Special Feature

Signalling by CD95 and TNF receptors: Not only life and death

CARINA MAGNUSSON and DAVID L VAUX

Walter and Eliza Hall Institute of Medical Research, Royal Melbourne Hospital, Parkville, Victoria, Australia

Summary Members of the TNF family of receptors play important roles in normal physiology and in defence. The recent rapid progress in the understanding of the mechanisms of apoptosis has been accompanied by assumptions that TNF family receptors such as CD95 (Fas/APO-1) only have a role in regulating cell survival. While regulation of cell death is one important function of TNF family receptors, they are capable of activating signal transduction pathways that have many other effects. The present review will focus on signalling of some TNF family receptors in the immune system, not only for apoptosis, but also for survival or activation.

Key words: apoptosis, CD95, NF-κB, signal transduction, TNF receptors.

TNF receptor family

The tumour necrosis factor receptor (TNFR)/nerve growth factor receptor (NGFR) family of molecules regulate a number of biological functions, such as growth, differentiation and apoptosis in multiple cell types. In the immune system, members of this receptor family are involved in the development of peripheral lymphoid organs, regulation of induced inflammatory responses and removal of cells at the end of an immune response.

The TNFR family consists of more than 15 different molecules. Most are type I membrane proteins which resemble each other largely in their extracellular regions, which all contain 2–6 characteristic cysteine-rich domains.1

The TNF family receptors are activated upon binding of their cognate ligands, most of which are trimers with a structure similar to TNF. Sometimes the ligands are cell bound type II membrane proteins, but several are cleaved off and appear as soluble trimers. Induction of trimers or higher order complexes of the TNF family of receptors allows their cytoplasmic domains to aggregate intracytoplasmic signalling molecules.

Signalling pathways controlled by TNF receptors

The cytoplasmic domains of the TNFR family, which are more diverse than the extracellular portions, do not have any intrinsic enzymatic activity, hence they signal by inducing aggregation of intracellular adaptor molecules (Fig. 1).

Death domains

The cytoplasmic domains of TNFR1 (p55), CD95 (Fas/APO-1), NGFR (p75), death receptor (DR) 3, TRAIL-R1 and TRAIL-R2 all bear a motif termed a ‘death domain’ (DD), so-called because it is required for these receptors to transmit apoptotic signals. The DD is a protein–protein interaction motif consisting of six alpha helices that allow two proteins with DD to bind to each other. Structurally the DD is related to two other homotypic interaction domains, the death effector domain (DED), and the caspase recruitment domain (CARD).2

Death domain adaptors: TRADD, FADD, RIP and RAIDD

Binding of TNF to TNFR1 induces recruitment of the DD-containing protein TRADD to the DD of TNFR1.3 Over-expression of TRADD alone also induces the TNF-regulated responses apoptosis and activation of the transcription factors NF-κB and Jun kinase (JNK), presumably because TRADD provides docking sites for downstream signalling proteins to the receptor complex.4

Two of the proteins that TRADD recruits to the signalling complex also bear death domains. One of these, RIP, has an N-terminal DD and a C-terminal kinase domain. Knockout studies have shown that RIP is required for induction of NFκB by TNF.5 The other, Fas-associated protein with death domain (FADD), has a C-terminal DD, and an N-terminal DED. The FADD is required for cell death signalling by TNFR1 and also by CD95, to which it binds directly via its death domain.6–8 The DED of FADD allows it to bind to DED in the pro-domain of caspase 8.

Through these interactions, ligation of TNFR1 or CD95 can result in the formation of a death-inducing signalling complex, which leads to activation of caspase 8, a cell death effector protease. Once activated, caspase 8 cleaves and activates downstream caspases, such as caspase 3, ultimately leading to cell death. Because cells from mice lacking caspase 8 are resistant to death induced by TNF receptors, CD95 and DR3, apoptosis triggered by all of these receptors must converge on this caspase.9 However, FADD must have other functions because FADD knockout mice die during embryogenesis, and lymphocytes from FADD-dominant negative transgenic mice do not proliferate normally in response to T cell mitogens in vitro.10–12

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Correspondence: DL Vaux, Walter and Eliza Hall Institute of Medical Research, Post Office Royal Melbourne Hospital, Parkville, Vic. 3050, Australia.

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RIP is an adaptor protein with a C-terminal death domain that can associate with the DD in the cytoplasmic domain of CD95. Via TRADD, RIP can also associate with the TNFR1. Cells from RIP knockout mice show increased susceptibility to TNF-mediated killing and fail to activate NF-κB in response to TNF. This indicates that RIP is required for NF-κB activation by TNF. Because RIP is a serine threonine kinase, it is likely to phosphorylate, and thereby activate, kinases that phosphorylate the inhibitor of NF-κB, IκB.

Interestingly, RIP knockout mice also have abnormal development of lymph nodes, similar to those in lymphoxygenin β (LTβ) receptor-deficient mice. Therefore it is possible that RIP also takes part in signalling from these receptors. However, because the LTβR lacks a DD, if it does signal via RIP then it must do so indirectly (see following).

Another DD-bearing adaptor molecule implicated in TNF signalling of apoptosis is ‘RIP-associated ICH-1/CED-3-homologous protein with a death domain’ (RAIDD). In addition to the DD, RAIDD has a CARD which allows it to bind to the CARD of procaspase 2. Overexpression of RAIDD in vitro induces apoptosis, suggesting that this interaction is functional. However, the significance of this pathway for induction of cell death is uncertain because neither CD95 ligand (CD95L) nor TNF are able to induce apoptosis in mice lacking FADD or caspase 8. In these mice, RAIDD and caspase 2 would presumably be able to function normally. Furthermore, TNF-α was still able to induce cell death in the absence of caspase 2.

**Figure 1** Structure and signalling from some tumour necrosis factor receptor (TNFR) family members and their intracellular adaptor proteins. Homologous motifs that interact with each other are shown with the same patterns. Due to limited space, all possible interactions are not shown. Some pathways that are not established to date are indicated with question marks; see text for discussion. Death domain; death effector domain; caspase recruitment domain. JNK, Jun kinase.

The group of TNF receptor-associated factors (TRAF) interact with members of the TNFR family. There are to date six TRAF proteins identified: TRAF1, TRAF2, TRAF3 (CRAF, LAP-1, CD40-bp), TRAF4 (CART1), TRAF5 and TRAF6 (review\(^\text{18}\)). With the exception of TRAF4, TRAF proteins interact with receptor molecules either directly, or indirectly through binding to other TRAF, or through binding to TRADD. The TNFR2 (p75), CD40, CD30 and lymphoxygenin-β receptor (LTβR) contain conserved, cytoplasmic TRAF binding motifs and are able to bind directly to TRAF proteins. Because TRAF2 can bind to TRADD, which in turn can associate with TNFR1, TRAF2 can indirectly participate in signalling from this receptor as well.

The TRAF molecules share similar C-terminal domains, designated the TRAF domain, which is involved in protein–protein interactions. TRAF2, TRAF3, TRAF5 and TRAF6 also bear an N-terminal RING finger, a zinc binding motif found in several types of intracellular proteins. TRAF proteins may signal from other receptors in addition to TNFR family molecules. TRAF6, which binds to CD40, is also involved in IL-1 receptor signalling through interaction with IRAK, a serine/threonine kinase that also has a DD. Studies of TRAF2 and TRAF3 knockout mice have shown that TRAF proteins are required for activation of Jun/AP-1 signalling by TNF receptors, and have important roles for normal development, since these mice die during early life.

**Inhibitor-of-apoptosis proteins**

The inhibitor-of-apoptosis (IAP) proteins were first discovered as baculovirus proteins, which were able to inhibit viral induced apoptosis in insect cells. Several homologues have been identified in different organisms including mammals, Drosophila, C. elegans and even yeasts. All the IAP contain one-to-three motifs termed ‘baculovirus IAP repeats’ (BIR). In addition, most contain a C-terminal RING finger and two (MIHB (cIAP1/hIAP2) and MIHC (cIAP2/hIAP1)) also contain a CARD. These two IAP proteins can also bind to TRAF1 and 2 and thereby become recruited into the TNFR complexes.

Overexpression of IAP protects cells from apoptosis induced by a variety of different pro-apoptotic stimuli. While the mechanism of action of IAP has not been determined with certainty, genetic and biochemical evidence from Drosophila suggest that they act upstream to prevent caspase activation, whereas biochemistry of mammalian IAP in vitro suggests that IAP proteins bind to activated caspases and thereby inhibit their action directly.

**Daxx**

In some cell types in vitro, ligation of CD95 is able to activate the JNK/SAPK pathway. A candidate for mediating this
activity is the CD95 ‘death domain-associated protein’ Daxx, which was identified in yeast two-hybrid experiments using the cytoplasmic tail of CD95, containing its DD, as bait. Daxx was also reported to bind to the death domain of TNFR1, although Daxx itself lacks a DD. When over-expressed in cell lines, Daxx activates the JNK pathway, possibly through activation of the ASK-1. The precise role of Daxx remains uncertain, because human Daxx is confined to the nucleus, where it would not be available for CD95 signalling. Furthermore, Daxx-stimulated apoptosis is completely inhibitable by Bcl-2, whereas CD95 and TNFR-stimulated death is not.

**FLIP**

Another factor involved in regulating signals from CD95 (and possibly other DD containing receptors) is FLICE-inhibitory protein (FLIP). FLIP resembles caspase 8 because it has two DED and a caspase-like domain, but it does not have any protease activity. FLIP interacts with FADD and thereby prevents FADD from activating caspase 8.

**NF-κB activation**

Activation of NF-κB is a frequent outcome of signalling by TNFR family members. NF-κB directs transcription for a large number of genes involved in regulation of cell growth, transcription factors, cytokines and cell surface receptors (review). The IAP proteins, which inhibit apoptosis, have been suggested as targets of NF-κB activation. Transcription of IAP proteins TRAF1 and 2 was found to be dependent on NF-κB. Knockout studies have shown that these pathways could influence susceptibility to cell death. The Jun-N-terminal kinase (JNK)/stress-activated kinase (MAPK) pathway, which leads to activation of the transcription factor AP-1. Knockout studies have shown that TRAF2 is required for JNK activation in response to TNF, but is not required for NF-κB activation. It is not known how TRAF proteins connect with JNK, but one candidate molecule that may link TRAF to JNK is the kinase apoptosis stimulating kinase-1 (ASK-1). However, because thymocytes from TRAF2 knockout animals showed increased susceptibility to TNF-induced apoptosis, TRAF are not likely to be involved in passing signals that induce cell death. In other studies, TNF-induced JNK activation was also not found to be involved in signalling to cell death, which suggests that JNK activation may preferentially promote cell survival.

**Roles of TNFR family members in vivo**

**TNF receptors**

TNFR1 is expressed on most cell types. It has been regarded as the primary signalling receptor for TNF-α inflammatory responses, because TNFR1 knockout mice are resistant to endotoxic shock. In addition, it has also been suggested that TNFR1 mediates signals required for proper formation of lymphoid structures such as primary B cell follicles and germinal centres, possibly through a defect in the development of follicular dendritic cells (FDC). TNFFR2, like TNFR1, is widely expressed. TNFFR2 binds soluble TNF poorly, and may only be activated by membrane bound TNFα. Unlike TNFR1 it has no cytoplasmic death domain, but it can directly bind TRAF1 and TRAF2, and in this way give activation signals and/or modulator activities of TNFR1. However, TNFR2 knockout mice have increased resistance to TNF-α-induced cell death and tissue necrosis, raising the possibility that some death signals are mediated also by this receptor.

**Lymphotoxin-β receptor**

The lymphotoxins are homo- or heterotrimeric molecules that bind to both TNFR (LTα1), TNFRI (LTα2β1) or LTβR (LTα1β2) (review). The cytoplasmic part of LTβR binds to TRAF3 and TRAF5 and these interactions have been reported to activate NF-κB. Lymphotoxin-β receptor knockout mice have substantial defects in peripheral lymphoid organs, because they lack lymph nodes and Peyer’s patches and fail to develop B cell follicles and germinal centres. Defective development of peripheral lymphoid organs and/or micro-anatomic compartments within organs have also been reported for LTα66-68 and LTβ69 knockout mice. It is possible that chemokines and cell adhesion molecules, which both may function in directing and/or maintaining cells to a specific site, are important factors in lymphoid organogenesis. Tumour necrosis factor-α and lymphotoxins are potent inducers of adhesion molecules and it has been reported that ligation of the LTβR induces production of chemokines. Moreover, transgenic expression of LTα in the pancreas induces inflammation and structures resembling lymph nodes. Considering the similar phenotypes found in the LTβR knockout and the RIP knockout mice, one may speculate that both these proteins participate in signalling pathways leading to expression of chemokines and/or adhesion molecules, which are involved in lymphoid organogenesis.
CD95 (Fas/APO-1)

CD95 triggers physiological cell death in many cell types and is involved in activation-induced cell death in mature lymphocytes. CD95-induced apoptosis requires the binding of the DD containing adaptor molecule FADD to the death domain of CD95. The importance of the CD95 or the CD95L molecules in regulating lymphocyte homeostasis is illustrated by lpr and gld mice, respectively (review72). Although lymphocyte development in bone marrow and thymus is fairly normal, these mice show massive peripheral lymphadenopathy, splenomegaly and autoimmune disorders when aged. Cells lacking genes for FADD and caspase 8 are just as resistant to induction of apoptosis as cells from lpr mice, indicating that all apoptosis signals from CD95 require FADD and caspase 8. Because lpr and gld mice develop lymphoid accumulations and autoimmune disease, but FADD knockout, caspase 8 knockout and crmA transgenic mice do not, these effects cannot be due to loss of cell death triggered by CD95, but must be due to some other activity signalled by it.10,73,74

The first suggestion that CD95 may also mediate activation or mitogenic signals came from experiments using monoclonal antibodies against CD95 on human T lymphocytes or thymocytes in vitro.75,76 That FADD does not only function in death-stimulating pathways was shown by studies using chimeric Rag-1 knockout/FADD knockout mice and mice carrying a dominant negative interfering FADD trans-gene, which lacks the DED and therefore is unable to transduce a death signal.11,12 In these animals, thymocytes and mature T lymphocytes were resistant to CD95-mediated killing. However, thymocyte numbers were reduced and negative selection appeared to be unaffected by disruption of FADD-mediated cell death. In addition, mature T cells were unable to proliferate normally in response to mitogens in vitro. While these results show that FADD must participate in some mitogenic pathways, they did not indicate whether the signals stemmed from CD95, TNFR1 or some other receptors. Interestingly, the effect of FADD deficiency on B lymphocyte development appears to be even more severe than for T cells, because an almost complete absence of peripheral B cells was seen in chimeric Rag-1 knockout/FADD knockout mice.

It is possible that in quiescent T cells, signals transmitted by FADD are directed towards an activation/proliferation pathway. Consistent with this, resting lymphocytes are relatively resistant to CD95-induced apoptosis, while IL-2-activated T lymphocytes become susceptible.77 It is possible that activation (or transformation) of lymphocytes shifts the CD95 signalling pathways towards induction of cell death, for example if levels of FLIP decline. Indeed, it was recently shown that IL-2 treatment suppresses transcription of FLIP.78

Conclusion

Tumour necrosis factor was first identified as a cytokine that induced cell death; namely necrosis of tumour cells. CD95 was first found as the target of monoclonal antibodies, chosen because they could induce apoptosis in lymphoid tumour lines. Death domains received their name because they were found on the cytoplasmic tails of both CD95 and TNFR1, and were required for them to transmit death signals. However, it is now clear that CD95 and TNFR1 and other members of the TNFR family can also stimulate other cellular responses, such as proliferation, activation or even lymphoid organogenesis. Similarly FADD, which contains only DD and DED, has important roles in addition to the activation of apoptosis. Other molecules, such as IRAK, myd88 and NF-kB p100 bear bonafide death domains79 and yet apparently do not play a role in induction of cell death. There is a natural tendency towards simplification, and in the absence of contradictory data, the simplest explanation is the one to choose. Despite our wishes, proteins resist simple behaviours and simple classifications, and the recent knowledge about the TNFR family of molecules reminds us not to stick to over-simplistic explanations.

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