

## Tumor Necrosis Factor Receptor-mediated Signaling Pathways

Renu A. Heller and Martin Krönke\*

The Institute of Biochemistry and Cell Biology, Syntex Discovery Research, Palo Alto, California 94304; and

\*Institut für Medizinische Mikrobiologie und Hygiene, Technische Universität München, 81675 München, Germany

**T**UMOR necrosis factor  $\alpha$  (TNF)<sup>1</sup> and tumor necrosis factor  $\beta$  (lymphotoxin) are two cytokines produced primarily by macrophages and lymphocytes, respectively. These cytokines are recognized by the same cell surface receptors and are associated with similar biological activities. Some of the well-known TNF effects include septic shock, cytotoxicity, inflammation, and cachexia. The role of TNF in the pathology of these disease states has been inferred from studies of: (a) the effects of TNF administration in both patients and animal models; (b) the effects of neutralizing anti-TNF antibody, (Fiers, 1991; Vassalli, 1992); and (c) the phenotype of mice made deficient for the 55-kD TNF receptor by gene targeting (Pfeffer et al., 1993; Rothe et al., 1993).

Of these major responses associated with TNF, the septic shock syndrome results from the reaction of the body to parasitic, bacterial or viral infections. Following endo- or exotoxic challenge, TNF is produced by macrophages and is believed to augment their production and their phagocytic/cytotoxic actions. The effects of septic shock can be mimicked by higher doses of TNF and these effects can be prevented by the prior injection of anti-TNF antibody (Vassalli, 1992). Direct cytotoxicity has been a widely cited effect to account for the harmful effects of TNF. However, TNF kills most cells only in the presence of metabolic inhibitors like actinomycin D and cycloheximide (CHX), conditions that are nonphysiological. Notwithstanding, the phenomenon of TNF-induced cell killing has attracted great attention and cells that are killed by TNF alone have become popular model systems for TNF cytotoxicity (see Heller et al., 1992). Inflammation as a general consequence of TNF action is likely to be related to TNF stimulation of arachidonic acid (AA) release, activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) and production of pro-inflammatory mediators like prostaglandins and leukotrienes. TNF's role in

osteo and rheumatoid arthritis may also be explained by the induction of matrix degrading proteins like interstitial collagenase and stromelysin (Fiers, 1992 and see below). Lastly, cachexia is a wasting syndrome associated with chronic diseases such as cancer and tuberculosis and results in weight loss, loss of muscle and fat, and an inability to develop these tissues. At the cellular level, lipid mobilizing enzymes such as lipoprotein-lipase prevent the uptake and storage of fat, while the expression of muscle- and fat-specific genes is suppressed (Tracey and Cerami, 1993).

TNF triggers the synthesis and secretion of most if not all proinflammatory mediators including IL-1, other cytokines, and lipid mediators like platelet-activating factor, to amplify its range of actions. Any picture of TNF biology deduced from its direct action on cells, inevitably is an underestimate of its pathophysiological role in host defense reactions and disease. A comprehensive account of the cellular responses to TNF and its protective role in various disease states such as autoimmune disease, cancer, bacterial, viral, and parasitic infections is presented in recent reviews (Vassalli, 1992; Tracey and Cerami, 1993).

TNF binds to two cell surface receptors, one is ~55 kD (p55 TNFR), and the other is 70–80 kD in size (p75 TNFR). The receptors are glycoproteins with a single membrane-spanning hydrophobic segment, and they are expressed on the surface of most cells, although in different amounts. Based mostly on similarities in their extracellular domains these receptors belong to a receptor superfamily which includes the low-affinity nerve growth factor receptor and the Fas-antigen or APO-1 (Nagata, 1993; Smith et al., 1994). Most of the known TNF responses occur by the activation of the p55 TNFR (Wiegmann et al., 1992) and this notion has been confirmed in vivo. Mice deficient for the p55 TNFR were resistant to lipopolysaccharide or *Staphylococcus aureus* enterotoxic shock yet proved compromised in their ability to clear intracellular bacteria like *Listeria monocytogenes* (Pfeffer et al., 1993; Rothe et al., 1993). However, thymocyte proliferation is associated with p75-TNFR (Tartaglia et al., 1991) and cytotoxicity may be a function of p75-TNFR acting alone or together with the p55-TNFR (Heller et al., 1992; Grell et al., 1993). Several review articles on TNF receptor structure and TNF responses have been published recently (Vilcek and Lee, 1991; Fiers, 1991; Tartaglia and Goeddel, 1992; Rothe et al., 1992), this article will therefore focus on the signaling pathways activated by these receptors and how they might interact to produce the diverse actions of TNF.

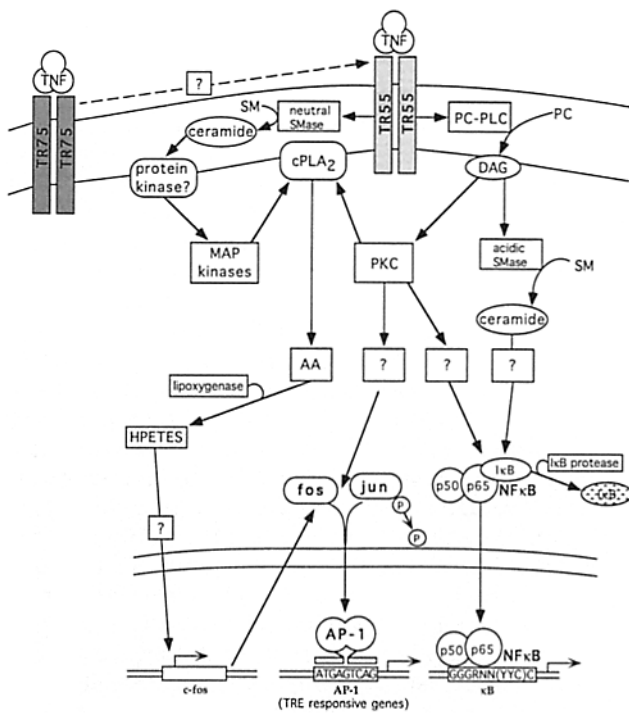
Address correspondence to R. A. Heller, The Institute of Biochemistry and Cell Biology, Syntex Discovery Research, 3401 Hillview Avenue, Palo Alto, CA 94304.

1. *Abbreviations used in this paper:* AA, arachidonic acid; CDC, cinnamyl-3,4-dihydroxy- $\alpha$ -cyano-cinnamate; CHX, cycloheximide; CO, cyclooxygenase; cPLA<sub>2</sub>, cytosolic phospholipase; LO, lipoxygenase; MAP, mitogen-activated protein; MBP, myelin basic protein; NDGA, nordihydroguaiaretic; NF $\kappa$ B, nuclear factor- $\kappa$ B; PC-PLC, phosphatidylcholine-specific phospholipase; PKC, protein kinase C; SMase, sphingomyelinase; TNF, tumor necrosis factor  $\alpha$ ; TPA, 12-*o*-tetradecanoyl phorbol 13-acetate.

A comparison of the intracellular sequences of the two TNFRs shows no obvious sequence similarity to known tyrosine- or serine/threonine kinase encoding sequences. Both receptors are rich in serine and threonine residues which may serve as sites of phosphorylation by other cellular kinases. The p55 TNFR also contains a tyrosine residue, a possible phosphorylation site for a heterologous tyrosine kinase. In the absence of any obvious catalytic function, one approach has been to screen for intermediates involved in signaling pathways of other cell surface receptors. Another approach is to use pharmacological agents to dissect TNF responses. The rapid induction by TNF of early response genes such as c-fos and c-jun or the activation of transcription factor like nuclear factor- $\kappa$ B (NF $\kappa$ B) have been used to monitor its intracellular signaling intermediates. Such efforts have identified several phospholipases, such as a phosphatidylcholine-specific phospholipase C (PC-PLC) and sphingomyelinases, both of which produce recognized second messenger molecules like DAG and ceramides, respectively. Central to the proinflammatory actions of TNF, might be the activation of cytoplasmic phospholipase A<sub>2</sub>, that generates arachidonic acid which in turn can be metabolized to leukotrienes and prostaglandins (Fig. 1).

### TNF Causes a Rapid Increase of Diacylglycerol via Hydrolysis of Phosphatidylcholine by Phospholipase C

Within minutes after binding to the p55 TNFR, TNF stimulates the production of diacylglycerol from membrane phospholipids by activation of a PLC. Under these conditions, diacylglycerol (DAG) production is accompanied by a lowering



**Figure 1.** Tumor necrosis factor receptor signaling. Proposed model for some of the pathways mediating TNF action from the cell surface to the nucleus. TR, TNFR; TRE, TPA responsive element; other abbreviations are defined in the text.

of cellular PC levels and increased phosphorylcholine content without changes in phospholipase D activity, phosphatidic acid phosphohydrolases, cellular Ca<sup>2+</sup> or inositol trisphosphate content. Such observations indicate a PC-PLC action (Schutze et al., 1991). PC hydrolysis by PC-PLC results in DAG generation without a concomitant increase in intracellular Ca<sup>2+</sup> levels. The DAG generated by PC-PLC appears central to the activation of at least two other signaling enzymes, protein kinase C (PKC), and acidic sphingomyelinase (SMase). Since Ca<sup>2+</sup> mobilization is not an accompanying feature, the DAG responsive PKC most likely belongs to a Ca<sup>2+</sup>-independent PKC isotype. However, a particular PKC isozyme activated by TNF remains to be identified particularly since PKC does mediate a number of TNF actions, most notably, the induction of JUN and FOS proteins that are components of the AP-1 transcription factor (Brenner et al., 1989).

### DAG Generated by a PC-PLC Activates an Acidic SMase

The DAG generated by the TNF-responsive PC-PLC leads to the activation of a C type phospholipase, the acidic SMase (Schutze et al., 1992). Of the two types of SMases found in cells, one, the acidic SMase, has a pH optimum in the acid range ~pH 5.0 and the other, the neutral SMase, ~pH 7.0. Diacylglycerols are known activators of protein kinase C, but by stimulating the activity of an acid SMase they cause a breakdown of sphingomyelin to producer ceramides. Although the phorbol esters, 12-*o*-tetradecanoyl phorbol 13-acetate (TPA), and phorbol 12,13-dibutyrate activate PKC, they do not alter SMase activity. The action of diacylglycerols on SMase is reported to be independent of its effects on PKC. In cells where PKC action is down regulated with phorbol esters, DAG can still increase SMase activity (Kolesnick, 1987).

The products of SMase hydrolysis of sphingomyelin are phosphorylcholine and ceramide. The latter acts as the second messenger mediating a number of cellular responses (Hannun, 1994). Activation by DAG of an acidic SMase, which presumably resides in acidic cellular compartments like lysosomes and endosomes, may occur via co-internalization of DAG with TNF/TNFR complexes, if PC-PLC action occurs in close vicinity to membrane receptors. Endosomal vesicles are rapidly acidified and provide optimal conditions for acidic SMase activity. Clearly, the significance of TNFR internalization for acidic SMase action needs further investigation.

### TNF Activates a Membrane-bound Neutral Sphingomyelinase

A neutral SMase has been demonstrated to play a role in TNF signaling (Kim et al., 1991). This TNF responsive neutral SMase hydrolyses membrane sphingomyelin to ceramide which activates a membrane-bound 97-kD kinase termed the ceramide-activated protein kinase (Liu et al., 1994). This enzyme phosphorylates peptide substrates containing the motif X-Ser/Thr-Pro-X, found in the EGFR and myelin basic protein (MBP), a motif that is a consensus sequence for the action of mitogen-activated protein (MAP) kinases (or extracellular signal-regulated kinases). The 97-kD kinase appears to be a member of this class of proline-

directed kinases but distinct from them in its membrane association and slightly different substrate preference. TNF reportedly also activates the 42- and 44-kD MAP kinases and increases their tyrosine phosphorylation as well as their ability to phosphorylate the standard substrate MBP (Vietor et al., 1993). The phosphorylation and activation of the 42-kD MAP kinase by TNF parallels an increase in cellular sphingomyelin hydrolysis and the addition of both bacterial SMase or cell-permeable ceramide analogs activate the p42 MAP kinase in a time-dependent manner, similar to TNF (Raines et al., 1993). Therefore, both the ceramide-activated kinase and the MAP kinases are activated by TNF and ceramides.

The distinction between acidic and neutral SMase seems both noteworthy and indisputable. These two C type phospholipases could exert functions, quite distinct, by virtue of their different compartments of localization. Due to its hydrophobicity, ceramide is not readily distributed within the cell so that ceramide signaling is likely to be confined to its site of production in the membrane. While ceramide produced by neutral SMase at or within the plasma membrane seems to trigger a plasma membrane-associated kinase (Liu et al., 1994), ceramide produced by the acidic SMase is associated with the endolysosomal compartments and therefore may have different consequences.

#### ***TNF Increases Phospholipase A<sub>2</sub> Activity***

AA release by TNF occurs in number of cell lines. Indeed, TNF mediates increases in both the secreted 14 kD (Oka and Arita, 1991) and the cellular 85-kD phospholipases (Hoeck et al., 1993), enzymes known for the production of AA by cells. Although the 14-kD sPLA<sub>2</sub> may not be specific for release of AA from the *sn*-2 position of membrane phospholipids, the 85-kD cPLA<sub>2</sub> is selective for AA (Lin et al., 1992) and provides a source for the production of biologically active lipid mediators such as prostaglandins and leukotrienes. This action could account for the proinflammatory properties of TNF. An earlier action of TNF on cPLA<sub>2</sub> is a phosphorylation of the enzyme, followed by a sustained production of new protein. TNF-induced phosphorylation of cPLA<sub>2</sub> causes a small increase in activity, but with Ca<sup>2+</sup> it produces a marked stimulation. In combination with Ca<sup>2+</sup> mobilizing agonists at sites of inflammation, the released AA could serve both a regulatory function and have a proinflammatory action. The effect of the antiinflammatory glucocorticoid dexamethasone is likely to be due to its inhibition of TNF-induced cPLA<sub>2</sub> production because it does not affect cPLA<sub>2</sub> activation by phosphorylation.

TNF-triggered release of AA provides a source for the production of eicosanoids. Although the pathway from the TNF receptor to cPLA<sub>2</sub> activation is not completely clear, the SMase/ceramide cycle possibly offers a link. cPLA<sub>2</sub> is phosphorylated on serine residues by protein kinase C and the p42 MAP kinase, and the MAP-kinase mediated phosphorylation is essential for receptor-induced cPLA<sub>2</sub> activation (Lin et al., 1993; Nemenhoff et al., 1993). Growth factors have been reported to induce MAP kinase activity through a cascade of signal transfer reactions including *ras* and *raf* proteins, and the serine/threonine MAP kinase kinases, which regulate the activity of the MAP kinase (reviewed by Thomas, 1992). Since the TNF/SMase initiated protein phosphorylation cascade stimulates a MAP kinase

pathway and the p42 MAP kinases activate cPLA<sub>2</sub>, it is conceivable that the ceramide-activated protein kinase is an intermediate in the MAP kinase action pathway, particularly if c-Raf could serve as a substrate for ceramide activated protein kinase (Liu et al., 1994).

#### ***The Cytotoxic Action of TNF and its Signaling Intermediates***

One of the prominent features of TNF is its ability to kill several cell lines. The loss of viability upon TNF treatment of certain cells is accompanied by sphingomyelin hydrolysis and ceramide production. In these cells both TNF and ceramides cause DNA fragmentation by a process identified as apoptosis. Thus, it appears that ceramides perhaps through their activation of kinases, phosphatases, inhibition of protein secretion or as yet unidentified processes could mediate the cytotoxic actions of TNF (Jarvis et al., 1994; Hannun, 1994). Apoptotic cell death is also caused by the Fas-antigen, a receptor structurally homologous to the TNFRs and is triggered by the Fas-ligand, a transmembrane protein structurally similar to TNF. Furthermore, the Fas-antigen and the p55 TNFR have a 24% sequence identity in a region of their cytoplasmic domains that is essential for cell death (Nagata, 1993). Whether these two related receptors use the ceramide cascade for signaling cell death remains an intriguing possibility. As discussed above, the ceramide cascade via its activation of MAP kinases is a reasonable pathway for TNF activation of the AA selective cPLA<sub>2</sub>. cPLA<sub>2</sub> has also been invoked in TNF cytotoxicity. Cells resistant to TNF cytotoxicity have lower levels of this enzyme while introduction of this activity into enzyme-deficient cells restores their sensitivity (Hayakawa et al., 1993). One mechanism for TNF cytotoxicity by cPLA<sub>2</sub> may be the generation of free radicals and lipid peroxides via the production of eicosanoids and their metabolites.

#### ***Activation of the Transcription Factor NFκB***

NFκB activation is a cellular response that links cell surface receptor activation to transcriptional events in the nucleus. Activation of NFκB involves the release of the inhibitory subunit IκB from a cytoplasmic complex containing the DNA-binding subunits p50 and Rel-A (formerly p65). This p50/Rel-A heterodimer is then translocated to the nucleus, where it directly binds to its DNA recognition sequence.

Exogenous PC-PLC or synthetic DAGs induce NFκB in both intact and in permeabilized Jurkat cells. It is tempting to speculate that DAG-mediated NFκB induction occurs through activation of PKC, but this mechanism has never been shown to occur in intact cells. Rather, in this case a DAG-responsive acidic SMase seems to mediate TNF activation of NF-κB since addition of exogenous SMase or ceramide could mimic this effect (Schutze et al., 1992). Ceramide production also occurs by a direct activation of neutral SMase without an involvement of PC-PLC and DAG production and ceramide generated by neutral SMase at the membrane has also been reported to activate NF-κB (Yang et al., 1993). TNF activation of NF-κB in at least BALB/c 3T3 cells has been shown to depend upon Ras and Raf activities (Finco and Baldwin, 1993) and if indeed, ceramides activate Raf proteins, this pathway could explain their activation of NF-κB. The differences in the type and mode of SMase activation

may reflect cell type- or tissue-specific responses. Recent studies indicate that rapid proteolytic degradation of I $\kappa$ B- $\alpha$  by a chymotrypsin-like protease (Henkel et al., 1993) is induced by TNF (Sun et al., 1993; Brown et al., 1993) and recently, I $\kappa$ B degradation was induced by exogenous SMase or ceramide in a cell-free system (Machleidt et al., 1994). Thus, ceramide may induce phosphorylation or another modification of the I $\kappa$ B protein, to make the inhibitor more susceptible to degradation by a constitutive protease or, alternatively ceramide may activate a protease that selectively degrades I $\kappa$ B.

### ***AA Metabolites Mediate TNF Induction of c-fos, As Well As Cell Killing in the Presence of CHX***

A role of AA in TNF cytotoxicity is indicated by experiments on murine TA1 cells. In these cells, TNF alone stimulates the release of AA and causes cell death when combined with CHX. A variant of these cells, TA1-R6, is resistant to TNF plus CHX killing and has reduced levels of AA in its phospholipid pools due to a defect in  $\Delta$ 6-desaturase, an enzyme of the AA biosynthetic pathway. Cytotoxicity is restored in the TA1-R6 cells by AA supplementation (Reid et al., 1991). Other roles of AA and its metabolites in TNF-mediated responses relate to the induction of c-jun and c-fos mRNA. Their protein products are components of the AP-1 transcription factor complex and specific AP-1 recognition sequence motifs are found in many TNF inducible genes, e.g., the matrix metalloproteinases, collagenase (Brenner et al., 1989), and stromelysin (Lee et al., 1990). In TA1 cells, TNF increases AA release and induces both c-fos and c-jun (Haliday et al., 1991). The addition of only AA or its hydroperoxides can also induce c-fos but not c-jun. AA is metabolized by both the lipoxygenase (LO) and cyclooxygenase (CO; prostaglandin endoperoxide synthase) enzyme systems and even though TNF induces the expression of CO (Jones et al., 1993) the induction of c-fos by TNF was blocked only by LO inhibitors. Nordihydroguaiaretic acid (NDGA) an inhibitor of 5-, 12-, and 15-LO, MK886 (a 5-lipoxygenase inhibitor), or BW755C (an inhibitor of both LO and CO activities) were all effective. Participation of the lipoxygenase products of AA has also been noted in TNF plus CHX killing because this cytotoxic action on TA1, HeLa and NIH-3T3 cells could be blocked by NDGA, MK886, and also by cinnamyl-3,4-dihydroxy- $\alpha$ -cyano-cinnamate (CDC) an inhibitor of all three 5-, 12-, and 15-lipoxygenases (Chang et al., 1992). Because some of these inhibitors have well-known antioxidant activity, the possibility remains that their ability to block eicosanoid biosynthesis may be less relevant than their antioxidant action in preventing these TNF-induced responses.

### ***The Lipoxygenase Pathway***

The metabolites of 5-, 12- and 15-lipoxygenase action have a wide spectrum of potent biological actions (Samuelsson et al., 1987). Their production by oxidation reactions of arachidonic acid generates compounds like hydroperoxy-, and hydroxy-eicosatetraenoic acids, dihydroxy-, epoxy-eicosatrienoic acids, and leukotrienes (HPETES, HETES, DHET, EET, and LT). Lipoxygenase metabolites have been implicated in TNF responses such as TNF cytotoxicity, induction of the transcription factor c-fos, and the mitochondrial superoxide radical scavenging enzyme manganous superoxide

dismutase or MnSOD (Haliday et al., 1991; Chang et al., 1992). TNF treatment of TA1 cells increases the level of the leukotriene 5-HETE and supplementation of hydroperoxy-lipoxygenase products, 5-, 12-, and 15-HPETES, causes c-fos induction. In contrast, the leukotriene metabolites LTB $_4$ , LTC $_4$ , LTD $_4$ , or HETES do not produce any increase in c-fos expression (Haliday et al., 1991), indicating specific functions of particular intermediates.

The HPETES can be converted into lipoxins and other metabolites and these lipid peroxidation products are a source of reactive-free radicals (Samuelsson et al., 1987). Oxygen radical generation by TNF (Meier et al., 1989; Zimmerman et al., 1989) may provide the essential cofactors for the induction of c-fos, MnSOD, and TNF-cytotoxicity (Yamauchi et al., 1989). Thiols and reactive oxygen species can induce the transcription factor NF- $\kappa$ B, and TNF induction of NF- $\kappa$ B is blocked by radical scavenging thiol agents (Schreck et al., 1991). TNF cytotoxicity can also be significantly reduced by radical scavenging thiol agents such as, N-acetyl-L-cysteine and pyrrolidine-dithiocarbamate to provide support for the idea that free radicals may be responsible for cell death (Chang et al., 1992). TNF actions caused by free radical production may occur via lipoxygenase metabolites but the precise pathways for the free radical generation and their transfer to mediate the diverse functions of TNF are currently not clear.

In summary, there is increasing evidence that TNF signaling operates via the activation of a SMase-mediated pathway through the generation of ceramides as second messengers. This pathway stimulates MAP-kinase-related serine/threonine kinases to cause the activation of cPLA $_2$ . On the other hand, TNF also activates a PC-PLC to generate DAG in the absence of Ca $^{2+}$ . PC-PLC-derived DAG can stimulate an endosomal acidic SMase that plays a major role in the activation of NF $\kappa$ B. In addition, DAG also mediates activation of a TNF-responsive PKC that likely triggers the activation of protooncogenes like c-myc, c-jun, and c-fos. An alternative pathway of c-fos induction involves AA metabolites generated by the combined actions of cPLA $_2$  and lipoxygenase. The active components of this pathway may be reactive oxygen or hydroxyl radicals eventually responsible for at least the induction of c-fos, MnSOD, and perhaps the cytotoxic effects of TNF. The proinflammatory action of TNF can be further explained by the induction of cPLA $_2$ , lipoxygenases, and prostaglandin endoperoxide synthase, that generate leukotrienes and prostaglandins. Moreover, c-fos and c-jun induce matrix metalloproteinases that are involved in inflammatory disease such as arthritis where TNF is a major mediator (Keffer et al., 1991).

Over the past five years, some of the participants in TNF signaling have been identified, but much work is needed to define their interactions. One major open question in TNF action is the role of the p75 TNFR. Apparently, p55 and p75 TNFR both have unique functions in cell proliferation and cytotoxicity (Tartaglia et al., 1991; Heller et al., 1992; Ikegami et al., 1993; Grell et al., 1993). TNF exhibits a wide variety of functions and the pathways mediating the actions of the two TNF receptors may turn out to be equally diverse.

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