shown to be abrogated in thymocytes obtained from TRAF2ΔN-expressing transgenic mice [31].

A second question arises from a simple but necessary consideration: although almost all of the cell types tested thus far are resistant to TNF-induced apoptosis unless sensitized by translational inhibitors or blockers of the TRAF2>NFκB pathway, it *makes sense* that conditions that allow the FADD>proteases apoptotic pathway to be activated do exist (otherwise the apoptotic potential of the receptor would never be used). Then, what are the physi-

ological or pathological conditions (other than artificial laboratory settings) that render TNF-R1 able to signal apoptosis? Detailed studies on the very few cell lines that are killed by TNFα in the absence of translational inhibitors may help provide some insights in this level of regulation. We can postulate the existence of blocks/defects in either the *afferent* (TRAF2>NFκB and other protective pathways) or the *efferent* (protective genes and their effectors) branches of the protective pathway. In the second group, mutations in relevant protective genes that could explain TNF sensitivity of some tumor cells may be included. Moreover, the activity of the protective proteins may be responsive to additional extracellular signals. In the first group may fall genetically acquired mutations of TRAF2 or other components of the protective apparatus of the receptor, as well as mutations of regulatory transcription factors involved in the protective response. Moreover, any point in this pathway may be subjected to control by extracellular signals modulating the activity/expression of its components. In this light, it would be important to define whether post-translational modification of components of the TRAF2/NFκB/protective genes axis able to affect their activity (for example phosphorylation of TRAF2 and regulated proteolysis) do exist. As already discussed, transmission of TNF-R1-originating signals seems to occur through a controlled series of protein–protein interactions. Following TNF-induced trimerization of the receptor, TRADD is recruited to TNF-R1 through a death domain to death domain interaction [7, 9]. In a subsequent step, TRAF2 is released from cytoplasmic complexes (in which TRAF2 is bound to I-TRAF, a general inhibitor of the activities TRAFs; see below) [32, 33] and recruited to the TNF-R1/TRADD complex. We do not know exactly what happens to the branch of the signal that leads to the activation of the FADD/FLICE pathway, but (as discussed above) it is highly likely that initially FADD is not recruited to the TNF-R1 complex. The mechanisms responsible for the regulation of these interactions, and in particular for the termination of each response, are unknown. We already know that each activated downstream effector can be individually down-regulated. As regards NFκB, IκBα degradation allows NFκB to enter the nucleus and to activate transcription; since the IκBα promoter contains NFκB binding sites, it is induced by NFκB, and the levels of IκBα are restored rapidly. Resynthesized IκBα enters the nucleus, interacts with DNA-bound NFκB, and probably exports it from the nucleus, thus contributing to the termination of the NFκB-dependent responses (reviewed in [34]). As regards the JNK/SAPK pathway, the level of JNK/SAPK activity following TNF treatment is maximal between 10 and 15 min following stimulation; JNK/SAPK activity then declines to non-stimulated levels 30 min after TNF treatment [20, 35]. Although this behavior is consistent with the activation of a JNK phosphatase, details on how this regulation occurs are not known. What is also unknown at present is the eventual existence of regulatory mechanisms modulating TRAF2-dependent responses at the TRAF2 level (or at the level of the TRADD/TRAF2 interaction). TRAF2 is composed of 501 amino acids organized in at least two distinct domains [14, 36]. The N-terminal region contains two zinc-binding domains, i.e. a ring finger and five zinc fingers [37, 38]. The COOH-terminal half of the molecule (TRAF domain) can be divided into the TRAF-N and TRAF-C subdomains [39], which are involved in both homodimerization and interaction with dimerization partners. While interaction with TRADD depends on sequences located at the COOH-terminus of TRAF-C, TRAF-N and the first three amino acids of TRAF-C (356 WKI 358) are required for both interaction with NIK and activation of NFκB [17, 36]. The N-terminal TRAF2 zinc-binding region is absolutely required for both NFκB and JNK/SAPK activation [16, 19–21]. However, no cellular proteins have been shown to bind to these domains to date, and it is not clear why they are required for the generation of TRAF2 signals. Although we cannot exclude the possibility that the deletion of the TRAF2 N-terminus simply causes conformational changes that render TRAF2 unable to signal, it is also possible to hypothesize that these domains mediate an (as yet unknown) activation step, targeting either TRAF2 itself or downstream effectors, such as NIK (discussed in [17]). An alternative possibility is that the failure to identify cellular proteins bound to this region only reflects the inadequacy of the currently available techniques.

One mechanism of TRAF2 regulation depends on a TRAF2-interacting protein known as I-TRAF or TANK [32, 33]. Although the proposed role of I-TRAF is to bind TRAF2 in the cytoplasm and to prevent its recruitment to the activated receptor [33], an alternative possibility is that I-TRAF may contribute to the regulation of TRAF2 after its recruitment to TNF-Rs. It will be interesting to study whether the expression of I-TRAF is constitutive or regulated and whether the several alternatively spliced I-TRAF variants ([33]; Natoli *et al.*, unpublished data) exert specialized roles, such as the selective inhibition of specific TRAF2 functions. Moreover, assuming that I-TRAF is constitutively bound to TRAF2 in unstimulated cells, the nature of the signal that determines I-TRAF dissociation from TRAF2 following TNF stimulation will merit further investigations.

A20, a zinc-finger protein that is expressed at rather low levels in most cell types, is induced rapidly by TNF and several other stimuli in an NFκB-dependent manner [40–44]. Although originally characterized as an inhibitor of TNF-induced cell death in stably expressing clones [45, 46], A20 seems to act mainly as a rather selective inhibitor of NFκB activation by TNF and several other stimuli without affecting either the apoptotic caspases pathway or the JNK/SAPK pathway [25, 47–49]. The mechanism of A20-dependent TRAF2 inhibition is still unknown. In this context, the most puzzling result is that although A20 is able to directly bind TRAF2, this interaction is not required for NFκB inhibition [25, 47]. Although this could suggest that A20 acts by inhibiting a downstream effector,

we have been unable to detect any effect of A20 on NFκB activation by overexpressed NIK, which is supposed to act at a step immediately downstream of TRAF2 in the NFκB pathway (Natoli *et al.*, unpublished data). Moreover, we have not been able to demonstrate the existence of a direct A20/NIK interaction in living cells or of trimolecular TRAF2/A20/NIK complexes. From a functional point of view, the fact that (similar to IκBα) A20 is such a powerful inhibitor of NFκB activation and that it is inducible by TNF-Rs/TRAF2-originated signals (thus representing the efferent branch of an auto-regulatory loop) suggests that additional regulatory mechanisms may not be required to effectively shut-down TRAF2 responses directed at activating NFκB. However, it will be important to directly test the eventual occurrence of TRAF2 post-translational modifications that could unveil additional levels of regulation.

It is not the aim of this commentary, which reflects the authors' personal views on an extremely rapidly evolving field, to draw any kind of definitive conclusion. What is rather clear is that TNF-R1 represents an extremely complex multifunctional molecule, that through evolution has acquired the ability to transmit several, sometimes antagonistic, signals, with each signal being subjected to multiple levels of regulation. Detailed characterization of these mechanisms of regulation will also allow us to design novel pharmacological agents that can modulate in a highly selective manner each TNF-dependent response. The impact of these agents in pharmacological modulation of inflammatory responses, demyelinating diseases, and in anti-cancer chemotherapy may be enormous.

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