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Cellular Responses to Tumor Necrosis Factor

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Abstract

TNF is a proinflammatory cytokine that plays an important role in many physiological and pathological conditions through the regulation of immunological reactions. Many of TNF functions have been proven to be cell type-specific, and the specificity of TNF-induced cellular responses in a given cell is determined by the specific intracellular signaling pathways that are activated by TNF. Although current information is insufficient to sort out how the cell type specificity is controlled by the different intracellular signaling pathways, a number of signaling pathways that are commonly activated in many types of cells by TNF have been revealed. This review weighs the current knowledge of these TNF-induced signaling pathways.

Introduction

Tumor necrosis factor α (TNF) is a proinflammatory cytokine produced mainly by activated macrophages or monocytes and plays an important role in diverse cellular events, such as the production of other cytokines, cell proliferation, differentiation and apoptosis (Beutler *et al.,* 1988; Tartaglia *et al.,* 1992; Rothe *et al.,* 1992; Tracey *et al.,* 1993; Beyaert *et al.,* 1994). TNF was originally identified as a factor that leads to rapid hemorrhagic necrosis of transplantable tumors in mice (Carswell *et al.,* 1975). It was identical to a factor named cachectin that was purified at almost the same time on the basis of its ability to suppress the expression of lipoprotein lipase in fat (Beutler *et al.,* 1984). Approximately one third of transformed cell lines were shown to be susceptible to the cytolytic action of TNF (Sugarman *et al.,* 1985). However, because of its toxicity in animals and humans, TNF did not fulfill the initial expectations that it would be useful in the treatment of cancer. It is clear that TNF also affects normal cells. TNF activates a variety of cells, such as neutrophils, endothelial cells, and fibroblasts. The cellular changes in response to TNF are cell-type dependent. For example, TNF may modify the anticoagulant properties of endothelial cells, promote T cell proliferation, cause bone resorption, and induce the release of other inflammatory cytokines in many different cells. Inappropriate production of TNF has been implicated in the pathogenesis of both acute and chronic inflammatory diseases such as septic shock, AIDS, arthritis and cancer (Beutler *et al.,* 1988; Tracey *et al.,* 1993). The studies in the last two decades have provided a large amount of information regarding the biological function of this important cytokine and have been reviewed by a number of excellent reviews (Beutler *et al.,* 1988; Tartaglia *et al.,* 1992; Rothe *et al.,* 1992; Tracey *et al.,* 1993; Beyaert *et al.,* 1994). The intracellular signaling pathways of TNF have been studied intensively in the past several years and a number of important components in these pathways have been identified. The commonality of TNF-induced cellular responses in different cells is that they are all initiated by the binding of TNF to receptors present on virtually all cells throughout the body (Tartaglia *et al.,* 1992; Rothe *et al.,* 1992). Though the downstream events may vary to some extent in different types of cells, current information does not allow us to compare the specific pathways in different types of cells. In this review we have highlighted the intracellular signal transduction pathways that are known to be activated in TNF-stimulated cells.

The Molecular Mechanism of TNF Signaling

TNF-induced cellular responses are mediated by either one of the two TNF receptors, TNF-R1 (p55) and TNF-R2 (p75), both of which belong to the TNF receptor super-family (Smith *et al.,* 1994; Nagata *et al.,* 1995). Almost all cell types express at least one of the two kinds of TNF receptors (Smith *et al.,* 1994; Nagata *et al.,* 1995). The two receptors display no significant homology in their intracellular domains, suggesting that the two receptors may elicit different intracellular signals. Genetically engineered mice lacking TNF-R1 are moderately resistant to the lethal effect of lipopolysaccharides but highly susceptible to the infection by *Listeria monocytogenes* (Pfeffer *et al.,* 1993). TNF-R2 knockout mice are moderately resistant to the lethal effect of TNF itself (Erickson *et al.,* 1994). A double knockout of TNF-R1 and TNF-R2 results in a sum of these phenotypic effects. Thus, the two TNF receptors have different functions in vivo. The first step of TNF signaling is believed to be ligand-induced oligmerization of the receptor molecules. Initiation of signaling occurs by recruitment of cytosolic effector proteins that associated with the cytoplasmic domains of the TNF receptors. To date, most studies related with TNF-induced cellular responses were on TNF-R1-mediated pathways. For TNF-R1 signaling, the first molecule recruited to TNF-R1 is known as TRADD, a death domain protein (Hsu *et al.,* 1995). In response to TNF, TRADD is recruited to TNF-R1 through the interaction between the death domains of these two proteins (Hsu *et al.,* 1995). TRADD subsequently recruits other effectors, such as TRAF2, RIP, FADD, cIAP1, cIAP2 and A20, to the TNF-R1 complex (Hsu *et al.,* 1996a; Hsu *et al.,* 1996b; Shu *et al.,* 1996; Lin *et al.,* 1999; Devin *et al.,* 2000; Zhang

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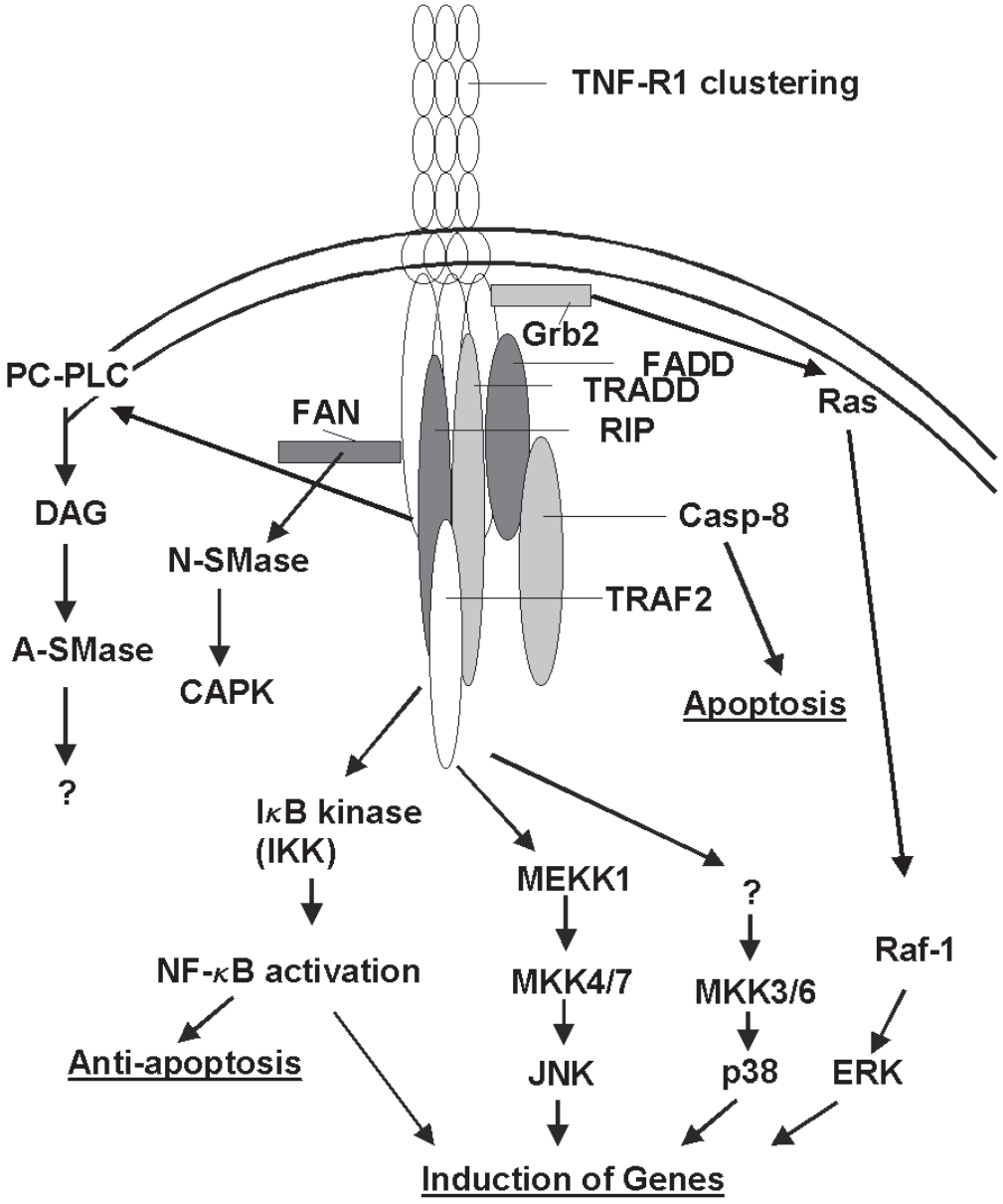

Figure 1. The intracellular signaling pathways downstream of TNF-R1. See text for details.

et al., 2000). These effector proteins then mediate the activation of proteases, phospholipases, protein kinases, and transcription factors respectively as shown in Figure 1. The signaling pathway of each TNF-induced cellular response is discussed separately below in detail.

NF-kB Pathway

NF-κB is one of the key transcription factors that mediate many TNF-induced cellular responses. In most types of cells, the activity of NF-κB can be potently elevated by TNF treatment (Israel *et al.,* 1989; Israel *et al.,* 1989). Inactive NF-κB is sequestered in the cytoplasm through its interaction with the inhibitory proteins, known as IκBs (Baeuerle *et al.,* 1996). In response to various stimuli including TNF, IκBs are phosphorylated by IκB kinases (IKKs) (Karin *et al.,* 2000). Then the Phosphorylated IκBs are polyubiquitinated and subsequently degraded by the proteasome (Karin *et al.,* 2000). The degradation of IκBs results in the release of NF-κB and allows its translocation into the nucleus and the subsequent activation of its target genes (Baeuerle *et al.,* 1996). Therefore, IKK activation is a key step in the activation of NF-κB. The molecular mechanism of TNF-induced IKK and NF-κB activation has been intensively studied in the last several years. The first identified effector molecule of the TNF-R1 signaling complex was TRAF2, although it was cloned as a binding protein of TNF-R2 (Rothe *et al.,* 1994; Rothe *et al.,* 1995).

After TRADD was isolated as the key adapter protein of the TNF-R1 signaling complex, TRAF2 was found to be recruited to the TNF-R1 signaling complex through its interaction with TRADD and to play an important role in TNF-R1-mediated NF-κB activation (Hsu *et al.,* 1996b). While the TRAF domain of TRAF2 is essential for its interaction with the N-terminal region of TRADD, the ring- and zinc-finger regions of TRAF2 are responsible for transducing the TNF signal to TRAF2's downstream targets (Baud *et al.,* 1999). Similarly, another critical component of the TNF-R1-signaling complex, RIP, a death domain kinase which was initially identified as a Fas binding protein (Hsu *et al.,* 1996a), was also found to be recruited to the TNF-R1 signaling complex by TRADD and to be a key effector of TNF-induced NF-κB activation (Hsu *et al.,* 1996a). It is believed that RIP is recruited to TRADD through the interaction between their death domains (Hsu *et al.,* 1996a). However, the kinase activity of RIP is not required for RIP to transduce TNF signaling (Hsu *et al.,* 1996a; Devin *et al.,* 2000). The important roles of TRAF2 and RIP in TNF-induced NF-κB activation have been confirmed by genetic deletion of these molecules in mice (Yeh *et al.,* 1997; Kelliher *et al.,* 1998).

Several other proteins, including A20, cIAP1 and cIAP2, were also found to be recruited to the TNF-R1 complex in response to TNF treatment (Shu *et al.,* 1996; Zhang *et al.,* 2000). A20 is a zinc finger protein induced by TNF and down-regulates NF-κB activation (Opipari *et al.,* 1990). Although A20 was found in the TNF-R1 complex, A20 does not inhibit TNF-induced nuclear translocation and DNA binding of NF-κB, suggesting that A20 functions downstream of the initiation of NF-κB activation (Heyninck *et al.,* 1999). Since A20 was found to interact with a protein known as ABIN, whose expression also inhibits NF-κB activation, it was proposed that A20 acts via its interaction with ABIN to suppress NF-κB-mediated transcription (Heyninck *et al.,* 1999). Genetic deletion of A20 resulted in the prolonged activation of IKK in response to TNF (Lee *et al.,* 2000). The function of cIAP1 and cIAP2 in NF-κB activation is still unclear. Interestingly, both cIAP1 and cIAP2 are ring-finger-containing proteins and have been shown to function as E3 ligases (Yang *et al.,* 2000). Therefore, it is possible that, much like the role of c-CBL in EGF signaling (Levkowitz *et al.,* 1999), cIAP1 and cIAP2 are involved in turning off TNF signaling.

Downstream of the effector proteins mentioned above, the mechanism that leads to the activation of IKK is less clear. It has been suggested that the MAP3Ks, such as NIK and MEKK1, mediate TNF-induced IKK activation (Chen *et al.,* 1996; Lee *et al.,* 1997; Malinin *et al.,* 1997; Regnier *et al.,* 1997). However, deletion of either of these two molecules genetically in mice did not affect TNF-induced IKK and NF-κB activation (Xia *et al.,* 2000; Yujiri *et al.,* 2000; Yin *et al.,* 2001). Therefore, it is unlikely that NIK and MEKK1 play any critical role in TNF-induced NF-κB activation, although the possibility that these kinases may have redundant functions in this process has not been completely ruled out. Most recently, another MAP3K, MEKK3, has been found to be involved in TNF-induced IKK and NF-κB activation (Yang *et al.,* 2001). In MEKK3 null MEF cells, the TNF-induced IKK and NF-κB activation has been severely impaired. Although it seems that MEKK3 functions downstream of TRAF2 and RIP and upstream of IKK, it is still not clear whether MEKK3 is the IKK kinase in response to TNF.

IKK is a kinase complex and is composed of three subunits: IKKα/IKK1, IKKβ/IKK2 and IKKγ/NEMO (DiDonato *et al.,* 1997; Mercurio *et al.,* 1997; Regnier *et al.,* 1997; Woronicz *et al.,* 1997; Zandi *et al.,* 1997; Yamaoka *et al.,* 1998; Rothwarf *et al.,* 1998). Both IKKα/IKK1 and IKKβ/IKK2 are catalytic subunits while IKKγ/NEMO is a regulatory subunit. Recently, studies reported that IKK was recruited to the TNF-R1 complex and was activated in response to TNF treatment (Devin *et al.,* 2000; Zhang *et al.,* 2000). The interaction between RIP and IKKγ/NEMO has been detected in the yeast two hybrid system as well as in overexpression experiments (Zhang *et al.,* 2000). But with TRAF2 null MEF cells, it has been found that TRAF2 is essential for the recruitment of IKK to the TNF-R1 signaling complex (Devin *et al.,* 2000). Most recently, it has been shown that TRAF2 recruits IKK to the TNF-R1 complex through its interaction with IKKα/IKK1 and IKKβ/IKK2 (Devin et al., 2001). Using NEMO/IKKγ deficient Rat-1 5R cells, it has also been shown that the interaction between RIP and IKKγ/NEMO plays a limited role in this recruitment process. Although both RIP and IKKγ/NEMO are required for IKK activation, the exact mechanism of this activation process is still unknown. It is possible that the RIP-IKKγ/NEMO interaction results in conformation changes in IKK and, in turn, leads to the autophosphorylation and subsequent activation of IKK. Another possibility is that RIP is required for recruiting the IKK kinase, most likely a MAP3K such as MEKK3, and then the interaction between RIP and IKKγ/NEMO primes the IKK kinase to activate IKK.

Several other kinases such as GSK3β and PKC were also suggested to be involved in TNF-induced NF-κB activation but they were not required for IKK activation (Hoeflich *et al.,* 2000; Sanz *et al.,* 1999). The role of these kinases in TNF-induced NF-κB activation seems to be limited to regulating NF-κB transcription activity. However, further examination into whether these kinases directly modulate NF-κB activity in response to TNF needs to be done.

ERK Pathway

ERK (extracellular signal-regulated kinases) pathway is one of the MAP kinase pathways that are activated by TNF. Since sphingomyelinase (SMase) and ceramide activate ERK, it was suggested that TNF induces ERK activation via lipid second messenger (Stout *et al.,* 1993). This idea was further supported by the studies aimed at dissecting the signaling pathway between acidic and neutral SMases (Wiegmann *et al.,* 1994). It was found that TNF-R1 deletion mutants displayed a loss-of-function phenotype with regard to activation of PC-specific phospholipase C (acidic SMase pathway), yet retained their capacity to signal stimulation of ERK, indicating that ERK activation is downstream of neutral SMase (Wiegmann *et al.,* 1994). The later work identified an adaptor protein FAN (factor associated with neutral SMase activation) that linked TNF-R1 to neutral

SMase (Adam-Klages *et al.,* 1996). There is evidence that ceramide generated by neutral SMase leads to the activation of ceramide-activated protein (CAP) kinase (also known as kinase of suppressor of Ras) and that c-Raf-1 is downstream of CAPK (Yao *et al.,* 1995; Zhang *et al.,* 1997).

Grb2 is an adapter protein and was found to bind to the tyrosine kinase receptor family members (Buday, 1999). Although TNF-R1 does not possess tyrosine kinase activity, Grb2 was found to interact with TNF-R1 using a two-hybrid screening (Hildt *et al.,* 1999). Using deletion mutants Hildt and Oess revealed that the C-terminal SH3 domain of Grb2 binds to a PLAP motif at amino acids 237 to 240 in TNF-R1 (Hildt *et al.,* 1999). The binding of Grb2 to the PLAP motif is essential for the activation of c-Raf-1 by TNF; disruption of the TNF-R1/Grb2 complex by cell permeable peptides inhibited TNF-induced c-Raf-1 activation and deletion of PLAP in TNF-R1 rendered TNF-R1 incapable of activating c-Raf-1 (Hildt *et al.,* 1999). Although the Grb2 and TNF-R1 interaction is required for c-Raf-1 and subsequently ERK activation, the signaling through Grb2 may not be sufficient for c-Raf-1 activation. In the same study, Hildt and Oess reported that interfering with neutral SMase pathway by disruption of the TNF-R1/FAN interaction also blocked c-Raf-1 activation (Hildt *et al.,* 1999). A model in which ERK activation requires two paralleled signals was proposed. However, conflicting results have been reported. A recent study using fan-/- cells revealed that TNF-induced ERK1/2 activation was not affected by the FAN knockout (Segui *et al.,* 2001).

JNK Pathway

JNK (c-Jun N-terminal kinase), also known as SAPK (stress-activated protein kinase), is another MAPK that is rapidly and potently activated by TNF in many types of cells (Derijard *et al.,* 1994). JNK is distantly related to the ERK, to which JNK exhibits about 40% identity. Three genes that encode JNK have been identified as *jnk1, jnk2* and *jnk3* by molecular cloning (Derijard *et al.,* 1994; Sluss *et al.,* 1994; Mohit *et al.,* 1995; Kallunki *et al.,* 1996). The alternative splicing of the transcripts of these three genes generates at least 10 JNK isoforms with molecular masses of 46 and 55 kDa (Gupta *et al.,* 1996). All of these isoforms of JNK can be activated by TNF. It is believed that JNK is activated through a MAP kinase cascade in response to TNF (Davis, 1999). Although MKK7/JNKK2 has been identified recently as a specific JNK kinase following TNF treatment (Tournier *et al.,* 2001), the corresponding MAP3K is still unknown. Several MAPKKKs, including MEKK1 and ASK1, have been suggested to mediate TNF-induced JNK activation (Liu *et al.,* 1996; Ichijo *et al.,* 1997). However, recent studies with genetic deletions of these genes have excluded their involvement in JNK activation in response to TNF (Yujiri *et al.,* 2000; Tobiume *et al.,* 2001).

It is not clear how the TNF signal is transduced from the TNF-R1 signaling complex to MAP3K. The roles of some effector molecules of TNF signaling including TRAF2, RIP and A20 have been examined and it has been shown that the dominant negative mutant of TRAF2 could completely block TNF-induced JNK activation (Liu *et al.,* 1996; Natoli *et al.,* 1997). When TRAF2 was genetically deleted, TNF-induced JNK activation dramatically diminished (Nguyen *et al.,* 1999). These studies indicated that TRAF2 plays an essential role in TNF-induced JNK activation. In contrast, the role of RIP in this process is less clear. Early studies with the dominant negative mutant of RIP suggested RIP was required for TNF-induced JNK activation (Liu *et al.,* 1996). But the study with genetic deletion of RIP detected only a minor decrease in JNK activation in RIP-/- cells in response to TNF (Kelliher *et al.,* 1998). Therefore, the role of RIP in this process needs further study. Although it plays a role in TNF-induced NF-κB activation, it seems that A20 is not involved in TNF-induced JNK activation (Zazgornik *et al.,* 1975).

p38 Pathway

p38 is a MAP kinase which has been identified as an important signaling molecule in inflammation (Han *et al.,* 1994). TNF is a strong activator of p38 in a variety of different cell types (Raingeaud *et al.,* 1995). To date, four different members of the p38 group MAP kinases have been identified in mammals: p38α (or p38, RK, CSBP), p38β (or p38-2), p38γ (or ERK6, SAPK3), and p38δ (or SAPK4) (Ono *et al.,* 2000). It appears that all of the four p38 isoforms are activated by TNF stimulation; however, the majority of the current data is derived from the research on the p38α activation in TNF-treated cells. It is known that the upstream MAP kinase kinases of p38α are MKK3 (or MEK3) and MKK6 (or MEK6) (Derijard *et al.,* 1995; Han *et al.,* 1996). The further upstream MAP3K in this pathway is not clearly understood. Since ASK1 and TAK1 were reported to be activated in TNF-stimulated cells, these two kinases may play a role in mediating p38α activation in the TNF signaling pathway. However, the most recent study with the genetic deletion of Ask1 indicated that Ask1 did not play an essential role in TNF-induce p38 activation (Tobiume *et al.,* 2001). Very little is known about the effectors downstream of TNF-R1 that lead to p38 activation. Although over-expression of TRAF2 can lead to p38 activation in various types of cells, the requirement of TRAF2 in mediating p38 activation has not been confirmed in TRAF2-/- cells. There is no information available as to whether other effectors, such as RIP, have any role in TNF-induced p38 activation. The signaling events between these effectors and the p38 MAP kinase cascade are completely unknown at the present time.

Acidic Sphingomyelinase (A-SMase) Pathway

Activation of the phospholipid transmission pathway by TNF was first reported nine years ago (Schutze *et al.,* 1992). In a study to explore the mechanisms of TNF-induced NF-κB activation, Schutze et al had found that PC-specific phospholipase C (PC-PLC) and A-SMase were activated in TNF treated U937 cells. Their data suggested that generation of 1,2-diacylglycerol produced by a TNF responsive PC-specific phospholipase C subsequently activated A-SMase. Ceramide, generated by sphingomyelin breakdown catalyzed by A-SMase, is the second messenger in triggering downstream NF-κB activation. Subsequent work from the same group of investigators

mapped the sequence in TNF-R1 which is required for A-SMase activation. As little as a 32 amino acid truncation of TNF-R1 at the C-terminus causes a defect in TNF-induced A-SMase activation (Wiegmann *et al.,* 1994). A later study showed that FADD is required for TNF-induced A-SMase activation (Wiegmann *et al.,* 1999). Although A-SMase is activated by TNF, its involvement in NF-κB activation became uncertain after further studies were performed. Inhibition of A-SMase by a specific inhibitor SR33557 had no effect on TNF-mediated NF-κB activation in ML-1a cells (Higuchi *et al.,* 1996). TNF-induced degradation of IκB-α and nuclear translocation of NF-κB in embryonic fibroblasts derived from an a-smase-/- strain is the same as in cells from the wild-type mice (Zumbansen *et al.,* 1997). Thus, it is unclear whether A-SMase has no role whatsoever in TNF-induced NF-κB activation or if its role in NF-κB activation is cell type dependent.

Neutral Sphingomyelinase (N-SMase) Pathway

TNF activates not only an endosomal A-SMase but also a membrane-associated N-SMase (Wiegmann *et al.,* 1994). The activation of A-SMase and N-SMase occurs through different mechanisms since the domain sequences in TNF-R1 required for their activation are different (Wiegmann *et al.,* 1994). As mentioned above, the A-SMase activation requires the C-terminus of the TNF-R1. However, the sequence that is required for N-SMase activation was mapped to amino acids 309-319, which is in the middle of the cytoplasmic domain of TNF-R1 (Adam *et al.,* 1996). Identification of FAN (factor associated with neutral SMase activation), which couples TNF-R1 to N-Smase, but not A-SMase, further supported the notion that N-SMase and A-SMase are two independent pathways (Adam-Klages *et al.,* 1996; Kreder *et al.,* 1999). In the same study, the ceramide generated by N-SMase, but not A-SMase, was suggested to activate the proline-directed serine/threonine protein kinase and phospholipase A2 (Wiegmann *et al.,* 1994). It was reported later that the ceramide activated protein kinase is downstream of ceramide and can activate the Raf-ERK pathway (Yao *et al.,* 1995). More recently, N-SMase was shown to be involved in TNF-induced cell death (Segui *et al.,* 2001). Dominant negative FAN abrogates TNF-induced ceramide generation and reduces caspase processing. In addition, fan-/- fibroblasts are resistant to TNF-induced cell killing (Segui *et al.,* 2001). Conflicting with the previous reports, activation of ERK was not altered in fan-/- cells (Segui *et al.,* 2001), indicating that the N-SMase pathway is not related with ERK activation.

TNF Signaling Related with Cell Death

Although TNF was named for its ability to cause tumor regression, it only selectively kills certain type of cells (Sugarman *et al.,* 1985; Beutler *et al.,* 1988; Rothe *et al.,* 1992; Tracey *et al.,* 1993; Beyaert *et al.,* 1994). It is now known that one of the reasons for this inefficiency is the activation of NF-kB in response to TNF treatment (Van Antwerp *et al.,* 1998). Studies from several labs have demonstrated that NF-κB activation protects cells against TNF-induced apoptosis (Liu *et al.,* 1996; Beg *et al.,* 1996; Van Antwerp *et al.,* 1996; Wang *et al.,* 1996). Inhibition of NF-κB activation rendered many types of cells TNF sensitive. Several of NF-κB's target genes, including cIAP-1, cIAP-2 and IEX-1L, have been suggested to have such anti-apoptotic effect (Wang *et al.,* 1996; Wu *et al.,* 1998). Recently, the existence of a TRAF2-dependent but NF-κB-independent anti-apoptotic pathway has been revealed through a genetic study (Yeh *et al.,* 1997).

Substantial evidence supports the view that engagement of TNF-R1 triggers apoptosis in many different cells. The pro-apoptotic effect of TNF-R2 was only found in some circumstances. Dependent on the type of target cell, TNF-induced cell death could be necrotic or apoptotic. Apoptotic cell death is morphologically characterized by membrane blebbing, condensation of both the cell and chromatin, DNA fragmentation, and finally fragmentation of the cell into discrete membrane bound particles (Cohen *et al.,* 1992; Kerr *et al.,* 1972). Such changes are seen in a number of different cells, such as U937, PC60 and KYM cells, after TNF treatment (Tewari *et al.,* 1995; Wright *et al.,* 1992). The morphological changes of necrotic cell death include cell swelling, destruction of organelles and cell lysis (Golstein *et al.,* 1991). TNF-treated murine L929 cells die with necrotic phenotype (Beyaert *et al.,* 1994; Fiers *et al.,* 1999). Both apoptosis and necrosis are initiated by TNF receptor I (TNF-RI) clustering and TRADD recruitment (Boldin *et al.,* 1996; Fiers *et al.,* 1999; Strasser *et al.,* 2000). As shown in Figure 1, FADD is required for caspase-8 autoactivation, which plays a key role in TNF-induced apoptosis (Li *et al.,* 1999). Active caspase-8 is an initiator caspase that either acts via cytochrome c (Cyt c) release or by the direct activation of effector caspases to execute apoptosis (Goossens *et al.,* 1995). At least in some cells, such as MCF-7, the cyt c release is associated with TNF-induced apoptosis (Srinivasan *et al.,* 1998). As with apoptosis, the TNF-induced necrotic pathway in L929 cells is also initiated by trimerization of the DD of TNF-RI (Vandevoorde *et al.,* 1997; Fiers *et al.,* 1999). Recruitment of TRADD also occurs in these cells. The two pathways may diverge downstream of TRADD since neither the known pro-apoptotic caspases including caspase-8, nor cyt c release are involved in this death pathway (Vercammen *et al.,* 1998; Fiers *et al.,* 1999; Goossens *et al.,* 1999). Moreover, the caspase inhibitor zVAD does not block TNF-induced L929 cell death, but in fact dramatically enhances TNF-induced cell killing (Vercammen *et al.,* 1998). It was recently suggested that RIP may be responsible for TNF-induced necrosis (Holler *et al.,* 2000). Despite the significant differences in the morphology of cell death, the apoptotic and necrotic pathways still share some common components downstream. Bcl-xL can prevent both apoptosis and necrosis (Kane *et al.,* 1993; Shimizu *et al.,* 1995). Metaxin, an outer mitochondrial membrane protein, was found to be required for both TNF-induced apoptosis and necrosis (Wang *et al.,* 2001). Thus, the TNF activated cell death pathway may not be a linear cascade. Apoptosis or necrosis may be determined by the balance among the different branches of the singaling pathway.

A number of studies suggested that acidic compartments, mainly constituted by lysosomes, have a

role in TNF-induced cell death (Liddil *et al.,* 1989; Deiss *et al.,* 1996; Monney *et al.,* 1998; Guicciardi *et al.,* 2000; Foghsgaard *et al.,* 2001). It was reported twenty years ago that the activity of tumor necrosis serum-induced cell death can be inhibited by lysosomtropic agents such as chloroquine (Kull *et al.,* 1981). In an analysis of a TNF-resistant L929 line, Liddil et al reported more than ten years ago that a TNF resistant L929 cell sub-line had a 50% reduction in total lysosomal protein levels in comparison with parental line (Liddil *et al.,* 1989). The lysosomal protease cathepsin D was identified to be required for TNF-induced cell death by random gene disruption (Deiss *et al.,* 1996). Cathepsin B, another lysosomal preotease, was recently reported to be involved in TNF-induced cell death by using cathepsin B -/- cells. (Guicciardi *et al.,* 2000). It was proposed that cathepsin B acts upstream of cyt c release from mitochondria. Another report suggested that lysosomal proteases may cleave bid, which in turn triggers cyt c release (Stoka *et al.,* 2001). In addition to the possible role of lysosomes in initiating the cell death process, we have found that the lysosomes were dramatically enlarged during TNF treatment (our unpublished results). The enlargement of lysosomes may represent a self-digestion during the cell death process, so lysosomes may also participate in the execution of cell death. As we have discussed above, acidic SMase, located in acidic endosomes and/or lysosomes, was reported to be activated by TNF (Nanda *et al.,* 1992). Resistance to radiation-induced apoptosis was reported in asmase-/- cells (Lozano *et al.,* 2001); however, whether TNF-induced cell death was affected in asmase-/- cells was not addressed in this study. In contrast, a recent report suggested that ceramide formed within or accumulated in lysosomes is not the second messenger of apoptosis induced by various stress stimuli, one of which is TNF. The cells derived from patients with Farber disease, which has a genetic defect of A-SMase, were equally sensitive to TNF-induced cell death as the wildtype cells (Segui *et al.,* 2000). So it appears that lysosomal SMase may not be involved in the initiation of apoptosis.

TNF Signaling Linked to Gene Induction

The pleiotropic effect of TNF is not only due to its cytotoxity in certain types of cells, but is also a consequence of the gene induction caused by this cytokine. The number of genes that can be up-regulated by TNF stimulation is unknown, but it is known that almost all pro-inflammatory cytokines are induced by TNF stimulation. Other molecules, such as matrix proteases, that are involved in inflammatory diseases are either directly or indirectly induced by TNF in vivo. The intracellular signaling pathways activated by TNF are essential for the gene induction.

We have mentioned above that a number of TNF-activated intracellular signaling pathways have been revealed. NF-κB is known to be the primary transcription factor involved in the gene induction of inflammatory molecules since the κB binding site(s) was found in the promoters of almost all TNF-inducible genes (Baeuerle *et al.,* 1996). TNF-activated JNK may be important in the activation of genes containing the AP-1 site(s), a cis-element found in many inducible genes (Shaw *et al.,* 1986: Whitmarsh *et al.,* 1996). ERK, JNK and p38 pathways also target other transcription factors such as CREB, ATF1, ATF2, ELK-1, Sap1, MEF2, etc. (Robinson *et al.,* 1997; Janknecht *et al.,* 1997; Ono *et al.,* 2000), that are directly or indirectly involved in TNF-induced gene activation. The relative role of the phospholipid transmission pathway activated by TNF in gene induction has proven difficult to establish unambiguously because conflicting results were reported regarding the activation of NF-κB by the A-SMase pathway and the activation of ERK by the N-SMase pathway (Schutze *et al.,* 1992; Wiegmann *et al.,* 1994; Yao *et al.,* 1995; Zumbansen *et al.,* 1997; Segui *et al.,* 2001). Nevertheless, the potential involvement of these phospholipid transmission pathways in TNF-induced gene induction cannot be excluded.

It is well known that many of the genes induced in inflammatory responses are subject to regulation at the levels of mRNA stability and protein translation (Guhaniyogi *et al.,* 2001). The AU-rich elements (ARE) in the 3'-untranslated region of mRNA play a key role in mediating its stability and translation (Han *et al.,* 1990; Kotlyarov A. *et al.,* 1999; Kontoyiannis *et al.,* 1999). It is worth noting that ARE can be found in the mRNA of almost all genes induced in inflammation. A number of ARE binding proteins have been identified, including AUF1, HuR and TTP (Peng *et al.,* 1998; Fan *et al.,* 1998; Carballo *et al.,* 1998; Piecyk *et al.,* 2000); however, information regarding whether and how these proteins regulate the ARE-bearing mRNA's stability or translation is very limited (Shyu *et al.,* 2000). It was proposed that this regulation was related to mRNA transport from the nucleus to the cytosol and perhaps also to the location of mRNA (Shyu *et al.,* 2000). A number of reports demonstrated the important role of p38 pathway in regulating mRNA stability and protein translation (Lasa *et al.,* 2000; Holtmann *et al.,* 2001; Kontoyiannis *et al.,* 2001; Faour *et al.,* 2001; Lasa *et al.,* 2001). A gene knockout of MAPKAPK2, a downstream kinase of p38, conformed that the p38 pathway has a regulatory role in ARE-mediated mRNA stability and translational regulation (Kotlyarov A. *et al.,* 1999). The involvement of JNK and ERK in regulation of mRNA stability and/or protein translation was also reported (Swantek *et al.,* 1997; Chen *et al.,* 1998; Sheng *et al.,* 2001). Whether such a role can be applied to TNF-induced genes requires further investigation. The biggest gap in our knowledge now is how the signaling pathway(s) links with the proteins that directly interact with ARE in the mRNAs.

TNF as a Target in the Treatment of Inflammatory Diseases

The investigation of TNF was driven largely by practical goals. The isolation of TNF was a result of searching for endogenous factors that would act to destroy tumor cells. Unfortunately, the application of TNF in tumor treatment was proven to be unsuccessful. Because of a close relationship between TNF and inflammation, extensive clinical trials have been performed to test the effects of TNF blockage in a number of inflammatory diseases.

Monoclonal antibodies that selectively neutralize TNF

were tested in treating septic shock. A randomized, controlled, double-blind, multicenter clinical trial showed no substantial benefit to the patients (Wherry *et al.,* 1993; Abraham *et al.,* 1995). One of the possible interpretations is that septic shock is a fulminate disease in which considerable damage may already have occurred before the initiation of therapy. Thus, blockage of TNF may not be an effective method to treat acute inflammatory diseases. In contrast, treatment of chronic inflammatory diseases, like rheumatoid arthritis (RA) and Crohn's disease, has been very successful (van Dullemen *et al.,* 1995; Bathon *et al.,* 2000). In the last two years, the US FDA and EU's Commission have approved etanercept and infliximab for use in the treatment of refractory RA. Etanercept is a fusion protein composed of Fc portion of IgG1 and the extracellular domain of TNF receptor II. Infliximab is a chimeric monoclonal antibody composed of murine variable and human constant regions. Both of them effectively bind to TNF and thereby inhibit its biological function. Intravenous injection of these TNF inhibitors rapidly decreased symptoms and slowed joint damage in patients more effectively than drugs such as methotrexate that are already in the market (Mikuls *et al.,* 2001). Clinical trials using combinations of these biological reagents with methotrexate have also proven to be beneficial (Kremer, 2001). As TNF certainly has beneficial roles in vivo, we would expect side effects of long term TNF blockage. Indeed, systemic inhibition of TNF activity can cause a lupus-like syndrome. About one percent of patients undergoing TNF blockage through the treatment with etanercept or infliximab develop reversible systemic lupus erythematosus, and nearly 10% develop anti-DNA antibodies (Charles *et al.,* 2000; Schaible, 2000). Since this syndrome is reversible, it did not prevent the application of etanercept and infliximab in RA patients.

Perspectives

TNF is one of the most intensively studied cytokines in the past twenty years, and this intensity will most likely not diminish in the coming years. Here we list a few possible directions of future TNF research. One emphasis of TNF research will be to translate our knowledge of this cytokine to clinical applications. As we have described above, directly targeting TNF has proven to be effective in treating some chronic inflammatory diseases. Targeting intracellular signaling molecules will be an alternative way to interfere with TNF functions. In addition, since many TNF-elicited cellular responses are cell-type dependent, interfering with the functions of different intracellular signaling molecules could provide opportunities to limit side effects of the treatment. Meanwhile, the study of the intracellular signaling pathways of TNF will continue to have great significance, since many gaps still exist in the signaling networks of this cytokine and, especially as we indicated above, the mechanism that controls the specificity of TNF signaling in different cell types has not been properly addressed at present time. Moreover, a number of TNF family members with distinguishing properties were identified in recent years and the studies of them have accelerated the research of TNF. The similarities and differences in the biological functions and the underlying mechanisms that control them among these TNF family members are also important issues to be addressed in the future.

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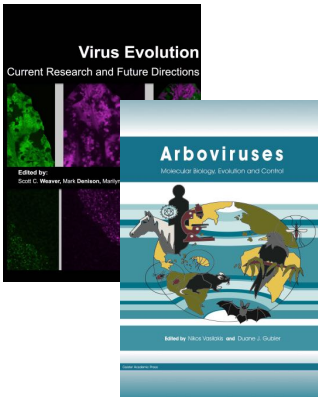

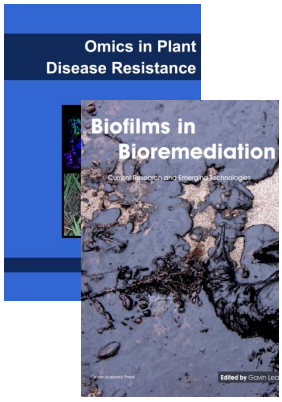

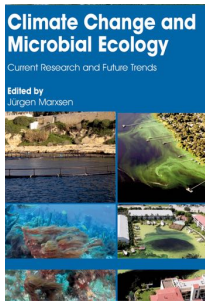

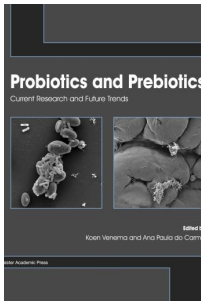

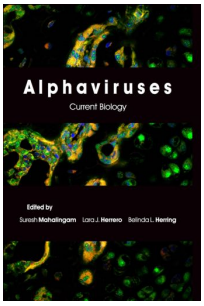

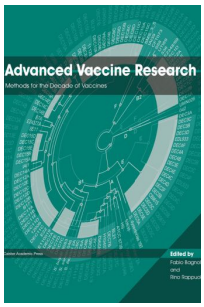

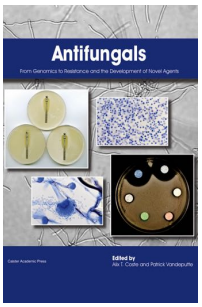

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