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Tumor necrosis factor (TNF) biology and cell death

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1. ABSTRACT

Tumor necrosis factor (TNF) was the first cytokine to be used in humans for cancer therapy. However, its role in the treatment of cancer patients is debated. Most uncertainties in this field stem from the knowledge that the pathways directly activated or indirectly affected upon TNF engagement with its receptors can ultimately lead to very different outcomes in terms of cell survival. In this article, we summarize the fundamental molecular biology aspects of this cytokine. Such a basis is a prerequisite to critically approach the sometimes conflicting preclinical and clinical findings regarding the relationship between TNF, tumor biology and anticancer therapy. Although the last decade has witnessed remarkable advances in this field, we still do not know in detail how cells choose between life and death after TNF stimulation. Understanding this mechanism will not only shed new light on the physiological significance of TNF-driven programmed cell death but also help investigators maximize the anticancer potential of this cytokine.

2. INTRODUCTION

Tumor necrosis factor (TNF, formerly referred to as TNF-alpha), which was the first cytokine to be employed for cancer biotherapy, is a pleiotropic protein that was first isolated from the serum of mice treated with bacterial endotoxin and was shown to replicate the ability of endotoxin to induce hemorrhagic necrosis of methylcholanthrene-induced sarcomas (1, 2). This discovery ended a long search for the active component of "Coley's toxin", which consisted of a crude bacterial filtrate developed by Willam Coley, a New York surgeon and pioneer of cancer biotherapy at the turn of the 20th century (3).

Coley's toxin-induced high fever and tumor necrosis in some patients affected with sarcoma, carcinoma and lymphoma. This phenomenon is now attributed, at least in part, to the effect of macrophage-derived TNF released upon activation of these leukocytes by the endotoxin contained within Coley's mixture.

Cloning of TNF gene in 1984 (4) led to the era of clinical experimentation for cancer therapy. As the cytokine minimum effective dose was greater than the maximum tolerated dose, systemic TNF administration was associated with substantial lack of objective tumor response and serious side-effects (i.e. shock-like syndrome) (5). In order to overcome this limitation, researchers investigated the delivery of TNF through the loco-regional route (i.e. isolated limb perfusion for the treatment of patients with locally advanced melanoma and soft tissue sarcomas), which culminated in a license from the European Medicine Evaluation Agency (EMEA) for the TNF-based treatment of limb-threatening soft tissue sarcomas (6-8).

Despite its approval for human use as anticancer agent in Europe, TNF is not approved in the USA and its role in cancer therapy is still debated (9-12), as its activity in advanced melanoma has been recently questioned (13) and no survival advantage has ever been demonstrated with its loco-regional administration (6-8).

In the present review, we summarize the fundamental molecular biology aspects of this cytokine, which is a prerequisite to critically approach the sometime conflicting preclinical and clinical findings regarding the relationship between TNF, tumor biology and anticancer therapy, which are described in detail in the other articles of this Frontiers in Bioscience issue dedicated to TNF and cancer.

3. THE TNF SUPERFAMILY

The TNF gene is located on chromosome 6p21.3 and is mainly expressed by activated macrophages, natural killer cells and T-lymphocytes, even though other cell types (e.g. fibroblasts, astrocytes, Kupffer cells, smooth-muscle cells, keratinocytes, and malignant cells) can express this cytokine as well, at least in some circumstances (14, 15).

In its soluble form, TNF acts as a homotrimer with a subunit molecular mass of 17 kDa (157 amino acids). TNF is first synthesized as a 26 kDa (233 amino acids) membrane-bound pro-peptide (pro-TNF) and is released after cleavage by the TNF-converting enzyme (TACE). This protease, which is a member of the a-disintegrin-and-metalloproteinase (ADAM) family (16), can also release TNF receptors from the cell surface: these circulating cytokine-binding proteins represent an important mechanism of negative regulation for the biological activity of soluble TNF (17).

Since its initial discovery, researchers have shown the existence of a superfamily of TNF protein homologues (with 15–20% identity to each other) consisting of 20 members that signal through 29 different receptors (Figure 1) (18).

The TNF superfamily ligands (except for lymphotoxin-alpha [LT-alpha, formerly known as TNF-beta] and vascular endothelial cell growth inhibitor [VEGI] that are secreted) are type-II transmembrane proteins with a carboxy-terminal extracellular domain (responsible for binding to the receptor), an amino-terminal intracellular domain and a single transmembrane domain. Receptor activator of nuclear factor kappa-B ligand (RANKL) and TNF (as above mentioned) are released from the cell surface by ADAM metalloproteases; CD95 ligand (CD95L, known also as FasL) by matrilysin; BAFF, EDA, TWEAK and APRIL by members of the furin family.

Most receptors are type-I transmembrane proteins (i.e., they feature an extracellular N-terminus and an intracellular C-terminus). BCMA, TACI, BAFF receptor (BAFFR) and XEDAR lack a signal peptide sequence, and thus are considered type-III transmembrane proteins. OPG and DcR3 lack a transmembrane domain and are therefore secreted.

From the functional viewpoint, TNF family receptors can be classified into three major groups. The first group contains a death domain (DD) in the cytoplasmic tail. Activation of these DD-containing receptors by their corresponding ligands leads to the recruitment of intracellular DD-containing adaptors such as Fas-associated DD (FADD, also known as MORT1) and TNF receptor associated DD (TRADD), which together form the so-called death inducing signaling complex (DISC). These molecules cause activation of the caspase cascade and induction of apoptosis, but can also recruit TNF receptor associated factor (TRAF) family members, which can have quite different effects (see below).

The second group of receptors contains one or more TRAF-interacting motifs (TIM) in the cytoplasmic tail. Activation of TIM-containing TNF receptors leads to the direct recruitment of TRAF family members, which ultimately activate multiple signal transduction key mediators, such as mitogen-activated protein kinases (MAPK) (e.g. c-Jun N-terminal Kinase [JNK], p38 [p38MAPK], extracellular signal-related kinase [ERK]), inhibitor of nuclear factor kappa-B (NFkB) kinase (IkB kinase, IKK) and phosphatidyl-inositol 3-kinase (PI3K).

The third group of TNF receptor family members does not contain functional intracellular signaling domains or motifs. Although these "decoy" receptors cannot provide intracellular signaling, they can effectively compete with the other two receptor groups for their corresponding ligands, which adds a further level of regulation to the TNF family ligands.

Interestingly, some receptors (CD27, CD30, CD40, 4-1BB, CD95, TNF receptor 1 [TNFR1], TNFR2) can be found in soluble form and act as circulating decoy receptors, which adds a level of regulation to the activity of the respective ligands.

4. THE TNF PATHWAY

TNF transduces its signal through two distinct receptors referred to as TNFR1 (also known as TNFRp55/p60, 60 kDa, expressed on all virtually cell types) and TNFR2 (also known as TNFRp75/p80, 80 kDa,

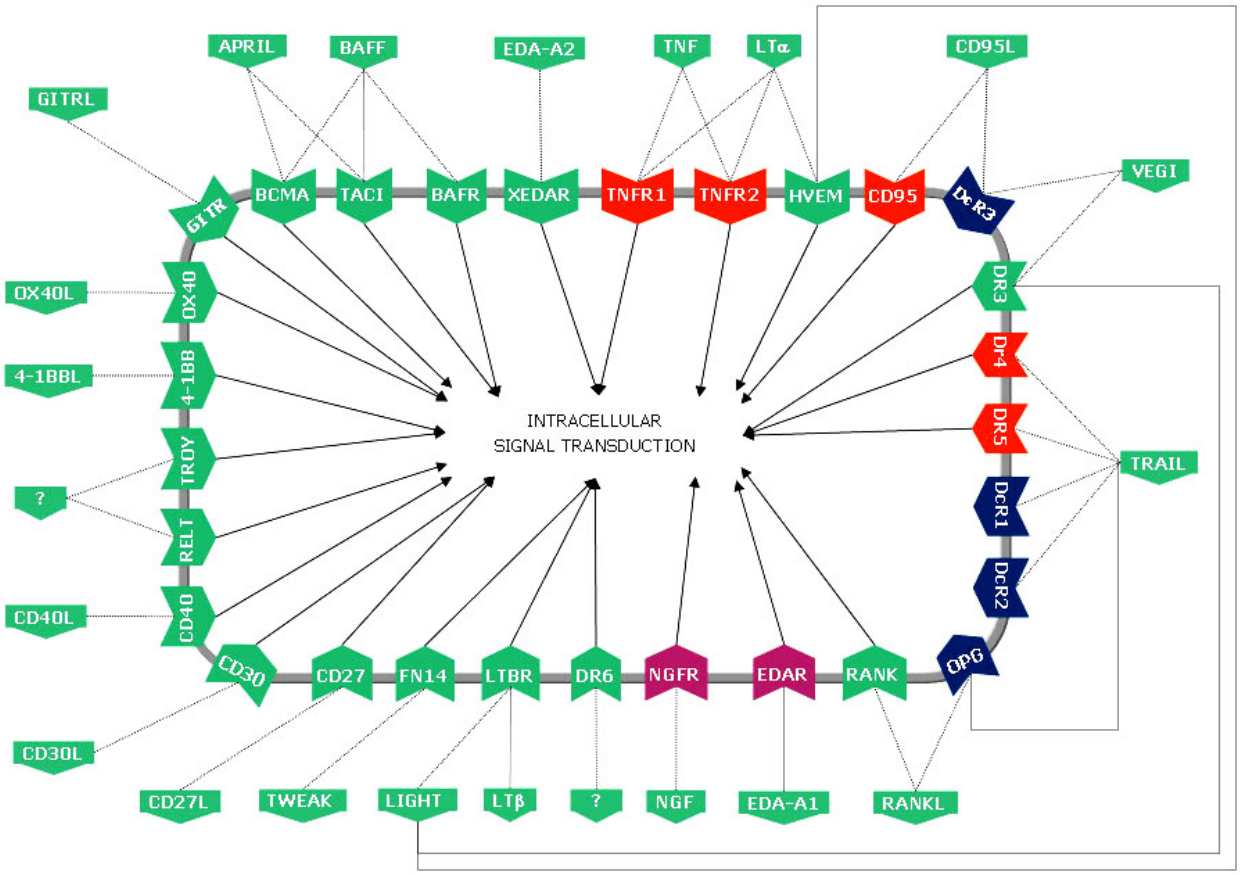

Figure 1. The TNF superfamily. Ligands, except LTa and VEGI (which are secreted), are type-II transmembrane proteins. TNF and RANKL are released from the cell surface by ADAM metalloproteases; CD95L by matrilysin; BAFF, EDA, TWEAK and APRIL by members of the furin family. Most receptors are type-I transmembrane proteins. BCMA, TACI, BAFFR and XEDAR lack a signal peptide sequence (type-III transmembrane proteins). OPG and DcR3 lack a transmembrane domain and thus are secreted. TNFR1, TNFR2, CD27, CD30, CD40 and 4-1BB can be found in soluble form (circulating decoy receptors). Death domain (DD) containing receptors in red; TRAF interacting motif (TIM) containing receptors in green; DD and TIM-containing receptors in violet; decoy receptors in blue. TNF: tumor necrosis factor (formerly TNFalpha), LTa/LTb: lymphotoxin alpha / beta (LTa: formerly TNFbeta); CD95L (FasL): CD95 ligand; TRAIL: TNF related apoptosis inducing ligand; VEGI: vascular endothelial cell growth inhibitor; CD27L, CD30L, CD40L, CD95L, OX40L, 4-1BBL, RANKL: ligands; BAFF: B-cell activating factor; APRIL: a proliferation inducing ligand; LIGHT: also called HVEML: EDA: ectodermal dysplasin; TWEAK: TNF-like weak inducer of apoptosis; GITRL: GITR ligand; NGF: nerve growth factor. TNFR1/TNFR2: TNF receptor 1/2; CD95: also called Fas; DR1-DR6: death receptors 1-6 (DR1: also called TNFR1; DR2: also called CD95; DR4/DR5: also called TRAILR1/TRAILR2); LTbR: LTbeta receptor; BAFFR: BAFF receptor; BCMA: B-cell maturation antigen; TACI: transmembrane activator and cyclophilin ligand interactor; RANK: receptor activator of NFkB; HVEM: herpes virus entry mediator; XEDAR: X-linked EDA receptor; RELT: receptor expressed in lymphoid tissues; GITR: glucocorticoid-induced TNF family receptor; EDAR: EDA receptor; NGFR: NGF receptor; DcRl/DcR2/DcR3: decoy receptors 1/2/3 (DcRl/DcR2: also called TRAILR3/TRAILR4); OPG: osteoprotegerin.

expressed only on some cell types, such as immune and endothelial cells) (2, 18, 19).

These two receptors possess no enzymatic activity: in fact, the signals they transduce are transmitted through the recruitment of more than a dozen of different signaling proteins, which together initiate signaling cascades leading to the activation of effector proteins (e.g. caspases) and protein kinases (e.g. MAPK, IKK), the latter being mainly involved in the activation of transcription factors (e.g. AP-1, NFkB).

Multiple experimental approaches have revealed that TNFR1 initiates the majority of TNF biological activities. The initial step in TNFR1 signaling involves the binding of the TNF homotrimer to the extracellular domain of the receptor, which induces TNFR1 trimerization and the release of the inhibitory protein silencer of death domains (SODD) from TNFR1 intracellular DD. SODD function is to repress spontaneous TNFR1 signaling by blocking the binding of adaptor proteins to the DD of TNFR1. After SODD release, TRADD can bind to the DD of TNFR1 and then recruit additional adaptor proteins, such as receptor

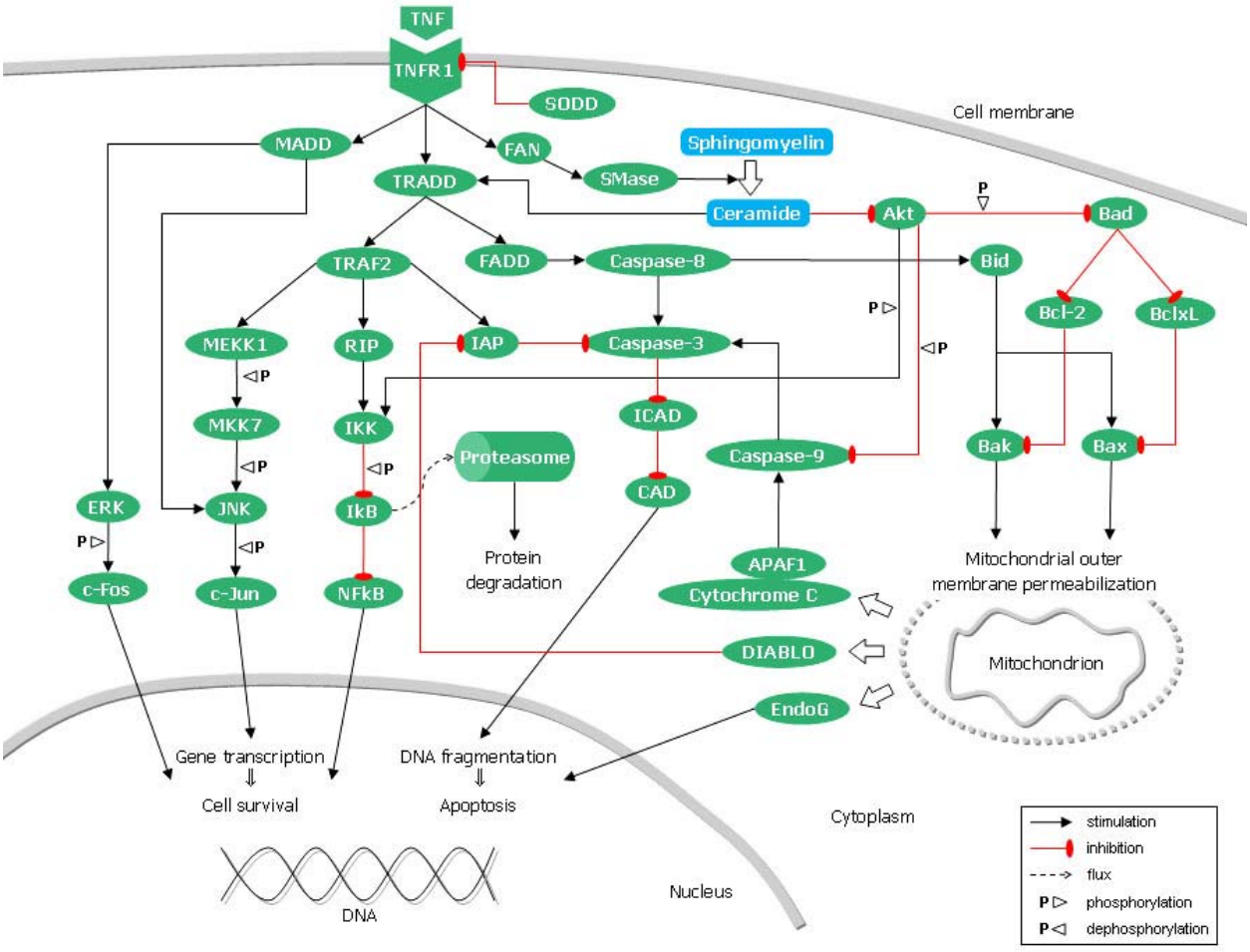

Figure 2. The TNF pathways, with special regard to those involved in cell death. Akt: protein kinase B (PKB), APAF1: apoptosis protein activating factor-1; Bad, Bcl-2, Bid, Bak, Bax, BclxL: mitochondrial apoptotic pathway-related factors belonging to the Bcl-2 (B-cell lymphoma-2) family, CAD: caspase-activated DNase, c-Fos c-Jun: transcription factors, DIABLO: direct IAP binding protein with low pl, endoG: endonuclease-G (mitochondrial DNase), ERK: extracellular signal-related kinase, FADD: Fas associated death domain, FAN: factor associated with neutral-sphingomyelinase, IAP: inhibitor of apoptosis protein, ICAD: inhibitor of CAD, IkB: inhibitor of NFkB, IKK: IkB kinase, JNK: c-Jun N-terminal kinase, MADD: MAPK activating death domain, MEKK1: MAPK/ERK kinase 1, MKK7: MAPK kinase 7, NFkB: nuclear factor kappa B, RIP: receptor interacting protein, SMase: neutral-sphingomyelinase, SODD: silencer of death domains, TNF: tumor necrosis factor, TNFR1: TNF receptor-1, TRADD: TNF receptor associated death domain; TRAF2: TNF receptor associated factor-2.

interacting protein (RIP), TRAF2 and FADD. These adaptors recruit other key proteins that are responsible for intracellular signaling events (Figure 2).

Caspase-8 (also called FADD-like IL-1 converting enzyme-like, FLICE) is recruited by FADD to the DISC, where it becomes activated, presumably by self-cleavage, and initiates a protease cascade leading to apoptosis. TRAF2 recruits inhibitor of apoptosis protein-1 (IAP-1) and IAP-2, and starts a pathway resulting in the phosphorylation/activation of transcription factors (c-Jun, c-Fos, ATF-2) via the activation of MAPK (JNK, p38MAPK and ERK). TRAF2 also activates the protein kinase RIP, which is critical to the activation of NFkB, a transcription factor composed of homodimeric and heterodimeric complexes of Rel/NFκB family members (c-Rel, RelA, RelB, NFkB1/p50, and NFkB2/p52). TNF-induced activation of NFkB hinges upon phosphorylation-dependent ubiquitination and consequent proteasome-mediated degradation of inhibitor of NFkB (IkB), which normally retains NFkB within the cytoplasm of non stimulated cells. Recently, the multiprotein IkB kinase (IKK) complex has been identified as the mediator of IkB phosphorylation (20). The IKK complex is recruited to TNFR1, where it becomes activated within minutes of TNF treatment in a RIP-dependent fashion.

TNFR2 signaling is largely confined to endothelial and hematopoietic cells and not much is known about its structure and function. Pro-TNF binds to TNFR2 through direct cell-to-cell contact and presents a higher affinity for this receptor than the soluble TNF form. As

above mentioned, TNFR2 lacks a DD and thus cannot stimulate the apoptotic process. However, TNFR2 interference with programmed cell death (PCD) via NFkB and JNK pathway activation (18) or TRAF2 inhibition (21) cannot be ruled out. In particular, the special role of the anti-apoptotic TRAF2 pathway in TNFR1 signaling could explain the observation that apoptosis induced by TNFR1, but not by CD95 and TNF-related apoptosis-inducing ligand (TRAIL), is enhanced by TNFR2, which recruits TRAF2 upon stimulation and triggers its proteasomal degradation. Thus, TNFR2 activation depletes the cell of TRAF2 and thereby liberates TNFR1 from the anti-apoptotic activity of this protein (22).

5. CELL DEATH AND DEATH RECEPTORS

A subgroup of the TNF superfamily receptors including TNFR1, cluster of differentiation 95 (CD95), death receptor 3 (DR3), TRAIL receptor-1 (TRAILR1), TRAILR2, ectodermal dysplasia receptor (EDAR) and the low affinity nerve growth factor receptor (p75NGFR) share a conserved protein-protein interaction domain in their cytoplasmic tail (the so called death domain, DD), which is necessary for direct activation of the apoptotic program of the cell by some (TNFR1, CD95, TRAILR1, TRAILR2) of these receptors. Due to the apoptosis-inducing capabilities of these receptors the whole subgroup has been named as "death receptors".

All death receptors trigger apoptosis under critical involvement of the cytosolic death domain-containing adapter protein FADD and the FADD interacting pro-caspase-8. In death receptor-induced apoptosis caspase activation occurs as a consequence of recognition of an extracellular ligand. Therefore, the apoptotic death receptor-FADD-caspase-8 pathway has been designated as the "extrinsic pathway"; by contrast, the "intrinsic pathway" is characterized by mitochondrial involvement in caspase activation and apoptosis. CD95 and the TRAIL death receptors TRAILR1 and TRAILR2 form a membrane-associated complex containing FADD and caspase-8, which has been named death inducing signaling complex (DISC) (23). By contrast, a TNF-induced DISC can only be demonstrated in an intracellular compartment (24). Recent studies with internalization deficient TNFR1 mutants have now revealed that a TNFR1 complex containing RIP and TRAF2 is formed at the plasma membrane and signals via the anti-apoptotic NFkB pathway, whereas internalization of TNFR1 is a prerequisite for formation of a pro-apoptotic signaling complex containing TRADD, FADD and caspase-8 (25). These data provide experimental evidence for a temporal and spatial separation of induction of the two disparate TNFR1 signaling pathways.

For all death receptor induced DISC, FADD mediates the assembly of active pro-caspase-8 dimers, which subsequently undergo maturation by autoproteolytic processing (26). In a first step the C-terminal p12 subunit of the caspase homology domain of caspase-8 is cleaved off and interacts with the remaining FADD-bound procaspase-8 molecule. In a second cleavage step the remaining caspase-8 p43/41 fragment is cleaved between the FADD-bound prodomain and the p20 subunit of the caspase homology domain. The resulting caspase-8 heterotetramer (p202p122) is released from FADD. Mature active caspase-8 can cleave and activate effector caspases such as caspase-3 and caspase-7, thereby triggering the execution steps of apoptosis. In addition, active caspase-8 can cleave the pro-apoptotic Bcl2 family member BID generating a fragment called truncated BID (tBID) (27). tBid in turn translocates to mitochondria and stimulates the intrinsic apoptotic pathway by inducing a conformational change in the pro-apoptotic Bcl2 family proteins Bax and Bak, which then allow by several, but not yet fully understood mechanisms the release of pro-apoptotic proteins from the mitochondria. Some of these proteins, such as apoptosis-inducing factor (AIF) and endonuclease-G, directly contribute to the execution steps of apoptosis or apoptosis-related processes, whereas other mitochondrial proteins such as cytochrome-C and second mitochondria-derived activator of caspase/direct IAP-binding protein with low pI (SMAC/Diablo) trigger caspase activation (28). After its release in the cytosol, cytochrome-C assembles together with ATP, apoptosis protein activating factor-1 (APAF1) and pro-caspase-9 to the apoptosome, a protein complex which delivers an environment where pro-caspase-9 becomes active by dimerization (29). Similar to mature caspase-8, apoptosome-associated pro-caspase-9 cleaves effector caspases and thus initiates the execution steps of apoptosis. The activity of effector caspases is further enhanced by SMAC/Diablo and some related proteins that interact with the inhibitor of apoptosis proteins and block these caspase inhibitory factors.

5.1. TNF-induced cell death

Two distinct forms of "extrinsic" (or death receptor-mediated) PCD can be mediated by TNF: 1) classical apoptosis, characterized by caspase-dependent chromatin condensation and fragmentation, membrane blebbing and generation of apoptotic bodies (30); 2) necrosis-like caspase-independent PCD, characterized by absent or marginal chromatin condensation, lack of nuclear fragmentation and disruption of membrane integrity (31).

The former is the best characterized type of TNF-driven PCD. As illustrated in Figure 2, activation of caspase-8 by FADD starts the caspase cascade. Caspases are synthesized as pro-enzymes and are activated by cleavage at specific aspartic acid residues. Besides causing DNA fragmentation through the activation of caspase-activated DNase (CAD), caspases can also initiate the mitochondrial apoptotic pathway (or "intrinsic" death pathway) leading to the release of several mediators (e.g. cytochrome-C) that further amplify the caspase cascade (32). Finally, another apoptosis execution pathway activated by TNF is the evolutionarily conserved lysosomal death pathway, which is mainly mediated by the cathepsin protease family (33).

In contrast to Fas (34) and TRAILR (35) that directly recruit FADD, TNFR1 only signals for PCD in certain circumstances (e.g. when protein synthesis is blocked, as occurs in experimental models using

cycloheximide). Most often, in already developed organisms TNFR1 induces the transcription and activation of inflammatory genes (36). This suggests that TNFR1 signaling also provides a mechanism to suppress the apoptotic stimulus. In effect, TNFR1 can recruit TRAF2 and RIP to activate the NFkB and JNK pathways, which can initiate inflammatory, proliferative and also anti-apoptotic responses. In particular, NFkB activation prompts the synthesis of IkB and anti-apoptotic factors, such as FLICE inhibitory protein (FLIP, also called IFLICE and FLAME) and IAP family members (e.g. IAP-1, IAP-2, survivin), which are potent inhibitors of PCD.

RIP, a key mediator of NFkB activation, offers another example of the crossroads connecting functionally opposite pathways: its cleavage by caspase-8 separates the N-terminal kinase domain (RIPn) from the C-terminal DD (RIPc) and results in ablation of the RIP-mediated activation of NFkB (37).

As regards JNK, its ultimate role in controlling TNF-driven PCD is less clear, as in mammals there is evidence for both pro- and anti-apoptotic activity (38).

Therefore, in an apparent dichotomy, TNFR1 assembles a signaling complex that can promote both cell death and survival. Physiological or disease-related alteration of virtually any intracellular apoptosis-related factor can tip the balance towards PCD or cell survival. Cells can be refractory to TNF-driven PCD owing to the over-expression of anti-apoptotic proteins, such as NFkB (39), FLIP (40), mitochondrial apoptotic pathway-related factors (e.g. Bcl2, Bcl-xL) (41) or the down-regulation of pro-apoptotic factors, such as caspases (42). However, the complex network of interrelations between all these molecules can make the prediction of the final outcome particularly difficult. For instance, TNF-induced apoptosis is unaffected in mice knockout for caspase-1, -2, -3 and -9. Moreover, FLIP - which interacts with a number of cell signaling proteins (e.g. FADD, caspases, Bcl-xL) - can even favor TNF-driven apoptosis under particular experimental circumstances (43).

Finally, the TNF signaling network intersects several other metabolic pathways that participate in the regulation of TNF-mediated PCD. For instance, generation of ceramide by TNF plays an important role in the induction of apoptosis by the cytokine (44). Ceramide exerts its pro-apoptotic effect by inhibiting the activity of anti-apoptotic factors (e.g. protein kinase-B [PKB/Akt]), and by stimulating TRADD recruitment to TNFR1 with subsequent increased activation of caspase-8 (45). Impairment of the activity of sphingomyelinases, which generate ceramide following TNF stimulation, reduces the ability of the cytokine to induce apoptosis (46). Additionally, short chain ceramide analogues increase cell sensitivity to TNF-induced PCD (45). These as well as other interactions represent an opportunity for investigators to polarize the killing activity of TNF selectively against malignant cells, as described in a dedicated article of this issue of Frontiers in Bioscience.

6. PERSPECTIVE

TNF is the only death receptor family member approved for clinical use as an anticancer agent. However, its pleiotropic nature leads not only to the activation of the apoptotic pathway, but also to the activation of inflammatory and anti-apoptotic pathways. In fact, while CD95 and TRAIL death receptors predominantly act as inducers of apoptosis (through the activation of protein-synthesis-independent pathways), TNFR1 functions are also related to the non-apoptotic gene translation inducing capabilities of this receptor.

This aspect is crucial to study the TNF ability of inducing tumor necrosis in several preclinical and clinical models. The differential behavior of TNFR1 as compared to CD95 and TRAIL death receptors in terms of pro-apoptotic activity is most likely due to the way these receptors are linked to FADD. As above outlined, CD95 and the TRAIL death receptors directly interact with FADD whereas TNFR1 indirectly associate with FADD via TRADD. The latter also mediates recruitment of TRAF2, which interferes with TNFR1-induced apoptosis by two mechanisms: first, TRAF2 and the related TRAF1 protein together with the cellular IAP-1 and -2 form a complex that inhibits caspase-8 activation; secondly, TRAF2 is implicated in TNFR1-induced activation of the protein-synthesis-dependent anti-apoptotic NFkB pathway. Accordingly, many attempts are being made to split this dichotomic feature of TNF so to fully exploit its anticancer potential (47). Overall, although the last decade has witnessed remarkable advances in this field, we still do not know in detail how cells choose between life and death after TNF stimulation (48, 49). Understanding this mechanism will not only shed new light on the physiological significance of TNF-driven PCD but also help oncologists maximize the therapeutic properties of this cytokine, as further discussed in the dedicated articles of this issue of Frontiers in Bioscience.

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Abbreviations: TNF: tumor necrosis factor; NK: natural killer; ADAM: a-disintegrin-and-metalloproteinase; VEGI: vascular endothelial cell growth inhibitor; RANKL: receptor activator of nuclear factor kappa-B ligand; DD: death domain; FADD: Fas-associated death domain; TRADD: TNF receptor associated death domain; DISC: death inducing signaling complex; TRAF: TNF receptor; associated factor; TIM: TRAF-interacting motif; MAPK: mitogen-activated protein kinase; JNK: c-Jun N-terminal Kinase; ERK: extracellular signal-related kinase; NFkB: nuclear factor kappa-B; IKK: inhibitor of NFkB kinase; PI3K: phosphatidyl-inositol 3-kinase; TNFR: TNF receptor; SODD: silencer of death domains; RIP: receptor interacting protein; FLICE: FADD-like IL-1 converting enzyme-like; IkB: inhibitor of NFkB; PCD: programmed cell death; TRAIL: TNF- related apoptosis-inducing ligand; TRAILR: TRAIL receptor; IAP: inhibitor of apoptosis protein

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