SIGNALLING PATHWAYS OF THE TNF SUPERFAMILY: A DOUBLE-EDGED SWORD

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Two different tumour-necrosis factors (TNFs), first isolated in 1984, were found to be cytotoxic to tumour cells and to induce tumour regression in mice. Research during the past two decades has shown the existence of a superfamily of TNF proteins consisting of 19 members that signal through 29 receptors. These ligands, while regulating normal functions such as immune responses, haematopoiesis and morphogenesis, have also been implicated in tumorigenesis, transplant rejection, septic shock, viral replication, bone resorption, rheumatoid arthritis and diabetes; so indicating their role as 'double-edged swords'. These cytokines either induce cellular proliferation, survival, differentiation or apoptosis. Blockers of TNF have been approved for human use in treating TNF-linked autoimmune diseases in the United States and other countries.

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Similar to most discoveries in science, the discovery of tumour-necrosis factor (TNF) is marked by a series of important landmarks. More than a century ago, a German physician, P. Bruns, reported the regression of tumours in humans after bacterial infection[1]. This led W. Coley, an American oncologist, to use bacterial extracts (referred as Coley's toxins) for the treatment of human cancers, with some success[2]. Gratia *et al.*[3] showed tumour regression by bacterial extracts in a guinea-pig model. In 1944, Shear *et al.*[4] isolated lipopolysaccharide (LPS) from the bacterial extracts and showed that this was responsible for the tumour regression. G. Algire, a French scientist, showed that LPS induces haemorrhagic necrosis of tumours by inducing systemic hypotension, collapse of tumour vasculature, tumour-cell anorexia and cell death[5]. Subsequently, O'Malley *et al.*[6] showed that the tumour regression effects of LPS are not direct, but are mediated through the induction of a factor in the serum; which they named tumour-necrotizing factor[6], renamed by L. Old's group as tumour-necrosis factor (TNF)[7]. Old and co-workers originally reported[7] that macrophages were the cellular source of this factor, but later they showed that a B-lymphoblastoid cell line was also a source[8]. This discrepancy is typical of the confusion that occurred at the time due to the crudeness of the preparations. Lymphotoxin (LT) was described in 1968 by G. Granger and co-workers[9] as a protein that is produced by lymphocytes that kills tumour cells.

Not until 1984, when my group purified the proteins to homogeneity[10,11], determined the amino-acid sequence[11,12] and cloned the complementary DNA[13,14], were the true chemical identities of TNF and LT, and their relationship revealed. Human LT was the first cytotoxic cytokine to be purified from a B-lymphoblastoid cell line[10]; its structure was determined by classic protein sequence methods[11]. Neutralization of LT activity by LT-specific antibodies led to the isolation of a second cytotoxic factor from a human myeloid-cell line that was known as TNF[12]. The determination of the amino-acid sequence of TNF indicated that the two proteins were homologous[12]. The binding of TNF to its receptor and its displacement by LT further confirmed the functional homology between the two proteins[15]. This was perhaps the earliest indication of the existence of a potential TNF superfamily. Once most of the protein sequence was known, the cDNAs for LT and TNF were isolated[13,14]. The sequence homology between these two proteins (30% amino-acid identity) and the existence of common cell-surface receptors led to

the renaming of TNF and LT to TNF-α and TNF-β, respectively. Soon after, mouse TNF-α was independently discovered as a factor that mediated LPS-induced wasting (cachexia) in mice by Beutler *et al.*[16], and as a myeloid differentiation factor by Takeda and co-workers[17]. These two cytokines laid the foundation for the isolation and identification of the larger family of cytokines, now known as the TNF superfamily (FIG. 1). Considerable advances have been made during the past two decades in our understanding of the biology and the clinical role of the TNF superfamily. This review focuses on these developments.

Characteristic features of ligands and receptors

At present 19 different ligands have been identified that belong to the TNF superfamily. Unlike TNF and LT, the ligands for FAS (CD95), CD27, CD30, CD40, 4-1BB, OX40 and herpes-virus entry mediator (HVEM) were identified and cloned not by protein sequencing, but by direct expression-cloning strategies[18–21]. This involved the use of specific antibodies, and studies of ligand–receptor interactions or biological activity. The description of the amino-acid sequences of several ligands and receptors of the superfamily led to the identification of certain regions of homology. Instead of an expression-cloning strategy, the availability of human genome sequences led scientists to use homology searches to identify several additional members of the TNF superfamily. TNF-related apoptosis-inducing ligand (TRAIL), also known as APO2L, was identified independently by two different groups[22,23], followed by the identification of receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL), also known as TRANCE (TNF-related activation-induced cytokine) or OPGL (osteoprotegerin ligand)[24–26], vascular endothelial cell-growth inhibitor (VEGI), also known as TL1A[27,28], and B-cell-activating factor (BAFF)[21,30], also known as β-lymphocyte stimulator (BLYS), all by similar approaches. Glucocorticoid-induced TNF receptor family receptor ligand (GITRL) was identified and cloned by using a glucocorticoid-induced differential display technique[31]. Because numerous groups discovered the same cytokine independently and simultaneously, numerous names exist for each TNF family member.

An intriguing feature of these ligands is that when certain ligands are shed, they inhibit the function of the ligand–receptor complex. For example, whereas the membrane-bound CD95 ligand (CD95L) kills human peripheral-blood T cells, soluble CD95L blocks this killing[36]. The fact that some soluble ligands act as agonists whereas others act as antagonists is intriguing. Furthermore, the transmembrane expression of most of the TNF-superfamily members indicates that they are meant to act locally. Only under non-physiological conditions, when these ligands are released, do they prove harmful. Perhaps the best example is TNF-α, which when released binds to various cell types to mediate various diseases.

The 19 ligands mediate their cellular response through 29 receptors that belong to the TNF receptor (TNFR) superfamily, and they are characterized by the presence of a cysteine-rich domain (CRD) in the extracellular portion (FIG. 1). These include four decoy receptors, known as DCR1, DCR2, DCR3 and osteoprotegerin (OPG), which reduce the ligand signalling. The ligands for death receptor 6 (DR6), receptor expressed in lymphoid tissues (RELT) and TROY have not yet been identified. Whereas germline mutations in the extracellular domain of TNFR1 suppress the secretion of the receptor leading to a family of dominantly inherited autoinflammatory syndromes[37], the secretion of OPG affects the regulation of bone density[38]. Receptors of the TNFR superfamily are broadly divided into two groups. Those with the death domain are known as death receptors. The death domain is a region of approximately 80 amino-acid residues first identified independently by two different groups in the cytoplasmic domain of CD95 and TNFRs; their deletion abolishes ligand-induced apoptosis[21,39,40]. Interestingly, a TNFR that has a cysteine-rich region in its extracellular portion and serine/threonine protein kinase activity in its cytoplasmic domain has been discovered in the maize plant[41]. This protein, known as CRINKLY4, is a TNFR-like receptor and has kinase activity that is involved in maize epidermal differentiation.

Additionally, a conserved domain in the extracellular region of TNFR1 and TNFR2 has been identified that mediates specific ligand-independent assembly of receptor trimers[42]. This pre-ligand-binding assembly domain (PLAD) is physically distinct from the domain that forms the main contacts with the ligand, but is required for the assembly of TNFR complexes that bind TNF and mediate signalling. Other members of the TNFR superfamily, including TRAIL receptor 1 (TRAILR1) and CD40, show similar homotypic associations. So, TNFRs and related receptors seem to function as preformed complexes rather than as individual receptor subunits that oligomerize after ligand binding.

The cellular expression patterns of various members of the TNF ligands and receptors, are shown in TABLE 1. Almost all of the TNF ligands are expressed by cells of the immune system, including B cells, T cells, NK cells, monocytes and dendritic cells, the only exception is VEGI, which is expressed mainly by endothelial cells[43]. The TNFRs, however, are expressed by a wide variety of cells. For example, no cell type in the body has yet been found that does not express TNFR1, whereas expression of TNFR2 is mainly by immune cells and endothelial cells. Numerous activities are assigned to TNF, perhaps because its receptors are expressed by all cell types. This might also account for the non-specific toxicity of TNF. Whereas most ligands bind to a single receptor, others bind to more than one. For example, TRAIL binds to as many as five receptors (DR4, DR5, DCR1, DCR2 and OPG), whereas BAFF binds to three receptors; transmembrane activator and cyclophilin ligand interactor (TACI), B-cell maturation antigen (BCMA) and BAFFR. Why there are several receptors for the same ligand is unclear. Both homomeric and heteromeric complexes of DR4 and DR5 have been described[44,45], leading to the recruitment of downstream cell-signalling intermediates. In the case of TRAIL, although DR4 and DR5 transduce most signals, DCR2 transduces partial

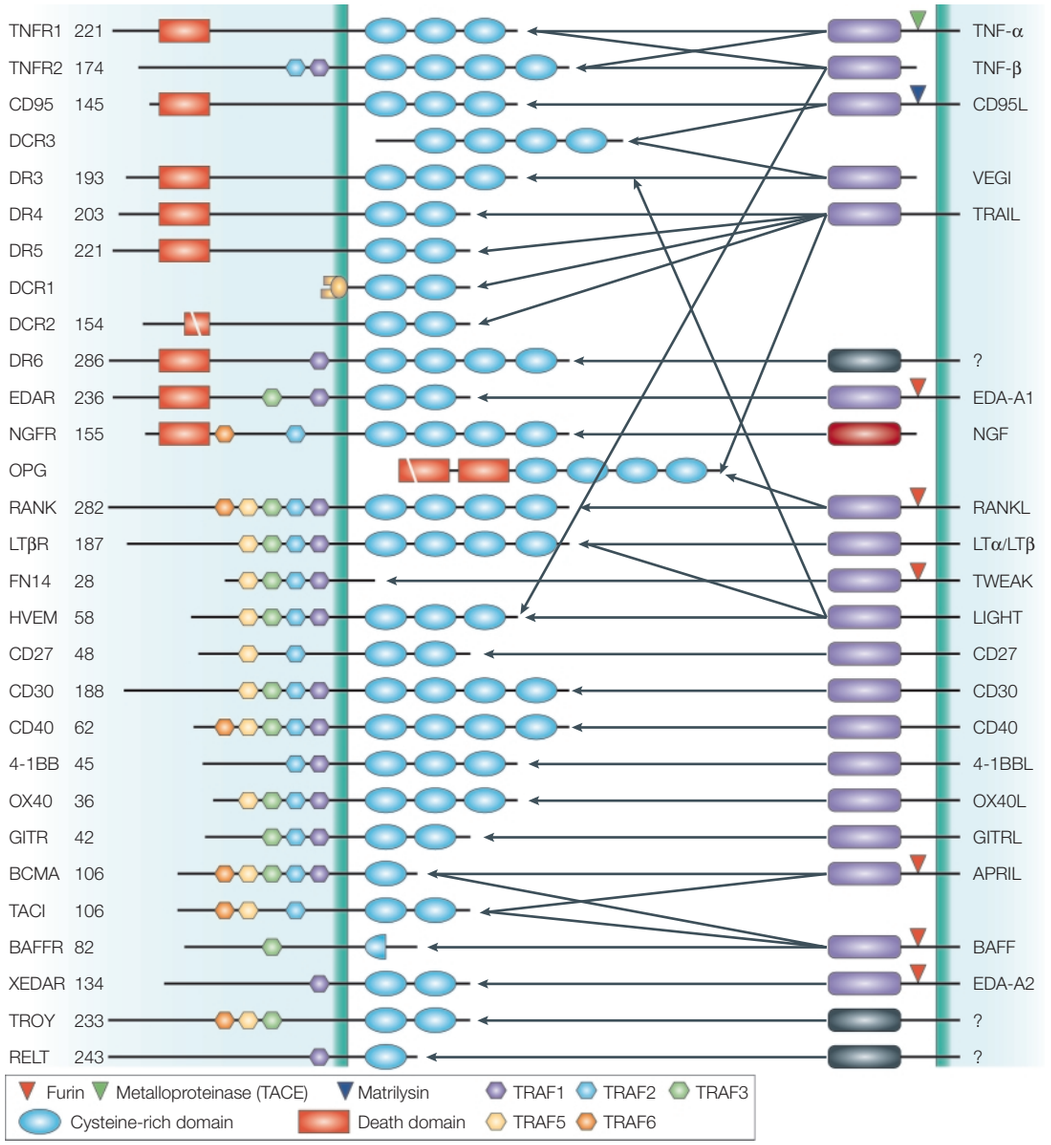

Figure 1 | **A diagrammatic representation of the ligands of the TNF superfamily and their receptors.** All ligands, except lymphotoxin-α (LTα) and vascular endothelial cell-growth inhibitor (VEGI) which are secreted, are type II transmembrane proteins with a carboxy-terminal extracellular domain, an amino-terminal intracellular domain and a single transmembrane domain. The C-terminal extracellular domain, known as the tumour-necrosis factor (TNF) homology domain, has 20–30% amino-acid identity between the superfamily members and is responsible for binding to the receptor. TNF-related apoptosis-inducing ligand (TRAIL) and CD95 ligand (CD95L) have the highest homology among the TNF-superfamily members, in parallel with their ability to induce apoptosis. Most members of the TNF superfamily are released from the cell surface by proteolysis through distinct proteases. TNF and receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) are released by metalloproteases of the ADAM (a disintegrin and metalloprotease domain) family[32,33], CD95L by matrilysin[34], and B-cell-activating factor (BAFF), ectodermal dysplasin (EDA), TNF-like weak inducer of apoptosis (TWEAK) and a proliferation-inducing ligand (APRIL) by members of the furin family[30,35]. Mutations in the furin consensus sequence have been shown to block proteolytic release of EDA and to cause X-linked hypohydrotic ectodermal dysplasia[35]. Unlike the ligands, the TNF receptors (TNFRs) are characterized as type I transmembrane proteins (extracellular N-terminus and intracellular C-terminus). Because they lack a signal peptide sequence, B-cell maturation antigen (BCMA), transmembrane activator and cyclophilin ligand interactor (TACI), BAFF receptor (BAFFR) and X-linked EDA receptor (XEDAR) belong to type III transmembrane protein group. Decoy receptor 1 (DCR1) is anchored by a covalently linked C-terminal glycolipid. Osteoprotegerin (OPG) and DCR3 lack a transmembrane domain and are therefore secreted as soluble proteins. Soluble forms of several receptors have been identified, which seem to have important functions, including CD27, CD30, CD40, TNFR1, TNFR2, CD95 and 4-1BB. Soluble CD95 and 4-1BB are generated by alternative splicing[18]. Until now, eight different death-domain-containing receptors have been identified, death receptor 1 (DR1, also known as TNFR1), DR2 (CD95), DR3, DR4 (also known as TRAILR1), DR5 (also known as TRAILR2), DR6, EDAR and low-affinity nerve growth-factor receptor (NGFR). Although structurally NGFR belongs to the TNFR superfamily, many of its biological actions differ from other members of the family. The numbers on the left represent the number of amino acids in the cytoplasmic domain of the receptor. GITR, glucocorticoid-induced TNFR family receptor; HVEM, herpes-virus entry mediator; RELT, receptor expressed in lymphoid tissues; TRAF, TNFR-associated factor.

signals only[46]. Through sequestration of the ligand, DCR1, DCR2, DCR3 and OPG have been shown to inhibit cell signalling. An OPG variant protein that has two death domains, but lacks a transmembrane domain, however, has also been shown to function as a ligand through an unknown receptor[47].

In the case of TNF, it has been suggested that although TNFR1 is a receptor for a soluble ligand, TNFR2 mediates the signalling of the membrane-bound ligand[48]. Whereas some reports indicate that TNFR1 mediates apoptosis and TNFR2 mediates proliferation, others suggest that the two TNFRs transduce their signals cooperatively[49,50]. The genetic deletion of either receptor blocked most of the signals that are transduced by TNF[50]. There is also evidence of crosstalk between receptors for different ligands of the TNF superfamily. For example, CD95L[18], TNF-like weak inducer of apoptosis (TWEAK)[51], CD30L and CD40L[52] were found to trigger through their cognate receptors the upregulation of expression of endogenous TNF, which subsequently activates TNFR1. Gene-deletion experiments have shown that crosstalk between TNFRs and LPS receptors might occur, indicating an overlap in cell-signalling pathways between different receptors[53].

Cell signalling. After binding to the receptor, members of the TNF superfamily either mediate apoptosis (such as TNF, LT, CD95L, TRAIL, VEGI, TWEAK and LIGHT), survival (such as RANKL and BAFF), differentiation (such as TNF, RANKL and DR6) or proliferation (such as TNF, CD27L, CD30L, CD40L, OX40L, 4-1BBL, APRIL and BAFF) through the activation of pathways involving NF-κB, JUN N-terminal kinase (JNK), p42/p44 mitogen-activated protein kinase (MAPK) and p38 MAPK. Signalling through TNF and CD95 has been a paradigm for most other members of the TNF superfamily. None of the receptors of the mammalian TNF superfamily has any enzymatic activity. By using the yeast

Table 1 | **Cellular expression of ligands and receptors of the tumour-necrosis factor superfamily**

Cytokine	Cells	Receptor	Cells
LTα	NK, T and B cells	TNFR1 TNFR2	Most normal and transformed cells Endothelial cells and immune cells
TNF	Macrophages, NK, T and B cells	TNFR1, TNFR2	See above
LTβ	DCs, macrophages, T, B and NK cells	LTβR	NK cells, $CD4^+$and $CD8^+$ T cells
CD95L	Activated splenocytes, thymocytes and non-lymphoid tissues, such as the eye and testis	CD95	Most normal and transformed cells
		DCR3	Lung and colon cells
TRAIL	NK cells, T cells and DCs	DR4, DR5 DCR1, DCR2, OPG	Most normal and transformed cells Most normal and transformed cells
TWEAK	Monocytes	FN14	Endothelial cells and fibroblasts
CD27L	NK, T and B cells	CD27	$CD4^+$ and $CD8^+$ T cells
CD30L	T cells and monocytes	CD30	Reed–Sternberg cells
CD40L	T and B cells	CD40	Reed–Sternberg cells
4-IBBL	B cells, DCs and macrophages	4-IBB	Activated T cells, monocytes and NK cells
OX40L	T and B cells	OX40	T cells
APRIL	Macrophages, lymphoid cells and tumour cells	BCMA TACI	B cells, PBLs, spleen, thymus, lymph nodes, liver and adrenals B cells, PBLs, activated T cells, spleen, thymus and small intestine
BAFF	T cells, DCs, monocytes and macrophages	TACI BCMA BAFFR	See above See above B cells, PBLs, resting T cells, spleen and lymph nodes
LIGHT*	T cells, granulocytes, monocytes and DCs	HVEM LTβR	T cells Non-lymphoid haematopoietic cells and stromal cells
VEGI*	Endothelial cells	DR3, DCR3	Activated T cells
GITRL	N. D.	GITR	$CD4^+CD25^+$ T cells
RANKL	Activated T cells and osteoblasts	RANK OPG	Osteoclasts, osteoblasts and activated T cells Osteoclast precursors, endothelial cells and others
EDA1	Skin	EDAR	Ectodermal derivative
EDA2	Skin	XEDAR	Ectodermal derivative
N. D.		DR6	Resting T cells
N. D.		RELT	Lymphoid tissues
N. D.		TROY	Embryo skin, epithelium, hair follicles and brain

*Binds to DCR3/TR6. VEGI is also known as TNF superfamily ligand (TL1) and TL1A is a longer variant of TL1; TROY is also known as TAJ (toxicity and JNK inducer). APRIL, a proliferation-inducing ligand; BAFF; B-cell-activating factor; BCMA, B-cell maturation antigen; DC, dendritic cells; DCR, decoy receptor; DR, death receptor; EDA, ectodermal dysplasin; EDAR, ectodyplasin-A-receptor; GITR, glucocorticoid-induced TNFR family receptor; L, ligand; LT, lymphotoxin; N. D., not determined; NK, natural killer; OPG, osteoprotegerin; PBL , peripheral-blood lymphocytes; R, receptor; RANK, receptor activator of nuclear factor-κB; RELT, receptor expressed in lymphoid tissues; TACI, transmembrane activator and cyclophilin ligand interactor; TNF, tumour-necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-like weak inducer of apoptosis; VEGI, vascular endothelial cell-growth inhibitor; XEDAR, X-linked ectodermal dysplasia receptor.

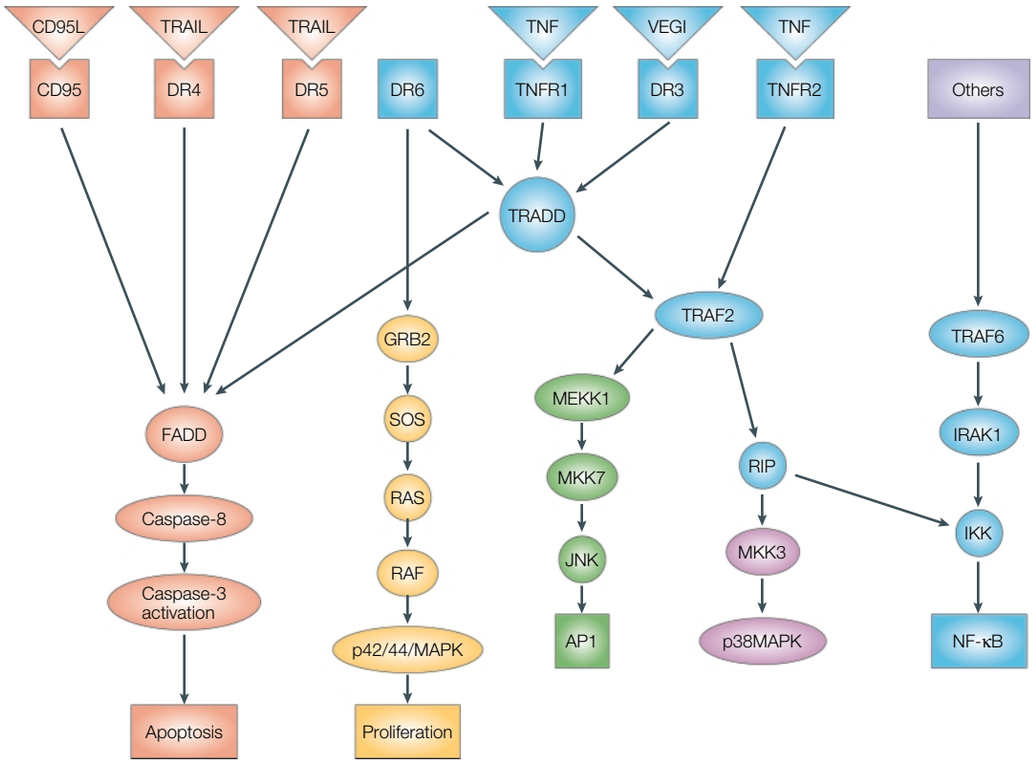

Figure 2 | **Cellular signalling pathways leading to activation of the main cellular responses by members of the TNF superfamily.** Tumour-necrosis factor receptor 1 (TNFR1) binds to TNFR-associated death domain (TRADD) protein, factor associated with neutral sphingomyelinase (FAN), TNFR-associated protein 1 (TRAP1), TRAP2, mitogen-activated protein kinase (MAPK) activating death domain (MADD) and growth-factor receptor-bound protein 2 (GRB2)[54,55]. Through its death domain, TNFR1 sequentially recruits TRADD, TNFR-associated factor 2 (TRAF2), receptor-interacting protein (RIP) and inhibitor of nuclear factor-κB (NF-κB) kinase (IKK), leading to the activation of NF-κB; and the recruitment of TRADD, FAS-associated death domain (FADD) and caspase-8, leads to the activation of caspase-3, which in turn induces apoptosis[56]. A recent report indicated that FADD and caspase-8, although required, were not recruited to the TNFR1 signalling complex during TNF-induced apoptosis[57]. CD95, death receptor 4 (DR4) and DR5 activate apoptosis through sequential binding of FADD to caspase-8 and caspase-10 (REFS 45,58). Whether DR4/DR5 recruit FADD through TRADD is controversial. Earlier reports indicate that both DR4 and DR5 bind to TRADD[44,59], but recent reports indicate that DR4/DR5 bind to FADD independently of TRADD[45,57]. The role of FADD in TNF-related apoptosis-inducing ligand (TRAIL) signalling is also controversial. Although an earlier report indicated that TRAIL-induced apoptosis is FADD independent[59], later reports indicated that FADD is required[45,57]. Besides TNFR1, DR3 and DR6 are also known to bind TRADD and mediate the activation of apoptosis, NF-κB and JUN N-terminal kinase (JNK)[28,60]. TNFR1 activates JNK through the sequential recruitment of TRAF2, MAP/ERK kinase kinase 1 (MEKK1) and MAPK kinase 7 (MKK7). The activation of p38 MAPK by TNFR1 is less well understood and involves the recruitment of RIP by TRADD, which in turn recruits MKK3 leading to p38 MAPK activation[54–56]. AP1, activator protein 1; IRAK1, interleukin-1-receptor-associated kinase 1; VEGI, vascular endothelial cell-growth inhibitor.

two-hybrid system for protein–protein interaction, co-precipitation and dominant-negative approaches, several proteins that mediate TNF signalling have been identified (FIG. 2).

Almost all of the members of the TNFR superfamily signal by binding to one or more TNFR-associated factors (TRAFs)[61] (FIG. 3 and TABLE 2). The interaction of TRAF1, TRAF2 and TRAF5 with various cytoplasmic domains of TNFR-superfamily members requires a specific motif in the receptor (that is, Pro-Xaa-Gln-Xaa-Thr). By contrast, TRAF6 uses a distinct motif (Gln-Xaa-Pro-Xaa-Glu), which has been identified in CD40 and RANK[62,63]. However, of the known TRAFs, only TRAF2, TRAF5 and TRAF6 have been shown to mediate the activation of NF-κB and JNK. TRAF2 is known to bind to almost all of the members of the TNFR superfamily, it binds to TNFR1 through the TNFR-associated death domain (TRADD) protein. Interestingly, it was recently found that the binding of TNF to TNFR2 induces inhibitor of apoptosis 1 (IAP1)-mediated ubiquitination and proteasomal degradation of TRAF2 (REF. 64). TNFR2-induced degradation of TRAF2 provides further evidence of crosstalk between the two receptors.

Most members of the TNF superfamily activate NF-κB through ubiquitin-mediated degradation of its inhibitor, IκBα. This process is initiated by phosphorylation of IκBα by the IκBα kinase (IKK) complex, mainly by the IKKβ catalytic subunit, and requires the regulatory subunit IKKγ (also known as NEMO). BAFF, however, has been shown to activate NF-κB by ubiquitin-mediated processing of the NF-κB2 inhibitory protein p100, which depends on the phosphorylation of p100 by IKKα. For BAFF to activate this second pathway BAFFR, NF-κB-inducing kinase (NIK) and protein synthesis, but not

NEMO[65,66] are required. This NEMO-independent cascade is physiologically relevant for the survival and maturation of splenic B cells. Similar to BAFF, LTβ receptor (LTβR) has also been shown to promote the NF-κB2 processing pathway[67]. IKKα has also been found to be crucial for RANK-induced NF-κB activation[68].

Recent studies indicate that after TNF binding, TNFR1 translocates to cholesterol- and sphingolipid-enriched membrane microdomains, known as lipid rafts, in which it associates with the adaptor proteins TRADD and TRAF2, and the Ser/Thr kinase receptor-interacting protein (RIP), forming a signalling complex[69] (FIG. 2). In lipid rafts, TNFR1 and RIP are ubiquitylated, which leads to their degradation by the proteasome pathway. Interfering with lipid-raft organization not only abolishes ubiquitylation, but also switches TNF signalling from NF-κB activation to apoptosis, indicating that lipid rafts are crucial for the outcome of TNF-activated signalling pathways.

Refinement of cell signalling by gene deletion. As indicated earlier, the pathways through which various members of the TNFRs signal have been elucidated by investigations into protein–protein interactions, and overexpression and dominant-negative studies. These techniques, however, can produce misleading data. Gene-deletion studies have provided a different picture of the mechanism of signalling by the TNFRs. For example, deletion of the *TRAF2* gene had a minimal effect on TNF-induced NF-κB activation, but resulted in defective JNK signalling[70], indicating that TRAF2 is dispensable for NF-κB activation, but not for JNK activation.

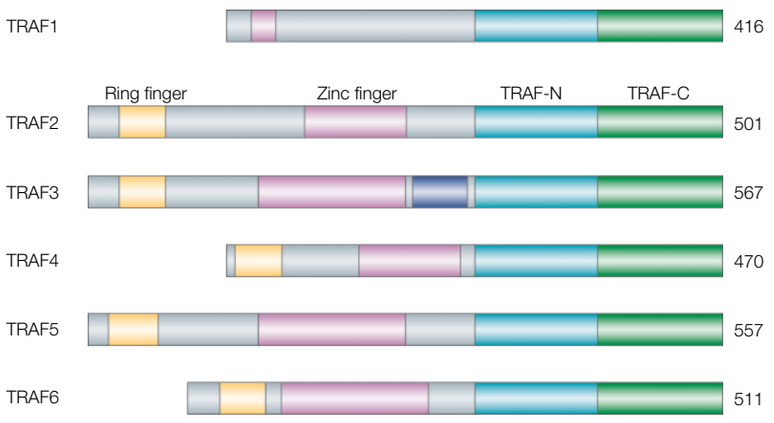

Figure 3 | **The structures of different TNF receptor-associated factors.** The tumour-necrosis factor receptor (TNFR)-associated factor (TRAF) family consists of six distinct proteins, each containing a ring finger domain (except TRAF1), a zinc-finger motif, an amino-terminal TRAF domain and a carboxy-terminal TRAF domain. TRAFs indirectly bind to the death domain of the receptors. TRAF2, TRAF5 and TRAF6 function as adaptor proteins that link the cell-surface receptors and downstream kinase cascades, which results in the activation of nuclear factor-κB (NF-κB) and activator protein 1 (AP1). Neither TRAF1 nor TRAF3 activates any kinases. The C-terminal half of the TRAF domain (TRAF-C) is highly conserved among members of the TRAF domain and it mediates homo- and heterodimerization of the TRAFs and association of TRAFs with various cell-surface receptors. The N-terminal portion of the TRAF domain (TRAF-N), predicted to form a coiled-coil structure, is less conserved. TRAF2, TRAF3, TRAF5 and TRAF6 contain five zinc-finger repeats, whereas TRAF4 has seven repeats. Apart from TRAF4, all of the TRAFs were identified by yeast two-hybrid screening using a cytoplasmic domain of various members of the TNFR family. So far, TRAF4 has no known function in the signalling of TNF or its family members.

However, TNF-induced NF-κB activation was markedly impaired in mouse embryo fibroblasts from TRAF2, TRAF5-double-knockout mice, and these cells were more susceptible to TNF-induced cytotoxicity[71]. These results indicated that both TRAF2 and TRAF5 are involved in TNF-induced NF-κB activation and protection from cell death. Deletion of the gene encoding RIP, however, abolished TNF-induced activation of NF-κB, but had minimal effect on JNK activation[72]. So RIP, which previously was not considered to be part of the TNF signalling mechanism, is now known to be a central mediator. Similarily, NIK was previously thought to be crucial for TNF-induced NF-κB activation[73], but was found by gene-deletion experiments to be dispensable[74]. Instead, NIK was found to be required for NF-κB2 activation by LTβR and BAFFR[74]. Although not previously recognized, gene-deletion studies have also indicated an essential role for MAP/ERK kinase kinase 3 (MEKK3) in TNF-induced NF-κB activation[75]. So, our understanding of how various members of the TNFR superfamily transduce their signals is becoming increasingly refined.

The fact that inhibitors of protein synthesis can enhance the cytotoxic effects of TNF and also make resistant cells sensitive to TNF has been well documented[12,76]. The mechanism for this, however, was unveiled only recently. Work in the past five years has indicated that TNF simultaneously activates both apoptotic and anti-apoptotic or cell-survival signals (FIG. 4). The apoptotic signals do not require active protein synthesis, whereas anti-apoptotic signals do, and the synthesis is mediated through the activation of NF-κB[77]. It is the balance between the pro-apoptotic and anti-apoptotic signals that eventually determines whether cells will undergo apoptosis, survive or proliferate in response to TNF or other family members. Cytokines such as TRAIL and CD95 that are poor activators of NF-κB induce apoptosis more rapidly than TNF does; whereas cytokines such as RANKL mainly provide survival signals through the activation of NF-κB. Interestingly, however, NF-κB does not just regulate anti-apoptotic signals, it also regulates pro-apoptotic signals through the regulation of death receptors (DR1, DR2, DR4, DR5 and DR6)[77–80], and death receptor ligands such as CD95L, TNF and TRAIL[77,80]. The pro-apoptotic effects of NF-κB could also be mediated through the upregulation of expression of p53 and c-MYC[81,82]. c-MYC, however, can sensitize cells to TNF-mediated apoptosis by inhibiting NF-κB transactivation[77]. This adds additional complexity to the role of NF-κB in the regulation of apoptosis.

Biological roles of the TNF-superfamily members. TNF and its family members represent a double-edged sword. Whereas physiologically they are important cytokines and required for normal responses, their inappropriate expression is harmful. Research during the past two decades has shown that TNF and its superfamily members have both beneficial and harmful activities. This is not unusual for most therapeutics (FIG. 5). First, I discuss the 'bright side' of TNF-superfamily members and then the 'dark side'. Several genetic diseases are linked to the TNF/TNFR superfamily, which are discussed later.

Table 2 | **Interaction of TRAFs with the TNFR-superfamily members**

Receptor	TRAF1	TRAF2	TRAF3	TRAF5	TRAF6
TNFR2	+	+	–	–	–
LTβR	+	+	+	+	N. D.
CD40	+	+	+	+	+
CD30	+	+	+	+	N. D.
CD27	N. D.	+	–	+	–
OX40	–	+	+	–	N. D.
4-1BB	+	+	–	–	N. D.
GITR	+	+	+	–	–
HVEM	+	+	+	+	–
RANK	+	+	+	+	+
TACI	–	+	–	+	+
BCMA	+	+	+	+	+
TROY	–	+	–	+	+
DR6	+	–	–	–	–
XEDAR	+	–	–	–	–
FN14	+	+	+	+	–
BAFFR	–	–	+	–	–
NGFR	–	–	–	–	+

BAFFR, B-cell-activating factor receptor; BCMA, B-cell maturation antigen; DR6, death receptor 6; GITR, glucocorticoid-induced TNFR family receptor; HVEM, herpes-virus entry mediator; LTβR, lymphotoxin-β receptor; N. D., not determined; NGFR, nerve growth-factor receptor; RANK, receptor activator of nuclear factor-κB; TACI, transmembrane activator and cyclophilin ligand interactor; TNFR, tumour-necrosis factor receptor; TRAF, TNFR-associated factor; XEDAR, X-linked ectodermal dysplasia receptor.

The bright side of the TNF superfamily

Anticancer potential. Although initially thought to be a potent anticancer agent, it is now generally believed that TNF has limited activity in the suppression of cancer, mainly because of its systemic toxicity[83]. If this systemic toxicity could be suppressed, the therapeutic usefulness of TNF might increase. For example, recent studies showed that the inhibition of matrix metalloproteinases blocks the lethal hepatitis and apoptosis induced by TNF and allows safe antitumor therapy[84]. Also, TNF is a successful treatment for locally advanced limb soft-tissue sarcomas and other large tumours when administered by isolated limb perfusion — this approach can avoid the need for amputation[85]. TNF was approved in Europe after a multicentre trial in patients with locally advanced soft-tissue sarcomas considered to be unresectable by an independent review committee; the response rate to isolated limb perfusion with TNF plus melphalan was 76% and the limb was saved in 71% of the patients. Moreover, the trial showed the efficacy of isolated limb perfusion of TNF and melphalan against various other limb-threatening tumours, such as skin cancers and drug-resistant bony sarcomas. Laboratory models of isolated limb perfusion have helped to elucidate the mechanisms of action and to develop new treatment methods. These studies have identified TNF-mediated vasculotoxic effects on the tumour vasculature and have shown that addition of TNF to the perfusate results in an increase of three to six times in uptake of the vasoactive drugs melphalan or doxorubicin by tumours. Moreover, isolated limb perfusion is an effective procedure for gene therapy mediated by an adenovirus vector. Various clinical phase I and phase II studies can be expected in the next few years. A multicenter, random-assignment trial of isolated limb perfusion that compares melphalan alone and melphalan with TNF in patients with melanoma, is also being carried out by the National Cancer Institute in the United States[86]. Alternative approaches to target TNF involve the use of agonistic antibodies specific for TNFR1 or gene therapy using TNFR1, which can also induce apoptosis of tumour cells when overexpressed.

In contrast to TNF, TRAIL has been found to specifically kill tumour cells without harming normal cells, and so it is being explored for the treatment of cancer. Animal studies investigating the anticancer potential of TRAIL have been promising[46,87]. In addition to TRAIL, agonistic antibodies specific for its receptors DR4 (REF. 88) and DR5 (REF. 89) are also being examined for their antitumour activities. The initial results with animal studies have been promising. The role of TRAIL and other members of the TNF superfamily in cancer immunotherapy has also been documented[90,91].

Regulation of the immune system. The immune system is regulated not only by cell proliferation and differentiation, but also by apoptosis. Loss-of-function mutations in the signalling molecules that are involved in apoptosis can cause hyperproliferation of cells. By contrast, amplification of the death cascade can cause the destruction of various tissues. Ligands of the TNF superfamily control and orchestrate the immune response at several levels[19]. TNF, LTα, LTβ and RANKL provide crucial signals for the morphogenesis of secondary lymphoid organs. Pro-apoptotic members, such as TNF, CD95L and TRAIL, contribute to the function of cytotoxic effector cells in the recognition and destruction of virus-infected cells.

CD95–CD95L interactions are important for regulating the immune system in several ways. Activated T cells express CD95 and CD95L, and they are sensitive to CD95-induced apoptosis, indicating that the activated T cells undergo suicide or kill each other (fratricide) to downregulate the immune response[92]. CD95-induced apoptosis might also be involved in clonal deletion of thymocytes that are highly reactive to antigens expressed in the thymus[93]. Several autoimmune diseases have been identified in which CD95–CD95L interactions are non-functional[94]. Elevated serum levels of CD95L have been seen in patients with natural killer (NK) cell large granular lymphoma leukaemia, SYSTEMIC LUPUS ERYTHEMATOSUS (SLE), rheumatoid arthritis, SJOGREN'S SYNDROME, lymphohistocytosis, myocarditis and acute GRAFT-VERSUS-HOST DISEASE[58].

Studies of the naturally occurring CD95-mutant mice (*lpr*, lymphoproliferation) and CD95L-mutant mice (*gld*, generalized lymphoproliferative disease) have enhanced our understanding of the role of these TNF-superfamily members in the regulation of the immune system. Mature T cells from *lpr* or *gld* mice do not die after activation. In addition to T-cell defects, CD95-deficient mice accumulate B cells and have

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE). A disease of unknown origin in which tissues and cells are damaged by the deposition of pathogenic antibodies and immune complexes. Patients generally have abnormal B- and T-cell functions.

SJOGREN'S SYNDROME (SS). An autoimmune disease characterized by diffuse lymphoid-cell infiltrates in the salivary and lacrimal glands, resulting in dry eyes and mouth due to insufficient secretion.

GRAFT-VERSUS-HOST DISEASE (GVHD). Tissue damage in a recipient of allogeneic transplanted tissue (usually a bone-marrow transplant) that results from the activity of donor cytotoxic T cells that recognize the recipient's tissue as foreign. GVHD varies markedly in severity, but can be life threatening in severe cases. Typically, damage to the skin and gut mucosa leads to clinical manifestations.

IMMUNE-PRIVILEGED SITE
Immune-privileged sites are areas in the body with a decreased immune response to foreign antigens, including tissue grafts. These sites include the brain, eye, testis and uterus.

elevated levels of immunoglobulin of various classes[95]. It has been shown that CD95L-expressing T cells kill CD95-expressing activated B cells. The eye is an IMMUNE-PRIVILEGED SITE, which ensures that inflammation does not destroy the tissue. This is mediated through the expression of CD95L by the cornea cells, which kills the inflammatory cells and is, therefore, responsible for protection of the eye from inflammation[96].

TRAIL has also been shown to have an important role in regulation of the immune system. TRAIL can regulate lymphocyte functions in the periphery by inhibiting cell-cycle progression by T cells[97]. Apoptosis of thymocytes is defective in mice that are deficient for TRAIL, such that thymic deletion induced by T-cell receptor ligation is markedly impaired. TRAIL-deficient mice are also hypersensitive to collagen-induced arthritis and streptozotocin-induced diabetes, and develop heightened autoimmune responses[98,99]. So, TRAIL mediates thymocyte apoptosis and is important in the induction of autoimmune diseases[99]. The potential role of TRAIL and other members of the TNF superfamily in organ transplantation have also been documented[90].

Balance between life and death

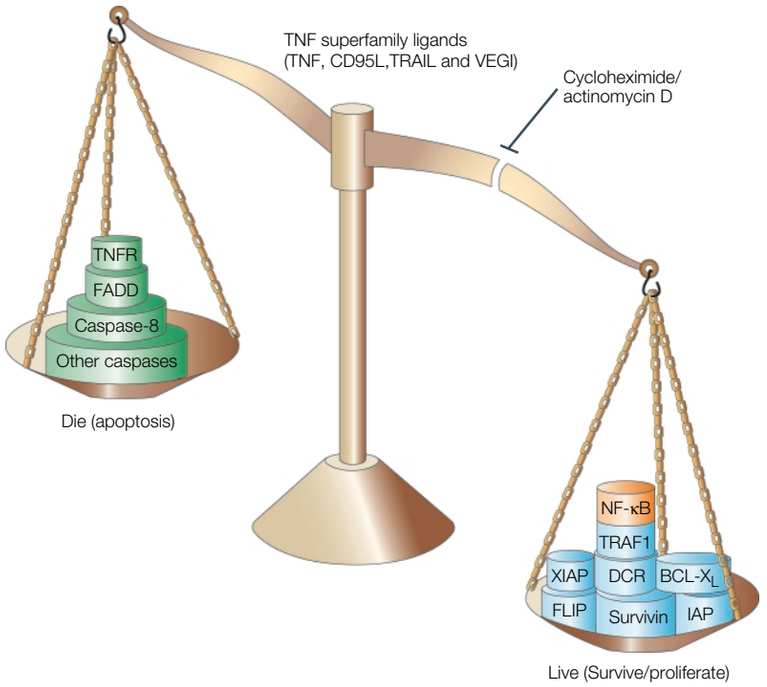

Figure 4 | **The balance between life and death mediated by TNF-superfamily members .** Apoptotic signals do not require active protein synthesis, whereas anti-apoptotic signals do and the synthesis is mediated through the activation of nuclear factor-κB (NF-κB)[77]. Several NF-κB-regulated proteins that can suppress apoptosis have been identified. These include tumour-necrosis factor receptor (TNFR)-associated factor 1 (TRAF1), inhibitor of apoptosis 1 (IAP1) and IAP2, survivin, FAS-associated death domain-like interleukin-1 converting enzyme inhibitory protein (FLIP), X-chromosome linked inhibitor of apoptosis protein (XIAP), decoy receptor (DCR) and BCL-X_L. These proteins inhibit different steps in the apoptotic pathways. For example, DCR sequesters the receptors, whereas FLIP blocks the activation of caspase-8, survivin inhibits caspase-3 activation, IAP suppresses caspase-9 activation, XIAP inhibits caspase-3 activation, and BCL-X_L prevents the release of cytochrome-c from the mitochondria and so blocks caspase-9 activation.

Haematopoiesis. Almost all of the TNF ligands are produced by haematopoietic cells (TABLE 1) and interactions mediated by these ligands between haematopoietic cells, such as T cells–B cells, T cells–monocytes and T cells–T cells have a crucial role in regulating their proliferation. For example, CD95L is involved in peripheral T-cell homeostasis; 4-1BBL preferentially co-stimulates $CD8^+$ T cells; CD27 co-stimulates T cells; CD30L is important in processes mediated by T helper 2 (T_H2) cells; CD40L is essential for T-cell-dependent antibody responses and OX40 co-stimulates T cells. All B cells also express receptors for CD30, CD40, TNF, LT, APRIL, BAFF and CD27L. Three different receptors for BAFF are expressed by B cells and control the maturation and survival of these cells. B-cell development is completely blocked in mice that are deficient for BAFF or BAFFR. Additionally, both TNF and LT have been implicated in B-cell proliferation[90,91]. BCMA deficiency does not lead to any severe B-cell phenotypes, indicating that BCMA is dispensable. TACI deficiency resulted in increased numbers of B cells, but the ratio of mature B-cell subsets in the spleen was normal. The activation of immune precursor cells to fully competent effector cells also depends on CD40L for B cells; 4-1BBL, OX40L and CD27L for T cells; and CD40 and RANKL for dendritic cells.

Protection against microbial infection. An effective host response against infection with bacteria depends partly on the ability to produce an appropriate T_H1-type cytokine profile, but TNFR signalling is also involved. It has been shown that deletion of either TNF or TNFR1 in mice leads to increased susceptibility to *Listeria monocytogenes*, rapid death from infection and resistance to LPS-mediated septic shock, indicating a crucial role of this cytokine in protection from infection. Antibodies specific for TNF (such as infliximab) and soluble TNFRs (such as enbrel) have been approved for human use in the treatment of Crohn's disease and rheumatoid arthritis, respectively. Anti-TNF therapies, however, have considerably increased the risk of development of certain diseases, such as tuberculosis[100]. This is not surprising considering the important role of TNF in killing *Mycobacterium tuberculosis* by macrophages.

The dark side of the TNF superfamily

Several of the harmful activities of TNF and its superfamily members are thought to be mediated through the activation of NF-κB. All members of the TNF ligand superfamily can activate NF-κB, although some members are more potent and ubiquitous than others. So far, over 200 genes have been shown to be regulated by NF-κB, and their products have been implicated in a wide variety of diseases. In general, the pathology that is linked to TNF and its family members is similar to that associated with inappropriate NF-κB activation.

Cancer. As mentioned earlier, TNF was discovered as a cytokine that could kill tumour cells, however, it is now clear that TNF can contribute to tumorigenesis by mediating the proliferation, invasion and metastasis of tumour cells. TNF has been shown to be an autocrine

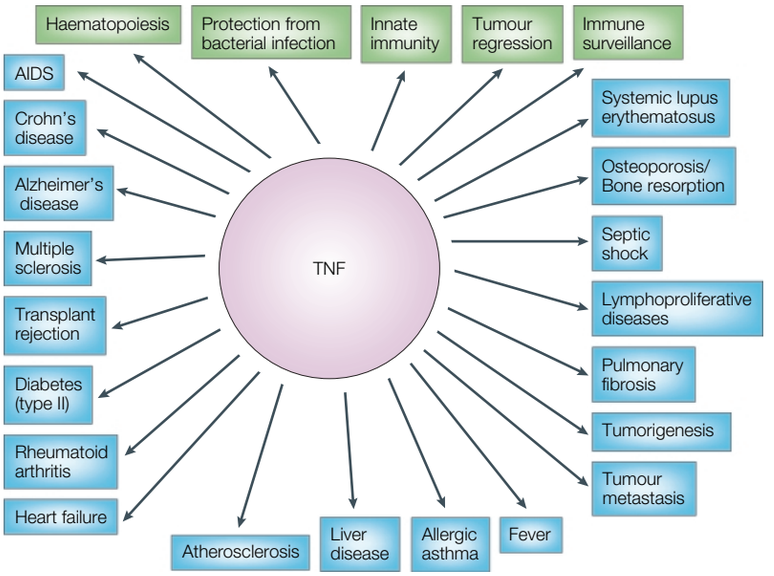

Figure 5 | **The main physiological and pathological effects linked to members of the TNF superfamily.** Different studies indicate that although tumour-necrosis factor (TNF) and its superfamily members are essential for haematopoiesis, protection from bacterial infection, immune surveillance and tumour regression (indicated in green); its dysregulation leads to various diseases (indicated in blue). This type of role as a 'double-edged sword' is typical of most members of the TNF superfamily.

growth factor for a wide variety of tumours. Through the activation of NF-κB, TNF induces the expression of various genes that are involved in invasion and metastasis, including adhesion molecules, urokinase plasminogen activator (UPA), matrix metalloproteinase 9 (MMP9), cyclo-oxygenase 2 (COX2) and vascular endothelial growth factor (VEGF). In addition, activation of NF-κB can suppress apoptosis, which further contributes to tumorigenesis. Several reports indicate that TNF is highly carcinogenic and mice deficient for TNF are resistant to skin carcinogenesis[101]. However, by contrast, a recent report showed that NF-κB blockade can trigger invasive epidermal neoplasia[102].

Autoimmunity. Several TNF ligands have been implicated in the development of autoimmunity. For example, transgenic overexpression of BAFF results in many autoimmune symptoms in mice[91]. These mice have an increased number of mature B cells, splenomegaly, DNA-specific antibodies, proteinuria and glomerulonephritis[91]. These phenotypes mimic those of SLE-like symptoms in humans. So, BAFF inhibitors might prove useful in regulating autoimmunity.

It is also now becoming apparent that TNF has an important role in the pathogenesis of type II diabetes mellitus. TNF has been shown to interfere with an insulin-signalling mechanism by inhibiting the tyrosine kinase activities of the insulin receptor and serine phosphorylation of the insulin receptor substrate 1 (REF. 103). These inhibitory effects of TNF on insulin signalling seem to be mediated through the upregulation of expression of suppressor of cytokine signalling 3 (SOCS3)[104]. The role of TNF has also been explored using mice in which the *TNF* or *TNFR* gene has been deleted[105]. The absence of TNF resulted in markedly improved insulin sensitivity in diet-induced obesity and in the ob/ob model of obesity. The TNF-deficient obese mice had lower levels of circulating free fatty acids, which are associated with the development of insulin resistance, and were protected from the obesity-related reduction in insulin receptor signalling in muscle and fat. This indicates that TNF is an important mediator of insulin resistance in obesity through its effects on several important sites of insulin action.

Other diseases. Besides the diseases indicated earlier, ligands of the TNF superfamily have been linked with various other diseases including chronic heart failure, bone resorption, AIDS, Alzheimer's disease, transplant rejection, atherosolerosis and hepatotoxicity.

The development of chronic heart failure involves phenotypic changes in several homeostatic systems so that, as the disease advances, chronic heart failure might be seen as a multi-system disorder with its origins in the heart extending to many extra-cardiac manifestations. Higher levels of TNF are found in the blood and myocardium of patients with chronic heart failure than in controls, and TNF has been implicated in several of the pathophysiological processes that are thought to be important in the progression of chronic heart failure[106,107]. Therapies directed against this cytokine therefore provide an approach to control heart failure. TNFR fusion proteins have been developed that target circulating TNF itself.

Both TNF and RANKL have been shown to have a crucial role in bone destruction[108]. These ligands induce osteoclastic differentiation of macrophages and myeloid progenitor cells. OPG protects bone integrity by downregulating osteoclastogenesis, therefore supporting the hypothesis that RANKL mediates bone loss. Further evidence comes from gene-deletion studies, which showed marked OSTEOPETROSIS after the deletion of either RANK or RANKL[109,110]. Conversely, the deletion of OPG leads to OSTEOPOROSIS[111]. Several of these activities are mediated by TNF ligand-induced activation of NF-κB.

Genetic mutations in the TNF/TNFR superfamily

Mutations in both ligands and receptors of the TNF superfamily have been found in humans. These include mutations in TNFR1, CD95/CD95L, RANK, ectodermal dysplasin (EDA) and CD40L. Missense mutations of TNFR1 lead to autosomal dominant periodic fever syndromes that are characterized by unexplained episodes of fever and severe localized inflammation[37]. The mutation disrupts the conserved extracellular disulphide bonds, causing a decrease in the level of soluble TNFR1 in the plasma of patients. Leukocytes from individuals with a Cys52Phe mutation in TNFR1 express increased levels of membrane TNFR1 and showed reduced receptor cleavage after stimulation. The autoinflammatory phenotype results from impaired downregulation of membrane TNFR1 and diminished shedding of potentially antagonistic soluble receptor. The existence of TNFR1-associated periodic syndromes (TRAPS) establishes an important class of TNFR mutations[112].

OSTEOPETROSIS
A hereditary bone disease with intense positive balance of body calcium. Autosomal recessive osteopetrosis is a rare, fatal disease characterized by the accumulation of excessive bone mass due to defective bone resorption. The pathogenesis of osteopetrosis is controversial. Defects in osteoblast–osteoclast interactions, incorrect differentiation of osteoclasts, abnormal contact between osteoclasts and the extracellular matrix, and abolished signalling can occur in this disease.

OSTEOPOROSIS
A condition that involves loss of bone due to an increase in the number of osteoclasts.

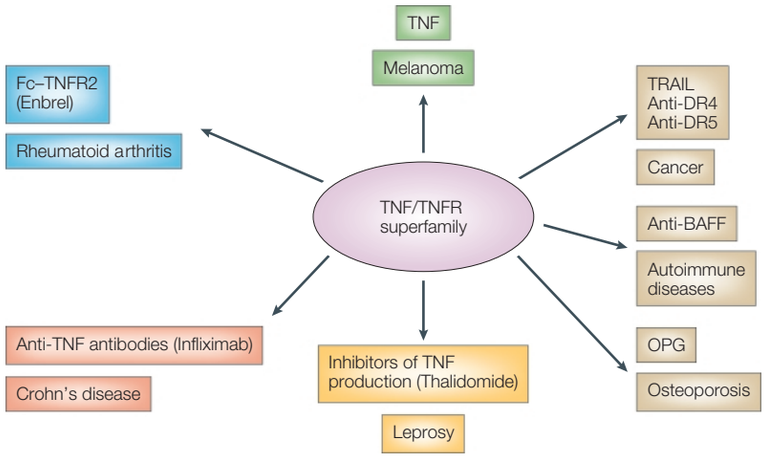

Figure 6 | **Present and future therapeutics based on members of the TNF superfamily and their receptors.** Based on tumour-necrosis factor (TNF) and its receptors, several therapeutic agents have been approved for the treatment of inflammatory diseases (such as rheumatoid arthritis and Crohn's disease), cancer (melanoma) and others (leprosy, autoimmunity and osteoporosis). Therapeutics based on TNF-related apoptosis-inducing ligand (TRAIL) and its receptors are presently being tested for the treatment of cancer. BAFF, B-cell-activating factor; DR, death receptor; OPG, osteoprotegerin; R, receptor.

Individuals with CANALE–SMITH SYNDROME or AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS) have phenotypes that are similar to those of *lpr*/*gld* mice, which have mutations in CD95 and CD95L, respectively. This is a good example of a case in which the identification of a mouse mutation has led to the understanding of a human disease[113]. These patients have an increased risk of developing T- and B-cell lymphomas[114]. Their risk of non-Hodgkin's lymphoma and Hodgkin's disease, respectively, was 14 and 51 times greater than expected, due to defective lymphocyte apoptosis, implicating that CD95-mediated apoptosis is important for preventing B-cell and T-cell lymphomas. CD95 mediates apoptosis through the activation of caspase-8. Human family members with an inherited genetic deficiency of caspase-8 have been described[115]. Homozygous individuals have defective lymphocyte apoptosis and homeostasis but, unlike individuals with ALPS, they also have defects in the activation of T cells, B cells and NK cells, which leads to immunodeficiency. So, caspase-8 deficiency in humans is compatible with normal development and shows that caspase-8 has a postnatal role in immune activation of naive lymphocytes.

Interactions between the B-cell surface antigen CD40 and its ligand expressed by activated T cells have a crucial role in isotype switching. This is illustrated by the failure of isotype switching in patients with X-linked hyper-IgM syndrome in who the *CD40L* gene is mutated[116].

Familial expansile osteolysis is another rare, autosomal dominant bone disorder that is characterized by focal areas of increased bone remodelling. The osteolytic lesions, which usually develop in the long bones during early adulthood, show increased activity of OSTEOBLASTS and OSTEOCLASTS. Two heterozygous insertion mutations in exon 1 of RANK were identified in patients with the disease. One was a duplication of 18 bases and the other a duplication of 27 bases, both of which affected the signal-peptide region of RANK. Expression of recombinant forms of the mutant RANK proteins indicated disruptions to the expression levels and lack of normal cleavage of the signal peptide. Both mutations caused an increase in RANK-mediated NF-κB signalling *in vitro*, consistent with the presence of an activating mutation[117].

Hypohydrotic ectodermal dysplasia is a genetic disease seen in humans and mice, characterized by the loss of hair, sweat glands and teeth. The main X-linked form results from mutations in EDA — a TNF-like ligand. A phenotypically indistinguishable autosomal form of the disease results from mutations in the receptor for EDA (EDAR). EDAR signalling is mediated by the activation of NF-κB. EDA–EDAR signalling mediates cell interactions in the ectoderm and regulates the initiation and morphogenesis of hair and teeth. It is also required for the development of fish scales, indicating that this pathway and its function have been conserved during the evolution of ectodermal organs[118].

Additionally, mutations in death receptors have been found in various cancers. For example, mutations in CD95 have been found in thyroid lymphoma, prostatic intraepithelial neoplasia and testicular germ-cell tumours[115–121]. Nucleotide substitution in the ectodomain of DR4 has been associated with lung cancer, and head and neck cancer[122].

Therapeutic implications

From this description, it is clear that members of the TNF superfamily and their receptors have a wide-ranging role in many cellular and physiological functions. These roles have been translated into new generation therapies (FIG. 6). TNF has been approved, in Europe, for human use in the treatment of sarcomas and melanomas[85,86]. Antibodies specific for TNF have been approved for the treatment of Crohn's disease[123] and are also being exploited for the treatment of various cancers[124]. Anti-TNF therapy using soluble TNFR2 has been approved for rheumatoid arthritis[125]. Several other therapies, including treatment with TRAIL or agonistic antibodies specific for DR4 and DR5 are being tested for anticancer properties at present. Due to the important role of BAFF in various autoimmune diseases, inhibitors of this cytokine are also under development.

Conclusion

Almost two decades ago, TNF was thought to be a single molecule, now it is clear that it belongs to a superfamily of cytokines that have a wide variety of activities. Although over 50,000 papers have been published on TNF since its initial isolation in 1984, there is little information about the other members of this superfamily. As all of the TNF-superfamily members, except LT, are transmembrane proteins, how these proteins are released from the cell surface remains to be elucidated. The ligands for receptors such as DR6, RELT and TROY remain to be identified. Future studies will indicate the differences in the signal transduction mechanisms between the various members of the TNF superfamily, which might allow for the development of more specific

CANALE-SMITH SYNDROME (CSS). An inherited disease characterized by massive lymphadenopathy, hepatosplenomegaly and systemic autoimmunity to erythrocytes and platelets.

AUTOIMMUNE LYMPHOPROLIFERATIVE SYNDROME (ALPS). ALPS is characterized clinically by chronic non-malignant lymphoproliferation and autoimmunity, and is caused by a genetic defect in apoptosis. Most patients with ALPS have heterozygous mutations in the *CD95* gene.

OSTEOBLASTS
Cells that are responsible for the formation of bone.

OSTEOCLASTS
Cells that are responsible for bone resorption. They are rare cells with only 2–3 cells seen per 1 mm^3 of bone. However, the loss of function in osteoclasts, problems with their differentiation and decrease in their number lead to bone osteosclerosis/osteopetrosis. Conversely, an increase in their number or function induces bone osteoporosis, indicating that osteoclasts have a pivotal role in bone homeostasis.

therapeutics. Several therapeutics based on TNF have been approved for use in humans, and more are likely to be found in the future based on other members of this family. Experience indicates that all of the TNF-superfamily members are double-edge swords and more studies are required to determine whether it is their agonist or antagonist feature that should be targeted for therapy.

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