British Journal of Pharmacology (2002) 135, 855–875

www.nature.com/bjp

REVIEW

TNF ligands and receptors – a matter of life and death

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British Journal of Pharmacology (2002) **135,** 855–875

Keywords: Cytokine; receptor; subtypes; signal transduction; kinase; lipase; G-protein; cancer

Abbreviations: Apaf, apoptosis protease activation factor; CAD, caspase-activated DNAse; caspase, cysteine aspartate-directed protease; CAPK, ceramide-activated protein kinase; CARD, caspase recruitment domain; c-IAP, inhibitor of cellular apoptosis; $cPLA_2$, cytosolic phospholipase A_2; DcR, decoy receptor; DD, death domain; DED, death effector domain; DIF, differentiation inducing factor; DISC, death-inducing signalling complex; DR, death receptor; EDG, endothelial differentiation gene; FADD, Fas-associated DD; FAN, factor associated with neutral sphingomyelinase activation; FLICE, FADD-like interleukin-converting enzyme; FLIP, FLICE-like inhibitory protein; IκB, inhibitor of κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; LT, lymphotoxin; MADD, MAPK-activating DD protein; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MEKK, MEK kinase; mTNF, membrane-bound TNF; NIK, NF-κB-inducing kinase; NF-κB, nuclear factor-κB; PARP, polyadenosine ribosyl polymerase; RIP, receptor-interacting protein; PKA, cyclic AMP-dependent protein kinase; PKB, Akt/protein kinase B; PKC, protein kinase C; PLAD, pre-ligand assembly domain; ROS, reactive oxygen species; S-1-P, sphingosine-1-phosphate; SAPK, stress-activated protein kinase; SODD, silencer of DD; TACE, TNF-α converting enzyme; TNF, tumour necrosis factor-α; TNFR, TNF receptor; TNFR1, type I 55 kDa TNFR; TNFR2, type II 75 kDa TNFR; TNFSF, TNF superfamily nomenclature; TNFRSF, TNFR superfamily nomenclature; TRADD, TNFR-associated DD; TRAF, TNFR-associating factor; TRAK, TNFR-associated kinase

It had been known for some time that tumour masses which had become contaminated by a bacterial infection would on occasion regress and disappear. It was thought that the bacteria were releasing a factor which would make the tumour necrotic and whither. This factor was termed tumour necrosis factor (TNF) (Old, 1985). It was not until more recent advances in immunology that it became clear that antigens from the bacterial invader (notably lipopolysaccharide LPS) were causing the release of the patient's own TNF which could cause the tumour regression. The hunt was on to isolate this TNF which could be used as a magic therapy to control cancer cell growth and persistence, plus enhance the academic understanding of the ways by which a cell could die. It was discovered that TNF and lymphotoxin (LT) were products from macrophages and lymphocytes that were capable of lysing many cell types including some tumour cells (Carswell *et al*., 1975; Granger *et al*., 1969). TNF was also found to be identical to the protein cachectin, which was known to be involved in the fever and muscle wastage seen in cancer patients (Beutler *et al*., 1985). Hence, the role TNF played in a range of physiological actions was important and the hunt was on to identify TNF and related molecules such as LT. Modern techniques have since allowed the isolation, characterization and cloning of the genes for TNF which is structurally related to LT plus an expanding family of TNF-like ligands (Table 1). These cytokine molecules include ligands such as Fas, CD40 and RANK which cause wide-ranging long-term cellular activities in cells such as differentiation, proliferation or death. Evolution has created this TNF superfamily of cytokines to control and manipulate the immune system, modulating processes such as haematopoiesis, antibody production, or short- and long-term immunity. It is only through its quirky tumour-killing characteristic that TNF cytokine was first identified and may still hold the key to effective tumour therapy. As the majority of information has been gained about TNF and it is the archetypal cytokine of the superfamily, displaying the greatest range of cellular actions, this review will focus on the molecular aspects and biological role of TNF signalling.

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TNF ligand

Biochemically isolated in 1984, TNF has since been found to be a pleiotropic agent produced mostly by activated macrophages and monocytes, but also by many other cell types including B lymphocytes, T lymphocytes and fibroblasts. It is expressed as a 26 kDa transmembrane protein that can be cleaved by the metalloprotease TNF-α-converting enzyme (TACE) to release a 17 kDa soluble TNF form (Idriss & Naismith, 2000). TNF has also referred to as TNFα, cachectin or differentiation inducing factor (DIF) (Aggarwal, 2000). Its superfamily of nearly 20 different homologues including TNF-β (lymphotoxin-α), lymphotoxin-β, and other more specific ligands such as RANKL and TRAIL which are involved more particularly in bone tissue and lymphocyte processes. All the TNF receptor superfamily of ligands are thought form non-covalently-bound homo-trimers which are capable of becoming a secreted form (Figure 1). These TNF ligand are primarily designed for cell–cell contact transfer of signalling information between neighbouring cells, but cleavage into their soluble forms may

allow more dispersed cytokine effects. TNF as well as causing necrotic cell death may also cause apoptotic cell death, cellular proliferation, differentiation, inflammation, tumourigenesis, and viral replication (Figure 2). TNF's primary role is in the regulation of immune cells, but is known to be heavily involved in pathogenic disorders such as rheumatoid arthritis, asthma, septic shock, irritable bowel disorder, haemorrhagic fever and cachexia (Locksley *et al*., 2001).

Table 1 TNF ligand superfamily

Ligands	*Alternative names*
TNF	cachectin, DIF, TNFA, TNFSF2
4-1BB ligand	4-1BBL, TNFSF9
APRIL	TALL2, TNFSF13
CD27 ligand	CD27L, CD70, TNFSF7
CD30 ligand	CD30L, TNFSF8
CD40 ligand	CD40L, CD154, GP39, HIGM1, IMD3, TNFSF5, TRAP
Fas ligand	APT1LG1, FasL, TNFSF6
GITR ligand	AITRL, GITRL, TL6, TNFSF18
LIGHT	HVEM ligand, TL1, TNFSF14
LTα	LT, TNFB, TNFSF1
LTβ	TNFC, TNFSF3, p33
OX40 ligand	gp34, OX40L, TNFSF4, TXGP1
RANK ligand	ODF, OPGL, RANKL, TNFSF11, TRANCE
THANK	BAFF, BLYS, TALL1, TNFSF13B
TRAIL	Apo2 ligand, TL2, TNFSF10
TWEAK	Apo3 ligand, DR3L, TNFSF12
VEG1	TL1, TNFSF15

TNF receptors

Just as modern molecular techniques have identified a superfamily of TNF-like cytokine ligands, their receptor molecules consist of a superfamily of proteins that can be activated by one or more ligands (Table 2). TNF receptors are an family of proteins that consist of, to date, at least 27 members characterized by their repeated cysteine-rich extracellular sequence homology, and include LT receptor, Fas, CD40, the low affinity nerve growth factor receptor, TRAIL receptors, RANK and death or decoy receptors (Darnay & Aggarwal, 1999). Many of these members go by multiple names and are activated by specific ligands, however TNF only has the ability to bind two of these receptors which are also activated by LTα.

TNF ligand achieves all its different cellular and pathological effects by its binding to either the TNFR1 or TNFR2 receptor subtype. They are single transmembrane glycoproteins with 28% homology mostly in their extracellular domain with both containing four tandemly repeated cysteine rich motifs. Their intracellular sequences are largely unrelated with almost no homology between each other, and early work suggested delineation of their signalling functions (Grell *et al*., 1994). They contain several motifs with known functional significance. Both TNFR1 and TNFR2 contain an extracellular pre-ligand-binding assembly domain (PLAD) domain (distinct from ligand binding regions) that pre-complexes receptors and encourages them to trimerize particularly upon activation by TNF ligand (Chan *et al*.,

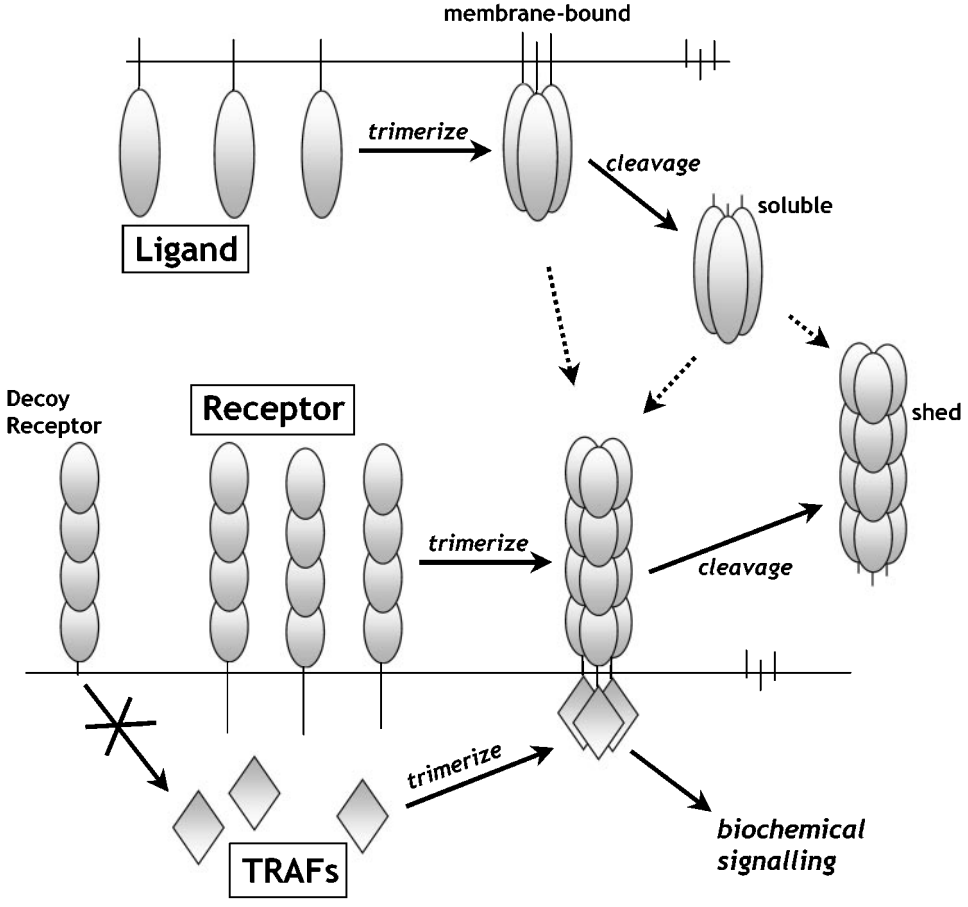

Figure 1 TNF superfamily ligand-receptor interactions.

Table 2 TNF receptor superfamily

Receptor	*Alternative names*	*Ligands*	*Associated TRAFs* (and associating proteins)
TNFR1	TNFRSF1A, CD120a, p55TNFR, TNF-R55, p60, TNF-R-I, TNFAR, TNFRβ	TNF, LTα	2 (TRADD, RAIDD, RIP, FAN, BRE, SODD, Grb2, MADD, PIP5K, p60TRAK)
TNFR2	TNFRSF1B, CD120b, p75TNFR, TNFR-75, p80, TNF-R-II, TNFBR, TNFRα	TNF, LTα	1,2,3 (RIP, p80TRAK)
4-1BB	CD137, TNFRSF9, ILA	4-1BB ligand	1,2,3
AITR	TNFRSF18	AITRligand	1,2,3
BCMA	TNFRSF17, BCM	APRIL, THANK	1,2,3
CD27	TNFRSF7, Tp55, S152	CD27 ligand	2,3,5
CD40	TNFRSF5, p50, Bp50	CD40 ligand	2,3,5,6
Death receptor-3	DR3, DDR3, TNFRSF12, TRAMP, WSL-1, WSL-LR, Apo3, LARD	Apo3 ligand, TWEAK	(TRADD)
Death receptor-6	DR6, TR7		
Decoy receptor-3	DcR3, TR6, TNFRSF6B	Fas ligand, LIGHT	
EDAR		EDA	
Fas	CD95, TNFRSF6, APO-1, APT1	Fas ligand	(FADD)
GITR		GITR ligand	2
HVEM	TNFRSF14, ATAR, TR2, HveA, LIGHTR	LIGHT, LTα	1,2,3,5
LTβ-R	TNFRSF3, TNFR2-RP, TNFCR, TNF-R-III	LTα, LTβ, LIGHT	3,5
OPG	TNFRSF11B, FDCR-1, OCIF, TR1, osteoprotegerin	TRAIL, RANKL	
OX40	CD134, TNFRSF4, ACT35, TXGP1L	OX40 ligand	1,2,3,5
p75NGFR	TNFRSF16	NGF, BDNF, neurotrophins	1,2,3,4,5,6
RANK	TNFRSF11A, TRANCE-R	RANKL	1,2,3,5,6
TACI	CAML interactor	APRIL, THANK	2,5,6
TRAIL-R1	Death receptor-4, DR4, TNFRSF10A	TRAIL	(FADD, TRADD, RIP)
TRAIL-R2	Death receptor-5, DR5, TRICK2A, TRICKB TNFRSF10B, KILLER, Apo2	TRAIL	(FADD,TRADD, RIP)
TRAIL-R3	Decoy receptor-1, DcR1, TNFRSF10C, LIT, TRID	TRAIL	
TRAIL-R4	Decoy receptor-2, DcR2, TNFRSF10D, TRUNDD, TR6	TRAIL	
Troy	TNFRSF19, Taj		2,5,6
XEDAR	EDA-A2R	EDA	1,3,6

2000). TNFR1 contains a death domain (DD) motif of approximately 80 amino acids in length towards the carboxyl-end of the receptor and is critical in the death-inducing activity of TNFR1 (Tartaglia *et al*., 1993a). The death domain is present on a number of associating proteins and related molecules that are primarily involved in signalling for cell death. One such molecule is the silencer of death domain protein SODD, which binds to the DD in TNFR1 and prevents other DD-interacting proteins from taking hold (Jiang *et al*., 1999). SODD dissociates from the TNFR1 DD upon TNF stimulation allowing other activator proteins to access the DD receptor module. TNFR1 also contains an intracellular sequence that is known to bind the adaptor protein FAN, a key stimulatory element in the activation of neutral sphingomyelinase (Adamklages *et al*., 1998a; Adam *et al*., 1996) which catalyses the degradation of sphingolipids into smaller ceramide-containing molecules which are key signalling intermediates (Kolesnick & Kronke, 1998). Moreover, a stress responsive 44 kDa protein expressed highly in brain and reproductive organs, BRE, specifically interacts with the juxtamembrane region of TNFR1 (Gu *et al*., 1998). BRE, like SODD, acts to inhibit and dampen TNFR1-stimulated signalling.

TNFR1

More information is known about TNFR1 than TNFR2. This is due to a number of factors including differences in affinity and efficacy of its ligands. The affinity of TNF ligand for TNFR1 receptor varies depending on the study from approximately 100 pM on native receptor (Tsujimoto *et al*., 1985; Kull *et al*., 1985; Vanostade *et al*., 1993), to estimations on cloned receptors from 100–600 pM (Schall *et al*., 1990; Hohmann *et al*., 1990; Pennica *et al*., 1992a; Loetscher *et al*., 1993; Grell *et al*., 1993; Moosmayer *et al*., 1994). Another study on native receptor indicated that soluble TNF, as is used experimentally, rapidly binds to TNFR1 with high affinity (Kd of 19 pM) and a slow dissociation from the receptor once bound ($t1/2 = 33$ min), a process which efficiently activates the receptor (Grell *et al*., 1998b). Such kinetics of ligand association are different from TNFR2 association (see below). Stimulation of TNFR1 leads to its internalization with inhibition of its long-term actions (Higuchi & Aggarwal, 1994) (Figure 3). Phosphorylation of TNFR1 occurs at a consensus MAPK site on its cytoplasmic domain or through tyrosine phosphorylation (Darnay & Aggarwal, 1997; Cottin *et al*., 1999), although it is not fully understood how these phosphorylations control receptor processing. TNFR1 is expressed on the cell surface but large amounts are found localized at the perinuclear-Golgi complex, as is TRADD, but which only associates with TNFR1 once at the plasmamembrane (Jones *et al*., 1999; Ledgerwood *et al*., 1999).

TNFR2

TNFR2 does not contain a DD motif but still recruits adaptor proteins including TRAF2. TNFR2 is thought to be

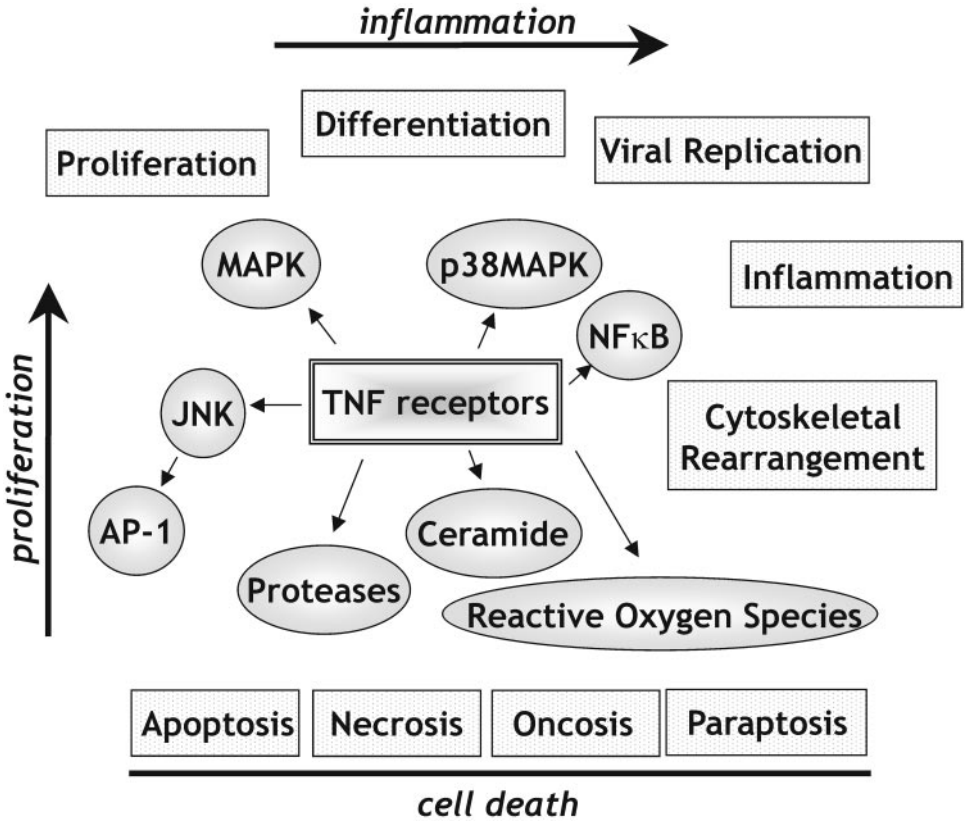

Figure 2 TNF receptor-mediated cellular responses.

able to signal apoptosis directly (Heller *et al*., 1992; Declercq *et al*., 1995) or through a so-called 'ligand-passing' mechanism by which TNFR2's greater affinity and half-life of TNF binding, holds ligand, increases the local TNF concentration in the vicinity of TNFR1 receptors which accept TNF ligand from TNFR2 and are themselves activated, signalling the TNFR1 apoptotic machinery (Tartaglia *et al*., 1993b). Others believe that additionally, TNFR2 signals for cell death through its cytoplasmic domain to induce mTNF expression, which then signals apoptosis *via* TNFR1 (Vercammen *et al*., 1995; Lazdins *et al*., 1997; Haas *et al*., 1999; Grell *et al*., 1999; Weiss *et al*., 1998). The kinetics of TNFR2 activation by TNF are different from TNFR1 (Vandenabeele *et al*., 1995). The dissociation kinetics of TNF from native TNFR2 is approximately 20–30 fold faster than from TNFR1 (Grell *et al*., 1998b), with workers finding the affinity of TNF for TNFR2 significantly greater (Tartaglia *et al*., 1993b) or less (Grell *et al*., 1998b) than the ligand's affinity for TNFR1. It is not clear how the binding characteristics of membrane-bound TNF at TNFR1 and TNFR2 compare to soluble TNF. Slight structural changes in the TNF sequence can lead to dramatic changes in its binding characteristics to TNF receptors. For example, murine TNF activates mouse TNFR1 and TNFR2 equally well, whereas human TNF acts on mouse TNFR1 but does not bind mouse TNFR2 (Lewis *et al*., 1991). Such observations led to studies in which mutant proteins ('muteins') of soluble TNF were developed that displayed reduced affinity towards TNFRs compared to wild-type TNF, however these muteins showed marked selectivity between TNFR1 and TNFR2 (Table 3) helping to uncover the role of each TNFR (Loetscher *et al*., 1993; Vanostade *et al*., 1993).

TNFR2 was fully cloned after TNFR1 and its structural and functional characterization is less well understood. The main reason for the relative lack of signalling information about TNFR2 is that, generally, it is not efficiently activated *in vitro*. It was assumed that recombinant 17 kDa soluble TNF (as is provided commercially) was an efficient activator of TNFR2. However, it was uncovered that the membrane-bound 26 kDa form of TNF (mTNF) was greater than soluble TNF is activating TNFR2 (Grell *et al*., 1995) leading to qualitatively different responses and new insight into TNFR2 function (Decoster *et al*., 1995). TNFR1 is activated equally well by soluble and mTNF. TNF ligand acts in the immune system whereby it would activate TNFRs through cell–cell interactions. As such, most of the TNF effects *in vivo* may be mediated by mTNF (TNFR1 = TNFR2 activation) rather than soluble TNF (TNFR1 > TNFR2 activation). As such, the physiological role of TNFR2 may be underestimated by most TNF research conducted in the laboratory which uses soluble TNF as the stimuli. Soluble TNF acts similar to a partial agonist on TNFR2 in that it binds to the receptor, but is not highly efficacious and efficient in its activation. Such limitations of stimulation are overcome by the use of agonistic antibodies capable of efficiently stimulating TNFRs (Grell *et al*., 1993; Tartaglia *et al*., 1991; Leeuwenberg *et al*., 1995; Paleolog *et al*., 1994; Wajant *et al*., 2000; Borset *et al*., 1996; Haridas *et al*., 1998; Jupp *et al*., 2001), although how these functionally relate to natural forms of TNF can only be assumed.

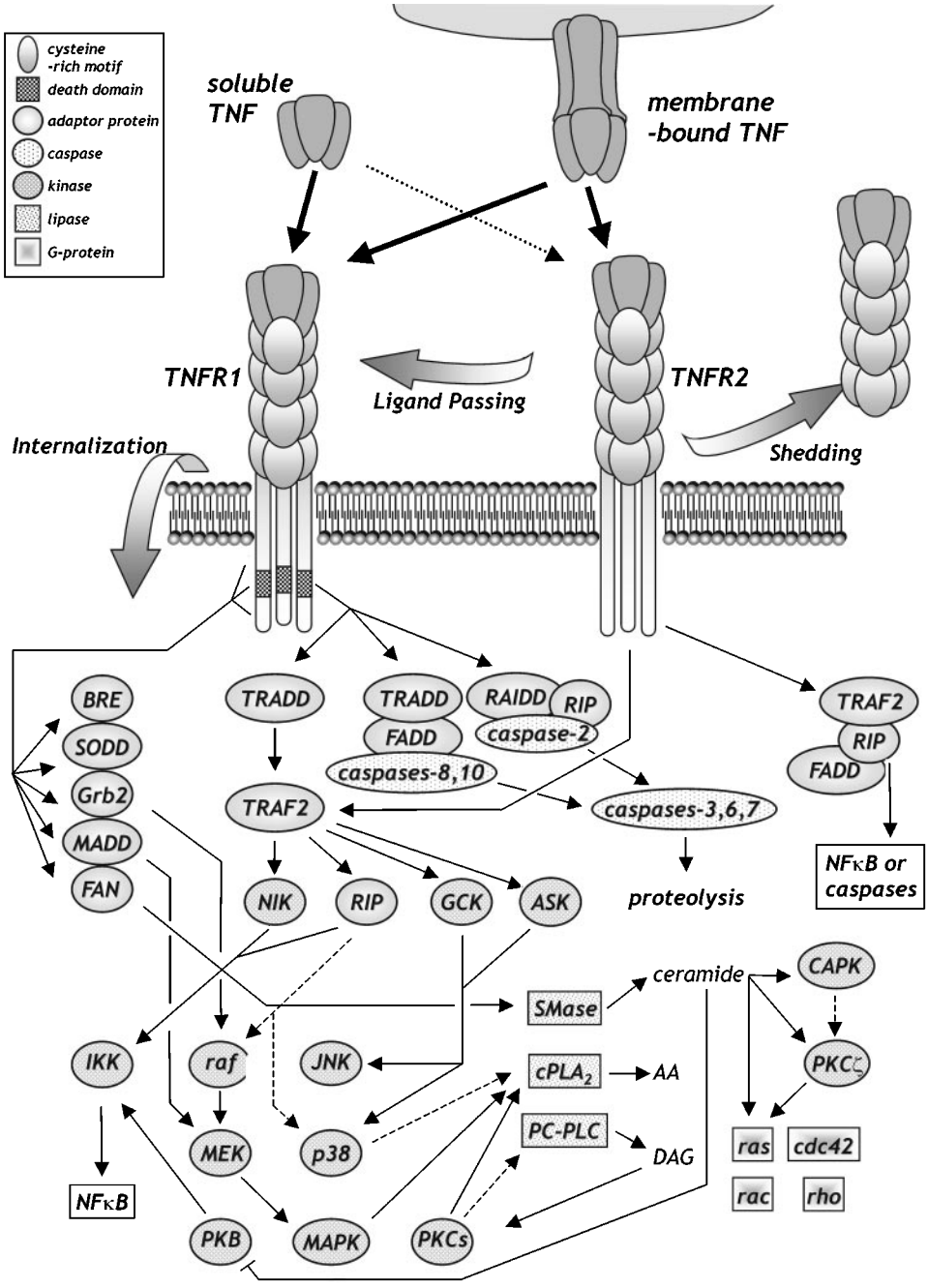

Figure 3 Major signalling pathways modulated by TNF receptor subtypes.

TNFR signalling mechanisms: TRAFs and other adaptor proteins

Both TNFR1 and TNFR2 possess sequences that are capable of binding intracellular adaptor proteins that link TNF receptor stimulation to activation of many signalling processes. These TNF receptor-associating factors (TRAFs) and adaptors are what transduce the TNF signal from the biochemically inert receptors to dramatic changes of the signalling molecules within target cells (Wajant *et al*., 2001). TRAF molecules all contain a RING finger and zinc finger motifs in their N-terminal, with their C-terminal regions possessing a TRAF domain sequence. To date six mammalian TRAF proteins have been identified. The first TRAFs to be uncovered, TRAF1 and TRAF2, were discovered by their ability to directly interact with the cytoplasmic domain of TNFR2 (Rothe *et al*., 1994). Work by the same group also identified the apoptotic adaptor proteins c-IAPI and c-IAP2 that bind to TNF receptor *via* a TRAF1/TRAF2 heterocomplex (Rothe *et al*., 1995a). Since then, it is now thought that mostly TRAF2 interacts with TNFR2 directly, with TRAF1 interacting indirectly and TRAF3 also able to associate (Table 2). TRAF2 is recruited to TNFR1 indirectly through a specific interaction with the protein TNF receptor-associated death domain (TRADD), a 34 kDa cytosolic adaptor protein that directly binds to TNFR1 through its

Table 3 Pharmacological modulation of TNF responsive signalling pathways

Signalling pathway	*Activator*	*Inhibitor*	*References*
TNF ligand	TNF lymphotoxin-α R32W, E146K or R32WS86T mutein	CDP571/humanized anti-TNF mAb etanercept/rTNF fusion protein	Fernandez-Botran, 2000; Couriel *et al*., 2000;
	TNFs (R1-selective)	InfliximAb/chimeric anti-TNF H398 mAb (R1-specific)	Vandenabeele *et al*., 1995;
	MR1-2 or htr-9 mAbs (R1-specific)	80M2 or utr-1 mAbs (R2-specific)	Jupp *et al*., 2001
	D143F or D143NA145R mutein TNFs (R2-selective)		
	MR2-1 mAb (R2-specific)		
5-lipoxygenase	FLAP	WY 50,295	Mayer *et al*., 1996
caspases		zIETD-fmk (caspase-8-selective)	Villa *et al*., 1997;
		zLEHD-fmk (caspase-9-selective)	Zhou & Salvesen, 2000
		YVAD-fmk (caspases-1,4-selective)	
		zDEVD-fmk (caspases-3,6,7,8,10-selective)	
		zVAD-fmk (caspases-1,3,4,7,8-selective)	
		zVEID-fmk (caspase-6-selective)	
		zVDVAD-fmk (caspase-2-selective)	
		CrmA protein (caspases1,8-specific)	
$cPLA_2$	melittin	manoalide	Mayer *et al*., 1996
$sPLA_2$		scalaradial	
cyclo-oxygenase		indomethacin	Mayer *et al*., 1996
JNK	anisomycin	CEP-1347/KT7515	Pyne & Pyne, 2000;
	ceramide	cyclosporin A	Murakata *et al*., 1996;
			Kujime *et al*., 2000;
			Kinloch *et al*., 1999;
			Maroney *et al*., 2001;
			Matsuda & Koyasu, 2000
MAPK		DL-threo dihydrosphingosine	Tolan *et al*., 1996
		Sphingosine	
MEK-1		PD98059	Kujime *et al*., 2000;
		PD184352	Kinloch *et al*., 1999;
		U0126	Dai *et al*., 2001
NF-κB		deoxyspergaulin	Lee & Burckart, 1998;
		gliotoxin	Ward *et al*., 1999;
		leflunomide	Manna & Aggarwal, 1999;
		silymarin	Manna *et al*., 1999;
		phenylarsine	Estrov *et al*., 1999
p38MAPK	anisomycin	SB203580	Schwenger *et al*., 1998;
	Na salicylate	SB202190	Kujime *et al*., 2000;
		cyclosporin A	Kinloch *et al*., 1999;
			Young, 1998;
			Matsuda & Koyasu, 2000
PC-PLC		D609	Adam *et al*., 1998
		desimipramine	
		imipramine	
PI-3K		wortmannin	Kinloch *et al*., 1999;
		LY294002	Young, 1998
PKC	phorbol esters	staurosporine	Mayer *et al*., 1996;
	DOPPA (β-selective)	Ro31-8220	Gescher, 2000;
	thymeleatoxin	sphingosine	Way *et al*., 2000
		UCN-01, CGP41251, Gö6983	
		LY333531 (β-selective)	
Rho		C3-exoenzyme	Bodie *et al*., 2001;
		EDIN	Bar-Sagi & Hall, 2000;
		Y27632 ($p160^{rock}$ inhibitor)	Gong *et al*., 1996
Serine/Threonine phosphatases 1 and 2		calyculin A	Barber *et al*., 1995;
		okadaic acid	Mayer *et al*., 1996;
		tautomycin	Matsuda & Koyasu, 2000
		FK506 (calcineurin-specific)	
		cyclosporin A (calcineurin-specific)	
		rapamycin (FKBP-selective)	
sphingomyelinases		SR33557	Kinloch *et al*., 1999
		glutathione	
Tyrosine kinase		genestein	Mayer *et al*., 1996;
		CGP53716	Young, 1998
		herbimycin A	Drummond & Holyoake, 2001;
		tyrphostin	O'Dwyer & Druker, 2001
		CP-118556 (Src-specific)	
		STI571/CGP57148B (Ab1-specific)	
Tyrosine phosphatase		phenylarsine oxide	Mayer *et al*., 1996;
		diamide	Morinville *et al*., 1998
		orthovanadate	

own death domain sequence (Hsu *et al*., 1995). TRADD recruits the downstream signalling adaptor molecules FADD (Fas-associated death domain) and RIP (receptor interacting protein). RIP originally identified as a Fas-associating molecule (Stanger *et al*., 1995) also interacts with TNF receptors (Hsu *et al*., 1996a; Liu *et al*., 1996). RIP contains a kinase sequence, but its role as a kinase enzyme is unclear at present. FADD contains a death effector domain (DED) sequence (Chinnaiyan *et al*., 1995), that interacts with the DED domain in caspase-8 (also known as FLICE or MACH) and a number of other DED-containing molecules that regulate cell death mechanisms (Muzio *et al*., 1996). Another DD-containing molecule RAIDD is recruited to TNFR1 and interacts with RIP and caspase-2, to allow its activation. RIP and FADD are also thought capable under certain conditions to be able to indirectly bind to TNFR2 *via* TRAF2 (Pimentel-Muinos & Seed, 1999).

TRAF2 is also capable of interacting with downstream signalling molecules such as NF-κB-inducing kinase (NIK) which is a member of the serine/threonine mitogen-activated protein kinase (MAPK) kinase (MEK) kinase (MEKK) family (Liu *et al*., 1996; Malinin *et al*., 1997). NIK phosphorylates its target at serine 176, an enzyme named inhibitor of κB (IκB) kinase (IKK) which is implicated in the activation of NF-κB transcription factor involved in transcriptional responses to stress and anti-apoptotic cellular action (Ling *et al*., 1998). NIK is also capable of interacting with TRAFs 1, 3, 5 and 6 (Wajant *et al*., 2001). One of the genes under the transcriptional control of NF-κB is the cellular inhibitor of apoptosis protein 2 (c-IAP2) which binds to TRAF2 and is capable of blocking caspase-8 activation and apoptosis. Similarly, A20 is an 80 kDa inducible protein that binds TRAF2 and is anti-apoptotic (Song *et al*., 1996). RIP and NIK are not the only kinases to interact with TRAF2, apoptosis-stimulating kinase (ASK1) (Nishitoh *et al*., 1998), germinal centre kinase (GCK) (Yuasa *et al*., 1998; Shi & Kehrl, 1997) and MEKK1 (Baud *et al*., 1999), have all been implicated in the activation of p38MAPK and c-Jun N-terminal kinase (JNK) stress kinases, as well as AP-1 and NF-κB transcriptional activation processes (Figure 3).

The activation of the MAPK family of kinase enzymes can be achieved by TNF receptor interaction with the factor associated with neutral sphingomyelinase activation (FAN) adaptor protein (Adam *et al*., 1996). FAN is responsible for neutral sphingomyelinase-mediated generation of ceramide-containing sphingolipids (Adamklages *et al*., 1998a) which are capable of activating ceramide-activated protein kinases (CAPK) (Mathias *et al*., 1991; Dressler *et al*., 1992) (also known as kinase suppresor of ras (Zhang *et al*., 1997) which activates Raf kinase (Yao *et al*., 1995), an upstream activator of the MEK and MAPK serine/threonine kinase family (Mathias *et al*., 1998). Interestingly, TNF receptors have been found to interact with the Grb2 adaptor and son of sevenless (SOS) exchange factor (Hildt & Oess, 1999; Adam *et al*., 1996). Activated Grb2 binds through its SH3 domain to a motif within the TNF receptor, allowing this activated complex to stimulate c-Raf-1 kinase. Another recently identified protein that interacts with FADD through a DED domain is the FLICE-like inhibitory protein (FLIP (Irmler *et al*., 1997; Thome *et al*., 1997) which blocks caspase-8 recruitment and activation. FLIP interacts with TRAFs and RIP to switch signalling pathways through more anti-apoptotic pathways such as NF-κB and Raf-1 kinase, resulting in marked MAPK activation (Kataoka *et al*., 2000). The death domain within TNFR1 is also able to bind MADD adaptor protein, which contains a DD sequence. MADD activates MAPK when overexpressed in cultured mammalian cells (Schievella *et al*., 1997; Kataoka *et al*., 2000; Brinkman *et al*., 1999). A 59 kDa TNFR2-associated serine/threonine kinase p80TRAK has been described that is TNF-stimulated (Darnay *et al*., 1994). p80TRAK binds to a 44 amino acid site in TNFR2 cytoplasmic domain in a similar fashion to casein kinase (Darnay *et al*., 1997).

Lipases

Some of the earliest TNF signalling research revealed the activation of several lipase activities (Dayer *et al*., 1985; Marquet *et al*., 1987; Beyaert *et al*., 1987; Clark *et al*., 1987; Godfrey *et al*., 1987). TNF receptors activated phosphatidylcholine-specific phospholipase C (PC–PLC) (Schutze *et al*., 1991; Wiegmann *et al*., 1992). PC–PLC stimulation by TNFR1 activates a downstream acidic membrane-bound sphingomyelinase activity. Stimulation of PC–PLC activity degrades phosphatidylcholine into choline (a molecule with no known signalling function) and diacylglycerol, the physiological activator of most protein kinase C (PKC) isoforms. Other reports have suggested TNF ligand stimulates phospholipase D (PLD) creating phosphatidic acid (Devalck *et al*., 1998; Kang *et al*., 1998), that may be converted to DAG by phosphatidic acid phosphohydrolase. Others have also implied TNF-stimulated phosphatidylino-sitol-specific phospholipase C (PI–PLC, generating inositol-1,4,5-trisphosphate and diacylglycerol second messengers) activity (Beyaert *et al*., 1993).

Much interest has centred on the ability of TNF to stimulate sphingomyelinase of which there are two types, neutral and acidic (Kolesnick & Kronke, 1998). These sphingomyelinases stimulate the breakdown of membrane sphingolipids into ceramide which may then be converted into other ceramide-containing lipids such as sphingosine and sphingosine-1-phosphate (S-1-P) (Hannun, 1994). TNF was the first stimuli found to induce ceramide generation (Okazaki *et al*., 1989; Mathias *et al*., 1991; Kim *et al*., 1991; Dressler *et al*., 1992). TNFR1 is responsible for the stimulation of membrane-associated neutral sphingomyelinase (Wiegmann *et al*., 1992, 1994), and *via* FAN adaptor protein, the stimulation of neutral sphingomyelinase (Adam *et al*., 1996). Exogenous ceramide is a highly specific yet destructive chemical in most cells with rapid stimulation of apoptotic cell death processes. These apoptosis-signalling processes include the sequential activation of Bad (Basu *et al*., 1998), then activation of CAPK which stimulates raf kinase (Yao *et al*., 1995) and MAPK activity (Raines *et al*., 1993; Bird *et al*., 1994). TNF-induced ceramide production may also stimulates the stress-activated kinase JNK (Coroneos *et al*., 1996). Sphingolipids are able to modulate PKC isoforms. For example, human leukaemia cells treated with ceramide or TNF caused the rapid translocation to the cytosol of PKC-δ and PKC-ε (Sawai *et al*., 1997). Similarly, in L929 rat fibroblasts, ceramide and TNF blocked PKC-α activity and translocation to the plasmamembrane (Lee *et al*., 2000). TNF-stimulated ceramide generation and apoptosis in HL-60 leukaemia cells was found to be crucially dependent

on PKC-β (Laouar *et al*., 1999), while ceramide has also been found to bind directly to the atypical isoform PKC-ζ which can activate ras, raf, MEK, MAPK and NF-κB transcription (Diazmeco *et al*., 1994); Lozano *et al*., 1994; Berra *et al*., 1995; Conway *et al*., 2000). TNF-stimulated sphingolipids are also capable of being converted into sphingosine-1-phosphate (S-1-P) which has intracellular actions including raising $[Ca^{2+}]_i$ from calcium stores and stimulating MAPK activity (Pyne & Pyne, 2000). As well as its intracellular actions, S-1-P is able to diffuse from the cell and act in an autocrine manner by stimulating endothelial differentiation gene (EDG) cell surface receptors (EDG1R – EDG8R), a family of heterotrimeric G-proteins receptors (Pyne & Pyne, 2000).

Another lipase strongly stimulated by TNF receptors is the 110 kDa hormone-sensitive, Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$) (Clark *et al*., 1991). $cPLA_2$ is responsible for the liberation of arachidonic acid from the *sn*-2 position of mainly phosphatidylcholine. The arachidonic acid again acts in an autocrine fashion and within its own cell of generation, to be converted into prostaglandins and leukotrienes to stimulate eicosanoid-sensing receptors. Moreover, these eicosanoids are responsible for the generation of oxygen radical-containing lipids and reactive oxygen species (ROS) that disrupt mitochondrial integrity and set in motion cell death mechanisms indicated above (Chang *et al*., 1992). Protective cellular enzymes such as superoxide dismutase counteract these disruptive ROS molecules (Wong *et al*., 1989; Wong & Goeddel, 1988). TNF receptor stimulation of MAPK and PKC isoforms leads to the phosphorylation and activation of $cPLA_2$ at specific residues (Nemenoff *et al*., 1993; Lin & Chen, 1998) (most notably serine 505 in the case of MAPK (Lin *et al*., 1993) resulting in a rapid liberation of arachidonic acid. Cytokine receptor activation of $cPLA_2$ through p38MAPK has also been implicated (Kramer *et al*., 1996) at serine residue 727, which leads to activation of the lipase (Borschhaubold *et al*., 1997, 1998), but which may be a route of $cPLA_2$ phosphorylation and activation restricted to platelets. A secondary mechanism of TNF-stimulated $cPLA_2$ gene induction also occurs, leading to the increased expression of $cPLA_2$ protein and activity which is sensitive to inhibition by protein synthesis blockers and glucocorticoids (Hoeck *et al*., 1993). It has been shown in several cell types that $cPLA_2$ protein and activity is crucial for TNF-mediated cell death (Hayakawa *et al*., 1993; Wu *et al*., 1998a, b; Devalck *et al*., 1998; Suffys *et al*., 1991; Jayadev *et al*., 1997). The mechanism of $cPLA_2$ stimulation by TNF is thought to be TNFR1-specific as only this receptor has shown the ability to activate the kinases involved in its phosphorylation (Boone *et al*., 1998; Jupp *et al*., 2001; McFarlane *et al*., 2001) with TNFR2 having a role in the regulation of $cPLA_2$ expression (MacEwan, 1996). Additionally, activated $cPLA_2$ has been shown to be the cleaved by caspases but the relative activity of the cleaved $cPLA_2$ fragments is not clear as this aspect of the reports are contradictory (Luschen *et al*., 1998; Adamklages *et al*., 1998b; Atsumi *et al*., 1998; Wissing *et al*., 1997; Voelkeljohnson *et al*., 1995).

Kinases and phosphatases

As mentioned above, through activation of PC – PLC, TNF receptors are capable of diacylglycerol generation and subsequent PKC activation (Bermudez & Young, 1987; Schutze *et al*., 1990). There exist at least 12 forms of PKC with differential activation characteristics and distinct tissue distribution, and some of these isoforms have been shown to be TNFR-stimulated. For example, PKC-α activation by TNF can occur in L929 fibroblasts, through an indirect activation process involving ceramide production (Lee *et al*., 2000). A variant of the HL60 human myeloid leukaemia cell line, HL525, that are deficient in PKC-β are resistant to TNF-induced apoptosis which can be reinstated by re-establishing PKC-β protein expression (Laouar *et al*., 1999). PKC-δ is a substrate for TNF-stimulated caspase-dependent cleavage and has also been shown to cause the serine phosphorylation of TNFR1 protein itself (Kilpatrick *et al*., 2000). The atypical PKCs-ζ, -λ, and -ι have also been shown to be regulated by more complex TNF-stimulated mechanisms that may involve lipid intermediates (Muller *et al*., 1995; Sanz *et al*., 1999; Bonizzi *et al*., 1999) and as mentioned above, in particular PKC-ζ directly bind TNF-generated ceramide to cause the subsequent activation of ras, raf, MEK, MAPK and NF-κB.

Much work has been concerned with the activation by TNF receptors of the extracellular signal-regulated protein kinase (ERK) superfamily of kinases, responsible for much of a cells reaction to a variety of mitogenic stimuli and stress responses (Paul *et al*., 1997). These enzymes are characterized by their activation process of dual phosphorylation on threonine and tyrosine residues in their sequences, with the motif Thr-X-Tyr, where X can be Glu for MAPK members, Gly for p38MAPK or Pro for the c-Jun N-terminal kinase (JNK) family of stress-responsive kinases (Fiers *et al*., 1996). All three of these ERK families are readily stimulated by TNF. Upstream kinases (MEKs) control their activation, which are themselves under the control of MEK kinases (MEKKs). They have a range of known substrates activated in an ERK family-selective manner, including transcription factors and downstream kinases (Davis, 1999). The first members of the ERK family to be identified, p42 and p44MAPK, are transiently activated by TNF through MEK-1 and MEK-2 phosphorylation (Vanlint *et al*., 1992; Minden *et al*., 1994) and one report in fibroblasts from TNFR-deficient mice indicate both receptor subtypes are capable of MAPK stimulation (Kalb *et al*., 1996) but more recent work has shown MAPK activation occurs through the TNFR1 receptor only (Jupp *et al*., 2001). Several pharmacological inhibitors of these MEKs such as PD98059 and U0126 have helped reveal a role for p42 and p44MAPK activation in TNF modulation of cell death (Chang, 2000; Rao, 2001; Cuvillier *et al*., 1996).

P38MAPK and JNK kinases are termed stress kinases as they are transiently activated by a range of stress stimuli including cytokines, heat- or osmotic-shock and UV irradiation. Upstream kinases responsible for p38MAPK activation include MEK-3 and MEK-6 (Enslen *et al*., 1998). The pharmacological tools SB203580 and SE239063 are relatively specific inhibitors of p38MAPK and have shown the role of p38MAPK in many TNF-induced cellular responses (Barone *et al*., 2001). One of the first reports to implicate p38MAPK in TNF signalling conclusively showed the role of the kinase in TNF-stimulated phosphorylation of heat-shock protein hsp27 and induction of NF-κB activity and interleukin-6 (Beyaert *et al*., 1996). Indeed, many of the actions of

p38MAPK are considered proinflammatory although the role of p38MAPK in cell death and survival is not absolutely clear (Xia *et al*., 1995; Eliopoulos *et al*., 1999; Cross *et al*., 2000). The JNK family of ERKs are a highly active arm of TNF receptor signalling mechanisms. JNK is activated mainly by MEK-4 (SEK-1), but also by MEK-7 (Davis, 1999). Both TNFR1 and TNFR2 are proficient activators of JNK (Weiss *et al*., 1998); Haridas *et al*., 1998; Jupp *et al*., 2001) perhaps through different pathways, but partially involving TRAF2. Unfortunately, widely-available JNK pathway inhibitor exists but the use of tools such as CEP-1347, or a dominant-negative form of MEK-4 have revealed an important role for JNK in TNF-mediated cellular processes including a probable role in apoptosis (Bozyczko-Coyne *et al*., 2001; Xia *et al*., 1995; Liu *et al*., 1996; Verheij *et al*., 1996; Doman *et al*., 1999; Helms *et al*., 2001).

TNF induction of inflammatory mediators and processes are critical steps in many pathological disorders including rheumatoid arthritis These inflammatory processes under the control of several promoter gene sequences but particularly the stimulation of NF-κB transcription factor (Aggarwal, 2000). The activation of NF-κB is controlled by its association with IκBα, whose degradation is controlled by its phosphorylation on serine 176 by NIK (Ling *et al*., 1998) and which is activated by the TNF stimulus (Malinin *et al*., 1997). Several of the TNF receptor-associating molecules, including TRAF2 (Hsu *et al*., 1996b) and RIP (Stanger *et al*., 1995; Liu *et al*., 1996), have been implicated as upstream NF-κB activators. TRAF2 was the first of these entities identified to activate NF-κB, with dominant-negative forms of TRAF2 (but not TRAF1 or TRAF3) able to block TNF-induced NF-κB activity (Rothe *et al*., 1995b). TRAF2 associated with NIK and was suggested to be crucial to NF-κB activation, however embryonic fibroblasts from TRAF2-deficient knock-out mice were still capable of TNF-induced NF-κB activation, but not JNK activation (Lee *et al*., 1997; Yeh *et al*., 1997). RIP-deficient mice on the other hand, were incapable of TNF-stimulated NF-κB activity, with JNK activation and apoptosis unaffected (Kelliher *et al*., 1998; Stanger *et al*., 1995). It appeared that RIP, but not TRAF2, was required for TNF-stimulated NF-κB activation, with TRAF2 needed for IKK recruitment and RIP being responsible for IKK activation (Devin *et al*., 2000). In a wide range of cell types, that both TNFR1 and TNFR2 are capable of NF-κB activation (Kruppa *et al*., 1992; Laegreid *et al*., 1994; Rothe *et al*., 1994; Weiss *et al*., 1997; Haridas *et al*., 1998; Amrani *et al*., 1999; McFarlane *et al*., 2001). Recently, RIP has thought to have a possible switching capability where it binds to both TNFRs and regulates their caspase or NF-κB-signalling (Kelliher *et al*., 1998; Pimentel-Muinos & Seed, 1999). The role of NIK in NF-κB activation processes has been brought into question, as unexpectedly normal activation of NF-κB by TNF was still present in NIK-deficient mice (Yin *et al*., 2001). Furthermore, a transactivation process of NF-κB stimulation has been identified whereby phosphorylation of IκB (or other signalling components of its activation machinery) results in its activation not necessarily with any characteristic proteolytic degradation (Nasuhara *et al*., 1999; Vandenberghe *et al*., 1998; Johannes *et al*., 1998). Thus, TNF-induced NF-κB activation processes probably occurs through multiple signalling pathways.

Protein kinase B (Akt) is a more recently discovered kinase that is recruited to the plasmamembrane by phosphatidylinositol-3,4,5-trisphophate (PtdIns(3,4,5)P_3) generated by phosphoinositide 3-kinases (PI3Ks) (Vanhaesebroeck & Alessi, 2000). PKB activation is completed by its phosphorylation by 3′-phosphoinositide-dependent kinase 1 (PDK1). PKB modulates signalling molecules controlling insulin actions as well as components thought to be important in regulating cell survival responses, including Bad, caspase-9, IKK, raf and ERK activities (Scheid & Duronio, 1998; Vanhaesebroeck & Alessi, 2000). Destruction of PKB by caspases allows inhibition of its survival signal (Widmann *et al*., 1998), with inhibition of PKB activity also achieved by ceramide lipids allowing apoptosis to occur (Zhou *et al*., 1998; Schubert *et al*., 2000). It was uncovered that PKB plays a role in TNF-induced stimulation of anti-apoptotic NF-κB activity with the activation of NIK dependent on PKB which phosphorylates IKKα at threonine 23 (Ozes *et al*., 1999). In addition another recently discovered kinase phosphatidylinositol-4-phophate 5-kinase (PIP5K-IIβ) responsible for the generation of phosphatidylinositiol-4,5-bisphosphate (substrate for PI-PLC-mediated inositol-1,4,5-trisphosphate and diacylglycerol second messengers) was found to interact with and be stimulated by TNFR1, but not TNFR2 (Castellino *et al*., 1997).

As well as TNF stimulating the production of ceramide to stimulate CAPK (see above), ceramide also has a role in the activation of ceramide-activated protein phosphatase (CAPP) of the type 2A (Dobrowsky *et al*., 1993). CAPP was found to be important in TNF-induced c-*myc* down-regulation as well as the dephosphorylation of c-Jun in TNF-stimulated A431 human epithelial cells (Reyes *et al*., 1996). Other reports have also indicated the possible involvement of serine/threonine (Totpal *et al*., 1992; Barber *et al*., 1995) or tyrosine-specific (Guo *et al*., 2000; Mishra *et al*., 1994) phosphatases in the regulation of cellular TNF responses. In particular, tyrosine phosphatases may be involved in the regulation of NF-κB activation processes that are controlled by TNF stimulation (Singh & Aggarwal, 1995; Dhawan *et al*., 1997). Thus serine/threonine and tyrosine phosphorylations or dephosphorylations are an aspect of TNFR signalling mechanisms.

Caspases: the key to death?

Members of the TNFR superfamily have the ability to cause proliferation, differentiation, cell death, or be blockers of apoptosis (Inoue *et al*., 2000). TNFR1 and TNFR2 are unique in that they can cause either a proliferative response or a cytotoxic response dependent on the cell type or its configuration (Figure 2). What the precise molecular switch is which defines whether activating a TNFR will lead to proliferative or destructive outcome is a matter for intense investigation. Certainly, the activation of destructive protease cascades will play a crucial part in this switching by TNFRs.

Cysteine-aspartate-directed proteases (caspases) are a group of at least 14 different enzyme forms, which orchestrate induction of most types of apoptotic cell death. As mentioned above, caspase-8 is part of the TNFR1 death-inducing signalling complex (DISC) by virtue of its DED domain allowing its interaction with the DD-containing TRADD/FADD machinery (Locksley *et al*., 2001). Caspases-2, -8, -9

and -10 are known as apoptotic initiator caspases that lie upstream of executioner procaspases-3, -6 and -7 which exist in a latent form until activated by its initiator through a process of cleavage, oligomerisation and autoactivation (Denecker *et al*., 2001). Caspase-1 is the enzyme that processes the proteolytic maturation of pro-interleukin-1β, and together with caspase-11 these enzymes are predominantly responsible for cytokine processing. Less is known about the function of caspases-4, -5, -12, -13 and -14. Evidence suggests a crucial role of caspases-3, -8 and -10 in TNF-induced apoptotic mechanisms with some viral products, such as CrmA cowpox modifier protein, blocking specific caspase forms and showing their absolute importance in TNF-mediated apoptotic (Hsu *et al*., 1995; Tewari *et al*., 1995), but not necrotic (Vercammen *et al*., 1998a, b) cell death. Much of the importance of caspase members in cell death has arisen from the use of cell-permeable pharmacological inhibitors of caspases, such as zVAD-FMK (Table 3) which have shown dramatic effects at blocking ligand-induced cell death (Kinloch *et al*., 1999). As with all pharmacological data, however, the claimed specificity of these agents does not match up to their actual inhibitions at the concentrations they are used at experimentally (Schotte *et al*., 1999), and as such, much of the precise role of each caspase family member remains uncertain. Transgenic mice deficient in caspases-1, -2, -3 and -9 showed TNF-induced apoptotic death that was largely unaffected, indicating multiple caspase pathways that may be commissioned by activated TNF receptors in each particular cell type (Kuida *et al*., 1995; 1996; 1998; Zheng *et al*., 1999). Much of the present thinking implicates only TNFR1 in its ability to activate caspase proteases (Figure 3), however, recent evidence investigating the variable ability of RIP adaptor protein (Holler *et al*., 2000) (itself a target for caspase-mediated degradation (Lin *et al*., 1999) to associate with TRAF2 and TNFR1 or TNFR2 suggests that cellular conditions may favour the switching of TNFR2 between anti-apoptotic NF-κB activation signalling and death induction through caspase mechanisms (Kelliher *et al*., 1998; Pimentel-Muinos & Seed, 1999).

Caspases initiate cell suicide by the controlled destruction of the cell's own repair mechanisms (Cohen, 1997). Mitochondria sense apoptotic signals via a family of Bcl-2 proteins that either inhibit (Bcl-2, Bcl-x_L) or promote (Bax, Bad, Bak, Bik, Bid) apoptosis (Chao & Korsmeyer, 1998). Caspase-8-mediated cleavage of Bid produces a truncated form (tBid) that translocates from the cytosol to the mitochondria. This caspase-mediated Bid activation step leads to reduced mitochondrial membrane potential and the release of cytochrome *c*, which binds the adaptor protein Apaf-1, a 130 kDa protein that contains an N-terminal caspase recruitment domain (CARD). With dATP/ATP and cytochrome *c* acting as cofactors Apaf-1 self-oligomerizes and binds procaspase-9 to form the apoptosome complex, which activates the executioner caspases-3 and -7 then other caspases to mediate apoptotic proteolysis of key repair and housekeeping proteins (Screaton & Xu, 2000). For example, caspase-dependent proteolysis cleaves and activates PKC-δ, and inactivates polyadenosine ribosyl polymerase (PARP) repair enzyme as well as the proteolytic activation of caspase-activated DNAses (CADs) that destroy the genome by excising genes leading to the characteristic DNA fragmentation and laddering associated with apoptotic death (Rich *et al*., 2000). Recent work on the Ca^{2+}-sensitive calpain group of proteases and cathepsins and granzymes has shown these destructive enzymes are brought into play during cell death (Squier & Cohen, 1996; Johnson, 2000; Utz & Anderson, 2000). They may be more important in forms of death associated with Ca^{2+} overload and excitotoxicity (Pornares *et al*., 1998a, b), but their role in general TNF signalling has been implicated (Diaz & Bourguignon, 2000; Han *et al*., 1999).

A role for G-proteins in TNFR signalling?

Although not one of the seven transmembrane G-protein-coupled class of receptors, TNF receptors can influence heterotrimeric G-protein activities. Bacterial toxin experiments in osteoblasts, breast cancer cells and hepatocytes implicated pertussis toxin-sensitive G-proteins in the sensitivity of these cells to TNF treatment (Branellec *et al*., 1992; Yanaga *et al*., 1992; Hernandezmunoz *et al*., 1997). In neutrophils, the involvement of the $G_i\alpha$ class of G-proteins has been implicated in TNF responses (McLeish *et al*., 1996). TNF priming of leukocytes also leads to regulation of G-protein activity and levels, particularly of the $G_{i2}\alpha$ class (Scherzer *et al*., 1997), whereas in airway smooth muscle cells, TNF treatment could lead to the regulation of $G_{i2}\alpha$ and $G_{i3}\alpha$ levels. TNF was recently found to selectively regulate the degradation of $G_q\alpha/_{11}\alpha$ class of G-protein in HeLa and L929 cell models of cytotoxicity (Pollock *et al*., 2000). Proteins that modulate G-protein function, such as GSP2 (G-protein pathway suppressor) and RGS16 (regulator of G-protein signalling), have also been found to be regulated by TNF activation (Fong *et al*., 2000; Jin *et al*., 1997).

Monomeric small G-proteins such as *ras* probably play a role in TNF signalling. For example, inhibition of *ras* activity by rap1 a tumour suppressor gene or rasN17 dominant-negative mutant, blocked TNF-induced apoptosis in fibroblasts (Trent *et al*., 1996). TNFR1 binds Grb2 adaptor protein which co-ordinates ras activation to stimulate the raf/MEK/MAPK signalling axis (Hildt & Oess, 1999). TNF-generated ceramides are capable of binding to and activating ras protein (Muller *et al*., 1998). Indeed, CAPK is a regulator of *ras* (Zhang *et al*., 1997) and is necessary for TNF stimulation of MAPK in intestinal epithelial cells (Yan & Polk, 2001). TNF has also recently been shown to stimulate cdc42 in fibroblasts (Puls *et al*., 1999) or Rac & cdc42 (Min & Pober, 1997) and rho (Petrache *et al*., 2001) monomeric G-protein pathway in endothelial cells. Such TNF-induced changes in endothelial cell cytoskeletal structures have also been reported by others to involve Rac, cdc42 and p21 rho monomeric G-protein (Wojciak-Stothard *et al*., 1998). In airway smooth muscle cells, TNF stimulates a rho-activation pathway that is involved in myosin light chain phosphorylation and contractile sensitivity (Hunter *et al*., 2001). Activation by TNF of transcription factors such as NF-κB and c-*fos* serum response element, have been shown to involve monomeric G-proteins such as *rho*, Rac and cdc42 (Perona *et al*., 1997; Kim *et al*., 1999). Thus, many aspects of TNFR signalling are probably significantly regulated by monomeric as well as heterotrimeric G-proteins.

A role for Ca^{2+} in TNFR signalling?

Inositol phosphates and Ca^{2+} were reported to by important in TNF-stimulated cellular responses (Beyaert *et al*., 1993; Denecker *et al*., 1997). TNF was shown to inhibit inositol phosphate action and cellular Ca^{2+} handling processes in a variety of cell types (Reithmann & Werdan, 1994; Yorek *et al*., 1999; Rosado *et al*., 2001), with a possible regulation of Ca^{2+}-mobilizing $InsP_3$ receptor subtypes by caspase-dependent processes (Diaz & Bourguignon, 2000). In cardiac myocytes, TNF regulated Ca^{2+} mobilization (Bick *et al*., 1997) and long-term potentiation (a Ca^{2+} handling phenomenon) was inhibited by TNF treatment (Cunningham *et al*., 1996). TNF is thought to have a primary role in the onset of asthmatic conditions (Thomas, 2001). In airway smooth muscle, TNF enhances Ca^{2+} mobilization in a process that is thought to be crucial to the airway hyper-responsiveness (Amrani *et al*., 1995). TNFR1 is the receptor isoform responsible for the observed hyper-responsiveness (Amrani *et al*., 1996) which also signals for MAPK and NF-κB activities (Amrani *et al*., 2000; McFarlane *et al*., 2001). It was uncovered that TNF was rapidly stimulating a novel pathway resulting in the enhanced phosphorylation of myosin light $chain_{20}$, resulting in greater contractile force at the same $[Ca^{2+}]_i$ (Parris *et al*., 1999). Prolonged TNF stimulation has itself been shown to raise $[Ca^{2+}]_i$ in L929 fibroblasts which undergo TNF-induced necrosis (Kong *et al*., 1997), however these Ca^{2+}-raising effects of TNF are not evident in a range of cell types which undergo TNF-induced cell death (Pollock *et al*., 2000; McFarlane *et al*., 2000). Interestingly in sensory neurones, TNF has the ability to stimulate rapid transient waves of Ca^{2+} mobilization (Pollock *et al*., 2002). Although similar to $InsP_3$-induced release of intracellular Ca^{2+} stores, these TNF-induced Ca^{2+} spikes are probably through ryanodine-sensitive stores, brought about by S-1-P converted from ceramide and sphingomyelinase activity.

Physiological role of TNFRs

TNF is also known as cachectin because of its primary role in the muscle wasting disorder cachexia (Beutler *et al*., 1985). It is now becoming apparent that TNF plays an important role in metabolic disorders including type II diabetes mellitus (Saltiel, 2001). Early work on this topic revealed TNF interfered with insulin signalling mechanisms, by inhibiting the tyrosine kinase activities of the insulin receptor and serine phosphorylation of the insulin receptor substrate 1 (IRS-1) (Hotamisligil *et al*., 1993; 1994). It has since been shown that TNFR1 isoform plays the major role in the TNF-mediated insulin resistance (Sethi *et al*., 2000) which occurs in a variety of lipid-handling tissues, suggesting anti-TNF therapy or blocking TNFR1 activity may be helpful in diabetes (Uysal *et al*., 1997). The exact signalling mechanism for these insulin-modulating effects of TNF are not fully clear but have been proposed to include PLC-γ, PKC-ζ, PKB and the STAT5 transcription factor that controls interferon-stimulated gene activity (Storz *et al*., 1998; Ravichandran *et al*., 2001; Ermakova *et al*., 1999).

TNFR2 is readily cleaved by the metalloprotease TACE into its soluble shed form which is still capable of TNF binding, rapidly altering the number of functional TNFR2 receptors that can signal their proliferative or apoptotic actions (Pennica *et al*., 1992b; Porteu & Hieblot, 1994; Higuchi & Aggarwal, 1994). Both TNFRs protein expression levels are also regulated by a number of physiological or signalling mechanisms (Vandenabeele *et al*., 1995). Although regulation of TNFR protein expression is not restricted purely to TNFR2 (Manna & Aggarwal, 1998), generally the more restrictive tissue distribution of TNFR2 and the flexible TNFR2 protein regulation suggest a physiological role for TNFR2 regulation in modulating TNF-responsiveness. TNFRs form homotrimers upon activation by TNF without the assembly of receptor heterotrimers (Moosmayer *et al*., 1994), however the TNFR1 : TNFR2 protein ratio has been found to be important in the way a cell predetermines its TNF response (Medvedev *et al*., 1996; Declercq *et al*., 1998; Baxter *et al*., 1999). Thus, largely unmodulated TNFR1 expression coupled to changeable TNFR2 levels in cells, alters the TNFR1 : TNFR2 ratio and controls the functional outcome to TNF of that cell, thus effectively altering the cellular and physiological responses that the same cytokine elicits.

Transgenic mice deficient in TNFR1 have greater sensitivity to infection by Listeria monocytogenes but are resistant to TNF or interleukin-1-mediated *in vivo* lethality, plus were resistant to models of endotoxic shock induced by lipopolysaccharide and D-galactosamine (Pfeffer *et al*., 1993; Rothe *et al*., 1993). TNFR1 has also been shown to control early graph versus host disease (Speiser *et al*., 1997). It has been noted that cleaved soluble TNFR1 is found in the sera of healthy patients, with higher levels in patients suffering leukaemia (Digel *et al*., 1992) with these shed forms of TNFRs may play a possible role in arthritis. Deletion of TNFR2 in transgenic mice has uncovered that this receptor subtype is important in low dose TNF-induced lethality (Erickson *et al*., 1994). In addition to a role in thymocyte proliferation (Grell *et al*., 1998a), TNFR2 plays an important role in models of cerebral malaria and microvascular endothelial cell damage (Lucas *et al*., 1997a, b; 1998). Langerhans cell migration was depressed in mice lacking TNFR2 (Wang *et al*., 1996), whereas TNFR2 plays a critical role in multiorgan inflammation (Douni & Kollias, 1998). Experimental hepatitis involves both TNFRs (Kusters *et al*., 1997) and TNFR2 was seen to have a minor role in Mycobacterium bovus (BCG) immunity in knock-out mice (Jacobs *et al*., 2000). Clearly, TNFR2 has a role in certain tissues and diseased states, but the validity of direct comparisons between TNFR-null transgenic mice and normal cells and tissues which ubiquitously express TNFRs at altering TNFR1:TNFR2 ratios, has to be considered when analysing the physiological role of TNFR1 and TNFR2.

Other members of the TNF ligand and receptor superfamilies have, in most cases, only recently been identified, and as such their full physiological role has still to be fully appreciated (Locksley *et al*., 2001). Nonetheless, many diseased states or transgenic studies have indicated a wide range of physiological responsibilities for these ligands and receptors. For example many of these ligands and receptors contribute critically to the adaptive immune response, co-ordinating the direction and magnitude of any immunological reaction. The receptors Fas, CD40 and OX40 have a crucial role in T cell responses including activation-induced apoptosis, whereas RANK receptor or RANKL are

expressed selectively of $CD4^+$ precursor cells and contribute towards haemotopoiesis and peripheral or mesenteric lymph node maturation (Dougall *et al*., 1999). Moreover, lack of functional RANK and RANKL cause osteoporosis as monocyte differentiation into osteoclasts is defunct. Likewise RANK and highly related receptors such as OPG control bone formation and integrity resulting in various bone density disorders (Wuyts *et al*., 2001; Kim *et al*., 2000; Li *et al*., 2000; Hughes *et al*., 2000; Kong *et al*., 1999). Neural development and hair follicle and sweat gland formation require the action of p75NGFR and other receptors including XEDAR and Troy also play a role in such tissue development (Locksley *et al*., 2001). Thus, TNF ligand and receptor superfamilies play and important function in a diverse range of physiological activities, hence further characterization and understanding of the signalling controlled by these receptors will hopefully lead to the development of pharmacological tools as therapies for a multitude of human disorders.

Therapeutic implications

Clearly TNF and its related ligands and receptors play a wide ranging role in a multitude of cellular and physiological acts. These roles have recently translated into a new generation of therapies for several common human disorders (Kollias *et al*., 1999). TNF itself has recently been tested as a tumour killing agent in the clinic on perfused isolated limbs to treat soft tissue sarcomas and melanomas (Couriel *et al*., 2000; Moore *et al*., 1999). Work is progressing to use lower doses of TNF ligands to minimize toxic side effects on healthy tissue (Van Der Veen *et al*., 2000). The most notable successes in controlling TNF's effects have been with the use of anti-TNF therapies to treat patients suffering from rheumatoid arthritis (Taylor, 2001; Feldmann & Maini, 2001) or Crohn's disease and severe irritable bowel syndrome (MacDonald *et al*., 2000; McDermott, 2001; Van Assche & Rutgeerts, 2000). These profitable multi-billion dollar ventures have shown the significance of TNF in human diseases, and the importance of the development of pharmacological tools to modulate cytokine activities.

Other research at an earlier developmental stage has shown a fundamental role for TNF in diseased states such as asthma and chronic obstructive pulmonary disorder (COPD) (Thomas, 2001), septic shock (Waage *et al*., 1987; Schluter & Deckert, 2000), meningitis (Schluter & Deckert, 2000), and even chronic heart failure (Bolger & Anker, 2000; Ferrari, 1999). TNF is also thought to be important in inflammatory diseases including malaria (Odeh, 2001) or lupus (Kontoyiannis & Kollias, 2000), and neural demyelinating conditions such as encephalomyelitis and multiple sclerosis (Probert *et al*., 2000; Kassiotis & Kollias, 2001). Osteoclast formation and actions are heavily controlled by the TNF ligands RANKL and OPG, which may be the new generation of cytokines used to treat bone diseases such as osteoporosis and Paget's disease (Horowitz *et al*., 2001). It is hoped that future development of drugs which modulate TNF ligand actions, or small molecular weight antagonists of the signalling pathways specifically controlled by TNF receptors, will lead to new and exciting strategies for the therapeutic intervention in a wider range of human diseases.

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(Received October 29, 2001
Revised December 6, 2001
Accepted December 7, 2001)