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Invited Review

Signaling pathways mediated by tumor necrosis factor α

K.G. Leong[1] and A. Karsan[1,2]

Departments of [1]Experimental Medicine and [2]Pathology and Laboratory Medicine, University of British Columbia, Vancouver, British Columbia, Canada

Summary. Tumor necrosis factor α (TNFα) has been shown to trigger many signaling pathways. Following oligomerization by TNFα, the receptors TNF-RI and TNF-RII associate with adapter molecules via specific protein-protein interactions. The subsequent recruitment of downstream molecules to the receptor complex enables propagation of the TNFα signal. Two cellular responses to TNFα have been well documented, the induction of cell death and the activation of gene transcription for cell survival. TNFα-induced apoptosis involves the activation of caspase cascades, which culminate in the cleavage of specific cellular substrates to effect cell death. TNFα has also been implicated in various caspase-independent cell death processes. Two transcription factors activated by TNFα are nuclear factor κB (NFκB) and activating protein 1 (AP-1). Pathways that promote the activation of these transcription factors involve signaling molecules such as kinases, phospholipases, and sphingomyelinases. In addition to increased survival (anti-apoptotic) gene expression, NFκB and AP-1 also induce the expression of genes involved in inflammation, cell growth, and signal regulation. The past decade has witnessed the identification of numerous signaling intermediates implicated in TNFα cellular responses. This article reviews the molecular mechanisms of TNFα signal transduction. In particular, pathways involved in cell death and transcription factor activation are discussed.

Key words: Tumor necrosis factor, Signal transduction, Apoptosis, NFκB, AP-1

Abbreviations

AIF: apoptosis inducing factor; AP-1: activating protein 1; ASK1: apoptosis signal-regulating kinase 1; ASMase: acidic sphingomyelinase; CARD: caspase activating recruitment domain; CARDIAK: CARD containing ICE-associated kinase; c-IAP: cellular inhibitor of apoptosis; CLAP: CARD-like apoptotic protein; CRD: cysteine-rich domain; DED: death effector domain; ERK: extracellular signal regulated kinase; FADD: Fas associated death domain; FAN: factor associated with NSMase; GCK(R): germinal center kinase (related); ICE: interleukin-1ß converting enzyme; IRAK: interleukin-1 receptor-associated kinase; JNK: c-Jun N-terminal kinase; MAPK: mitogen activated protein kinase; NIK: NFκB-inducing kinase; NFκB: nuclear factor κB; NSD: NSMase domain; NSMase: neutral sphingomyelinase; RAIDD: RIP-associated ICH-1 homologous death domain; RIP: receptor interacting protein; SODD: silencer of death domains; TACE: TNFα converting enzyme; TANK: TRAF-associated NFκB activator; TNFα: tumor necrosis factor alpha; TNF-R: TNF receptor; TRADD: TNF receptor associated death domain; TRAF: TNF receptor associated factor

Offprint requests to: Aly Karsan, Department of Pathology and Laboratory Medicine, UBC McDonald Research Laboratories, St. Paul's Hospital, Room B292, 1081 Burrard St., Vancouver, BC. Canada V6Z 1Y6. e-mail: akarsan@mrl.ubc.ca

Introduction

The effects of tumor necrosis factor α (TNFα) have been implicated in numerous physiological and pathological conditions. Until recently, knowledge concerning the global effects of TNFα far outweighed that of its cellular effects. Over the last few years, key players involved in mediating the cellular effects of TNFα have been identified. We now know that TNFα activates cell death pathways as well as transcription factors that signal survival pathways. This review will focus on the TNFα signaling pathways that enable cell death and transcription factor activation to occur.

The role of tumor necrosis factor α in health and disease

In 1893, a New York surgeon by the name of W.B. Coley was the first to document the effects of TNFα (Orlinick and Chao, 1998), although TNFα itself had not yet been identified. In 1975, an endotoxin-induced factor isolated from macrophages was discovered (Carswell et

al., 1975). This factor was named tumor necrosis factor alpha (TNFα), because of its ability to kill tumor cells *in vitro* and to cause hemorrhagic necrosis of solid tumors *in vivo*. Concurrent with the discovery of TNFα, another macrophage-derived factor known as cachectin was identified (Beutler et al., 1985b). Cachectin is responsible for the development of cachexia, a wasting syndrome commonly associated with chronic disease states (Beutler and Cerami, 1987; Tracey and Cerami, 1993). Such diseases may include chronic parasitic infection, acquired immunodeficiency syndrome (AIDS), malaria, tuberculosis, and cancer. Cachexia is characterized by the prevention of fat storage and the suppression of muscle- and fat-specific gene expression leading to weight loss (Tracey and Cerami, 1993). Sequence comparison between TNFα and cachectin revealed the two factors to be identical (Beutler et al., 1985a). Since then, TNFα has been purified and crystallized, and its tertiary and quaternary structures characterized.

TNFα is a pleiotropic cytokine involved in a wide variety of physiological conditions. Endogenous TNFα is an important mediator of natural immunity, and has been shown to be essential for the development of a successful response to microbial infection (Echtenacher et al., 1990). Its production at sites of bacterial or viral invasion serves to recruit and activate defense mechanisms. TNFα has also been implicated in inflammatory conditions, by inducing the synthesis of proinflammatory mediators. Other diverse responses influenced by TNFα include cell proliferation and differentiation (Heller and Kronke, 1994), cell death (Kerr et al., 1972; Famularo et al., 1994; Ware et al., 1996), neuroprotection (Cheng et al., 1994), and neurotransmission (Tancredi et al., 1992).

The strength and duration of TNFα expression greatly influences the effect of this cytokine. When produced inappropriately, pathological conditions may ensue. The development of septic shock (Kramer et al., 1995) and autoimmune diseases such as rheumatoid arthritis (Elliott et al., 1994; Feldmann et al., 1995; Taupin et al., 1997) have been correlated with high systemic levels of TNFα. In addition, sustained activation of TNFα is associated with multiple organ failure (Landow and Andersen, 1994), multiple sclerosis (Selmaj et al., 1991; Raine, 1995), cardiac dysfunction (Meldrum, 1998), atherosclerosis (Rus et al., 1991), ischemia-reperfusion injury (Meldrum and Donnahoo, 1999), insulin resistance (Hotamisligil et al., 1995; Peraldi and Spiegelman, 1998), and inflammatory bowel diseases (Brynskov et al., 1994). Unraveling the molecular mechanisms of TNFα action may contribute to the development of novel and effective therapies for treatment of TNFα-related diseases.

The TNF ligand family

TNFα is the prototypical member of a large family of ligands designated the TNF ligand family. The original family consisted of two members: the macrophage-derived TNFα and the T lymphocyte-derived LT-α (lymphotoxin-α/TNFß). Additional members have since been added to the family. These include FasL/CD95L, CD40L, CD27L, CD30L, LT-ß, OX40L, 4-1BBL, Apo2L/TRAIL, Apo3L/TWEAK, RANKL/TRANCE/ODF/OPGL, and GITRL (Smith and Ross, 1993; Armitage, 1994; Gruss and Dower, 1995; Nocentini et al., 1997; Gurney et al., 1999; Takahashi et al., 1999). With the exception of LT-α, all of the other family members are type II membrane proteins. Characteristics include a C-terminal extracellular domain, a single transmembrane domain, followed by a short N-terminal cytoplasmic domain. All members share 20-25% homology in a 150 amino acid stretch in the extracellular domain, required for binding to their cognate receptors (reviewed in Baker and Reddy, 1996; Orlinick and Chao, 1998).

The human genome contains a single copy of the TNFα gene, located within the major histocompatibility complex (MHC) locus on chromosome six (Nedwin et al., 1985a; Browning et al., 1993). In addition to macrophages/monocytes, TNFα is also produced by lymphocytes, mast cells, fibroblasts, and hepatocytes (Vassalli, 1992; Vandenabeele et al., 1995; Wallach, 1997; Zhang et al., 1997; Yan et al., 1999). Secretion from macrophages usually occurs in response to an inflammatory stimulus such as lipopolysaccharide (LPS) (Pauli, 1994). Activation of antigen receptors on T lymphocytes can induce TNFα production, whereas aggregation of high-affinity IgE receptors on mast cells is sufficient to trigger cytokine secretion (Zhang et al., 1997). The fact that activated mast cells secrete TNFα implicates TNFα as a major player in immediate inflammatory responses (Thomas et al., 1996). Production of TNFα can occur within 2 to 3 hours post induction, with maximum synthesis achieved after 48 hours (Nedwin et al., 1985b). The rate of production has been shown to increase by a factor of several thousand in macrophages activated by LPS (Bazzoni and Beutler, 1996). Enhanced TNFα synthesis functions as a distress signal both in an autocrine and paracrine fashion, triggering protective mechanisms designed to cope with infection.

TNFα is initially produced as a 26 kDa membrane-bound proform (proTNFα) (Perez et al., 1990). The extracellular domain of proTNFα contains a membrane-proximal metalloprotease cleavage site (Blobel, 1997). Specifically, a metalloprotease of the disintegrin family named TACE (TNFα converting enzyme, also known as ADAM-17) is responsible for cleaving proTNFα to the 17 kDa soluble TNFα form (Black et al., 1997; Moss et al., 1997). Both forms of TNFα are active and capable of binding to their cellular receptors, although distinct roles have been demonstrated for the individual forms of TNFα (Perez et al., 1990).

The TNF receptor superfamily

Members of the TNF ligand family interact with their cognate receptors, all of which belong to a group of

receptors collectively known as the TNF receptor superfamily. The criteria for classifying cell surface receptors are based on structural and mechanistic features. With respect to the TNF receptor family, all members are type I membrane proteins with their C-terminal domains located in the interior of the cell. Furthermore, all members contain highly conserved cysteine-rich domains (CRDs) in their extracellular region (Beutler and van Huffel, 1994). Although the number of CRDs within different receptors varies from two to six, each CRD contains six cysteine residues interspersed within a 40 amino acid stretch (Smith et al., 1994; Ashkenazi and Dixit, 1998). The TNF receptor superfamily consists of TNF-RI (TNF-R55, p55-TNFR, CD120a), TNF-RII (TNF-R75, p75-TNFR, CD120b), TNF-RIII (TNF-RP), Fas (Apo1), OX-40, 4-1BB, CD27, CD30, CD40, poxvirus gene products PV-T2 and PV-A53R, p75 NGFR, TRAMP (Apo3, DR3, LARD, Ws1), CAR1, TRAIL-RI (DR4), TRAIL-R2 (Apo2, DR5, KILLER, TRICK2), DR6, and RANK (Itoh and Nagata, 1993; Smith and Ross, 1993; Armitage, 1994; Gruss and Dower, 1995; Brojatsch et al., 1996; Kitson et al., 1996; Pan et al., 1997a,b; Ashkenazi and Dixit, 1998). In addition, TRAIL has at least three decoy receptors, DcR1 (TRAIL-R3, TRID), DcR2 (TRAIL-R4), and DcR3 (Ashkenazi and Dixit, 1999).

TNFα interacts with two receptors, TNF-RI and TNF-RII (Loetscher et al., 1990; Nophar et al., 1990; Schall et al., 1990; Smith et al., 1990). Both receptors can be found on the surface of most cells (Vandenabeele et al., 1995), although they are expressed in different amounts. On a per cell basis, the number of TNF-RII receptors far surpasses that of TNF-RI (Fiers et al., 1995). The fact that TNF-RI expression is constitutive and non-inducible and that TNF-RII expression is inducible may account for the varying expression levels (Newton and Decicco, 1999). Another difference between these receptors involves their localization within the cell. During steady-state conditions, the majority of TNF-RI molecules are found in the perinuclear Golgi complex (Jones et al., 1999). In contrast, most TNF-RII molecules are expressed on the cell surface. TNF-RI and TNF-RII molecules localized to the cell membrane exist as glycoproteins. Their extracellular domains contain four CRDs, all of which are essential for efficient ligand binding (Marsters et al., 1992). Both receptors contain a single transmembrane domain, but there is no homology evident in their cytoplasmic domains (Tartaglia and Goeddel, 1992). In addition, neither TNF-RI nor TNF-RII contains intrinsic tyrosine or serine/threonine kinase domains. Differences between their cytoplasmic domains account for the different signaling pathways initiated by the two receptors.

Ligand-receptor interactions

Membrane-bound and soluble forms of TNFα are both capable of forming homotrimers, due to the presence of a ß-sheet sandwich structural motif within each monomer (Browning et al., 1993). Only trimeric TNFα can interact with TNF-RI and TNF-RII receptors. The interfaces between the TNFα subunits comprising the trimer are required for receptor binding (Banner et al., 1993). With respect to both receptors, structural studies have demonstrated a role for the CRDs present in the extracellular domain for ligand binding (Beutler and van Huffel, 1994). Trimerization of either TNF-RI or TNF-RII by trimeric TNFα triggers a cellular response.

TNF-RI and TNF-RII have similar affinities for soluble TNFα (Grell et al., 1995). Dissociation rates, however, vary between the two receptors. TNFα bound to TNF-RII has a fast off rate relative to TNFα bound to TNF-RI (Tartaglia et al., 1993b). Because concentrations of TNFα in normal physiological conditions are low (Adolf et al., 1994), TNFα should favor the activation of TNF-RI. This may explain why TNF-RI is involved in a majority of the actions mediated by TNFα. Membrane-bound TNFα, on the other hand, exhibits a greater affinity for TNF-RII (Grell, 1995; Grell et al., 1995). Therefore, TNF-RII may be important during local inflammatory reactions that involve intercellular contacts.

Binding of TNFα to either TNF-RI or TNF-RII has been shown to induce shedding of the receptor extracellular domain. A cleavage site has been localized to a small region flanked by the CRD and transmembrane domains (Brakebusch et al., 1994). Shedding represents a mechanism for regulating the surface expression of both receptors. A second regulatory mechanism is also established upon receptor cleavage. Because the soluble forms of the receptors retain their ability to bind TNFα (Gatanaga et al., 1990), the shed extracellular domains function as inhibitors of TNFα and downregulate the bioavailability of the cytokine (Van Zee et al., 1992; van der Poll et al., 1995; Aderka, 1996). Binding of TNFα to either receptor can also lead to internalization of the TNFα-receptor complex, followed by subsequent degradation of the ligand (Tsujimoto et al., 1985; Boldin et al., 1995a; Bradley et al., 1995). Hence the ability of TNFα to bind to receptor and initiate intracellular signaling mechanisms is strictly regulated.

Signal transduction by TNFα

Numerous signaling pathways have been characterized for TNFα in multiple subcellular compartments, which include the plasma membrane, cytosol, endosomes, mitochondria, and nucleus. Among the many diverse cellular effects of TNFα, two effects have garnered the most attention: (1) the initiation of cell death, and (2) the activation of transcription factors leading to the expression of genes, some of which promote cell survival.

Cell death pathways activated by TNFα

The demise of a cell can occur by two mechanisms: necrosis and/or apoptosis. TNFα can elicit both

mechanisms of cell death. The specific death pathway activated by TNFα in a cell is dependent on such variables as cell type, TNFα dosage, and the local microenvironment. Necrosis occurs when a cell is subjected to overwhelming external injury, either from physical stress or toxic agents (Kerr et al., 1972; Majno and Joris, 1995). Necrotic cell death involves activation of the complement system or stimulation of granule release from cytotoxic T lymphocytes. In both situations, the integrity of the target cell lipid bilayer is challenged. An osmotic imbalance is created that leads to cell swelling, followed by a sudden collapse of the cell (Grooten et al., 1993). Cellular contents are then released into the cell surroundings. Apoptosis is a form of programmed cell death where the cell actively participates in its own destruction. Apoptotic death plays an important role in the regulation of development and growth (Tomei and Cope, 1994), as well as the elimination of unwanted cells (Hebert et al., 1996; Jacobson et al., 1997). Characteristics of apoptosis include chromatin condensation, DNA degradation into oligonucleosomal fragments, and membrane blebbing (Duvall and Wyllie, 1986). An important difference between apoptosis and necrosis is the confinement of cellular contents within membrane-bound particles during apoptosis. These particles can then be phagocytosed without the risk of intracellular enzyme leakage (Majno and Joris, 1995; Hart et al., 1996).

In most cell types, induction of apoptosis by TNFα cannot occur without inhibiting the expression of new genes (Itoh and Nagata, 1993). This can be achieved through the addition of RNA synthesis inhibitors such as actinomycin D, or protein synthesis inhibitors such as cycloheximide. The inhibition of new gene expression is essential because TNFα has been shown to induce the expression of anti-apoptotic proteins (Karsan, 1998; Malek et al., 1998). In general, activation of the TNFα survival pathway inhibits activation of the death pathway (Natoli et al., 1998). In transformed cells or virally-infected cells, however, TNFα alone is capable of inducing cell death (Beyaert and Fiers, 1994). The increased sensitivity of these cells to TNFα may be due to the lack of a transient protective factor that renders normal cells resistant to TNFα-induced cytotoxicity.

The role of TNF-RI in apoptosis

Apoptotic signaling is primarily mediated by TNF-

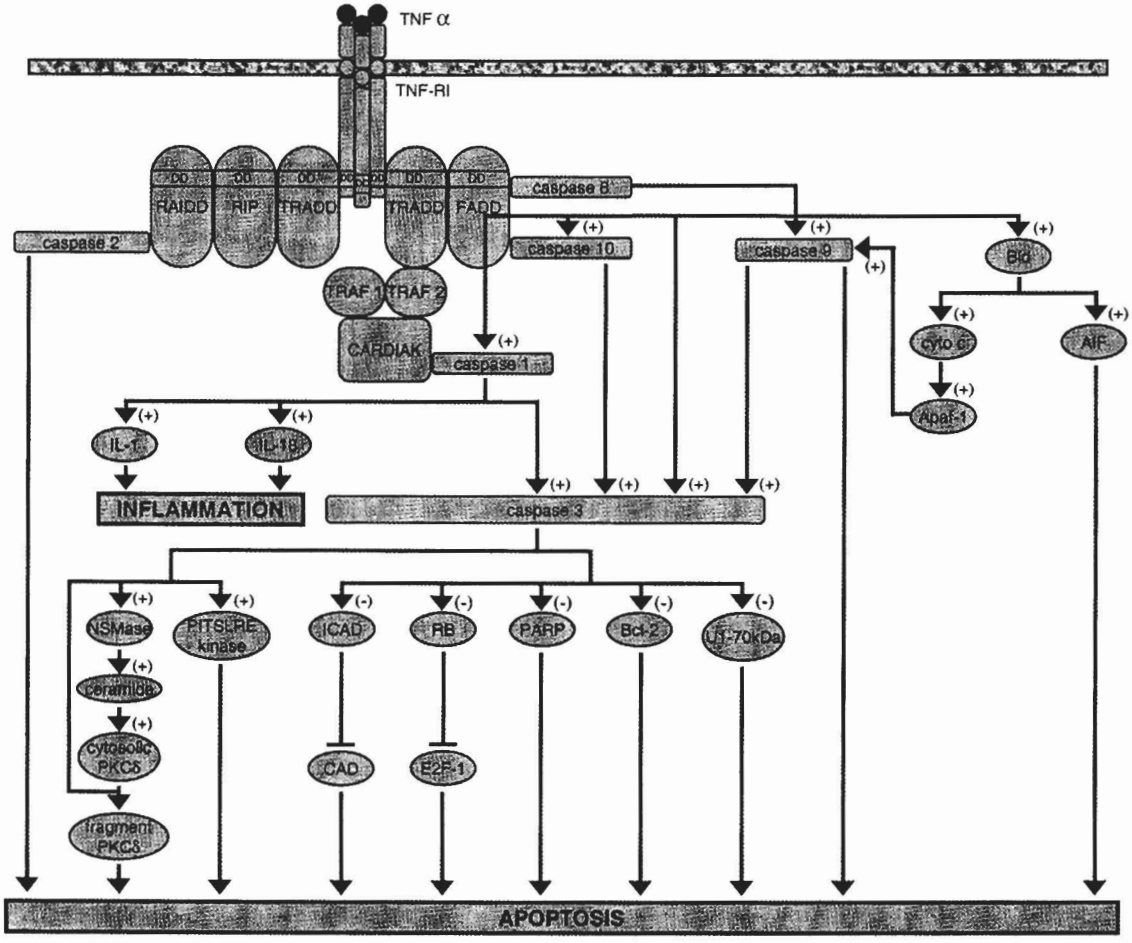

Fig. 1. Caspase activation pathways. These signal transduction pathways are specific for TNF-RI. Trimerization of TNF-RI by TNFα initiates the signaling cascade. The net effect of caspase activation is apoptotic cell death. Refer to the text for abbreviations of signaling components.

RI (Tartaglia et al., 1993c). Because TNF-RI is devoid of catalytic activity, it utilizes cellular proteins as adapter molecules for propagating the death signal. The cytoplasmic domain of TNF-RI contains a region termed the death domain, which has been shown to be essential for TNFα-induced cytotoxicity (Tartaglia et al., 1993a). The death domain folds into a series of antiparallel amphipathic α-helices with exposed charged residues, which functions as a dimerization motif (Huang et al., 1996). Associations between various death domain-containing proteins are facilitated by this motif, thus enabling propagation of the death signal within the cell. Subsequent activation of protease cascades leads to the apoptotic demise of the cell. The proteases involved belong to a family of aspartyl-directed cysteine proteases termed caspases.

The caspase activation pathways. In the basal state without bound TNFα, a protein named SODD (silencer of death domains) is associated with the death domain of TNF-RI and prevents signal transduction (Jiang et al., 1999). Upon binding trimeric TNFα, however, SODD rapidly dissociates and enables aggregation of the TNF-RI death domains to occur. A death domain-containing protein named TRADD (TNF receptor associated death domain), which exhibits high affinity for the aggregated TNF-RI death domains, is recruited to the receptor complex (Hsu et al., 1995, 1996b) (Fig. 1). TNF-RI - TRADD complexes are relatively stable. Similar to TNF-RI, TRADD is devoid of enzymatic activity and thus functions as an adapter protein. The death domain of TRADD directly interacts with the death domain of FADD (Fas associated death domain, also known as MORT1) (Boldin et al., 1995b; Chinnaiyan et al., 1995). Although TRADD-FADD complexes are stable, TNF-RI-TRADD-FADD multimeric complexes require additional proteins for stability (Hsu et al., 1996b). The C-terminal death domain of FADD does not play a role in the further propagation of the death signal; instead, an N-terminal domain termed the DED (death effector domain) is required (Boldin et al., 1995b; Chinnaiyan et al., 1995). The interaction between TRADD and FADD exposes the DED of FADD (Muzio et al., 1996).

A domain analogous to the DED, called the CARD domain (caspase activating recruitment domain), is found in the prodomain of various upstream caspases (Hofmann et al., 1997). Procaspase 8 (FLICE/MACH/Mch5) contains two DED (CARD) domains at its N-terminus (Boldin et al., 1996; Muzio et al., 1996). FADD and procaspase 8 bind through their DED (CARD) domains. Recruitment of procaspase 8 to the receptor signaling complex results in oligomerization of procaspase 8, thereby enabling activation of its mild autoproteolytic activity (Yeh et al., 1998; Zhang et al., 1998). The induced proximity of two or more procaspase 8 molecules results in the excision of the procaspase 8 prodomain (Muzio et al., 1998), followed by further cleavage to fragments of approximately 20 kDa and 10 kDa. These fragments form a dimer, and two dimers combine to form a tetramer, thus yielding an active caspase.

Active caspase 8 is capable of activating procaspase 9 (Pan et al., 1998). Processing of procaspase 9 to its active form, however, may not be a direct action of caspase 8. Instead, a signaling pathway involving the mitochondria is involved. Caspase 8 cleaves the cytosolic protein Bid, releasing a C-terminal fragment (Li et al., 1998; Luo et al., 1998). Translocation of this Bid fragment to the mitochondria causes loss of mitochondrial transmembrane potential and release of cytochrome c (Li et al., 1998). Normally, pH and electrical gradients exist across the inner mitochondrial membrane (Kroemer et al., 1997). When the inner mitochondrial membrane becomes permeable to water and solutes, however, a reduction in mitochondrial transmembrane potential occurs, which triggers the release of AIF (apoptosis inducing factor), cytochrome c, and procaspases 2, 3, and 9 (Liu et al., 1996a; Susin et al., 1996, 1999; Cai et al., 1998). AIF translocates to the nucleus to effect the degradation of DNA into large fragments (Lorenzo et al., 1999). Because Apaf-1 has a CARD domain, it is able to interact with procaspase 9 (Mch6/APAF3) in the presence of cytochrome c and dATP (Zou et al., 1997; Pan et al., 1998). The induced proximity of two or more procaspase 9 molecules causes autoactivation of procaspase 9 and subsequent cleavage to its active form (Srinivasula et al., 1998). Active caspase 9 cleaves and activates procaspase 3 (YAMA/CPP32/apopain) (Budihardjo et al., 1999). Recently, a CARD domain-containing protein named CLAP (CARD-like apoptotic protein) has been shown to function downstream of caspase 8 (Srinivasula et al., 1999). CLAP was able to activate the Apaf-1-caspase 9 pathway. Furthermore, the CARD and C-terminal domains of CLAP demonstrate low levels of proapoptotic activity. The CARD domain also exerts a dominant negative effect on TNFα-induced anti-apoptotic activity. Hence CLAP may enhance the induction of apoptosis by TNFα. In addition to the activation of procaspase 9, caspase 8 also cleaves and activates procaspase 3 (Tewari et al., 1995b), which is directly involved with the manifestation of the apoptotic phenotype (Yuan, 1997; Hirata et al., 1998; Stennicke et al., 1998). Caspase 8 may also cleave and activate procaspase 1 (ICE, interleukin-1 ß converting enzyme) (Beutler, 1999), which is required for processing proIL-1b and proIL-18 into their active forms (Schonbeck et al., 1997; Saha et al., 1999). Therefore, caspases are involved in both death and inflammatory signaling by TNFα.

In addition to interacting with procaspase 8 during downstream death signal propagation, FADD can also interact with procaspase 10 (Vincenz and Dixit, 1997). Binding is mediated by DED (CARD) domains located at the N-terminus of both proteins (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997). Activation of procaspase 10 occurs in a manner similar to that of procaspase 8, where receptor oligomerization leads to

the aggregation of procaspase 10 molecules, and hence autocatalytic cleavage into active caspase 10 (Fernandes-Alnemri et al., 1996; Vincenz and Dixit, 1997; Yuan, 1997; Ashkenazi and Dixit, 1998). Receptor oligomerization can also function to bring procaspase 8 and procaspase 10 into close proximity, thus facilitating the activation of these caspases by each other (Vincenz and Dixit, 1997). Active caspase 10 can activate the executioner procaspase 3 (Stennicke et al., 1998). To date, three isoforms of caspase 10 have been identified, caspase 10a (Mch4), caspase 10b (FLICE2), and the recently identified caspase 10c (Ng et al., 1999). TNFα specifically stimulates the expression of caspase 10c mRNA through an unidentified pathway. Caspase 10c is insoluble and forms filamentous structures known as DEFs (death effector filaments) (Siegel et al., 1998; Ng et al., 1999). DEFs are postulated to be involved in intracellular caspase recruitment and activation, thus effectively enhancing the apoptotic response of TNFα.

The adapter protein TRADD also recruits the death domain-containing protein RIP (receptor interacting protein) to the TNF-RI receptor signaling complex, mediated by the death domains of the two proteins (Hsu et al., 1996a). The domain structure of RIP consists of a C-terminal death domain and an N-terminal kinase domain (Stanger et al., 1995), separated by a unique intermediate domain (Hsu et al., 1996a). RIP is an active serine/threonine protein kinase (Hsu et al., 1996a). Propagation of the death signal, however, does not require kinase activity. Instead, RIP utilizes its death domain and functions as an adapter protein to recruit RAIDD (RIP-associated ICH-1 homologous death domain, also known as CRADD) to the receptor complex (Ahmad et al., 1997; Duan and Dixit, 1997). Because RAIDD possesses a DED (CARD) domain, it is able to interact with procaspase 2 (ICH-1). Subsequent activation of caspase 2 may assist in the apoptotic demise of the cell, although the direct substrates of caspase 2 have yet to be identified. A RIP-like kinase called RIP3 has been shown to interact with RIP and to activate various caspases (Yu et al., 1999). Similar to RIP, the kinase domain of RIP3 is not involved in the induction of apoptosis. RIP, in addition to playing a role in apoptosis, is also involved in TNFα-induced anti-apoptotic responses through the activation of transcription factors (Hsu et al., 1996a; Kelliher et al., 1998). Caspase 8 can cleave RIP to the cleavage product RIPc, thus effectively blocking the induction of the anti-apoptotic response (Lin et al., 1999). Furthermore, RIPc can strengthen the interaction between TRADD and FADD, thereby enhancing the cytotoxic effects of TNFα.

Finally, TRADD can recruit TRAF (TNF receptor associated factor) proteins to TNF-RI (Hsu et al., 1996b). Although TRAFs primarily participate in the activation of anti-apoptotic signals induced by TNFα, they are also involved in death signaling. Six different TRAFs (TRAF1-6) have been identified (reviewed in Arch et al., 1998). Among these, only TRAF1 and TRAF2 have been shown to be involved in apoptosis thus far (Hsu et al., 1996b). All TRAF proteins exhibit homology in a conserved C-terminal 150 amino acid region called the TRAF domain (Rothe et al., 1994). The TRAF domain itself is divided into N-terminal and C-terminal portions, TRAF-N and TRAF-C, respectively. Dimerization of TRAF proteins is mediated by the TRAF domains. Despite the ability of TRAF1 and TRAF2 to homodimerize *in vitro*, only TRAF1-TRAF2 heterodimers have been detected *in vivo*. Interaction between TRADD and TRAF1-TRAF2 heterodimers occurs via the N-terminal TRAF-binding domain of TRADD and the TRAF-C domain of TRAF2 (Hsu et al., 1996b). Downstream signaling involves association of the TRAF1-TRAF2 heterodimer with the serine/threonine kinase CARDIAK (CARD containing ICE-associated kinase, also known as RIP2/RICK) (Thome et al., 1998). Via a C-terminal DED (CARD) domain, CARDIAK can interact with a homologous domain in procaspase 1. This interaction results in the activation of procaspase 1, independent of the kinase activity of CARDIAK (McCarthy et al., 1998; Thome et al., 1998). TNFα treatment is capable of inducing apoptosis by activating procaspase 1 in the nucleus (Mao et al., 1998). This novel TNFα action has led to the identification of a nuclear localization signal within the prodomain of procaspase 1. TNFα is able to induce nuclear translocation of procaspase 1, perhaps by facilitating the removal of repressor proteins that mask the nuclear localization signal. Subsequent activation of procaspase 1 within the nucleus may lead to cleavage of nuclear death substrates (Cohen, 1997; Nicholson and Thornberry, 1997).

Execution of the apoptotic death sentence. The main executioner caspase in TNFα-induced apoptosis is caspase 3. It can cleave numerous cytoplasmic and nuclear substrates within the cell, thus manifesting the apoptotic phenotype. One such substrate is the anti-apoptotic protein Bcl-2 (Cheng et al., 1997). Cleavage of Bcl-2 into N-terminal and C-terminal products acts as a positive feedback mechanism to enhance cell death. The C-terminal Bcl-2 cleavage product acts as a proapoptotic protein. However, preventing Bcl-2 cleavage does not alter the kinetics of TNFα-induced death (Johnson and Boise, 1999). PITSLRE kinase, another substrate of caspase 3, is cleaved into its active form upon TNFα treatment (Lahti et al., 1995). The active kinase facilitates the execution of apoptosis. I-CAD is an inhibitor protein that binds to CAD (caspase-activated DNase) and sequestors CAD in the cytosol (Enari et al., 1998). Upon cleavage of I-CAD by caspase 3, CAD is able to migrate to the nucleus where it digests DNA into internucleosomal fragments, yielding the characteristic DNA laddering pattern of apoptosis (Sakahira et al., 1998). The 70 kDa component of the U1 small nuclear ribonucleoprotein (U1-70 kDa) is also cleaved by caspase 3 (Tewari et al., 1995a), which compromises the RNA splicing process. TNFα can induce RB

(retinoblastoma) protein degradation via activation of caspase 3 (Tan et al., 1997). RB is normally found in the cytosol complexed to E2F-1, a factor that can induce apoptosis when overexpressed (Shan and Lee, 1994). When cleaved by caspase 3, RB can no longer suppress the action of E2F-1, thus amplifying the death response to TNFα.

Caspase 3 is known to cleave PARP (Poly(ADP-ribose) polymerase) during TNFα-induced apoptosis (Nicholson et al., 1995; Tewari et al., 1995b). PARP is a repair enzyme that is activated in the presence of DNA damage (reviewed in D'Amours et al., 1999). During DNA repair, PARP consumes vast amounts of NAD^+, which leads to depletion of cellular ATP levels. It has been postulated that because apoptosis is a highly ATP-dependent process, insufficient ATP would shift the cell death pathway towards necrosis. Consequences would include damage to neighboring cells and the generation of inflammatory responses. To avoid such a fate, caspase 3 cleaves PARP into its inactive form and hence prevents apoptotic cells from entering necrosis.

Caspase 3 can activate neutral sphingomyelinase (NSMase), a membrane-bound enzyme involved in the generation of ceramide (Yuan, 1997). The upregulated production of ceramide induces a translocation of membrane-associated PKCδ to the cytosol (Emoto et al., 1995). Cytosolic PKCδ is susceptible to caspase 3 cleavage, thus forming a PKCδ fragment. Translocation of PKCδ to the cytosol is required for TNFα-induced apoptosis (Sawai et al., 1997).

Regulation of TNFα-induced apoptosis. A protein structurally similar to caspase 8, called c-FLIP (Casper/MRIT/FLAME/CASH), is involved in regulating the activation of caspase 8 (Goltsev et al., 1997; Han et al., 1997; Inohara et al., 1997; Irmler et al., 1997; Rasper et al., 1998). Although c-FLIP shares extensive homology with caspase 8, including the presence of a DED domain, c-FLIP is not a functional caspase because it lacks several key residues necessary for catalysis (Shu et al., 1997). c-FLIP has been reported to be anti-apoptotic as well as proapoptotic (Goltsev et al., 1997). Interactions of c-FLIP with both TRAF-2 and FADD have been demonstrated. Simultaneous binding of procaspase 8 and procaspase 3 to c-FLIP can also take place. Following recruitment to the TNF-RI receptor complex, the DED domain of c-FLIP may act in a FADD-like fashion to bind procaspase 8, thus preventing oligomerization and activation of caspase 8 by FADD (Hu et al., 1997; Shu et al., 1997; Thome et al., 1997). Another potential function of c-FLIP may be to localize procaspase 3 to the vicinity of procaspase 8, thus facilitating activation of procaspase 3 by procaspase 8 (Shu et al., 1997). Additional studies need to be performed to better determine the function of c-FLIP in apoptosis.

Several proteins belonging to the c-IAP (cellular inhibitor of apoptosis) family have been implicated in TNFα-induced apoptosis inhibition (reviewed in Clem and Duckett, 1997). c-IAP1 has been found to associate directly with the TNF-RI receptor signaling complex (Shu et al., 1996). In addition, both c-IAP1 and c-IAP2 can bind to TRAF1 and TRAF2 (Shu et al., 1996; Uren et al., 1996), as well as to CARDIAK (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998). The mechanism with which c-IAPs perturb the TNFα apoptotic signal may be at the level of caspase activation. Specifically, c-IAPs have been shown to directly inhibit some TNFα-activated caspases (Deveraux and Reed, 1999).

Mitochondria and cell death induction by TNFα. As mentioned previously, the propagation of the TNFα-induced apoptotic signal can involve the disruption of mitochondrial integrity, with the subsequent release of procaspases, AIF, and cytochrome c (Liu et al., 1996a; Susin et al., 1996). TNFα can also affect the mitochondria via a pathway independent of caspase 8 activation. Following treatment of susceptible cells with TNFα, the distribution of mitochondria within the target cell is modified (De Vos et al., 1998). Specifically, the mitochondria translocate from an initial dispersed pattern to a perinuclear cluster. The region of the cytoplasmic domain of TNF-RI immediately adjacent to the membrane is required for mitochondrial translocation. In an untreated cell, motor proteins called kinesins facilitate the intracellular movement of cargo along microtubules towards the cell periphery (Vale, 1987). TNF-RI with liganded TNFα sends a signal via its membrane-proximal region to inhibit kinesin function, perhaps by activating caspases (De Vos et al., 1998). Unable to direct mitochondrial movement away from the nucleus, perinuclear clustering of mitochondria occurs. The net effect of kinesin inactivity is an enhancement of cell death.

TNFα acting through TNF-RI can also induce necrotic cell death in various cell types (Vercammen et al., 1998). This process is independent of caspase activation. Instead, the mechanism may involve the delivery of exogenous TNFα directly to the mitochondria via a TNF-binding protein in the inner mitochondrial membrane (Ledgerwood et al., 1998). TNFα also induces the formation of reactive oxygen intermediates by the mitochondria, which may cause necrosis of the target cell (Schulze-Osthoff et al., 1992, 1993; Goossens et al., 1995; Vercammen et al., 1998).

The role of TNF-RII in apoptosis

TNFα binding to TNF-RI is the major ligand-receptor interaction involved in apoptotic signal transduction (Hohmann et al., 1990; Thoma et al., 1990; Tartaglia et al., 1993a). TNF-RII, unlike TNF-RI, does not possess a death domain and hence is unable to recruit death adapter proteins such as TRADD. Under some circumstances, however, TNF-RII may also be involved in inducing cell death (Heller et al., 1992; Bigda et al., 1994; Medvedev et al., 1994). For example, TNF-RII is

a major player in the apoptotic demise of mature $CD8^+$ T lymphocytes (Zheng et al., 1995). Two roles for TNF-RII in apoptotic signaling have been postulated. First, TNF-RII may increase the local TNFα concentration so as to enhance death signaling from TNF-RI. The dissociation rate of TNFα from TNF-RII is significantly greater than that for TNF-RI (Tartaglia et al., 1993b). Hence TNF-RII can perform a ligand passing function and bind many molecules of TNFα, which are then rapidly passed on to TNF-RI (Tartaglia et al., 1993b; Declercq et al., 1998; Haridas et al., 1998). Second, TNF-RII may induce degradation of TRAF2 or inhibit TRAF2 function, thereby preventing the activation of anti-apoptotic processes by TNFα (Duckett and Thompson, 1997; Weiss et al., 1998). Although TRAF2 is involved in TNF-RI death signaling, its major function is in the transduction of anti-apoptotic signals.

Transcription factor activation pathways of TNFα signaling

The activation of transcription factors is a major cellular effect induced by TNFα. These transcription factors regulate the expression of genes whose products are responsible for many of the physiological and pathological effects of TNFα. The principal transcription factors activated are NFκB (nuclear factor κB) and AP-1 (activating protein 1).

NFκB activation is associated with TNFα-induced cellular protection (Beg and Baltimore, 1996; Liu et al., 1996b; Van Antwerp et al., 1996; Wang et al., 1996). NFκB, a member of the Rel family of transcription factors, functions as a homo- or heterodimer formed from five possible subunits: p50/105 (NFκB1), p52/100 (NFκB2), p65 (RelA), RelB, and c-Rel (reviewed in Baeuerle and Henkel, 1994; Siebenlist et al., 1994). The p50 and p52 subunits are synthesized as inactive precursors, p105 and p100, respectively, that are subsequently processed into their active forms. All of the subunits share homology in a N-terminal 300 amino acid region, which is important for dimer formation, nuclear translocation, and DNA binding. The most common form of NFκB consists of a p50 and p65 heterodimer (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995). Heterodimeric NFκB is sequestered in the cytosol in an inactive state via noncovalent interactions with the IκB family of inhibitor proteins (Baeuerle and Baltimore, 1988, 1989; Beg et al., 1992; Baldwin, 1996). IκB effectively masks the nuclear localization signal on NFκB. When IκB is degraded, NFκB translocates to the nucleus and participates in the regulation of numerous genes, many of which are involved in anti-apoptotic and inflammatory responses (Baeuerle and Henkel, 1994).

AP-1 is a member of the basic region leucine zipper family of transcription factors (Karin et al., 1997). Similar to NFκB, AP-1 is also a homo- or heterodimer. Subunits for AP-1 include c-Jun, c-Fos, and members of the ATF (activating transcription factor) family. Activation of AP-1 is regulated by three of the five known mammalian MAPK (mitogen activated protein kinase) protein families, which are activated upon TNFα treatment (reviewed in Karin, 1995; Woodgett et al., 1996; Paul et al., 1997; Kyriakis, 1999). Specifically, JNK (c-Jun N-terminal kinase, also known as SAPK1), p38 (SAPK2), and ERK (extracellular signal regulated kinase) function to upregulate and phosphorylate the subunits of AP-1. Phosphorylated subunits are transcriptionally active, and can translocate to the nucleus and initiate gene expression (Karin, 1995). Various proinflammatory genes have been shown to be targeted by AP-1 (Reimold et al., 1996). Although some reports suggest that AP-1 is involved in TNFα-induced cellular protection mechanisms (Roulston et al., 1998),

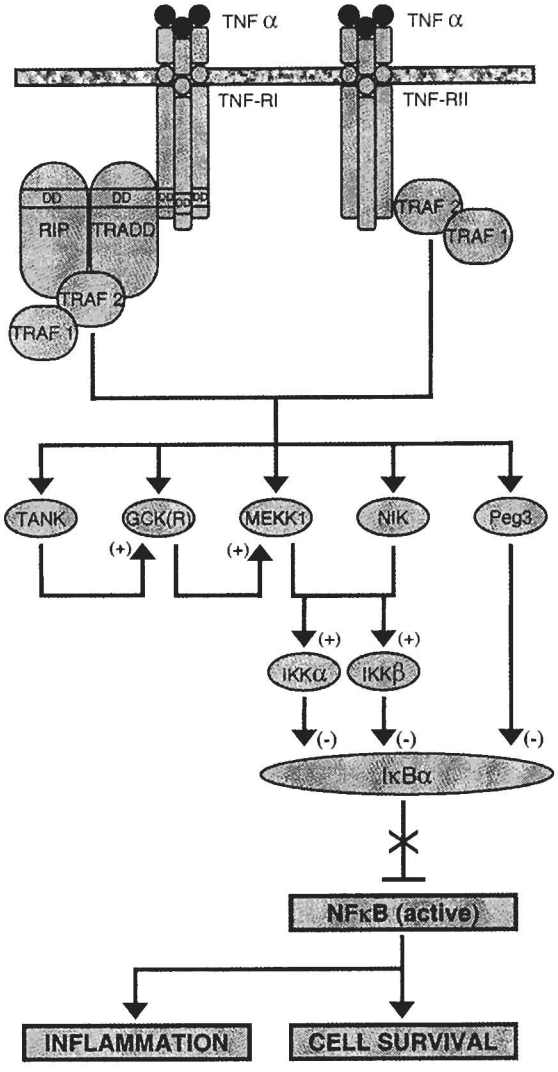

Fig. 2. NFkB activation pathways common to both TNF-RI and TNF-RII. Binding of TNFα to either receptor initiates the signaling cascade. Activation of NFkB results in expression of genes for cell survival and inflammation. Refer to the text for abbreviations of signaling components.

others have implicated AP-1 in the induction of apoptosis (Singh et al., 1995). Activation of the JNK pathway is often associated with stress-induced apoptosis (Hoeflich et al., 1999), although other studies do not show a distinct role for JNK in apoptotic cell death (Liu et al., 1996b). Further research may help to clarify these discrepancies.

The NFκB activation pathways

Pathways common to both TNF-RI and TNF-RII. Both TNF-RI and TNF-RII are capable of activating NFκB independently (Laegreid et al., 1994; Rothe et al., 1994, 1995; Hsu et al., 1995). TNFα binding to either receptor can lead to the degradation of IκB (Fig. 2). As noted previously, RIP plays a role in TNFα-induced apoptosis via interactions with the TNF-RI receptor complex. RIP is also critical for TNFα-induced NFκB activation (Hsu et al., 1996a). The intermediate domain of RIP, which enables RIP to act as a scaffold for TRAF1-TRAF2 heterodimers, is essential for this function (Hsu et al., 1996a). With respect to TNF-RII, this receptor can directly associate with TRAF1-TRAF2 heterodimers, mediated by interactions between TRAF2 and the C-terminal domain of TNF-RII (Rothe et al., 1994). Hence TRAF2 is recruited to both the TNF-RI and TNF-RII receptor complexes.

TRAF2 has been shown to interact with NIK (NFκB-inducing kinase) through the WK1 motif within the TRAF domain (Malinin et al., 1997; Song et al., 1997; Lin et al., 1998). NIK shares sequence homology with members of the MAPKKK family (Eder, 1997; Robinson and Cobb, 1997), but is unable to directly phosphorylate IκBα (Fischer et al., 1999). Instead, the activation of NFκB is regulated by a high molecular weight complex called the signalsome (also known as the IKK (IκB kinase) complex) (Woronicz et al., 1997). Components of the signalsome include NIK, IKKα (IKK1/CHUK), and IKKß (IKK2) (DiDonato et al., 1997; Malinin et al., 1997; Mercurio et al., 1997; Regnier et al., 1997; Woronicz et al., 1997; Zandi et al., 1997). Two adapter proteins are also present in the signalsome, IKKγ (NEMO/IKK associated protein 1) (Rothwarf et al., 1998) and IKAP (IKK complex associated protein) (Cohen et al., 1998), which may serve to stabilize the complex. NIK preferentially binds and phosphorylates IKKα, although interactions with IKKß do occur (Woronicz et al., 1997; Ling et al., 1998; Nakano et al., 1998; Fischer et al., 1999). When phosphorylated, IKKα and IKKß are activated and can phosphorylate IκBα on two serine residues (Ser32 and Ser36) (Chen et al., 1995; Traenckner et al., 1995; DiDonato et al., 1997). An IκBα-Ub (ubiquitin) ligase has a high affinity for phosphorylated IκBα, and can catalyze the ubiquitination of IκBα (Suzuki et al., 1999). Proteasome complexes then recognize the ubiquitinated protein and target it for degradation (Zandi et al., 1997). Because IκBα is no longer able to sequester NFκB in the cytosol, NFκB translocates to the nucleus and activates transcription.

Three additional proteins are recruited via TRAF2 to the receptor complex: TANK (TRAF-associated NFκB activator) (Cheng and Baltimore, 1996), GCK(R) (germinal center kinase (related)) (Yuasa et al., 1998; Chin et al., 1999), and MEKK1 (Baud et al., 1999). All three proteins participate in a relay mechanism towards NFκB activation. TANK activates GCK(R) (Chin et al., 1999), which in turn activates MEKK1 (Shi and Kehrl, 1997), which subsequently activates both IKKα and IKKß (Lee et al., 1998). Although MEKK1 can activate both IKKα and IKKß, activation of IKKα by MEKK1 far exceeds that of IKKß (Lee et al., 1997; Nakano et al., 1998). The fact that MEKK1 has been shown to be a component of the signalsome complex (Mercurio et al., 1997) has added credence to the proposal that MEKK1 is the principal kinase involved in NFκB activation (Lee et al., 1998). A fourth protein that interacts with TRAF2 is Peg3 (Pw1) (Relaix et al., 1998). This protein causes the dissociation of IκBα from NFκB, thus activating the transcription factor.

Pathways specific for TNF-RI. Several NFκB activation pathways mediated by TNF-RI alone have been characterized. One pathway involves IRAK (interleukin-1 receptor-associated kinase), which is a death domain-containing protein (Feinstein et al., 1995; Trofimova et al., 1996). IRAK is capable of direct interaction with TNF-RI via its death domain, and hence can initiate signaling independent of TRADD binding (Vig et al., 1999). Complex formation between IRAK and NIK has been demonstrated. Binding to NIK results in activation of IKKα and IKKß, and thus the activation of NFκB.

Activation of NFκB by the protein p62 specifically involves the TNF-RI receptor complex (Sanz et al., 1999). RIP, a component of the TNF-RI-TRADD-RIP trimeric complex, binds via its intermediate domain to p62. This intermediate domain also mediates interactions between RIP and TRAF2 (Hsu et al., 1996a). Association studies have revealed that the interactions of TRAF2 and p62 with RIP are not antagonistic (Sanz et al., 1999). p62 contains a dimerization domain upstream of its RIP-binding site, which is able to bind to members of the aPKC (atypical protein kinase C) family of proteins, namely PKCζ and PKCλ/i (Puls et al., 1997; Sanchez et al., 1998). Both aPKC proteins are recruited to the receptor complex, where they can activate IKKß and induce NFκB activation (Lallena et al., 1999).

A signal transduction pathway involving PI3K (phosphoinositide 3-kinase) is able to activate NFκB. PI3K is a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit (reviewed in Carpenter and Cantley, 1996). Formation of active PI3K can only occur if both subunits have been phosphorylated. TNFα binding to TNF-RI eventually leads to the activation of an as yet unknown tyrosine kinase activity, which is capable of phosphorylating the p85 subunit (Guo and Donner, 1996). The mechanism for p110 subunit activation has been characterized, and

involves the Grb2 protein. Grb2 is a cytoplasmic protein with two SH3 domains, one located at each terminus. An intracellular region of TNF-RI, designated the PLAP motif, binds to the C-terminal SH3 domain of Grb2. With its N-terminal SH3 domain, Grb2 interacts with SOS (Lowenstein et al., 1992; Hildt and Oess, 1999). SOS is a guanine nucleotide exchange factor that binds inactive Ras-GDP and converts it to the active Ras-GTP. Ras-GTP is then able to phosphorylate the p110 subunit. Association of the phosphorylated p85 and p110 subunits forms an active PI3K molecule. The substrate for PI3K is generated via another signaling pathway initiated by TNF-RI. The protein PIP5K (phosphatidylinositol 4-phosphate 5-kinase) has been shown to interact with the juxtamembrane region of TNF-RI (Castellino et al., 1997). PIP5K catalyzes the conversion of PtdIns4P (phosphatidylinositol 4-phosphate) to PIP_2 (phosphatidylinositol 4,5-bisphosphate). PI3K phosphorylates PIP_2 to form PIP_3 (phosphatidylinositol 3,4,5-trisphosphate). Both PIP_2 and PIP_3 have a high affinity towards proteins containing PH (pleckstrin homology) domains (Lassing and Lindberg, 1988; Hemmings, 1997; Klippel et al., 1997; Stokoe et al., 1997; Downward, 1998). Hence two PH domain-containing proteins, Akt and a specific serine/threonine kinase that activates Akt, are recruited to the receptor complex. Active Akt is able to activate IKKα and thereby induce NFκB activation (Ozes et al., 1999). Furthermore, Akt has been shown to phosphorylate and inactivate BAD, a pro-apoptotic molecule of the Bcl-2 family (Yang et al., 1995; White, 1996).

The JNK kinase activation pathways

Pathways common to both TNF-RI and TNF-RII. TRAF2 is a major conduit of JNK activation. Because both the TNF-RI and TNF-RII receptor complexes are able to recruit TRAF2, both receptors have been implicated in JNK activation (Rothe et al., 1995; Natoli et al., 1997). Both receptors are also able to recruit TRAF2 for NFκB activation (Laegreid et al., 1994; Rothe et al., 1994, 1995; Hsu et al., 1995). Hence it is not surprising that some signal transduction mechanisms are shared between the two activation pathways. One such mechanism is the activation of MEKK1, described earlier. Briefly, TRAF2 interacts with three proteins, TANK, GCK(R), and MEKK1. TANK activates GCK(R), which in turn activates MEKK1. It is at this point in the cascade where the two activation pathways bifurcate. MEKK1 has been shown to be a potent activator of the JNK pathway (Minden et al., 1994). MEKK1 activates MKK4 (JNKK/SEK1/SKK1/SAPKK1/Mek4), which in turn activates JNK (Liu et al., 1996b; Natoli et al., 1997).

Interaction between TRAF2 and ASK1 (apoptosis signal-regulating kinase 1) triggers another pathway for JNK activation (Nishitoh et al., 1998). ASK1, a member of the MAPKKK family, contains a C-terminal kinase domain. When ASK1 is in its latent state, the regions flanking the kinase domain interact with each other, thus effectively masking the kinase domain (Ichijo et al., 1997; Chang et al., 1998; Nishitoh et al., 1998). Upon binding to TRAF2, however, this inhibitory interaction is displaced. Active ASK1 directly activates two members of the MAPKK family, MKK4 (Ichijo et al., 1997) and MKK7 (Moriguchi et al., 1997). These kinases in turn activate JNK (Liu et al., 1996b; Moriguchi et al., 1997; Natoli et al., 1997). CARDIAK, another TRAF2-interacting protein, is also capable of activating the JNK pathway (Thome et al., 1998). The kinase domain of CARDIAK is not required for JNK activation. Instead, the CARD domain of CARDIAK is responsible. An additional function of CARDIAK is the activation of NFκB (Thome et al., 1998), although the mechanism for this activation has not been elucidated. Finally, JNK can also be activated by TRAF-2 interactions with caspase 8, caspase 10, or c-FLIP. This pathway requires the DED of the latter three proteins, but is independent of their protease activities (Chaudhary et al., 1999).

Active JNK can phosphorylate and activate various transcription factors, including c-Jun, ATF2, Elk-1, and CREB (Liu et al., 1996b; Natoli et al., 1997; Reinhard et al., 1997). CREB proteins may enhance the transcriptional activity of NFκB (Zhong et al., 1997). Elk-1 induces the expression of c-Fos (Karin, 1995). c-Jun, ATF2, and c-Fos are subunits of the AP-1 transcription factor. Hence the JNK pathway influences gene expression by promoting the formation of the AP-1 complex.

Pathways specific for TNF-RI. A TNF-RI-specific signaling pathway described previously, involving the activation of PI3K and the subsequent production of PIP_3, also assists in the activation of JNK. Upon TNFα treatment, a signaling cascade involving PI3K and the downstream mediator Rac has been shown to result in JNK activation (Kim et al., 1999). A molecule downstream from Rac, namely $cPLA_2$ (cytosolic phospholipase A_2), is also activated (Kim and Kim, 1997). $cPLA_2$ activation can result in increased c-Fos expression as well as JNK activation (Kim et al., 1999), both of which enhance the function of AP-1. More work is needed to identify the various players in this signaling cascade.

The p38 kinase activation pathways

Both TNF-RI and TNF-RII can stimulate pathways leading to p38 kinase activation. Following recruitment of TRAF2 to the receptor complex, ASK1 is known to bind TRAF2 (Nishitoh et al., 1998). ASK1 (a MAPKKK) can directly activate three MAPKKs that have been identified as activators of p38 kinase. These are MKK2, MKK3, and MKK6 (Derijard et al., 1995; Kyriakis and Avruch, 1996; Winston et al., 1997). In addition, evidence suggests that the intermediate domain of RIP may associate with a p38-specific MAPKKK

enzyme, although such an enzyme has not yet been identified (Yuasa et al., 1998). Substrates for p38 kinase include ATF2 and $cPLA_2$. Activation of both substrates assists in AP-1 transcription factor formation. Activation of $cPLA_2$ can also lead to NFκB activation. MAPKAP kinases 2 and 3 are also substrates for p38 kinase (Stokoe et al., 1992; Ludwig et al., 1996). When activated by p38 kinase, both MAPKAP kinases can directly phosphorylate mammalian hsp25/27 (heat shock protein 25/27) (Engel et al., 1995). Immediately following TNFα treatment, phosphorylated hsp25/27 proteins are known to aggregate to form large oligomers (Mehlen et al., 1995, 1997). These oligomers function to protect the cell from reactive oxygen species generated by TNFα (Park et al., 1998; Preville et al., 1998).

The ERK kinase activation pathways

The activation of ERK kinase is specifically mediated by TNF-RI. ERK activation involves a previously described receptor complex consisting of TNF-RI, Grb2, SOS, and Ras-GTP. The target of Ras-GTP in the ERK pathway is c-Raf-1 (Avruch et al., 1994). However, Ras-GTP alone cannot activate c-Raf-1 (Hildt and Oess, 1999). Rather, TNFα upregulates PKCζ kinase (Das et al., 1999), which acts in concert with Ras-GTP to phosphorylate and activate c-Raf-1 (Berra et al., 1995). Activated c-Raf-1 then activates MEK1 (Winston et al., 1995), which in turn activates ERK1/2 (Vietor et al., 1993). ERK1/2 can activate the transcription factor Elk1, thus promoting transcription of c-Fos and the subsequent formation of AP-1 (Karin, 1995).

The neutral sphingomyelinase pathway

The binding of TNFα to TNF-RI can induce the activation of neutral sphingomyelinase (NSMase), a C type phospholipase localized to the plasma membrane. A region adjacent to the death domain of TNF-RI, designated the NSD (NSMase domain), is necessary for NSMase activation (Adam et al., 1996). A WD-repeat protein called FAN (factor associated with NSMase) binds to the NSD domain of TNF-RI (Adam-Klages et al., 1996), and acts as an adapter to recruit NSMase to the receptor complex. NSMase cleaves sphingomyelin located in the plasma membrane, generating ceramide (Dressler et al., 1992; Wiegmann et al., 1994). It was thought that one of the functions of TNFα-induced ceramide was to induce apoptosis, although this view is now controversial (Kolesnick et al., 1994; Adam-Klages et al., 1996; Hannun, 1996). Signaling pathways mediated by ceramide have been linked to the activation of transcription factors (Schutze et al., 1992), as well as the production of proinflammatory metabolites (Heller and Kronke, 1994). TNFα-induced ceramide is known to increase c-Fos expression (Kim et al., 1999), to activate PLA_2 (Lin et al., 1993; Wiegmann et al., 1994), and to activate NFκB (Yang et al., 1993). Direct downstream targets of ceramide include CAPK (ceramide activated protein kinase) (Liu et al., 1994), CAPP (ceramide activated protein phosphatase) (Wolff et al., 1994), and PKCζ (Lozano et al., 1994; Muller et al., 1995).

Activation of CAPK by ceramide induces the binding of CAPK to c-Raf-1 (Yao et al., 1995). CAPK can then phosphorylate c-Raf-1. As mentioned before, activation of c-Raf-1 may require cooperative phosphorylation by more than one kinase. The Grb2-SOS-Ras pathway may provide this additional phosphorylation for activating c-Raf-1 (Hildt and Oess, 1999). Ceramide-activated PKCζ may also participate in c-Raf-1 activation (Berra et al., 1995). Some reports indicate that ceramide may be able to phosphorylate and activate c-Raf-1 directly (Pfeilschifter and Huwiler, 1998). Active c-Raf-1 targets MEK1 for activation (Yao et al., 1995), thus leading to the activation of ERK1/2 (Vietor et al., 1993; Bird et al., 1994). Downstream signaling events following ERK1/2 activation include the activation of both Elk-1 (Karin, 1995) and PLA_2 (Lin et al., 1993). Elk-1-induced c-Fos production leads to AP-1 formation, whereas PLA_2-induced phosphatidylcholine degradation leads to arachidonic acid production. Arachidonic acid can induce the activation of JNK (Rizzo and Carlo-Stella, 1996). In addition, it can act as a substrate to further propagate TNFα signaling.

Arachidonic acid is a substrate for both lipoxygenase and cyclooxygenase. Lipoxygenase oxidizes arachidonic acid to produce leukotrienes and other lipoxygenase metabolites (Samuelsson et al., 1987). Effects of these metabolites include the induction of c-Fos expression (Haliday et al., 1991) and the generation of reactive oxygen intermediates (Samuelsson et al., 1987). In fact, reactive oxygen intermediates can act as cofactors to promote c-Fos production (Yamauchi et al., 1989), as well as promote the activation of NFκB (Schreck et al., 1991). Conversely, the reactive oxygen intermediates produced may also potentiate the cytotoxic effects of TNFα (Chang et al., 1992). When arachidonic acid is targeted by cyclooxygenase, prostaglandins are produced. Prostaglandins, along with leukotrienes, are proinflammatory mediators that can increase blood flow and enhance capillary permeability. These effects can contribute to TNFα-induced inflammatory responses (Heller and Kronke, 1994).

TNFα binding to TNF-RI has been shown to stimulate the production of diacylglycerol (Schutze et al., 1991). The signaling pathway leading to diacylglycerol production involves activation of PC-PLC (phosphatidylcholine dependent phospholipase C) by the death domain of TNF-RI (Schutze et al., 1992; Wiegmann et al., 1994). Activated PC-PLC converts phosphatidylcholine to diacylglycerol, which in turn activates a TNFα-responsive isotype of PKC that triggers the induction of c-Jun and c-Fos (Fain and Berridge, 1978; Michell et al., 1979; Brenner et al., 1989).

Ceramide can be cleaved by ceramidase to generate sphingosine (Kolesnick and Golde, 1994; Hannun, 1996;

Spiegel and Merrill, 1996). Subsequent cleavage of sphingosine by sphingosine kinase yields sphingosine-1-phosphate. In this signaling pathway, activation of sphingosine kinase is mediated by the actions of either sphingomyelinase or ceramidase. Recently, a sphingomyelinase- and ceramidase-independent activation of sphingosine kinase by TNFα has been detected (Xia et al., 1999). The mechanism of this novel signaling pathway, however, is currently unknown. Sphingosine-1-phosphate activates anti-apoptotic and inflammatory pathways (Xia et al., 1999). In addition to activating NFκB and stimulating ERK activity, sphingosine-1-phosphate can also inhibit the function of caspase 3. Hence sphingosine-1-phosphate plays a role in TNFα-induced cell protection mechanisms.

The acidic sphingomyelinase pathway

Similar to NSMase, acidic sphingomyelinase (ASMase) is also a C type phospholipase. Activation of ASMase can only occur through TNF-RI. Furthermore, because ASMase is localized to acidic compartments of the cell such as lysosomes, TNF-RI must be internalized for ASMase activation to occur (Schutze et al., 1999). The ASMase pathway is initiated when TNF-RI, present at the plasma membrane, activates PC-PLC. Propagation of the signal can occur if the diacylglycerol produced from lipid hydrolysis is in close proximity with TNF-RI, so that some diacylglycerol is incorporated into endosomes during TNF-RI internalization. Following endosome formation, lysosomes containing ASMase fuse with the endosome, thereby effectively approximating TNF-RI and ASMase. The interior of the fusion vesicle is now optimal for ASMase activity to occur. Activation of ASMase requires both diacyl-glycerol and the extracellular domain of TNF-RI (which is located within the lumen of the vesicle) (Kolesnick, 1987; Schutze et al., 1992; Wiegmann et al., 1999). Also, TNF-RI must be associated with both TRADD and FADD for ASMase activation to occur (Adam-Klages et al., 1998). TRADD and FADD are believed to enhance the stability of TNF-RI, thus enabling the extracellular domain of TNF-RI to activate ASMase. Activation of ASMase leads to the generation of ceramide (Merrill et al., 1993; Spence, 1993), which can induce NFκB activation (Machleidt et al., 1994; Reddy et al., 1994). Ceramide can also bind and activate the lysosomal protease cathepsin D, thereby contributing to TNFα-induced cell death (Wickel et al., 1998).

Gene expression following TNFα-induced transcription factor activation

Four categories of genes are induced following TNFα treatment. These include anti-apoptotic genes, proinflammatory genes, mitogenic genes, and signal regulatory genes.

Anti-apoptotic gene expression. Concomitant with activation of apoptotic pathways, induction of protective proteins provides cells with resistance to TNFα-induced cytotoxicity. Both NFκB and AP-1 participate in cell survival. In response to TNFα, several anti-apoptotic proteins are induced. These include A1, an anti-apoptotic protein of the Bcl-2 family, the zinc finger protein A20, the reactive oxygen intermediate scavenger enzyme manganese superoxide dismutase, plasminogen activator inhibitor 2, c-IAPs, TRAF2, and IEX-1L (Krikos et al., 1992; Karsan et al., 1996; Uren et al., 1996; Deveraux et al., 1997; Moriguchi et al., 1997; Hu et al., 1998; Wu et al., 1998). Of interest, both A1 and A20 have also been reported to inhibit NFκB activation in addition to inhibiting apoptosis (Jaattela et al., 1996; Stroka et al., 1999).

Proinflammatory gene expression. Inflammation is a major physiological effect induced by TNFα. Both NFκB and AP-1 can participate in the production of proinflammatory mediators. This includes upregulation of cell adhesion molecules such as E-selectin, intracellular adhesion molecules 1 and 2, and vascular cell adhesion molecule 1 (De Luca et al., 1994; Barnes and Karin, 1997; Farina et al., 1997; Subramaniam et al., 1997), as well as production of various chemokines such as monocyte chemoattractant protein 1, interleukin-8, and macrophage inhibitory protein 2a (Barnes and Karin, 1997; Ueda et al., 1997; Roger et al., 1998). Additional proinflammatory-related genes induced by TNFα include interleukin-6, prostaglandin H synthase 2, platelet activating factor, and the matrix metalloproteases, collagenase and stromelysin (Barnes and Karin, 1997).

Mitogenic gene expression. Both NFκB and AP-1 can enhance the expression of growth promoting genes in response to TNFα treatment (Arch et al., 1998). One such gene is granulocyte-macrophage colony stimulating factor (Munker et al., 1986). Granulocyte-macrophage colony stimulating factor is known to enhance monocyte and granulocyte production, as well as to promote the growth of pluripotent and erythroid stem cells (reviewed in de Groot et al., 1998).

Signal regulatory gene expression. TNFα can activate regulatory systems within a cell to limit the magnitude and duration of TNFα-induced inflammatory responses. TNFα-activated NFκB and AP-1 can increase the synthesis of TNFα which may modulate the immune response in a positive or negative fashion (Takashiba et al., 1993). The increased production of TNFα may act to kill effector cells, and thus restrict the immune response (Swantek et al., 1997). On the other hand, cells already exposed to sublethal doses of TNFα are partially resistant to the cytotoxic effects of TNFα, which may potentiate the inflammatory response. NFκB can induce the synthesis of IκBα, which can rapidly restore the inhibitory protein levels to render NFκB activation transient (Baldwin, 1996). The expression of factors involved in a TNFα negative feedback loop, such as

interleukin-10 (Platzer et al., 1995), corticosteroids (Van der Poll et al., 1991), and prostanoids (Dayer et al., 1985), functions to suppress the production of TNFα. NFκB has also been shown to induce the expression of TRAF1 and TRAF2 (Wang et al., 1998; Schwenzer et al., 1999). TRAF expression has been postulated to play a role in feedback regulation of activated receptors.

Conclusions

A plethora of knowledge has accumulated over the past decade with respect to the mechanisms of TNFα signal transduction. Many of the intracellular signaling molecules have been identified. Detailed molecular pathways have been established from the point of signal initiation mediated by ligand-receptor interactions at the plasma membrane, to the point of gene transcription within the nucleus. The mechanisms involved in cell death induction by TNFα have also been characterized. Factors influencing the balance of opposing signaling pathways, however, remain to be identified. With this vast amount of data available at hand, potential clinical applications for TNFα may become more apparent. Understanding the complex nature of TNFα signaling allows the rational development of therapies to modulate TNFα function. It is important, however, to realize that many of the components of the signaling pathways and their interaction partners were identified under nonphysiological conditions, including overexpression studies and targeted null mutations in animals. Furthermore, signaling mechanisms may be cell-type specific, as evidenced by the contradicting results in the literature. Clearly, much remains to be learned about the relationship between the cellular and global effects of TNFα.

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