Targeting death and decoy receptors of the tumour-necrosis factor superfamily

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TARGETING DEATH AND DECOY RECEPTORS OF THE TUMOUR-NECROSIS FACTOR SUPERFAMILY

Avi Ashkenazi

Cancer cells often develop resistance to chemotherapy or irradiation through mutations in the p53 tumour-suppressor gene, which prevent apoptosis induction in response to cellular damage. Death receptors — members of the tumour-necrosis factor receptor (TNFR) superfamily — signal apoptosis independently of p53. Decoy receptors, by contrast, are a non-signalling subset of the TNFR superfamily that attenuate death-receptor function. Agents that are designed to activate death receptors (or block decoy receptors) might therefore be used to kill tumour cells that are resistant to conventional cancer therapies.

Since the late 1950s, the main strategy to treat cancer, besides surgery, has been radiotherapy or chemotherapy. These approaches work primarily by damaging proliferating cells at the level of DNA replication or cell division, and inducing apoptotic cell suicide as a secondary response to the damage. Although such treatments can be associated with tumour stasis or regression, they are rarely curative and are often hampered by the existence or emergence of resistant tumour cells. Mutations in *TP53* (which encodes p53 in humans) frequently underlie the resistance because they disable a key connection between sensors of cell damage and the apoptosis machinery. Moreover, radiotherapy and chemotherapy generally do not distinguish between malignant and non-malignant types of proliferating cell, and can cause undesired toxicity to normal tissues, such as the bone marrow, gut or kidney. In recent years, efforts to improve cancer therapy have centred on developing more selective, biological-mechanism-based approaches that can help to overcome tumour resistance as well as minimize toxic side effects. The Human Genome Project is already yielding exciting new targets for biological cancer therapy. Among these are some new members of the tumour-necrosis factor (TNF) receptor (TNFR) gene superfamily that can be targeted to trigger apoptotic killing of tumour cells.

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The name tumour-necrosis factor underscores a historical connection between the TNF gene superfamily and cancer therapy. This link goes back to observations that were reported in 1868 by Brunes, who noticed that some of his cancer patients' tumours spontaneously regressed after acute bacterial infection. In 1894, Colley observed that cell-free extracts from bacteria — later called Colley's toxins — could cause tumour shrinkage. By 1944, Shear and colleagues isolated a factor from gram-negative bacteria called endotoxin or lipopolysaccharide (LPS), which induced haemorrhagic tumour necrosis after injection into tumour-bearing mice.

In 1962, W. O'Malley *et al.* transferred serum from LPS-treated mice into tumour-bearing mice and found that this could induce tumour necrosis[1]. In 1975, E. Carswell, L. Old and colleagues confirmed this observation and coined the term tumour-necrosis factor[2]; they also showed that the LPS-induced serum factor could kill tumour cells in culture[3]. In 1984, D. Pennica *et al.* reported the cloning of a cDNA that encodes the TNF protein[4], whereas P. Gray *et al.* succeeded in cloning another cytotoxic protein called lymphotoxin (LT)[5]. Independently, B. Beutler and A. Cerami isolated a factor that mediated LPS-induced wasting (cachexia) in mice, which turned out to be the mouse orthologue of human TNF[6]. Surprisingly, the TNF and LT proteins showed about 30% sequence identity with each other,

Summary

- Tumour-necrosis factor (TNF) was discovered many years ago as a serum factor that was able to kill cancer cells in mice. The TNF receptor (TNFR) was shown to be expressed by mammalian cells years later, and led to the discovery of a superfamily of transmembrane proteins. These discoveries led to the identification of two gene families that include 18 ligands and 28 receptors, many of which are being targeted as anticancer therapies.
- TNFR signalling was discovered to be an important aspect of the immune response, and family members such as FASL and APO2L/TRAIL induce apoptosis through a p53-independent mechanism. The signalling members of the TNFR superfamily can be divided into two main subgroups on the basis of their cytoplasmic region. One class of receptors, called death receptors (DR), contains a cytoplasmic death domain, whereas the other class does not.
- APO2L/TRAIL has been shown to induce apoptosis in a wide variety of cancer cells, whereas most normal human cell types are resistant to APO2L/TRAIL-induced cell death.
- Some TNFR family members do not signal, but act as 'decoys' that compete with receptors for ligands. A number of tumour types overexpress decoy receptors.
- Treatment with factors that activate death-receptor signalling on cancer cells, and antibodies or small molecules that antagonize decoy receptors, might be an effective anticancer strategy.

providing the first hint of the existence of a gene family. The two proteins were renamed TNF-α and TNF-β; however, the subsequent discovery of a closer homologue of LT[7] reinstated the name TNF and led to renaming of the two closer homologues as LT-α and LT-β.

In 1985, B. Aggarwal *et al.* reported that TNF and LT-α bound to common receptors[8]. Clear evidence for the existence of a TNFR family, however, came several years later, in 1989, with the isolation from T cells of cDNAs that encode the CD40 antigen[9] and the 4-1BB antigen[10]; both of these proteins showed sequence similarity in their extracellular region to the previously cloned p75 nerve growth-factor receptor (NGFR)[11]. Several groups then isolated and cloned two related receptors that bound to TNF as well as to LT-α, called type 1 TNFR (also called TNFR1, p55-TNFR or CD120a) and type 2 TNFR (also called TNFR2, p75-TNFR or CD120b)[12]. In subsequent years, researchers identified several new TNF-family members, including CD40L, LT-β, CD27L, CD30L, 4-1BBL, FASL and OX40L, as well as additional TNFR-family members, including OX40, FAS/APO-1/CD95, CD27, CD30 and LT-βR[13]. The genes that encode these proteins were cloned on the basis of recognition by specific antibodies, ligand–receptor interaction or biological activity.

In 1995, the human genome project prompted a third wave of discovery, beginning with the identification and characterization of APO2 ligand/TNF-related apoptosis-inducing ligand (APO2L/TRAIL)[14,15]. The number of discoveries peaked in 1997/1998 with the cloning of five more TNF homologues and ten new TNFR relatives, including four closely related receptors for APO2L/TRAIL[16,17]. More recently identified molecules have expanded the TNFR family to more distant relatives, such as BR3/BAFFR[18,19] and FN14/TWEAK-R[20]. The two gene families are now recognized as superfamilies, with 18 ligands and 28 receptors (FIG. 1). A new nomenclature system has been proposed that denotes each ligand as TNFSFN and each receptor as TNFRSFN, where N is a specific number (see online links box). But how do TNF-superfamily members function?

TNF-superfamily-mediated apoptosis

TNF-superfamily ligands exert their biological effects primarily, though not exclusively, within the immune system, modulating innate as well as adaptive immunity[21]. Some of the ligands — for example, TNF — promote inflammatory responses to microbial infections. Others, such as LT-β, CD40L, LIGHT, RANKL and BLYS/BAFF, regulate specific aspects of cellular or humoral immunity, including the formation of lymphoid organs, activation of dendritic cells and stimulation or survival of T or B cells. Yet other ligands, such as FASL (also called APO1L or CD95L)

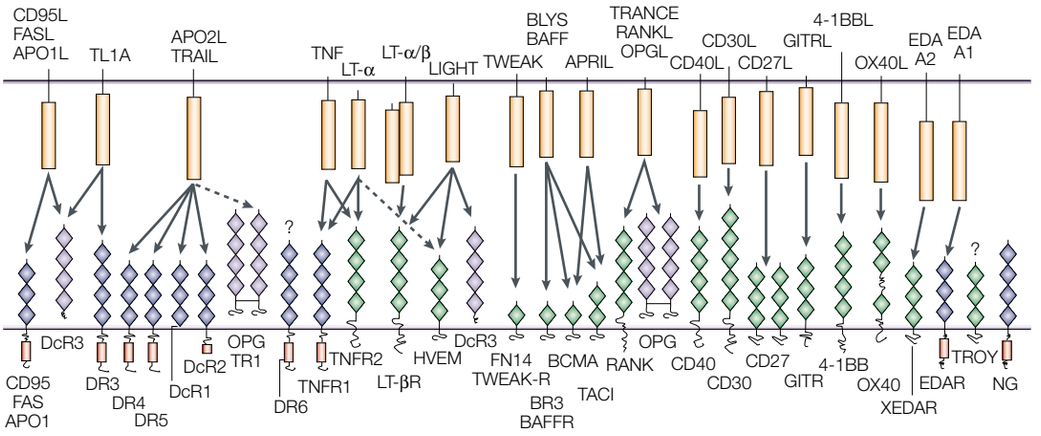


Figure 1 | **The TNF and TNFR superfamilies.** Ligands are shown in their schematic transmembrane form. Arrows indicate receptor interactions with solid lines for strong binding and dashed lines for low-affinity binding. Question marks indicate that cognate ligands have not yet been identified. Diamonds represent receptor cysteine-rich domains and red boxes denote receptor cytoplasmic death domains.

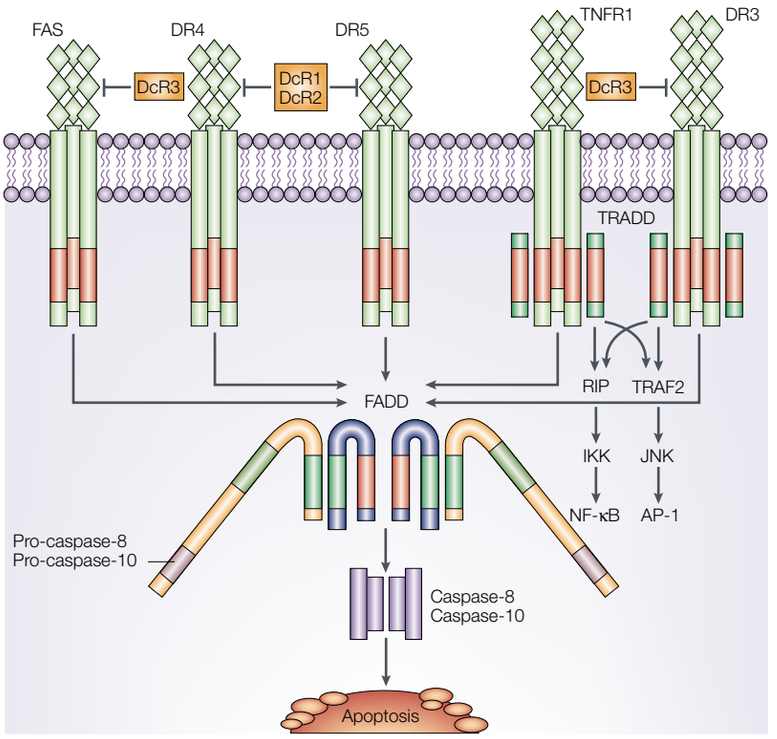


Figure 2 | **Signal transduction by death receptors.** After binding of FASL or APO2L/TRAIL, the death receptors FAS, DR4 or DR5 each assemble a death-inducing signalling complex (DISC): through the adaptor protein FAS-associated death domain (FADD), they recruit and activate the apoptosis-initiating proteases caspase-8 (REFS 129–132) and/or caspase-10 (REFS 133,134). The physical proximity of the recruited enzymes in the ligand-induced signalling complex leads to their autoactivation by proteolysis, thereby triggering intracellular signalling cascades that induce specific cellular responses. After binding tumour-necrosis factor (TNF), TNF receptor 1 (TNFR1) first recruits TNFR-associated death domain (TRADD) as a platform adaptor, and, in turn, assembles alternative signalling complexes through secondary adaptors[135,136]. One type of complex is a DISC that involves FADD and caspase-8 (and probably caspase-10) and triggers apoptosis in a manner similar to the other death receptors. Another complex involves the receptor-interacting protein (RIP), which links receptor stimulation to the inhibitor of κB-kinase (IKK) cascade, activating the nuclear factor of κB (NF-κB) transcription factor. A third complex involves TNFR-associated factor 2 (TRAF2), which couples receptor engagement to the JUN N-terminal kinase (JNK) cascade, stimulating the AP-1 transcription factor. Death receptor DR3, which was identified by virtue of its homology to TNFR1, assembles signalling complexes that are similar to those of TNFR1 (REFS 137–139). DR6 (not depicted), a similarly discovered death receptor, seems to signal mostly through the JNK/AP-1 pathway[140,141]. Death domains are indicated in red; death-effector domains in blue. Caspase domains are shown in green. Decoy receptors (DcRs) are members of the TNFR superfamily that are capable of competing with signalling receptors for ligand binding, thereby inhibiting their function. DcR1 and DcR2 compete with DR4 and DR5 for binding of APO2L/TRAIL. DcR3 competes with FAS for binding of FASL and with DR3 for binding of TL1A.

and APO2L/TRAIL, regulate activation-induced apoptosis of peripheral lymphocytes and mediate apoptosis-inducing activities of natural killer (NK) cells and cytotoxic lymphocytes against virus-infected or oncogenically transformed cells. Several TNF-superfamily members also have important roles in regulating tissues and organs, such as bone or skin, outside the immune system.

Each member of the TNF superfamily binds at least one receptor from the TNFR superfamily (FIG. 1). Some of the ligands bind several receptors. The most complex example of this is APO2L/TRAIL, which binds five different receptors. In addition, many of the ligands share some of their receptors with other ligands. For example, LIGHT shares LT-βR with LT-β, but binds selectively to another receptor called HVEM; BLYS/BAFF shares the receptors BCMA and TACI with APRIL, but binds selectively to BR3/BAFFR. The ligands are expressed by cells as homotrimeric type 2 transmembrane proteins — except LT-α, which is directly secreted as a soluble protein. LT-α and LT-β have an unusual property: they can form heterotrimeric complexes in which one or two soluble LT-α chains associate with the complementary number of transmembrane LT-β chains[7].

The extracellular, carboxy-terminal region of many of the TNF-superfamily ligands is proteolytically processed into a soluble protein that is released to the extracellular space. This region, which binds to the cognate receptors, is the most homologous between the ligands, having up to ~30% protein sequence identity. Among TNFR-superfamily members, the amino-terminal, extracellular region is the most similar, with up to ~65% protein sequence identity. The homology occurs mainly in cysteine-rich domains (CRDs). The number of CRDs ranges from one to six, but most commonly equals three or four. X-ray crystallography studies of soluble TNF[22], LT-α[23], APO2L/TRAIL[24], CD40L[25] and RANKL[26] confirm the homotrimeric subunit organization of these ligands. Similar studies show that the TNFR extracellular region folds as a string of CRDs in tandem; each CRD has an α-helical structure that is stabilized by one–three disulphide bonds between internal cysteines[27]. The basic signalling unit of the TNFR superfamily consists of three receptors bound by a trimeric ligand molecule. Recent evidence indicates, however, that TNFR1 and FAS pre-associate in the plasma membrane as homoligomers, independently of ligand; the association occurs through an amino-terminal region called the pre-ligand association domain (PLAD)[21]. This implies that, at least for some TNFR-superfamily members, the ligand might not actually induce receptor trimerization; rather, it might bind to a pre-associated trimeric receptor complex and change the conformation of the receptors in a manner that facilitates signalling. This model has yet to be tested in physiological settings.

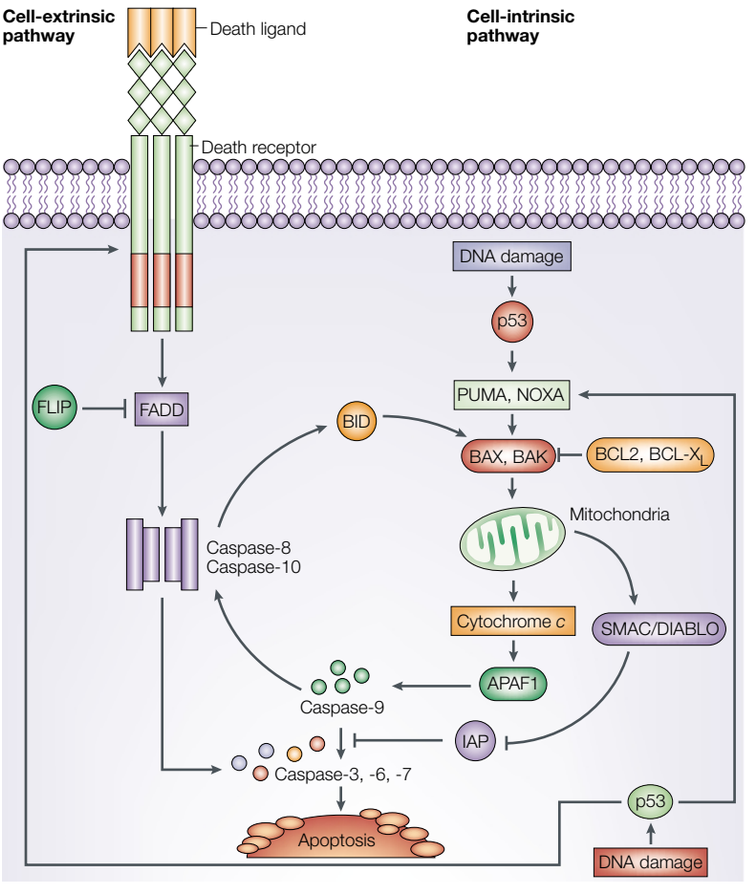
Most TNFR-superfamily members function as transmembrane signal transducers that respond to ligand binding. Some of the receptors, however, do not signal. Instead, they seem to act as decoys that compete for the interaction of cognate ligands with their signalling receptors. The signalling members of the TNFR superfamily can be divided into two main subgroups on the basis of their cytoplasmic region. One class of receptors, called the death receptors (DR)[16], contains a cytoplasmic death domain (FIG. 2); the other class does not. Death domains mediate interaction of death receptors with death-domain-containing adaptor proteins. The adaptors contain additional sequence modules that mediate binding to intracellular effector enzymes. One such adaptor — called FADD (FAS-associated death domain; also called MORT1)[28,29] — activates specific caspases that initiate apoptosis. Another adaptor —

Box 1 | **Crosstalk between apoptosis signalling pathways**

Death receptors can activate the cell-intrinsic pathway by caspase-8-mediated cleavage of the apical pro-apoptotic BCL2 superfamily member BID[114–117]. BID interacts with the pro-apoptotic BCL2 relatives BAX and BAK, which cause release of mitochondrial cytochrome *c* and SMAC/DIABLO, activating caspase-9 and -3 (REFS 118,119). This amplifies apoptosis induction through the cell-extrinsic pathway. Conversely, DNA damage can induce transcriptional upregulation of some death receptors, such as FAS and death receptor 5 (DR5), through p53-dependent as well as p53-independent mechanisms[120]. This upregulation increases cellular sensitivity to death-receptor ligands. In some cell types, death-receptor engagement of the cell-extrinsic pathway suffices for commitment to apoptotic death. In other cell types, commitment to apoptosis requires amplification of the death-receptor signal by the cell-intrinsic pathway[121].

Gene knockouts of *Bax* and/or *Bak* indicate that, in mouse hepatocytes, these proteins are necessary, although mutually redundant, for death-receptor engagement of mitochondria[122,123]. By contrast, somatic knockout of *BAX* in human HCT116 colon cancer cells[124] reveals an absolute requirement for BAX in death-receptor engagement of the mitochondria[125,126]. BAX is a frequent target of mutational inactivation in tumours that harbour DNA mismatch-repair deficiency, which constitute about 15% of colorectal, gastric and endometrial cancers[127]. Mismatch-repair-deficient tumours can acquire resistance to death-receptor ligands, such as APO2L/TRAIL, through mutational inactivation of *BAX*[125]. Pre-exposure to chemotherapy, which leads to upregulation of DR5 and BAK, rescues APO2L/TRAIL sensitivity[125].

Because death-receptor targeting and conventional agents induce tumour-cell apoptosis through different signalling pathways, combinations of the two approaches might facilitate killing of tumour cells that resist death induction through either one of the pathways. Combinations should also reduce the probability that tumour cells will develop resistance to either therapy. It might be possible to circumvent resistance further by adding agents that work through other mechanisms, such as tumour-targeted antibodies and anti-angiogenic drugs.



called TRADD (TNFR-associated death domain)[30] — stimulates protein kinases that control phosphorylation cascades, to induce transcription of immune-system modulation genes. Alternatively, TRADD can initiate apoptosis through FADD.

Two main signalling pathways initiate the apoptotic suicide programme in mammalian cells (BOX 1). The cell-intrinsic pathway triggers apoptosis in response to DNA damage, defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of survival factors or other types of severe cell distress. This pathway involves activation of the pro-apoptotic arm of the *BCL2* gene superfamily, which, in turn, engages the mitochondria to cause the release of apoptogenic factors such as cytochrome *c* and SMAC/DIABLO into the cytosol[31–33]. In the cytosol, cytochrome *c* binds the adaptor APAF1, forming an 'apoptosome' that activates the apoptosis-initiating protease caspase-9. In turn, caspase-9 activates 'executioner' proteases caspase-3, -6 and -7. SMAC/DIABLO promotes apoptosis by binding to inhibitor of apoptosis (IAP) proteins and preventing these factors from attenuating caspase activation[34,35].

Most chemotherapy agents and irradiation trigger tumour-cell apoptosis through the cell-intrinsic pathway, as an indirect consequence of causing cellular damage. Engagement of this pathway usually requires p53 function. Inactivation of p53, either directly through *TP53* mutations or indirectly through p53 modulators such as the MDM2 protein, occurs in many human cancers. Without p53 function, tumour cells evade apoptosis and can continue to proliferate, despite genetic instabilities that are caused by chemotherapy or irradiation.

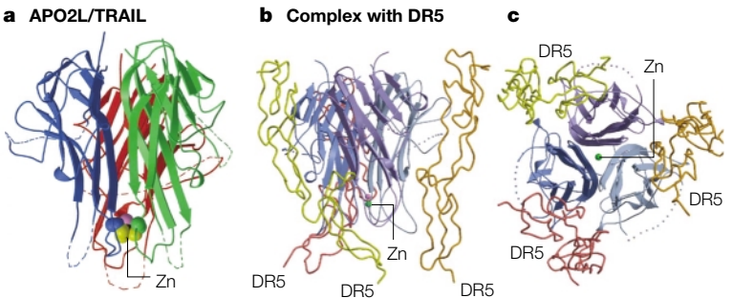
The cell-extrinsic pathway triggers apoptosis in response to engagement of death receptors by their ligands. This pathway stimulates the apoptotic caspase machinery independently of p53. Ligand-induced activation of cell-surface death receptors leads to rapid assembly of a death-inducing signalling complex (DISC) and activation of the apoptosis-initiating proteases caspase-8 and caspase-10. These caspases, in turn, activate the same set of executioner caspases that are activated by the cell-intrinsic pathway through caspase-9. The cell-extrinsic pathway is becoming recognized as an important mechanism that is used by NK cells and cytotoxic T lymphocytes to trigger apoptosis in virus-infected cells and in tumour cells. These two apoptosis signalling pathways communicate with each other (BOX 1).

The second subgroup of signalling members of the TNFR superfamily consists of receptors that lack death domains in their intracellular portion. Instead, these receptors display homologous sequence motifs that mediate interaction with specific adaptors from the TNFR-associated factor (TRAF) family[36]. TRAFs link these receptors to serine/threonine protein kinase cascades that regulate gene transcription, namely the inhibitor of κB kinase (IKK), JUN N-terminal kinase (JNK)/p38 and ERK/MAPK (extracellular-signal-regulated kinase/mitogen-activated protein kinase) pathways.

The non-signalling members of the TNFR superfamily seem to act as decoys that compete with signalling receptors for ligand binding. Osteoprotegerin (OPG) is a

Box 2 | **Structure of APO2L/TRAIL and its complex with DR5**

In panel a, APO2L/TRAIL is shown as a ribbon diagram that depicts β-strands, with each subunit in a different colour. A zinc ion (yellow spheres) — coordinated by three cysteines at position 230 of each subunit (blue, pink, green spheres) — stabilizes the ligand's homotrimeric structure[24,74,75]. Each polypeptide subunit in the ligand folds into a jellyroll-like structure that is composed of ten β-strands and nine connecting loops that assemble into three β-sheets. In the absence of zinc, two of the three cysteines randomly form a disulphide bond, creating an asymmetric molecule that is less stable and soluble than the zinc-bound trimer[94]. Panels b and c show APO2L/TRAIL ligand–receptor complex in side (b) and top (c) views. The crystal structures of complexes between lymphotoxin-α (LT-α) and tumour-necrosis-factor receptor 1 (TNFR1) (REF. 128) or APO2L/TRAIL and death receptor 5 (DR5) (REF. 74) shed some light on the principles that govern interactions between TNF- and TNFR-superfamily members. In these structures, one ligand homotrimer (ribbon diagram) binds three receptor molecules (worm diagrams), each docking in the groove between two of the ligand's subunits. There are two distinct interaction patches, located on the receptor side in cysteine-rich domain (CRDs) 2 and 3 (REF. 74). Residues in the CRD3 binding patch are conserved throughout the TNFR superfamily and, therefore, this site seems to be important for binding affinity. By contrast, the CRD2 binding patch contains features that are unique to each ligand–receptor pair, so it seems to be important for binding selectivity.



soluble decoy for RANKL/OPGL[37] and possibly also for APO2L/TRAIL[38]. DcR1 and DcR2, which are closely related in their CRDs, are decoys for APO2L/TRAIL[16]. DcR1 is attached to the plasma membrane through a glycophosphatidylinositol anchor. DcR2 is a transmembrane protein with a truncated death domain in its cytoplasmic tail that is unable to signal apoptosis. DcR3 — the closest relative of OPG — is a soluble decoy for FASL[39], LIGHT[39,40] and TL1A[41].

Tumour-necrosis factor. TNF was discovered on the basis of its cytotoxic activity; however, it is now well established that TNF can elicit a variety of biological effects[36]. Mouse *Tnf* and *Tnfr1* gene knockout studies show that TNF has an essential role in protection against infection by bacterial, fungal, parasitic and, probably, viral pathogens[36]. TNF is produced by a wide range of immune cell types, including monocytes, macrophages, T cells, B cells and NK cells, consistent with its immunomodulatory role. At the cellular level, TNF's main signalling function is the transcriptional induction of pro-inflammatory cytokines, such as interleukin-1, -6 and -8, and of leukocyte adhesion molecules. Although TNF is important for normal immunity, TNF overproduction is a key pathological factor in autoimmune inflammatory diseases, such as rheumatoid arthritis and Crohn's disease. Indeed, TNF blockade by TNFR2-Fc fusion protein or anti-TNF antibodies has proved to be clinically effective against these disorders.

The cloning of the human TNF cDNA, as reported in 1984, made it possible to generate recombinant soluble protein of sufficient quality and quantity for the investigation of safety and efficacy in preclinical cancer models and, later, in clinical trials. In these trials, TNF was found to be unsuitable for systemic administration at clinically relevant doses, because it had toxic side effects that were associated with its strong pro-inflammatory activity, including fever, lung or liver failure, increased blood clotting and hypotension[42]. These side effects were probably triggered by TNF induction of nuclear factor of κB (NF-κB)-dependent gene transcription. Subsequent studies, however, showed that TNF can be administered safely and effectively in the setting of isolated limb perfusion, which prevents the protein from entering the systemic blood circulation[43].

Recently, the European Medicine Evaluation Agency approved TNF for isolated limb perfusion in combination with melphalan chemotherapy for the treatment of non-resectable, high-grade sarcomas. In the study that supported regulatory registration, 74% of 196 patients had complete or partial objective tumour responses, leading to limb salvage in 71% of the patients[43]. The American College of Physicians is conducting a multicentre, random-assignment trial of isolated limb perfusion that compares melphalan alone with melphalan plus TNF in patients with melanoma[44]. TNF is also being evaluated for treatment of non-resectable liver tumours by isolated hepatic perfusion[44].

Studies in mouse models indicate that the hepatotoxic side effects of TNF can be prevented by inhibitors of matrix metalloproteinases[45], suggesting a potential way to expand TNF's therapeutic window. New experimental strategies to deliver TNF to tumours more safely might include gene therapy, liposomal formulations and fusion to tumour-targeted monoclonal antibodies. Studies with mouse fibrosarcomas indicate that TNF induces tumour necrosis by activating signalling through TNFR1 on endothelial cells, rather than through direct action on the tumour epithelium[46].

APO2L/TRAIL and its death receptors. By searching the human genome database for sequences with homology to TNF, researchers at the biotechnology companies Genentech and Immunex independently identified expressed sequence tags that led to the cloning of a novel TNF-superfamily member. Because of its protein sequence homology to FAS/APO1 ligand (23% identity) and TNF (19% identity), the newly discovered protein was named APO2 ligand (APO2L)[15] or TNF-related apoptosis-inducing ligand (TRAIL)[14]. Functional studies showed that the new ligand had a potent ability to trigger apoptosis in a variety of tumour cell lines regardless of p53 status. As the sequencing of the human genome progressed, researchers identified several receptors for APO2L/TRAIL: two death receptors, DR4 and DR5 (also

called TRAIL-R1 and TRAIL-R2)[47–50], and two decoy receptors, DcR1 and DcR2 (also called TRAIL-R3 and TRAIL-R4)[48,49,51–53]. A third decoy receptor, OPG, which was identified initially as a receptor for RANKL/OPGL, was shown later to bind APO2L/TRAIL[38]. The physiological relevance of OPG as a receptor for APO2L/TRAIL is unclear, however, because the affinity for this ligand at physiological temperatures is very low[54].

Although the main biological activity of APO2L/TRAIL seems to be the induction of apoptosis, the complete physiological role of this ligand is not yet fully understood. The relatively complex receptor interaction pattern of APO2L/TRAIL is unmatched not only in the TNF superfamily, but also in other cytokine systems. Mouse gene-knockout studies indicate that APO2L/TRAIL has an important role in antitumour surveillance by immune cells[55]. Mouse experiments in which APO2L/TRAIL was blocked by the administration of neutralizing antibodies implicate APO2L/TRAIL specifically in interferon-γ-dependent antitumour function of NK cells[56]. Indeed, activated NK cells[57,58], monocytes[59] and $CD4^+$ or $CD8^+$ T cells[60–64] express APO2L/TRAIL and use this ligand to trigger apoptosis in tumour target cells. APO2L/TRAIL might also be an important mediator of apoptosis-inducing activity of type I interferons in renal-cell carcinoma[65] and multiple myeloma cells[66], and of retinoids in leukaemia cells[67]. The APO2L/TRAIL pathway might have arisen as part of the immune defence against some as yet unspecified pathogen(s). The presence of a transcriptional regulatory element in the APO2L/TRAIL gene that is responsive to interferons supports this hypothesis[68]. Furthermore, infection of cells with reovirus[69] or herpesvirus[70] upregulates APO2L/TRAIL expression, and NK cells can use APO2L/TRAIL, induced through type I interferons, to kill virus-infected cells[71].

Lymphocytes express APO2L/TRAIL as a transmembrane protein, and can release this ligand in a vesicle-associated form[72] or in a soluble form that is generated through enzymatic shedding of the protein's extracellular, carboxy-terminal portion[73]. Structural studies show that an internally bound zinc ion, coordinated by a cysteine side chain from each of the ligand's subunits, stabilizes the soluble APO2L/TRAIL homotrimer[24,74,75] (BOX 2).

Recombinant soluble APO2L/TRAIL induces apoptosis in cell lines from a broad spectrum of human cancers, including colon, lung, breast, prostate, pancreas, kidney, central nervous system and thyroid cancer, as well as leukaemia and multiple myeloma, indicating that this ligand might be useful for the treatment of many cancers[76–83]. In athymic or SCID mice that bear human tumour xenografts derived from colon carcinoma[84,85], breast carcinoma[77], multiple myeloma[86] or glioma[87,88], administration of recombinant soluble APO2L/TRAIL exerts marked antitumour activity without systemic toxicity. Furthermore, combinations of APO2L/TRAIL and certain DNA-damaging drugs[76,89] or radiotherapy[90] have synergistic antitumour activity in several xenograft mouse models.

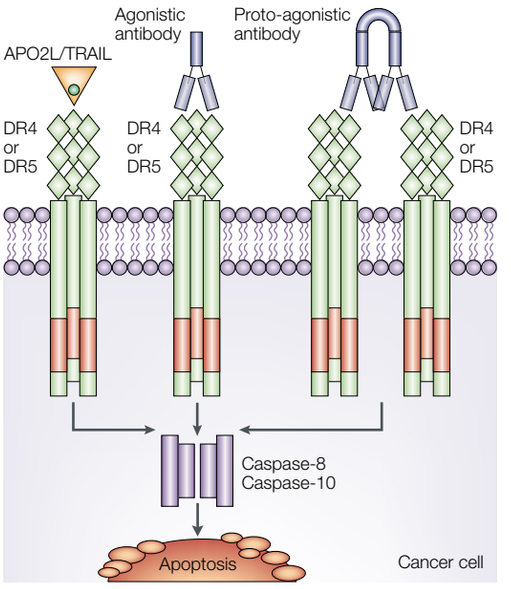


Figure 3 | **Molecular approaches to death-receptor activation.** The death receptors DR4 and/or DR5 can be functionally engaged by recombinant soluble APO2L/TRAIL in its zinc-bound, trimeric form. Antibodies that bind to the extracellular portion of either receptor can also stimulate caspase-mediated apoptosis, with or without a requirement for receptor crosslinking.

APO2L/TRAIL shows efficacy not only in cell-line-based tumour models but also in models that involve tumour cells that are taken directly from patients and propagated only briefly in culture before transplantation into mice (E. Repasky, personal communication). Like APO2L/TRAIL, monoclonal antibodies that functionally engage DR4 or DR5 also show antitumour activity in certain xenograft studies[91,92]. One type of death-receptor-activating monoclonal antibody (mAb), the proto-agonistic type, shows agonistic activity after crosslinking through the FC DOMAIN[91] (FIG. 3). A second type seems to possess intrinsic agonistic activity[92]; however, this might be caused by the presence of antibody (Ab) aggregates, as is the case for anti-FAS/APO1 mAbs[93]. Both types of antibody show apoptosis-mediated tumoricidal activity in xenograft mouse models[91,92], although the exact mechanism by which they trigger apoptosis *in vivo* remains unclear.

Various recombinant versions of human APO2L/TRAIL have been generated. One version contains APO2L/TRAIL amino acids 114–281 fused to an amino-terminal POLYHISTIDINE TAG[15]. A second variant contains amino acids 95–281 fused amino terminally to a modified yeast Gal4 leucine zipper (LZ), which promotes trimerization of the ligand[77]. A third version contains residues 95–281 fused to an amino-terminal 'Flag' EPITOPE TAG; crosslinking of this tagged protein with anti-flag antibodies enhances its activity against certain cell lines such as Jurkat T leukaemia[75]. At present, a fourth recombinant version of the ligand is probably the most preferred for clinical application: it contains amino acids 114–281 of human APO2L/TRAIL without any

FC DOMAIN
The antibody molecule can be proteolytically cleaved into two pieces — the $F(ab')_2$ fragment, which contains the antigen-binding activity, and the Fc domain, which carries out the effector function of the immunoglobin molecule.

POLYHISTIDINE TAG
A type of epitope tag that is made up of six histidine residues (6X-His).

EPITOPE TAG
A short amino-acid sequence that is added, in frame, to either end of a gene. This allows the recombinant protein to be easily detected and purified using antibodies against the tag. Commonly used tags include MYC, glutathione-*S*-transferase, and FLAG.

added exogenous sequences[84]. This latter version is therefore the least likely to be immunogenic in human patients. The production in bacteria of non-tagged recombinant APO2L/TRAIL as a stable, soluble homotrimer has been optimized by the addition of zinc and reducing agent to the cell-culture media and extraction buffers, and by formulation of the purified protein at neutral pH[85,94].

Most normal human cell types tested so far, including epithelial, endothelial, fibroblastic and smooth muscle cells, are refractory to the optimized recombinant version of APO2L/TRAIL[76,94]. Some normal cell types, such as astrocytes[76,77], hepatocytes[94,95] and keratinocytes[96], are resistant to the non-tagged, zinc-bound recombinant APO2L/TRAIL, but show significant sensitivity to apoptosis induction by the tagged and antibody-crosslinked recombinant variants. One potential explanation for this difference is that commitment of these normal cells to apoptosis might require high-order multimerization of DR4 and DR5. The tagged versions of the ligand, which are not optimized for zinc content, have a low solubility and tend to aggregate and/or precipitate at high concentrations, as does the antibody-crosslinked ligand (A. A., unpublished observations). Therefore, these ligand preparations might over-multimerize death receptors, leading to a signal that surpasses the high threshold for apoptosis activation in the normal cells. A study that compared Flag-tagged APO2L/TRAIL in crosslinked or non-crosslinked form indicated that DR4 is responsive to both forms of the ligand, whereas DR5 responds only to crosslinked APO2L/TRAIL[97]. However, data on the biochemical characteristics of the soluble ligand used in these experiments were not provided (for example, its zinc content, presence of intersubunit disulphides and aggregates), making it difficult to assess the results. Moreover, several cell lines that express DR5 but not DR4 are killed quite efficiently by non-tagged, zinc-bound APO2L/TRAIL. These cell lines include G55 and G142 glioblastoma cells (A. A., unpublished observations).

The non-tagged, zinc-bound APO2L/TRAIL, by contrast, is highly stable and soluble as a trimer — this ligand probably forms only trimeric death-receptor complexes, which are not sufficient for triggering apoptosis in normal cells. This hypothesis is supported by the observation that polyhistidine-tagged APO2L/TRAIL binds irreversibly to cultured hepatocytes, whereas non-tagged, zinc-bound APO2L/TRAIL has reversible binding[94,95]. In tumour cells, however, the threshold for apoptosis induction is usually lower than in normal cells[98] — death-receptor activation with non-tagged, zinc-bound trimeric APO2L/TRAIL potently triggers apoptosis in numerous cancer cell lines[76,94], supporting the clinical potential of this optimized recombinant ligand form.

Nevertheless, given that certain versions of APO2L/TRAIL can induce hepatocyte apoptosis[94,95], the preclinical assessment of the ligand, as well as of other agents that target DR4 or DR5, must be done with the utmost diligence. Initial studies in non-human primates — such as cynomolgus monkeys and chimpanzees — show that short-term intravenous administration of non-tagged, zinc-bound APO2L/TRAIL is well tolerated even at high doses[85,94]. Further animal safety studies are needed to determine the impact of long-term administration of APO2L/TRAIL as a single agent and in combination with chemotherapy or radiotherapy.

Antibodies that functionally engage death receptors DR4 or DR5 (REFS 91,92) provide potential alternatives to recombinant APO2L/TRAIL. Studies with an agonistic DR5 mAb indicated that this receptor is not expressed in cultured human hepatocytes, and that antibody engagement of DR5 does not induce apoptosis *in vitro* in these normal cells, but does so in certain cancer cell lines[92]. Other studies, however, showed DR5 mRNA expression in hepatocytes and/or in liver tissue[48,49,95], so it is unclear whether DR5 protein is significantly expressed on hepatocytes. It is possible that both DR4 and DR5 must be engaged on hepatocytes in order to trigger apoptosis. If so, then antibodies with selectivity for DR4 or DR5 might be inactive toward liver cells. Nevertheless, it will be important to verify the safety of such antibodies *in vivo* using an appropriate preclinical model, because such agents might have unique properties that could differentiate them from the ligand and have implications for clinical safety and efficacy. Among these are a longer plasma half-life; the potential to activate Fc-mediated antibody effector functions, such as antibody-dependent cell-mediated cytotoxicity or complement-dependent lysis against tumour and/or normal cells that express the target antigen; and lack of modulation by decoy receptors, which might protect against APO2L/TRAIL in certain normal cell types[48,99–101].

The patient population that is likely to benefit from therapeutics that are designed to target DR4 or DR5 might be broader for APO2L/TRAIL, which can act on tumours that express either DR4 or DR5, or both. Cancer cell lines express DR5 more frequently than DR4, indicating that DR5 mAbs might be more widely useful than DR4 mAbs. Combinations of DR4 and DR5 antibodies or mAbs that recognize both receptors could potentially enhance activity against tumours that express both receptors. So, recombinant soluble APO2L/TRAIL, in its zinc-bound trimeric form, and antibodies that engage death receptors DR4 and/or DR5 *in vivo* might be used as single agents in cancer therapy, or in combination with other treatments — especially chemotherapy. Preclinical work is underway to further evaluate and optimize these various approaches. Researchers have also begun to test APO2L/TRAIL gene therapy in experimental models[102].

DcR3. Decoy receptor 3 (DcR3) was discovered in a search of the human genome database for sequences with homology to the TNFR superfamily[39,40], as well as in a screen designed to identify new secreted proteins[103]. The full-length DcR3 cDNA encodes a protein that contains a signal sequence but no transmembrane domain, indicating that DcR3 is a secreted, soluble molecule. DcR3 is 300 amino acids long, with four

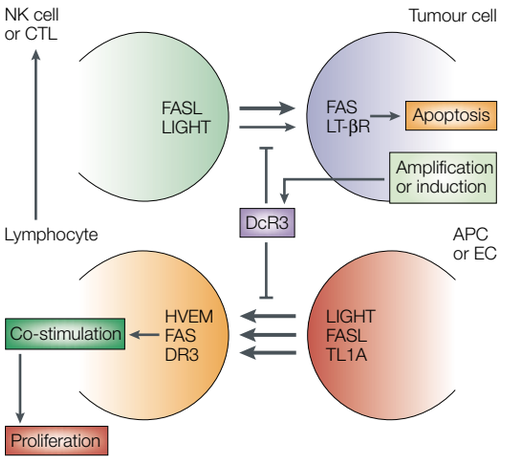


Figure 4 | **Hypothetical model of tumour-DcR3 action.** Various tumour types overproduce DcR3 through gene amplification or transcriptional upregulation. The DcR3 protein, which is secreted into the extracellular space, binds to the tumour-necrosis factor (TNF)-superfamily ligands FASL, LIGHT and TL1A, inhibiting their interaction with cognate signalling receptors. FASL and LIGHT might be involved in tumour-cell apoptosis induction by natural killer (NK) cells and cytotoxic T lymphocytes (CTL). DcR3 inhibition of these molecules might also interfere with expansion of tumour-reactive lymphoctes, which is induced by antigen-presenting cells (APC). TL1A, a ligand that is expressed by endothelial cells (EC), is capable of stimulating T-cell proliferation (and of inducing apoptosis in erythroleukaemic cells). The ability of DcR3 to block these ligands might provide cancer cells with a useful mechanism to evade immune surveillance. Therapeutic agents that neutralize DcR3 might counteract this type of immune evasion by tumours.

CRDs and an extended carboxy-terminal region; its closest sequence homologue in the TNFR-superfamily is OPG[37]. Ligand-binding studies show that DcR3 recognizes three TNF-superfamily members: FASL[39], LIGHT[40] and TL1A[41]. DcR3 competes for the binding of these ligands to their respective signalling receptors — FAS for FASL, HVEM and LT-βR for LIGHT, and DR3 for TL1A. A DcR3-encoding gene exists in human, chimpanzee and cynomolgus primates, but seems to be absent in mice (A. A., unpublished observations). The human DcR3 gene maps to chromosome 20q13 (REFS 39,103). DcR3 mRNA is expressed in several normal human tissues including spleen, lung and the gastrointestinal tract, as well as in umbilical-vein endothelial cells; expression in peripheral-blood T cells is upregulated after antigenic stimulation[39,40,103]. Because FASL, LIGHT and TL1A have or seem to have immunomodulatory functions, it is possible that DcR3 has evolved as a means of regulating these ligands in connection with immunity against some infectious agent(s) that specifically affect primates.

There is strong evidence that several types of tumour overexpress DcR3. Analysis of about 35 primary lung and colon tumours by quantitative polymerase chain reacion showed substantial amplification of the DcR3 gene in nearly half of the samples[39]. *In situ* hybridization analysis showed DcR3 mRNA overexpression in 6 out of 15 lung tumours, 2 out of 2 colon, 1 out of 1 gastric and 2 out of 5 breast tumours[39]. Subsequent studies showed DcR3 mRNA and protein overexpression in nearly half of 68 adenocarcinomas of the oesophagus, stomach, colon and rectum, in some cases without DcR3 gene amplification[103]. Investigation of virus-associated lymphoma specimens revealed DcR3 gene amplification and overexpression in 19 out of 45 cases[104]. Analysis of gliomas indicated an apparent correlation between DcR3 expression and malignancy: 11 grade II astrocytomas showed no expression, whereas 15/18 grade IV glioblastomas showed significant DcR3 immunoreactivity[105]. These observations indicate that tumour cells that overexpress DcR3 might acquire a relative advantage of growth or survival that is conferred by DcR3's ability to inhibit the putative antitumour effects of FASL, LIGHT and, perhaps, TL1A.

FASL seems to be important for immune surveillance against tumours: NK cells and cytotoxic T cells can use FASL to induce apoptosis in FAS-expressing tumour-cell targets[106,107]. Another main cytotoxic mechanism that is used by these cells involves secretion of the pore-forming protein perforin and entry of specialized proteolytic enzymes that activate caspases into the target cell[108]. Evidence consistent with a central role for FASL, and perhaps other death-receptor ligands, in antitumour immune surveillance comes from studies with FLIP (FLICE (FADD-like interleukin-1β-converting enzyme)-like inhibitory protein), a cytoplasmic inhibitor of death-receptor-mediated caspase activation[107]. FLIP-transfected mouse cancer cell lines formed tumours *in vivo* more frequently and aggressively than controls without FLIP; this difference was observed in mice that had an intact immune system, as well as in perforin-deficient mice, but not in immunodeficient mice[109,110].

More recent work in mouse models shows that epigenetic silencing of FAS can occur in tumour cells, allowing immune evasion and tumour growth; restoration of FAS expression leads to NK-cell-dependent tumour rejection (H. Maecker and A. Giaccia, personal communication). There is also evidence that FASL can co-stimulate T-cell proliferation[111], which might contribute to the expansion of tumour-reactive lymphocytes. These findings support a potentially crucial role of FASL–FAS interactions in immune surveillance against tumours.

LIGHT co-stimulates T-cell proliferation through HVEM in conjunction with antigen presentation and T-cell-receptor activation[112]. Therefore, LIGHT might also contribute to the expansion of tumour-reactive lymphocytes. Consistent with this possibility, transgenic expression of LIGHT in mouse cancer cells enhanced tumour-specific cytotoxic T-cell activity and inhibited tumour establishment in syngeneic mice[112]. Injection of these mice with a second challenge of tumour cells resulted in complete rejection, indicating that LIGHT promoted the establishment of a sustained antitumour immunity. LIGHT also can stimulate tumour-cell apoptosis through interaction with LT-βR[113].

The interaction of DcR3 with TL1A was discovered only recently, so knowledge about the potential relevance of TL1A to cancer is limited. Nonetheless, TL1A, which is

expressed primarily on endothelial cells, promotes T-cell co-stimulation, as well as inducing apoptosis in erythroleukemic cells[41], presumably acting through DR3. Together, the data with FASL, LIGHT and TL1A indicate that overexpression of DcR3 in tumours might confer an ability to evade mechanisms of immune surveillance that are modulated by some or all of these ligands. DcR3 might protect tumour cells against apoptosis induction by cytotoxic leukocytes, as well as by attenuating the co-stimulation of tumour-reactive T cells (FIG. 4).

Antibodies or small molecules that antagonize DcR3's ligand interactions might therefore have significant use for cancer therapy. Such agents should promote apoptosis through p53-independent mechanisms, so it will be interesting to investigate whether they can cooperate against tumours with conventional therapies. Moreover, it will be intriguing to examine further the interaction of DcR3 inhibition with therapeutic antibodies that target tumour antigens, such as ERBB2 (also know as HER2/neu) or the epidermal growth-factor receptor (EGFR), given that the activity of such antibodies might involve the recruitment of cytotoxic leukocytes to kill cancer cells. Because the gene that encodes DcR3 does not seem to exist in the mouse genome, it has been difficult to investigate its importance by using rodent models of cancer. DcR3 transgenic mice might help in further assessing the functional link between DcR3 and cancer.

Conclusions

Apoptosis induction in response to cell damage often requires the function of the tumour-suppressor p53, which engages the cell-intrinsic apoptosis signalling pathway. In most human cancers, conventional treatments eventually select for tumour cells in which p53 is inactivated, resulting in resistance to therapy. Death receptors can instruct tumour cells to commit apoptotic suicide independently of p53. So, targeting of death receptors in cancer might be a useful therapeutic strategy. In tumours that retain some responsiveness to conventional therapy, death-receptor engagement in combination with chemotherapy or irradiation might lead to synergistic apoptosis activation, and reduce the probability that tumour cells that are resistant to either type of agent will emerge. In tumours that have lost p53 function, death-receptor targeting might help to circumvent resistance to chemotherapy and radiotherapy.

The apoptosis-inducing activity of TNF is useful for cancer treatment, although systemic inflammatory side effects have, so far, limited the use of TNF to isolated limb perfusion of high-grade sarcomas. Researchers are working to develop new strategies to deliver TNF to tumours selectively, and to protect normal tissues from TNF's side effects.

Recombinant soluble APO2L/TRAIL in its non-tagged, zinc-bound trimeric form, or antibodies that engage this ligand's death receptors, DR4 or DR5, trigger apoptosis in a variety of cancer cells, while having little or no effect on normal cells. Work is underway to continue the evaluation of safety and efficacy of these agents in preclinical models and to identify suitable candidates for clinical investigation.

Various tumours overexpress the decoy receptor DcR3, which binds to FASL, LIGHT and TL1A. Because these ligands might be involved in antitumour immune surveillance, it is possible that DcR3 overproduction by tumour cells serves as a means of immune evasion. Studies in animal models are in progress to examine further whether neutralization of DcR3 enhances immune-cytotoxic attack against tumours. The identification of more TNF- and TNFR- superfamily members through the Human Genome Project has yielded novel apoptosis-based approaches that might expand cancer therapy in an exciting new direction.

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