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Minireview

Life And Death Decisions: Secondary Complexes and Lipid Rafts in TNF Receptor Family Signal Transduction

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Signaling by receptors in the TNF receptor (TNFR) superfamily mediate biological outcomes ranging from inflammation to apoptosis and other forms of programmed cell death. How receptor signaling mediates these divergent responses is just beginning to be understood. Here, we discuss how receptor submembrane localization and the formation of alternate signaling complexes can alter the fate of cells stimulated through TNFRs with a death domain, also known as "death receptors."

The Tumor Necrosis Factor receptor (TNFR) superfamily has grown to 32 members and 19 ligands (a downloadable table can be accessed at http://www.irp.niams.nih.gov/ImageStore/Test/WORD/AB/IRG/tnfchart.doc). TNF family receptors orchestrate many aspects of immune cell function, including lymphoid organ development, acute inflammation, and lymphocyte costimulation (Bodmer et al., 2002). Blocking TNF-TNFR1 interactions has emerged as a powerful and clinically effective disease-modifying therapy for autoimmune conditions such as rheumatoid arthritis and inflammatory bowel disease, and therapies based on blocking the actions of other members of the TNF family are under development.

Divergent Signaling by TNFR Family Members

One of the most fascinating aspects of TNFR biology is the diversity of responses triggered through each receptor. The presence of a particular TNFR family member on the cell surface does not generally predict efficacy or biological outcome of signaling by that receptor. Instead, signaling by these receptors is dependent on cell-type and environmental factors. For example, TNFR1 can trigger cellular activation via NF-κB or apoptosis via activation of apical caspases. In most instances, TNFR1 signaling results in NF-κB activation. However, cell death can be triggered by TNFR1 under conditions where new protein synthesis is blocked or where NF-κB activation is specifically inhibited prior to TNF stimulation (Varfolomeev and Ashkenazi, 2004). In vivo, when NF-κB activation is blocked, endogenous TNF signaling through TNFR1 can lead to embryonic lethality due to massive liver cell death during development (Beg and Baltimore, 1996).

Unlike TNFR1, Fas is relatively weak at inducing NF-κB and primarily induces programmed cell death. However, susceptibility to Fas-mediated apoptosis can be regulated independently of receptor expression. Mixing experiments with Fas-expressing activated $CD4^+$ T cells have shown that only T cells receiving TCR restimulation become susceptible to FasL-dependent apoptosis while bystander activated T cells of different antigenic specificity in the same cultures do not undergo apoptosis (Combadiere et al., 1998). This "competency to die" signal can be triggered by weak TCR agonists, is independent of CD28 costimulation, and does not appear to require new RNA or protein synthesis.

Recent data has shed new light on how membrane proximal events control fate decisions in signaling by two members of the TNFR family, TNFR1 and Fas. Here, we review how distinct signaling complexes can trigger alternative fates of cells after TNF receptor triggering. In addition, we discuss how receptor localization in lipid raft microdomains can alter proximal signaling events by TNF family receptors and ultimately affect cell fate. These new findings have significant implications for our understanding of the function of TNF family members in the immune system.

Secondary Complexes in TNFR Signaling

After TNF binding to TNFR1, the adaptor protein TRADD is recruited through interactions between the death domains in the receptor cytoplasmic tail and the C-terminal portion of TRADD. It has been shown that FADD and caspase-8 are required for TNFR1-mediated cell death. However, direct association of these molecules with endogenous TNFR1 has not been seen. In addition, although many studies have shown that inhibition of NF-κB activity can sensitize cells to die through TNFR1, it was not clear how NF-κB activation by TNFR1 could act quickly enough to inhibit apical caspase activation through the same receptor.

A solution to this paradox has been proposed in the recent finding that TNFR1 signaling involves assembly of two molecularly and spatially distinct signaling complexes that sequentially activate NF-κB and caspases (Figure 1) (Micheau and Tschopp, 2003). Within a few minutes of TNF binding to TNFR1, a signaling complex termed "complex I" forms. This complex contains the receptor itself, RIP1, TRAF2, and TRADD. Complex I transduces signals that lead to NF-κB activation through recruitment of the I-κB kinase "signalsome" high molecular weight complex (Poyet et al., 2000; Zhang et al., 2000).

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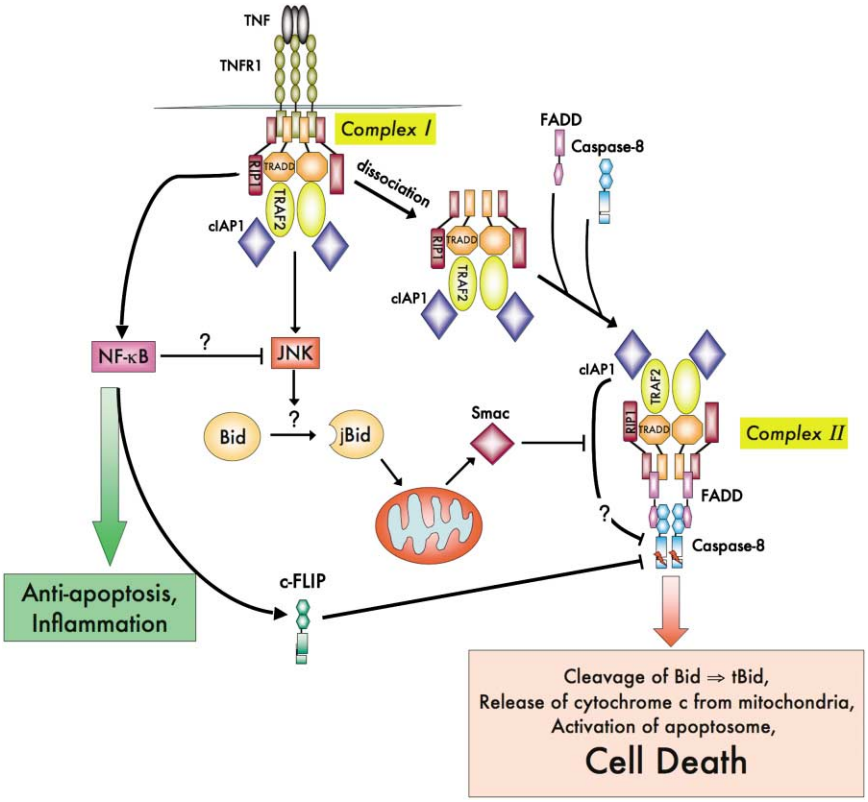

Figure 1. Alternate Complexes and Role of JNK in TNFR1 Signaling

Early after TNF binding, RIP1, TRADD, TRAF2, and cIAP1 are recruited to TNFR1 to form complex I. Complex I transduces signals leading to NF-κB translocation. At later time points, RIP1, TRADD, and TRAF2 dissociate from TNFR1 and recruit FADD and caspase-8 to form complex II. In the absence of NF-κB activity from complex I, complex II can initiate caspase-8 activation and cell death. NF-κB inhibits cell death through upregulation of antiapoptotic genes such as c-FLIP, which directly inhibits caspase activation in complex II or through suppression of JNK activity, possibly through upregulation of A20. Sustained TNFR1-mediated JNK activation results in the cleavage of Bid to jBid, which then induces the preferential release of SMAC from the mitochondria. Released SMAC interferes with cIAP1-mediated inhibition of caspase-8.

At late time points (>2 hr) after TNFR1 triggering, possibly after receptor internalization, RIP1, TRAF2, and TRADD dissociate from the receptor and recruit FADD and caspase-8 into a secondary complex (complex II). In situations where complex I or other exogenous factors trigger sufficient NF-κB signaling, gene expression of antiapoptotic proteins is induced and the activation of apical caspases in complex II is inhibited. However, when NF-κB activity is deficient, these gene products are not made, and complex II can signal for apoptosis. Because of the long delay in complex II assembly, there is time for antiapoptotic proteins to be synthesized and complete the negative feedback loop. In this way, the ability of complex I to activate NF-κB early after TNF binding acts as a checkpoint to control whether complex II can induce apoptosis a few hours later.

Several NF-κB target genes have been proposed to inhibit TNFR1-induced apoptosis. The gene for the cellular FLICE-like inhibitory protein (c-FLIP) is upregulated by NF-κB and can inhibit caspase-8 activation in the Fas signaling complex (Irmler et al., 1997; Micheau et al., 2001). In TNF-stimulated cells, c-FLIP is recruited to complex II, where it inhibits caspase activation (Micheau and Tschopp, 2003). Indeed, MEFs that are deficient for c-FLIP undergo cell death in response to TNF whereas wt MEFs do not (Yeh et al., 2000). Other candidates for negative regulators of TNFR1 apoptosis signaling are the BIR-repeat containing proteins cIAP-1 and cIAP-2 and the TRAF and RING finger-containing proteins TRAF1 and TRAF2 (Wang et al., 1998).

In addition to activating antiapoptotic genes, NF-κB can suppress apoptosis by inhibiting sustained activation of the MAP-kinase family member Jun-Kinase (JNK) (Tang et al., 2001). Although interfering with JNK activation can suppress apoptosis, sustained JNK activation itself is dependent on the mitochondrial permeability transition, a central feature of apoptosis. Thus it was not clear whether JNK activation precedes or depends on mitochondrial changes in apoptosis. A recent study proposed a novel mechanism to connect JNK activation with mitochondrial events in apoptosis (Deng et al., 2003). When NF-κB was inhibited, TNFR1 signaling induced sustained JNK activation that results in the caspase-8-independent cleavage of Bid to a novel cleavage product termed jBid. jBid induced the preferential release of the proapoptotic factor SMAC/Diablo from the mitochondria. SMAC/Diablo may then relieve the inhibition of cIAP1 on caspase-8 and positively feed back on the apoptotic protease cascade (see Figure 1) (Deng et al., 2003). Although a direct association between cIAP1 and caspase-8 has not been demonstrated, it may be possible that this inhibition is indirect.

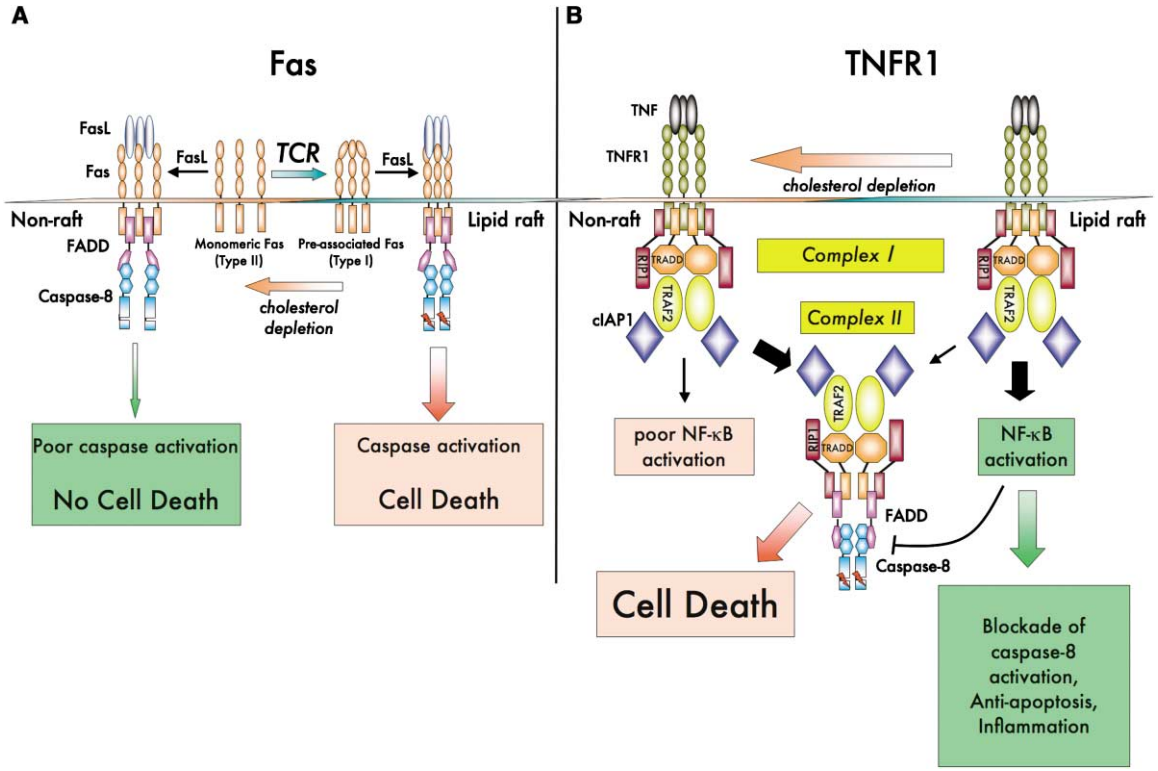

Figure 2. Examples of How Lipid Rafts Can Modify Early Events in TNFR Family Signaling

(A) In activated $CD4^+$ T cells, Fas is excluded from lipid rafts. Outside of lipid rafts, Fas is likely to be monomeric. After engagement by FasL, although FADD and caspase-8 are recruited to Fas, they do not signal efficiently for death. Upon TCR restimulation, Fas translocates into lipid rafts where it tends to preassociate. Within lipid rafts, upon FasL binding, FADD and caspase-8 are recruited to Fas where caspase-8 can autoactivate and trigger cell death.

(B) Upon TNF binding, TNFR1 translocates into lipid rafts wherein complex I formation takes place and results in the activation of NF-κB. Through mechanisms described in Figure 1, NF-κB can inhibit caspase activation within complex II. When cholesterol is depleted, lipid raft structure is disrupted and complex I forms outside of lipid rafts and cannot signal efficiently for NF-κB and therefore cannot inhibit death induced by complex II. The thickness of the arrows indicates relative efficiency of each signaling pathway.

A number of NF-κB target genes have been proposed to suppress the sustained JNK activity that is associated with apoptosis. GADD45β and XIAP were isolated in genetic screens for NF-κB target genes that can protect from cell death and do perform this function when overexpressed in cell lines (De Smaele et al., 2001; Tang et al., 2001). However, primary cells from GADD45β- and XIAP-deficient mice are not hypersensitive to TNF-induced cytotoxicity, suggesting that there may be functional redundancy in these pathways (Amanullah et al., 2003; Harlin et al., 2001). Targeted disruption of XIAP did sensitize a human colon cancer cell line to cell death induced by the TNF-related apoptosis-inducing ligand (TRAIL), indicating that the role of XIAP may be different in transformed cells (Cummins et al., 2004). Another NF-κB target gene, the cytoplasmic zinc finger protein A20 (TNFAIP3), dramatically affects cell fate in response to TNF. A20-deficient thymocytes and MEFs undergo increased apoptosis in response to TNF despite sustained NF-κB and JNK activation. A20-deficient mice exhibit lymphocyte-independent systemic inflammation and have increased liver cell death (Lee et al., 2000). Thus A20 is a good candidate for a TNF-induced negative regulator of JNK.

Many of these observations were initially made in non-immune cells, and understanding how alternative TNFR1 signaling complexes are regulated in the immune system is an important goal. TNF can act as a costimulator of T cell activation through TNFR2, a non-death domain containing TNFR, but in activated T cells TNF can contribute to TCR-induced cell death in a TNFR2-dependent manner (Zheng et al., 1995). TNFR2 has been shown to sensitize T cells to die via induced ubiquitin-dependent degradation of TRAF2 (Chan and Lenardo, 2000; Li et al., 2002). In light of the findings discussed here, TRAF2 downregulation may promote cell death by enhancing the activity of complex II. Interestingly, a number of bacterial effector proteins, such as *Yersinia* YopJ, appear to target NF-κB signaling and sensitize cells to apoptosis, whereas activation of NF-κB by intracellular pathogens can protect infected cells from death receptor-induced apoptosis. Anti-inflammatory therapeutic agents that can inhibit NF-κB, such as glucocorticoids, sulfasalazine, salicylates, or thalidomide derivatives, may also sensitize cells to TNF-induced apoptosis.

Lipid Rafts as Dynamic Modulators of TNFR Family Signaling

Liquid-ordered microdomains enriched in sphingolipids and cholesterol constitute a distinct biophysical plasma

membrane compartment. They have been termed lipid rafts based on their ability to float in a discontinuous density gradient after lysis in nonionic detergents (Munro, 2003; Simons and Toomre, 2000). It has been proposed that lipid rafts serve as signaling platforms for BCR, TCR, and Fcε antigen receptors, bringing the receptors into proximity with activating kinases that are constitutive residents of lipid rafts (Dykstra et al., 2003). Although membrane-anchored kinases do not appear to participate in TNFR family signaling, the local environment of the membrane may influence the efficiency of signaling and composition of receptor signaling complexes.

Recent findings have delineated distinct roles for lipid rafts in Fas and TNFR1 signaling (Figure 2). After TNF binding in the human fibrosarcoma cell line HT1080, TNFR1 translocates to lipid rafts within 2 min, where RIP1, TRADD, and TRAF2 are recruited (Legler et al., 2003). TRAF2, the adaptor molecule that links TNFR1 to NF-κB activation, has been reported to be constitutively present in lipid rafts. When lipid rafts are disrupted by cholesterol depletion, phosphorylation of IκBα in response to TNF is inhibited and apoptosis is induced (Legler et al., 2003). These findings may be cell-type dependent. In the myeloid cell line U937, TNFR1-induced apoptosis has been reported to depend on lipid raft integrity, while in primary mouse macrophages, lipid rafts appear to allow TNFR1 to signal for ERK activation, but not NF-κB (Doan et al., 2004; Ko et al., 1999).

For Fas, where a death signaling complex is recruited directly to the cytoplasmic tail of the receptor, the reported localization of Fas in lipid rafts and a requirement for lipid raft integrity in Fas-mediated apoptosis has varied widely (Algeciras-Schimnich et al., 2002; Eramo et al., 2004; Hueber et al., 2002; Muppidi and Siegel, 2004). Some of these differences may be explained by variation in the potency of Fas ligands and anti-Fas antibodies used in these studies. More importantly, the mechanism of Fas signaling itself appears to vary between cell types. In cell lines termed "type I," the death-inducing signal complex (DISC), consisting of Fas, FADD, and caspase-8, is easily detected, and Fas-induced cell death cannot be blocked by overexpression of anti-apoptotic Bcl-2 family members. In type II cell lines the DISC is less easily detected, and overexpression of antiapoptotic Bcl-2 family members blocks Fas-induced cell death (Scaffidi et al., 1998).

We have recently correlated the mode and efficiency of Fas signaling with the submembrane localization of the receptor. In type I cells, a portion of Fas resides constitutively in lipid rafts, while in type II cells, the receptor is excluded from lipid rafts during early signaling (Muppidi and Siegel, 2004). The presence of Fas in lipid rafts allows type I cells to undergo apoptosis in response to weak bivalent anti-Fas stimulation, which cannot induce apoptosis in type II cell lines. Disruption of lipid raft structure by cholesterol depletion reduces signaling efficiency in type I cells, but not in type II cells. Lipid rafts may modulate signaling efficiency of Fas by modulating receptor preassociation, which has been previously been shown to be important in Fas-mediated apoptosis (Siegel et al., 2000). Crosslinking studies showed that Fas is preassociated in type I, but not type II, cells. In type I cells, preassociated Fas is preferentially distributed within lipid rafts (Muppidi and Siegel, 2004). Other groups have also recently found similar alterations in the lipid raft localization of Fas between type I and type II cell lines (Eramo et al., 2004).

Modulation of lipid raft localization of Fas may be relevant to the "competency signal" for apoptosis delivered by the TCR in activated $CD4^+$ T cells. Like type II cell lines, human $CD4^+$ T cell blasts are relatively insensitive to bivalent anti-Fas antibodies or endogenously produced FasL. Fas in these cells does not partition into lipid rafts. However, TCR restimulation induces a ligand-independent redistribution of Fas into lipid rafts that renders $CD4^+$ T cells sensitive to these stimuli. Cholesterol depletion inhibits this TCR-mediated sensitization to Fas-induced apoptosis (Muppidi and Siegel, 2004).

The concept that Fas association with lipid rafts varies between cell types may resolve some of the apparent discrepancies in the literature regarding the role lipid rafts in Fas signaling. For example, in thymocytes, which are type I cells, Fas localizes to lipid rafts and cholesterol depletion, which disrupts lipid raft structure and can inhibit Fas-induced cell death (Hueber et al., 2002). However, in the human fibrosarcoma cell line HT1080, which is likely type II, Fas is excluded from lipid rafts, and cholesterol depletion does not inhibit cell death induced by crosslinked anti-Fas or FasL stimuli (Legler et al., 2003).

It may seem paradoxical that disruption of lipid rafts can desensitize cells to Fas-induced cell death and yet sensitize cells to TNFR1-induced cell death unless one considers how signals are transduced through these receptors. TNFR1 signals for NF-κB via the receptor-associated complex I and cell death via complex II, which is not receptor associated (Figure 1). However, Fas can trigger cell death via the receptor-associated DISC. Thus the divergent outcomes of lipid raft disruption may reflect the function of lipid rafts to enhance the efficiency of signaling in the receptor-associated primary signaling complex (Figure 2). Indeed, lipid raft disruption blocks the ubiquitination of the adaptor proteins RIP1 and TRADD in the TNFR-1 signaling complex and prevents NF-κB induction by complex I (Legler et al., 2003).

Future Challenges

The studies discussed here show that far from being "hard-wired" to elicit a certain response, TNFR signaling should be seen as a dynamic process that is subject to the influence of other signaling pathways, environmental, and genetic factors. Whether findings from Fas and TNFR1 are applicable to the receptors in the TNFR superfamily that do not have a cytoplasmic death domain and bind directly to TRAFs is not yet clear. Yet it is likely that further studies of the TNFR superfamily will yield more interesting receptor biology and targets for immunotherapy.

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Note Added in Proof

Schneider-Brachert et al. (Immunity *21*, 415–428) have recently reported that in U937 cells, TNFR1 internalization precedes formation of a signaling complex containing TRADD, caspase-8, and FADD. This differs from the situation with Fas, where the DISC assembles rapidly and receptor internalization can be prevented by caspase inhibitors (Algeciras-Schimnich et al., 2002 and Spiegel et al., J. Cell Biol., in press).