# Exploring the TRAILs less travelled:

## TRAIL in Cancer Biology and Therapy

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The discovery that the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) can induce apoptosis of cancer cells without causing toxicity in mice has led to the in-depth study of pro-apoptotic TRAIL receptor (TRAIL-R) signalling and the development of biotherapeutic drug candidates that activate TRAIL-Rs. The outcome of clinical trials with these TRAIL-R agonists has, however, been disappointing so far. Recent evidence indicates that many cancers, in addition to being TRAIL-resistant, employ the endogenous TRAIL–TRAIL-R system to their own advantage. However, novel insight at two fronts: how resistance of cancer cells to TRAIL-based pro-apoptotic therapies might be overcome, and how the pro-tumourigenic effects of endogenous TRAIL might be countered, gives reasonable hope that the TRAIL system can be harnessed to treat cancer. In this review we assess the *status quo* of our understanding of the biology of TRAIL–TRAIL-R system – as well as the gaps therein – and discuss the opportunities and challenges in effectively targeting this pathway.

Although unknown at the time, tumour necrosis factor (TNF) first entered the world stage of cancer therapy towards the end of the 19th century when William Coley found that sarcomas shrunk with certain bacterial infections[1,2]. It was not until well into the 20th century that this effect was found to be due to the induction of TNF,[3] which caused tumours to become necrotic, a feature that coined the name of the protein. Initial enthusiasm following the discovery of TNF was, however, dampened by the demonstration that systemic TNF treatment induced a lethal inflammatory shock syndrome[4]. In search for another molecule with similar anti-tumour properties, the attention turned to CD95 (also known as FAS and APO-1) , a receptor homologous to TNF receptor 1 (TNFR1) and TNFR2 that can potently trigger apoptosis in many cancer cells[5,6]. However, systemic treatment with CD95 agonists led to fulminant liver toxicity in mice within hours of treatment[7,8], again excluding a TNF-like molecule for therapeutic use. Third time lucky: another TNF superfamily (TNFSF) member termed TNF-related apoptosis-inducing ligand (TRAIL, also known as TNFSF10 and APO2L) was discovered a few years later[9, 10], and this factor was capable of killing tumour cells, importantly however without causing the lethal adverse effects encountered with TNF or CD95 agonists[11,12].

Although these promising findings resulted in the development of TRAIL-receptor (TRAIL-R) agonists for clinical use, this happened at a time when toxicity of pro-apoptotic TNF-like factors in general, but also of TRAIL specifically[13,14], was a concern as some recombinant forms of TRAIL had shown potential for liver toxicity at high doses[13-15]. Moreover, when the decision to take particular molecular entities forward for clinical development was made, the biology of TRAIL and its receptors in cancer, as well as inflammation and immunity, was still underexplored and could therefore not adequately be taken into consideration. Since then, this has substantially changed. It is therefore timely to take a step back and revise our current understanding of the biology of the TRAIL–TRAIL-R system in order to come forward with novel and effective therapeutic strategies harnessing this system for cancer therapy.

## [H1] The TNF superfamily and TRAIL–TRAIL-Rs

### *[H3] The TNF superfamily*

TNF is the canonical member of the TNFSF of which TRAIL and the CD95 ligand (CD95L, also known as FASLG and APO-1L) are closely related members. Apart from lymphotoxin-α (also known as TNFSF1) and vascular endothelial growth inhibitor (also known as TNFSF15), which are encoded as soluble proteins, all other members of this family are encoded, and if not further cleaved, expressed as type II transmembrane proteins [16]. Some members, including TNF, CD95L and TRAIL, can subsequently be released from the cell surface through the action of proteases, and therefore can occur as both membrane-bound and soluble proteins. The proteases ADAM10 (a disintegrin and metalloproteinase domain-containing protein 10,) and ADAM17 (also known as TACE) have been identified to cleave CD95L and TNF to generate their respective soluble forms in a process termed shedding[17,18]. The generation of soluble TRAIL through shedding also involves cysteine protease activity[19], but the identity of the responsible protease(s) remains unknown. Soluble TRAIL is present in the plasma of a healthy adult at approximately 100 pg/ml[20], a concentration at which TRAIL fails to induce apoptosis in most cell lines *in vitro*[21].

For CD95L, only the membrane-bound protein can induce apoptosis whilst the soluble form has cancer-promoting effects[22]. For TRAIL, this is less clear. It has, however, been shown that liposome-bound TRAIL, which mimics membrane association, is more active in killing cancer cells than its soluble counterpart[23,24]. In this context it is conceivable that recombinant forms of TRAIL that comprise the extracellular domain fused to motifs that enable stabilisation and multimerisation might mimic the membrane-bound conformation. This is likely the reason why different recombinant forms which contain such motifs[11,13,25] are, by several orders of magnitude, more potent inducers of apoptosis than recombinant TRAIL preparations that lack such additional motifs[12].

TNFSF members bind to a corresponding family of receptors, referred to as the *TNFR superfamily* (TNFRSF), which comprises more members than the TNFSF. Hence, some ligands have several receptors. Eight TNFRSF members, including TNFR1 (also known as TNFRSF1A), CD95, TRAIL-R1 (also known as DR4 and TNFRSF10A) and TRAIL-R2 (also known as DR5 and TNFRSF10B)[26] contain an intracellular domain required for cell death induction, consequently referred to as the death domain (DD).

*[H3] The TRAIL–TRAIL-R system*

Amongst the TNFSF, human TRAIL is unique in that it binds four membrane receptors and one soluble receptor (Figure 1a). The human TRAIL-Rs can be subdivided into two classes: the full-length intracellular DD-containing receptors TRAIL-R1[27] and TRAIL-R2,[28-34] which are capable of inducing apoptosis and are most widely expressed, and the alternative receptors TRAIL-R3 (also known as DCR1 and TNFRSF10C)[33-36] TRAIL-R4 (also known as DCR2 and TNFRSF10D)[37,38] and osteoprotegerin (OPG, also known as TNFRSF11B), which also functions as a soluble receptor for receptor activator of nuclear factor-κB ligand (RANKL, also known as TNFSF11)[39]. TRAIL-R3 is glycosylphosphatidylinositol (GPI)-anchored to the plasma membrane, hence lacks an intracellular domain, and TRAIL-R4 contains a cytoplasmic domain capable of inducing nuclear factor-κB (NF-κB) activation but not apoptosis as it only encodes a truncated DD. At 37°C, TRAIL binds TRAIL-R2 with higher affinity than the other membrane-expressed TRAIL-Rs[40]. It is therefore likely that under physiological conditions binding to TRAIL-R2 would be favoured, especially when endogenous TRAIL is limited.

All of the alternative TRAIL-Rs were proposed to act as TRAIL “decoys”, i.e. their binding to TRAIL would lower the concentration of TRAIL available for binding to the pro-apoptotic receptors TRAIL-R1 and TRAIL-R2 and, thereby, negatively regulate apoptosis induction by TRAIL. Whereas *in-vitro* overexpression results and additional correlative data in favour of this concept were presented[41], it remains to be seen whether this function is indeed exerted by any of these receptors in cancer cells under endogenous expression levels[42-45].

When TNFR1 was first crystallised complexed with its ligand, it formed receptor trimers with a ligand trimer located in its core[46]. However, when not bound by its ligand, TNFR1 formed dimers[47]. Similar to TNFR1, TRAIL-Rs also exist as preassembled multimers. In the TRAIL–TRAIL-R system, however, receptor dimers are ligand-induced and present in high molecular weight fractions together with ligand-induced trimers[48]. Adding another level of complexity, TRAIL-R1 and TRAIL-R2 can homo- and heterotrimerise to form higher-order complexes. It has been suggested that such complexes can either involve trimer

multimerisation or crosslinking of neighbouring trimers via dimerisation between receptor interfaces which are located opposite of the ligand-binding interfaces resulting in a hexameric honeycomb-like structure[48]. The latter model received support through two recent studies showing that non-stabilised, untagged TRAIL synergised with TRAIL-R2-specific antibodies to kill cells and that this was achieved through a ternary complex crystal structure resembling the above mentioned honeycomb [21,49].

The most obvious molecular difference between the two DD-containing TRAIL-Rs is that there is only one splice variant for TRAIL-R1 whereas there are two for TRAIL-R2[50]. The long isoform of TRAIL-R2 contains an additional 29 extracellular amino acids, which are located immediately adjacent to the membrane. As this polypeptide, rich in threonine, alanine, proline and glutamine (TAPE), also referred to as the TAPE domain[34], is thought to form a rigid stalk as described for a highly homologous polypeptide in TNFR2[51], it is likely that its presence results in protrusion of the long isoform from the glycocalyx **[G]**. It is therefore tempting to speculate whether only TRAIL-Rs whose extracellular domains protrude at similar stalk-dependent heights may effectively heterotrimerise. If that were the case, TRAIL-R1, TRAIL-R4 and the short isoform of TRAIL-R2, in addition to forming homotrimers, would be capable of forming heterotrimers amongst each other whereas the long isoform of TRAIL-R2 would only form homotrimers. According to this model, with five consecutive repeats of the TAPE domain[34] TRAIL-R3 would hover high above the other TRAIL-Rs and therefore also only form homotrimers.

Despite surface expression of TRAIL-R2 in cell lines derived from pancreatic cancer, chronic lymphocytic leukaemia or mantle cell lymphoma, these cells only employ TRAIL-R1 for apoptosis induction by TRAIL[52-54]. In addition, apoptosis induction via TRAIL-R2 requires crosslinking of untagged soluble TRAIL[55] implying that TRAIL-R2 might have a higher apoptotic threshold than TRAIL-R1 . In several leukaemia and lymphoma cell lines, however, antibody-mediated TRAIL-R2 triggering appears sufficient to induce apoptosis without additional crosslinking[56]. Together, these data highlight that human TRAIL-R1 and TRAIL-R2 fulfil partly overlapping but also distinct functions, of which many remain to be discerned.

In contrast to humans, mice only express a single TRAIL-R (mTRAIL-R, also known as MK) with an intracellular DD which shares almost the same level of identity with human TRAIL-R1 (43% sequence homology) and human TRAIL-R2 (49% homology); like its human counterparts, mTRAIL-R is capable of inducing apoptosis[57]. Two further mouse TRAIL-Rs (mDcTRAIL-R1, also known as TNFRSF23 and mDcTRAIL-R2, also known as TNFRSF22) were later described, but these lack an intracellular DD[58] (Figure 1b). They differ substantially in their amino acid sequence from human TRAIL-R3 and TRAIL-R4 and do not induce apoptosis or NF-κB activation upon overexpression[58]. Notably, human TRAIL binds only weakly to mTRAIL-R whereas mouse TRAIL has high affinity for the human TRAIL-Rs[59]. These findings need to be considered when designing tolerability studies in mice.

Although studies in mTRAIL-R-deficient mice have shed light on the relevance of many TRAIL-R-induced pathways *in vivo*, it remains mysterious why humans have evolved to express two DD-containing receptors for TRAIL. One option to study this question further would be to develop a “humanised” mouse expressing human DD-containing TRAIL-Rs.

## [H1] TRAIL-induced signalling pathways

Like other members of the TNFSF, TRAIL can trigger a variety of biological responses in cancer and normal cells. Besides the induction of cell death by apoptosis or necroptosis (Box 1) this also includes the activation of non-cell death pathways that in turn trigger a plethora of cellular processes.

### *[H3] Pro-apoptotic TRAIL signalling*

In 1999, two groups independently showed that systemic treatment of mice bearing xenograft tumours with recombinant forms of human TRAIL resulted in tumour regression[11,12]. This discovery, together with the demonstration that systemic treatment with high-dose leucine zipper (LZ) mouse TRAIL, which was capable of killing mouse cells *in vitro*, was well tolerated[11], formed the basis for the clinical development of TRAIL-R agonists. Moreover, it sparked great interest in investigating the mechanisms by which TRAIL-R1 and TRAIL-R2 initiate apoptosis, what prevents this in TRAIL-resistant cancers and how TRAIL resistance can be broken.

Upon TRAIL binding, the intracellular DDs of three ligand-crosslinked receptors adopt a conformation that enables them to recruit the intracellular adaptor molecule FAS-associated protein with death domain (FADD) via its DD. FADD contains a death effector domain (DED) that enables recruitment of the initiator caspases 8 and 10 via their DEDs. The membrane-associated complex resulting from these interactions is termed the TRAIL death-inducing signalling complex (DISC)[60-63] (Figure 2).

TRAIL DISC formation induces proximity-induced activation of caspase-8 and caspase-10, thereby further amplifying their activation through mutual cleavage. Part of the aggregation required for full caspase-8 activity can be facilitated by Cullin-3-mediated non-degradative ubiquitination of caspase-8, which leads to p62 binding and aggregation of caspase-8 at the DISC[64]. Moreover, degradative K48-linked ubiquitin chains were shown to be attached to the cytosolic p18 fragment of active caspase-8 in a TNF receptor-associated factor 2 (TRAF2)-dependent manner, leading to proteasomal degradation of active caspase-8 in the cytosol, thereby serving as its shut-off timer[65]. Active caspases 8 and 10 are released into the cytosol were they cleave downstream effector caspases such as caspase-3. Importantly, albeit for reasons not entirely understood, caspase-10 cannot compensate for loss of caspase-8 despite effective recruitment to the TRAIL DISC in the absence of caspase-8[63], assigning caspase-8 a central role in the initiation of extrinsic apoptosis **[G]** by TRAIL.

FLICE-like inhibitory protein (FLIP, also known as CFLAR) is a caspase-8 homologue that can compete with caspase-8 for binding to FADD but does not contain catalytic activity and is consequently frequently upregulated in cancers to mediate resistance against DISC activation and apoptosis[66,67]. Irrespective of FLIP levels, in some cells DISC activation is insufficient to trigger extrinsic apoptosis. Here, cross-signalling to the mitochondria via cleavage of BH3-interacting domain death agonist (BID)[68] and assembly of the caspase-9-activating apoptosome **[G]** [69] are essential (Figure 2). Apoptosome formation and the resulting caspase-9 activation in turn enhance caspase-3 cleavage and activity. Activation of effector caspases, including caspase-3, induces cleavage of a plethora of cellular proteins, ultimately resulting in the execution of apoptosis.

*[H3] Non-canonical TRAIL signalling*

Apart from inducing cell death, binding of TRAIL to TRAIL-R1, TRAIL-R2 and also TRAIL-R4 has been shown to induce activation of NF-κB [29,38], a transcription factor involved in pro-inflammatory immune responses[70]. Receptor-interacting serine/threonine-protein kinase 1 (RIPK1), apart from executing necroptosis, is also involved in TRAIL-mediated NF-κB induction[71] by activating the inhibitor of κB (IκB) kinase-complex (IKK-complex) which in turn phosphorylates IκB leading to its degradation and NF-κB nuclear translocation. Although TRAIL-induced NF-κB activation was initially suggested to simply mediate resistance to TRAIL-induced apoptosis, more recent evidence shows that NF-κB activation can serve other purposes in TRAIL-resistant cells. Accordingly, TRAIL can induce proliferation in TRAIL-resistant Jurkat cells via NF-κB as demonstrated by using RIPK1-deficient or NEMO (also known as IKKγ deficient cells[72]. In apoptosis-resistant cholangiocarcinoma cells, TRAIL promotes NF-κB-dependent tumour cell migration and invasion without influencing proliferation[73]. In addition, RIPK1 is present in the native TRAIL-DISC where it can induce NF-κB when caspases are inhibited[74]. Interestingly, these are also conditions under which necroptosis can be induced.

Furthermore, TRAIL-induced NF-κB activation is decreased in the absence of FADD and increased during co-treatment with caspase inhibitors, which is, at least in part, thought to be due to the fact that caspases cleave RIPK1, thereby rendering it unable to activate NF-κB [75]. The anti-apoptotic protein FLIP also modulates TRAIL-induced NF-κB activation, but precisely how it achieves this is still controversial. Overexpression of FLIP increases basal NF-κB activation to the same extent as caspase-8 and FADD overexpression[76] whereas it also inhibits TRAIL-R-mediated NF-κB activation[75].

Intriguingly, the formation of a secondary intracellular signalling complex following DISC formation has been proposed to activate not only NF-κB, but also the JUN N-terminal kinase (JNK) and p38 MAPK, pathways[77]. The implications of kinase pathway activation by TRAIL were recently reviewed[78]. Another member of the MAPK family, ERK, seems to be intimately involved in TRAIL-induced non-apoptotic effects[79]. TRAIL induces ERK-mediated proliferation in caspase-8-deficient small-cell lung cancer (SCLC) cells in a TRAIL-R2-dependent manner[80]. Interestingly, in certain cancer cells TRAIL-R2, which is normally expressed at the plasma membrane, was found in the nucleus where it promotes proliferation by interacting with accessory proteins of the microprocessor complex **[G]** leading to impaired maturation of the microRNA let-7, a known negative regulator of *KRAS* mRNA[81]. It therefore appears that subcellular compartmentalisation of TRAIL-R2 may determine distinct TRAIL-R2 signalling outputs (reviewed by Bertsch et al. [82]).

Another study demonstrated that TRAIL is expressed in highly vascularised soft tissue sarcomas and, intriguingly, soluble TRAIL induced endothelial cell migration and vessel tube formation to a similar extent as vascular endothelial growth factor (VEGF)[83]. Low-dose TRAIL was shown to trigger migration in non-small cell lung cancer (NSCLC) cell lines *in vitro* in a manner dependent on RIPK1, SRC and signal transducer and activator of transcription 3 (STAT3)[84]. Moreover, oncogenic KRAS signalling rendered colorectal cancer cells resistant to TRAIL and CD95L through suppression of RHO-associated protein kinase (ROCK) activity and enabled their migration[85 86]. Of note, the membrane-proximal domain (MPD) of TRAIL-R2, a short, ten amino-acid-long stretch juxtaposed to the plasma membrane, was sufficient to

promote cell-autonomous RAC1 activation and migration of NSCLC cell lines with an oncogenic KRAS mutation in response to constitutive stimulation by endogenous TRAIL[86]. Interestingly, this domain is at least partially conserved between TRAIL-R2 and CD95, which can also activate RAC1 in neurons via its MPD[87].

**[H1] TRAIL and its receptors in cancer**

The TRAIL–TRAIL-R system affects many physiological and pathophysiological processes in both immunology and cancer. With respect to the immune system, TRAIL and its receptors are expressed on various human innate and adaptive immune cell types. TRAIL expression levels on these different cells depend on the stimulation status of the immune cell. In innate immune cells, TRAIL is expressed on monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells, after lipopolysaccharide (LPS) or pro-inflammatory cytokine stimulation, and it is involved in effector mechanisms in these cells [88-90]. Regarding adaptive immune cells, the TRAIL–TRAIL-R system plays an important role in preventing aberrant T-cell activation and is required for immune homeostasis in normal physiology[91-94]. Regarding cancer, depending on the type of malignancy and its particular oncogenic make-up, this system can mediate either immunosurveillance against pre-malignant cells or pro-tumourigenic effects.

*[H3] Pleiotropic effects of* TRAIL–*TRAIL-Rs in mouse models of tumourigenesis and metastasis*

One of the first indications for a role of endogenous TRAIL in regulating tumour growth came when it was shown that *Trail*-deficient mice are more susceptible to transplanted mouse A20 lymphoma, as they died prematurely due to an increased number of lymphoma nodules in the liver[95]. The influence of the TRAIL–TRAIL-R system on cancer in mouse models is summarised in Table 1. Moreover, administration of neutralising antibodies against TRAIL or *Trail*-deficiency promoted tumour development in mice treated with the chemical carcinogen methylcholanthrene (MCA)[96,97]. The protective effect of TRAIL in this model was at least partially dependent on interferon-γ (IFN-γ)-mediated upregulation of TRAIL on NK cells. TRAIL expression on NK cells is an important mechanism used by the immune system to kill cancer cells[98,99] (Figure 3), however it cannot be excluded that TRAIL expression on other immune effector cells could also contribute to the protection against tumour development. Additional evidence for a role of TRAIL in host immune-surveillance against the development of primary tumours came from studies with *Trp53*$^{+/-}$ mice in which loss of TRAIL predisposes to development of a greater number of spontaneous tumours, including disseminated lymphomas and sarcomas[96,100]. *Trail*-deficiency also rendered immunocompetent mice more susceptible to experimental and spontaneous liver metastasis and tumour growth resulting from intrasplenically injected syngeneic renal carcinoma cells[95,101]. Similarly, primary growth and spontaneous liver metastasis of syngeneic breast cancer cells injected into the mammary glands also increased in *Trail*-deficient mice[97]. The anti-metastatic effects observed in the spontaneous liver metastasis models could be mainly explained by the lack of TRAIL expression on hepatic NK cells as the *ex vivo* cytotoxicity of liver NK cells from *Trail*-deficient mice was dramatically reduced compared with those from control mice[97]. Taken together, these findings further demonstrated an important role of TRAIL expressed on NK cells as an anti-tumour effector molecule.

Several mouse models of carcinogen-induced or genetically engineered malignancies conducted in *Trail-r*-deficient mice have further highlighted the central role of the TRAIL–TRAIL-R system in carcinogenesis. In a mouse model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis, *Trail-r* deficiency promoted development of macroscopic liver lesions[102]. The same study also showed that in mice *Trail-r* deficiency promoted *Eμ-Myc*-driven lymphomagenesis as well as lung and liver metastasis as a result of loss of mTRAIL-R–mediated cell death in lymphomas. In addition, mTRAIL-R acts as a specific suppressor of metastases in an autochthonous model of skin carcinogenesis. In this model, metastasis suppression occurred without affecting primary epithelial skin tumourigenesis and was due to TRAIL sensitisation of detached cancer cells[103]. Since TRAIL expression on NK cells contributes to immune surveillance, it seems likely that TRAIL-expressing NK cells could be the effector cells responsible for the killing of detached skin carcinoma cells via TRAIL-R-mediated apoptosis[104].

In contrast, cancer cell-expressed endogenous mTRAIL-R was shown to promote progression, invasion, and metastasis of autochthonous *KRAS*-driven pancreatic and lung cancer in a cell-autonomous manner[86]. In contrast to previous studies, cancer cell-restricted deletion of mTRAIL-R in the presence of intact TRAIL–mTRAIL-R signalling in all other cells was studied here for the first time. It is, however, possible that intact TRAIL–mTRAIL-R signalling in non-tumour cells contributes to overall cancer promotion. In line with this notion, it is interesting that shorter survival of tumour-supportive myeloid-derived suppressor cells (MDSCs) and tumour-associated macrophages (TAMs), referred to as type 2 myeloid cells, is caused by caspase-8-dependent apoptosis via TRAIL-R2[105,106]. These data suggest that at least some of the effects seen in cancer models studying whole-body *Trail-r*-deficient mice could be due to increased numbers of MDSCs and, thereby, immune regulation and tumour promotion rather than only the absence of TRAIL-induced apoptosis in cancer cells. In addition, activation of TRAIL-R on tumour endothelial cells was shown to induce their apoptosis, causing vascular disruption with consequent reduction in tumour growth in a genetically engineered mouse model of pancreatic cancer[107].

Interestingly, TRAIL-induced stimulation of TRAIL-Rs on cancer cells has also recently been shown to induce the secretion of cytokines, most importantly C-C motif chemokine ligand 2 (CCL2), resulting in recruitment of tumour-supporting type 2 myeloid cells that express chemokine (C-C motif) receptor 2 (CCR2), thereby contributing to tumour growth[108]. Of note, unlike RAC1 activation, induction of the TRAIL-induced cancer secretome requires FADD and the scaffold function of caspase-8 but not its enzymatic activity, implying that cancer cell-expressed TRAIL-Rs can trigger distinct but parallel signalling pathways to promote cancer[108,109]. Interestingly, the linear ubiquitin chain assembly complex (LUBAC) was recently identified to regulate TRAIL-induced gene activation and cell death and to be required for TRAIL-induced cytokine production downstream of FADD, caspase-8, cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2[110].

TThe results from these experimental mouse models of cancer highlight that the role of TRAIL-TRAIL-R system in cancer biology is diverse and can only be fully understood through cell-population-specific deletion, an undertaking that is far from complete and therefore an area of research that deserves further attention. Importantly, as mentioned above mTRAIL-R is homologous to both human TRAIL-R1 and TRAIL-R2, also highlighting that not all aspects of human TRAIL-R biology can be studied in the murine system. Therefore, also here the development of a mouse expressing a “humanised” TRAIL–

TRAIL-R system could be an interesting approach to further dissect the pro- and anti-tumour signalling pathways triggered by human TRAIL-R1 as compared to TRAIL-R2.

## [H1] Clinical trials of TRAIL-R agonists

As noted above, TRAIL's capability to induce apoptosis selectively in cancer cells[11,12] led to the clinical development of several agonists for TRAIL-Rs. They fall into two categories: recombinant forms of TRAIL and agonistic antibodies against TRAIL-R1 and TRAIL-R2 (current clinical trials employing TRAIL-R agonists are summarised in Table 2). However, to date none of these agonists have yielded a clinical benefit in cancer patients[111]. It appears that there are three main contributors to the failure of clinical trials conducted so far with TRAIL-R agonists: insufficient agonistic activity of the drug candidate in question, resistance of many primary cancer cells to monotherapy with TRAIL-R agonists[49,112] and, lastly, lack of suitable biomarkers for identifying patients who are more – or less – likely to respond to a particular TRAIL-R agonist-comprising therapy[113].

### *[H3] Recombinant forms of TRAIL*

Several recombinant TRAIL formulations have been developed with the aim to increase stability and/or tumour-specific delivery of TRAIL. Stabilization of the TRAIL trimer has been attempted by the addition of N-terminal tags such as poly-Histidine[10], FLAG epitope[9], leucine zipper[11] and isoleucine zipper (iz) motifs [13], the fusion of TRAIL to the Fc portion of human immunoglobulin G (IgG)[114] and to human serum albumin[115]. In order to increase the tumour delivery of TRAIL and, concomitantly, reduce TRAIL dilution in circulation, two main drug delivery approaches have been followed: passive targeting based on coupling TRAIL to nanoparticles and active targeting via antibody fragments or peptides that specifically target cancer cells or components of the tumour microenvironment (reviewed by de Miguel[116]).

The only recombinant form of TRAIL developed to date for clinical application is the non-tagged APO2L.0 (also known as dulanermin and AMG-951), which comprises of amino acids 114-281 of the extracellular domain of human TRAIL[12]. Dulanermin, as opposed to receptor-specific antibodies, has the advantage of targeting both TRAIL-R1 and TRAIL-R2, rendering it less dependent on expression of only one of them. However, despite encouraging preclinical results with this protein[12,13,15,117], clinical trials failed to show any significant anti-cancer activity[111,118,119]. Two particular characteristics of dulanermin are most likely the main culprits for this failure: its reported short half-life of approximately 30 minutes *in vivo* [117,120] and its weak capacity to induce higher-order clustering of TRAIL-Rs[21,49] which we will revisit in detail later. In addition, any recombinant form of TRAIL, including dulanermin, may also engage the non-apoptosis-inducing TRAIL-Rs (TRAIL-R3, TRAIL-R4 and OPG). Despite the fact that the putative decoy function of these receptors in cancer cells remains to be demonstrated in non-overexpression systems[41], it is likely that in cancers in which the ratio of non-cell death-inducing TRAIL-Rs to death-inducing TRAIL-Rs is particularly high, the apoptosis-inducing capacity of any recombinant form of TRAIL would be lessened.

### *[H3] Agonistic TRAIL-R-specific antibodies*

Antibodies developed as specific TRAIL-R1 or TRAIL-R2 agonists are more stable and have substantially longer half-lives than dulanermin. However, despite encouraging anti-cancer activity achieved in preclinical models, TRAIL-R1- and TRAIL-R2-specific antibodies neither improved objective response rates, nor increased overall patient survival[121-126]. The clinical failure of antibodies as death receptor agonists might, however, be explained by their bivalent mode of receptor binding; in the early 1990's it was shown in the context of the death receptor CD95 that non-crosslinked bivalent antibodies against CD95 are insufficient to induce apoptosis as, at the very least, trimerisation of the receptor is required as discussed above[127]. Although TRAIL-R2-agonistic antibodies can trigger DISC formation, this is enhanced by crosslinking[128], highlighting that higher-order complex formation is still needed for high levels of apoptosis induction.

Consequently, novel TRAIL-R agonists have been designed with the aim to render them more potent in activating the apoptosis-inducing capacity of TRAIL-R1 and/or TRAIL-R2[129]. Amongst them, TAS266 was first to reach the clinic. TAS266 is a novel agonistic tetravalent nanobody **[G]** targeting TRAIL-R2, consisting of four identical humanised high affinity heavy chain domain (VHH) antibody fragments, occurring naturally in camelid species, connected through three linkers of 35 amino acids each[130]. Since each VHH domain can bind to TRAIL-R2 with high affinity, TAS266 has the potential to cluster four TRAIL-R2 molecules simultaneously leading to efficient DISC formation and apoptosis induction[131]. However, when a phase I clinical study in patients with advanced solid tumours was initiated to evaluate the safety and tolerability of TAS266, the trial unfortunately had to be terminated early due to the rapid elevation of liver enzyme values indicative of acute toxicity in three patients[130]. Although not investigated in depth, the acute but reversible toxicity likely stemmed from the fact that these patients had pre-existing anti-camelid antibodies binding TAS266 and thereby suffered from an anti-drug antibody (ADA) response **[G]** which apparently increased the agonistic activity of TAS266[130].

Of note, administering dulanermin to cynomolgus monkeys induced the production of ADAs. These ADAs were directed against the only four amino acids in which human and cynomolgus monkey TRAIL differ and were shown to be responsible for the observed liver toxicity[132]. This striking result emphasises the importance of considering the immunogenic potential of a novel biotherapeutic, especially when it is designed to act as an agonist. Specifically for TRAIL-R agonists, the results with TAS266 in humans and dulanermin in cynomolgus monkeys also imply that the desirable level of activity likely is not the highest possible, but an intermediate one which perhaps best mimics the activity exerted by cell surface-expressed TRAIL. Therefore, the clinical success of a novel TRAIL-R agonist will likely depend on the right mix of increased agonistic activity and a suitable safety profile. Consequently, when designing novel TRAIL-R agonists, apart from optimising their agonistic activity, it will be important to minimise possible adverse effects; potential immunogenicity is a crucial factor to consider. It will be interesting to see whether the two TRAIL-R agonists that are most advanced in preclinical development[25,133] fulfil these requirements once they are tested in humans.

*[H3] Combination of TRAIL-R agonists*

As TRAIL-R binding of a supposedly agonistic TRAIL-R-specific antibody might interfere with the interaction between endogenous TRAIL and its receptors, the treatment with such antibodies might

even be counterproductive by preventing the killing of cancer cells by endogenous TRAIL. Surprisingly, soluble untagged TRAIL in the form of dulanermin and the agonistic TRAIL-R2-specific antibody AMG-655, both only exhibiting limited single-agent activity in killing cancer cells, synergised in the killing of cancer cells[21,49]. This result was due to a concomitant binding of TRAIL and AMG-655 to TRAIL-R2, which led to enhanced multimerisation of TRAIL-R2 and, consequently, increased formation of the TRAIL-DISC. Recently, the mere stabilisation of higher-order DISCs was shown to be sufficient to sensitise cancer cells, but importantly not primary hepatocytes, to TRAIL-induced apoptosis[21,49]. Intriguingly, together they were about as active as an iz-tagged stabilised trimeric form of recombinant TRAIL. Importantly, both dulanermin and AMG-655 have been used in several clinical trials, either alone or combined with other drugs, without causing acute adverse effects, including no ADA response. Their combined clinical application should therefore be feasible, offering the possibility of a clinical trial testing their efficacy in combination.

*[H3] Resistance and Biomarkers*

Although TRAIL can specifically kill many cancer cells, it is now well established that the majority of primary cancers are resistant to TRAIL-R agonistic monotherapy. Primary epithelial cancer cells isolated from colon, breast and lung carcinomas, as well as primary olfactory neuroblastoma and leukaemia cells are TRAIL-resistant[72,112,134]. Furthermore, the majority of primary high-grade serous ovarian cancer cells derived from ascites of patients with chemotherapy-resistant disease are also resistant to TRAIL-induced apoptosis[21,49,135-137]. The identification and drug-mediated removal of factors causative for this resistance is crucial to sensitise cancer cells to TRAIL-induced apoptosis (previously reviewed[111,116,138]). Interestingly, resistance to TRAIL can also arise through cell-to-cell variability in initial TRAIL sensitivity within clonal cell populations[139] (Box 2). To date, many TRAIL-sensitising strategies have been tested such as the combination of TRAIL-R agonists with proteasome inhibitors (reviewed by de Wilt et al.[140]), standard chemotherapeutic agents, SMAC (also known as DIABLO) mimetics, BH3 mimetics to antagonise anti-apoptotic BCL-2 family members, or different kinase inhibitors (for example, those that inhibit AKT or PI3K) (previously reviewed[111,113]). However, many of these studies only show limited therapeutic activity *in vivo* and likely also underestimate potential *in-vivo* toxicity[99].

Recently, inhibition of cyclin-dependent kinase 9 (CDK9) was described as the most potent TRAIL sensitisation strategy discovered to date. CDK9-inhibitory drugs, of which several are currently in clinical development, exquisitely sensitise NSCLC cell lines to TRAIL-induced apoptosis via the concomitant downregulation of two anti-apoptotic factors, MCL1 and FLIP, thereby simultaneously increasing DISC-generated caspase-8 activity and removing a mitochondrial block to maximal apoptosis induction[141]. As a result, the combination of iz-TRAIL with the CDK9 inhibitor SNS-032 rendered many TRAIL-resistant cancer cells highly TRAIL-sensitive whilst the sensitisation of primary human hepatocytes was more limited[141]. Thus, success of future combination therapies comprising an optimised TRAIL-R agonist will not only depend on simultaneous neutralisation of different factors which together cause TRAIL resistance but also on the cancer cell selectivity of the apoptosis sensitisation.

The identification of valid biomarkers that will predict which patients will benefit from TRAIL-based therapy is also an important aspect to consider when using TRAIL-R agonists. At present, the expression of TRAIL-R1 and/or TRAIL-R2 serves as the only marker to identify patients who are likely to benefit from a TRAIL-R-agonist-comprising therapy. High expression of the O-glycosyl transferase GALNT14 was proposed to be a signature of TRAIL sensitivity[142]. However, GALNT14-positive patients had only a trend towards increased progression-free survival and overall survival, and there was no significant correlation between tumour GALNT14 expression and clinical response to dulanermin in a randomised phase II study of patients with advanced NSCLC[119]. This may, however, not be the same for more potent TRAIL-R agonists. In summary, the task to identify a useful marker or marker set for deciding which patients may benefit from a TRAIL-R-agonist-comprising therapy is far from complete. Approaches involving the use of high-throughput screening of large cell line collections with known mutations[143] may turn out to be useful for the identification of such markers.

In the context of biomarkers it should also be noted that high expression of TRAIL-R2 correlated with parameters of malignancy in patients with *KRAS*-mutated colorectal or pancreatic cancer[86] and high TRAIL expression correlated with higher probability to develop metastases in *KRAS*-mutated NSCLC patients[144]. Therefore, *KRAS* mutation should be considered an exclusion criterion for stand-alone treatment with a TRAIL-R agonist. Importantly, , in KRAS-mutated cancer the TRAIL–TRAIL-R system can promote the progression, invasion and metastasis, suggesting that in these cancers the pro-tumourigenic function of TRAIL-R signalling might be positively selected for[86]. Thus, for patients with *KRAS*-mutated cancers, the inhibition of TRAIL or TRAIL-R2 should be explored as a therapeutic approach instead.

*[H3] Apoptosis-inducing therapies harnessing the endogenous TRAIL–TRAIL-R system*

Apart from the above discussed strategies of activating TRAIL-Rs on cancer cells through agonists with improved activity or, alternatively, blocking them in the context of *KRAS*-mutated cancers, another therapeutic strategy might be to make use of the fact that several US Food and Drug Administration (FDA)-approved drugs and newer therapeutics in advanced clinical trials have been shown to activate caspase-8, suggesting activation of a TNFRSF death receptor pathway. Interestingly, the cyclooxygenase 2 (COX2, also known as PTGS2) inhibitor celecoxib has been shown to induce TRAIL-R2-dependent caspase-8 activation and apoptosis in NSCLC cell lines[145]. It is therefore not surprising that celecoxib treatment also sensitised malignant glioma and hepatocellular carcinoma cell lines to apoptosis induced by exogenously added TRAIL[146,147]. Of note, celecoxib-mediated sensitisation of lymphoma cells to TRAIL-induced apoptosis was subsequently shown to be independent of COX2 inhibition [148].

Treatment of leukaemia cells with all-trans-retinoic acid (ATRA) upregulated TRAIL expression, which contributed to apoptosis induction by ATRA[149]. In addition, the anti-tumour activity of trabectedin, a DNA-binding molecule produced by the sea squirt, partially involved killing of TAMs through TRAIL-mediated caspase-8 activation[106]. More recently, the endoplasmic reticulum (ER) stress-inducing agents thapsigargin and brefeldin-A were shown to converge on TRAIL-R2 during ER stress to induce caspase-8-dependent cell death[150]. Moreover, the small molecule ONC201 (also known as TIC10) was identified to induce *TRAIL* gene expression, and had potent anti-tumour cell activity *in vitro*[151,152].

These findings, together with the fact that many cancers highly express endogenous TRAIL-Rs, suggest that one strategy to harness the TRAIL–TRAIL-R system for therapy might be through sensitisation to endogenous TRAIL-R-mediated apoptosis. This strategy would also allow the expression of TRAIL, its apoptosis-inducing receptors and perhaps some key pro-apoptotic factors involved in TRAIL-induced apoptosis, such as FADD and caspase-8, to be potentially used as clinical markers for patient selection.

*[H3] The TRAIL–TRAIL-R system in cancer immunotherapy – to block or to activate?*

It is now well established that the immune system is crucial for both tumour progression and treatment response[153]. Blockade of the immune checkpoint molecules cytotoxic T lymphocyte associated antigen 4 (CTLA4) and/or programmed cell death protein 1 (PD1)–PD1 ligand 1 (PDL1) has shown striking therapeutic efficacy in patients with advanced melanoma and now also several other cancers including NSCLC.However, in a substantial proportion of patients suffering from these cancers, and certainly in several other cancer entities, the therapeutic effect of immune checkpoint blockade is more limited. Although conventional or targeted anti-cancer therapies mostly act by killing cancer cells, many of these therapies also affect the immune system, which will potentially contribute to their therapeutic efficacy. In a recent study, it was shown that COX1 and COX2 inhibition enhanced the efficacy of a PD1-blocking antibody suggesting that production of immune-suppressive factors by the tumour is a potent additional mechanism of tumour immune escape[154]. As celecoxib induces tumour cell apoptosis through TRAIL-R2 as discussed above[145], it would be interesting to investigate whether the TRAIL–TRAIL-R system contributes to, or synergizes with, the combination therapy of COX inhibition with PD1 blockade.

Several studies have shown that TAMs express TRAIL-Rs, suggesting that TRAIL-Rs could be potential targets for selectively eliminating these cells[105,106]. Moreover, TRAIL was recently shown to significantly reduce the number of intratumoural regulatory T cells (Tregs)[155] by promoting their apoptosis, and Treg depletion has proven to be an effective therapeutic strategy to elicit anti-tumour immunity in a wide variety of mouse models of cancer[156-160]. Therefore, and as the TRAIL–TRAIL-R system cannot only induce the killing of cancer cells but also of tumour-supportive immune cells, TRAIL-R agonists could perhaps be combined with immunotherapy for the treatment of certain cancers (Figure 3)**.**

TRAIL has, however, also been shown to promote an immune-suppressive cancer microenvironment, to induce proliferation of Tregs and to act as one of the mechanisms by which Tregs suppress effector immune cells[92,93,161]. TRAIL can also contribute to a T cell-suppressing microenvironment by inducing pro-inflammatory cytokines that enable myeloid cell polarisation towards MDSC and fully differentiated M2 macrophage phenotypes[108,162]. Therefore, in certain cancers antagonising endogenous TRAIL–TRAIL-R signalling might not only exert a beneficial effect through suppression of cancer cell-autonomous promotion of cancer[86] but also by interfering with Treg and type 2 myeloid cell activity. Hence, TRAIL–TRAIL-R blockade might be an alternative, perhaps additional therapeutic strategy to consider in the context of immune checkpoint inhibition (Figure 3).

Clearly, the last two paragraphs provide rather divergent, indeed opposing principles for future development. Yet, it is entirely possible that certain cancer patients will benefit from therapies

comprising a TRAIL-R agonist whilst for others a TRAIL antagonist-comprising therapy may prove more suitable. It is therefore crucial to uncover the underlying biological principles and mechanisms that determine whether a cancer patient is likely to benefit from one versus the other therapeutic concept and to identify biomarkers that can guide such therapeutic decisions.

## [H1] Conclusions and Perspective

Based on the results obtained to date and the above discussion, we propose four therapeutic concepts harnessing different systemic functions of the TRAIL–TRAIL-R system in a cancer context-dependent manner (Figure 4). First, developing and using optimised multimeric variants of TRAIL or other TRAIL-R1 or TRAIL-R2 agonists in combination with potent sensitisers to TRAIL-induced apoptosis to overcome cancer cell resistance to current TRAIL-based therapeutic approaches. Second, employing FDA-approved drugs that are capable of inducing tumour cell death via engagement of the endogenous TRAIL–TRAIL-R system. Third, blocking TRAIL–TRAIL-R systemically to neutralise both its autocrine and paracrine tumour-supportive roles in *KRAS*-mutated cancers. Finally, combining any of these three concepts with immune checkpoint blockade could prove efficacious. Determining which patients will benefit most from each of these strategies and deciphering the underlying biology thereof will be the crucial feats of the future.


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## Box 1

### *Non-apoptotic cell death signalling by TRAIL*

A long-standing conundrum in cell death research was the counterintuitive finding that genetic ablation of the DISC components *Fadd* and caspase-8 (*Casp8*) in mice resulted in embryonic lethality accompanied by signs of excessive cell death in embryonic tissues[163,164]. Recently, however it was discovered that apoptotic DISC components play an integral part in suppressing a previously unrecognised form of cell death now known as regulated necrosis or necroptosis. Necroptosis depends on the kinase activities of RIPK1 and RIPK3[165-167]. Importantly, embryonic lethality in *Casp8* and *Fadd* knockout mice could be prevented by co-ablation of *Ripk3* or *Ripk1*, demonstrating that aberrant necroptosis is responsible for prenatal death of these animals[168-172]. Despite an early study identifying a

caspase 8-independent, RIPK1-dependent cell death pathway induced by CD95L, TNF and TRAIL[165], the field initially focussed on investigating this phenomenon mainly for TNF. Much later, it was found that necroptosis triggered by CD95L and TRAIL is enabled under certain circumstances, including by acidic pH[173,174] or depletion of cellular inhibitor of apoptosis (cIAP) proteins or TRAF2[175-177]. It was further shown that necrostatin, a small molecule inhibitor of RIPK1, can block this kind of cell death[178]. Recently, a wide variety of human cell lines have been found to undergo necroptosis upon combined treatment with TRAIL and chemotherapeutic drugs[179]. It shall be interesting to explore further how induction of TRAIL-induced apoptosis versus necroptosis will affect tumour immunogenicity and how this may in turn impact tumourigenesis as well as the efficacy of cancer immunotherapy.

**Box 2**

***Mechanisms causing resistance to TRAIL-induced apoptosis: a cell population-based perspective***

Cancer cells have developed various mechanisms of resistance that operate at different points along the extrinsic apoptosis pathway. For example, upregulation of FLIP blocks apoptosis at the DISC, upregulation of anti-apoptotic BCL-2 family members blocks apoptosis at the mitochondria; there are many more, and these have been extensively reviewed elsewhere[111,113,138,180] so will not be reiterated here.

More recently however, a cell population-based approach has demonstrated that cell-to-cell variability in TRAIL resistance in clonal cell populations is caused by varying levels of BCL-2 family protein expression at any one time[139]. This model suggests that emerging TRAIL resistance upon persistent TRAIL exposure might be a consequence of selection of pre-existing high BCL-2-protein-expressing cells rather than the actual upregulation of these anti-apoptotic proteins. This concept also suggests that the frequently encountered TRAIL resistance of primary cancer cells[112] might have been caused by prior exposure to selection via the extrinsic apoptosis pathway. Interestingly, inactivating caspase-8 (*CASP8*) mutations are frequently found in biopsies across various solid tumour entities suggesting that this might be a key strategy of tumours to escape extrinsic apoptosis induced by cytolytic immune infiltrates[181]. Moreover, epigenetic silencing of *CASP8* is a frequent event in small cell lung cancer (SCLC)[182] again suggesting that selective pressure via the extrinsic apoptosis pathway might be responsible for TRAIL resistance of these cells[80]. Thus, studying TRAIL resistance arising during clonal evolution within cancer cell populations will likely uncover both naturally occurring selection mechanisms within tumours as well as novel therapeutic options that might eliminate pre-existing resistant clones and, consequently, the resistance driven by them.

**Figure legends:**

Figure 1 **Human and mouse TRAIL-receptor systems. a** The human TRAIL–TRAIL-receptor (TRAIL-R) system. Humans express three receptors with an intracellular domain containing a DD, or in the case of

TRAIL-R4, a truncated DD. TRAIL-R2 is expressed as a long (L) and a short (S) isoform which differ by the presence or absence of a single TAPE domain, respectively. Moreover, TRAIL-R3 is linked to the membrane via a GPI-anchor, expresses five TAPE domains but is devoid of an intracellular domain. [OPG serves as a low-affinity soluble receptor for TRAIL. **b** The mouse TRAIL-TRAIL-R system. mTRAIL-R is homologous to human TRAIL-R1 and –R2, whereas mDcTRAIL-R1 and –R2 differ significantly and the latter is also found as a soluble form. Similar to human OPG, mOPG serves as a low-affinity soluble TRAIL receptor.

Figure 2 **TRAIL-induced signalling pathways. a** Pro-apoptotic TRAIL-signalling. Upon binding of TRAIL, TRAIL-R1 and/or TRAIL-R2 assemble to form the DISC in a receptor:FADD:pro-caspase-8 stoichiometry of approximately 3:1:9-10 [183]. At this step, FLIP can competitively bind FADD and thereby limit Caspase-8 recruitment. Caspase-8 is ubiquitinated by Cullin-3 enhancing its clustering and activation. In cells referred to as type I, DISC formation is sufficient to trigger the full caspase cascade resulting in apoptosis. In cells referred to as type II, full activation of caspase-3 which requires a maturation step following the initial caspase-8-mediated cleavage[184] is inhibited by high levels of the X-linked inhibitor of apoptosis (XIAP) protein[185]. To overcome this, caspase 8 cleaves BID[68] which in its truncated form (tBID) translocates to mitochondria and activates BAX and BAK to execute mitochondrial outer membrane permeabilisation (MOMP)[186]. MOMP results in release of a natural antagonist for XIAP, SMAC, thereby relieving effector caspases from XIAP-imposed inhibition and enabling their full activation[187]. Along with SMAC, Cytochrome C is released which enables the adaptor molecule Apoptotic protease activating factor 1 (APAF1) to assemble the apoptosome, an activation platform for the intracellular initiator caspase 9[69]. Apoptosome formation and the resulting caspase 9 activation in turn enhance caspase 3 cleavage and activity. **b** Non-canonical TRAIL signalling. TRAIL can trigger the formation of a secondary cytosolic complex retaining FADD, TRAF2 and NEMO. This complex activates NF-κB, p38, JNK and ERK. RIPK1 also associates with TRAIL-Rs when caspase 8 is inhibited and is required for TRAIL-induced SRC and STAT3 activation and promotion of migration. LUBAC is present in both, complex I and complex II in TRAIL signalling where it limits caspase 8 activation and enables recruitment of the IKK complex allowing for NF-κB activation[110].

Figure 3 **The influence of the TRAIL–TRAIL-R system on the cancer immune-environment. a** The TRAIL–TRAIL-R system can induce direct killing of tumour-supportive immune cells. TRAIL reduces the number of tumour-associated macrophages (TAMs), polymorphonuclear MDSCs (PMN-MDSCs), mononuclear MDSCs (M-MDSCs) and regulatory T cells (Tregs) by promoting their apoptosis, which in turn facilitates the activation of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells within the tumour microenvironment. The killing of tumour-supportive immune cells can also indirectly block the binding of programmed cell death protein 1 (PD1) on CTLs to PD1 ligand 1 (PDL1) and PDL2 to prevent immune checkpoint activation. Collectively these effects lead to an accumulation of CTLs in the tumour microenvironment which facilitates the restoration of an immune response against the tumour. Moreover, TRAIL-expressing NK cells can promote killing of cancer cells via TRAIL-R-mediated apoptosis. **b** The TRAIL–TRAIL-R system can promote an immune-suppressive cancer microenvironment. TRAIL-

induced stimulation of TRAIL-Rs on cancer cells induces the secretion of several cytokines that regulate the recruitment of TAMs, PMN-MDSCs, M-MDSCs and Tregs to tumours. TRAIL on Tregs can suppress CTL activation.

Figure 4 **Proposed therapeutic concepts utilising the TRAIL–TRAIL-R system**. We propose four options: drugs that sensitize cells to the pro-apoptotic activity of TRAIL-comprising therapies (e.g. the combination of optimised TRAIL-R agonists with small-molecule kinase inhibitors, such as CDK9 inhibitors) (**a**); FDA-approved drugs that activate cell death through the endogenous TRAIL–TRAIL-R system (e.g. treatment with ATRA, celecoxib, trabectedin, ONC201, thapsigargin and brefeldin-A) (**b**); systemic TRAIL or TRAIL-R blockade to antagonise cancer-promoting effects of endogenous TRAIL–TRAIL-R stimulation on cancer cells including CCL2 production and RAC1-driven migration (**c**); and combining either of these three concepts with cancer immunotherapy (e.g. with immune checkpoint inhibitors such as anti-CTLA-4 and/or anti-PD1/PDL1) (d).

Table 1: The role of the TRAIL–TRAIL-R system in tumourigenesis and metastasis in mice

| Mouse Model | TRAIL and/or TRAIL-R status of the mice | TRAIL and/or TRAIL-R status of the tumour cells | Disease outcome | Refs |
|---|---|---|---|---|
| **Transplanted B cell lymphoma (A20)** | *Trail*-deficient mice | mTRAIL-R-expressing mouse A20 B lymphoma cell line | Increased lymphoma load in the liver | [95] |
| ***Trp53*$^{+/-}$** | *Trail*-deficient mice<br>Injection of TRAIL-blocking antibodies | TRAIL-expressing and TRAIL-deficient mouse lymphoma and sarcoma cells | Increased predisposition to disseminated lymphoma and sarcoma | [96,100] |
| ***Eμ-Myc*-induced lymphoma** | *Trail-r*-deficient mice | *Trail-r*-deficient mouse lymphoma cells | Increased lymphomagenesis and metastasis formation | [102] |
| **DEN-induced hepatocarcinogenesis** | *Trail-r*-deficient mice | *Trail-r*-deficient hepatocellular carcinoma cells | Larger number of macroscopic liver nodules | [102] |
| **MCA-induced fibrosarcoma** | *Trail*-deficient mice<br>Injection of TRAIL-blocking antibodies<br>*Trail-r*-deficient mice<br>Injection of TRAIL-R agonist | TRAIL-deficient fibrosarcoma cells<br>TRAIL-R-expressing and TRAIL-R-deficient fibrosarcoma cells | Increased frequency of sarcoma<br>TRAIL treatment induced dysregulation of vascular integrity, intratumoural haemorrhage and reduced tumour growth. | [97]<br>[107] |
| **Experimental liver metastasis** | *Trail*-deficient mice<br>Injection of TRAIL- | *Trail-r*-expressing mouse renal cancer cell line | Higher susceptibility to liver metastasis | [101] |

| | | | | |
|---|---|---|---|---|
| | blocking antibodies | | | |
| **Autochthonous (DMBA/TPA)-induced squamous cell carcinoma** | *Trail-r*-deficient mice | *Trail-r*-deficient squamous cancer cells | Enhanced formation of metastasis to lymph node | [103] |
| ***KRAS*-driven genetically engineered model of pancreatic and lung cancer** | KPC* x conditional *Trail-r*-deficient mice<br><br>KP** x conditional *Trail-r*-deficient mice | *Trail-r*-deficient lung and pancreatic cancer cells | Diminished cancer formation, progression and metastasis | [86] |
| **Genetically engineered model of pancreatic ductal adenocarcinoma** | Injection of TRAIL-R agonist | *Trail-r*-expressing pancreatic cancer cells | Disruption of tumour vasculature, increased haemorrhage | [107] |

*$Kras^{G12D}$; $p53^{R172H}$; PDX-1-Cre (or KPC); **$Kras^{G12D}$;$p53^{R172H}$(or KP)

Table 2: Active TRAIL–TRAIL-R-based therapy in clinical trials

| **TRAIL-R agonistic antibodies** | **Combination** | **Cancer** | **Clinical Trials Identifier (www.clinicaltrials.gov)** |
|---|---|---|---|
| Mapatumumab (HGS-ETR1) | Sorafenib (multikinase inhibitor) | Advanced Hepatocellular Carcinoma | NCT01258608 |
| Conatumumab (AMG-655) | FOLFOX6 (chemotherapy), Ganitumab (anti-IGF-1R), Bevacizumab (anti-VEGF) | Advanced Solid Tumors | NCT01327612 |
| Tigatuzumab (CS-1008) | Abraxane | Patients With Metastatic, Triple Negative Breast Cancer | NCT01307891 |
| DS-8273a | Nivolumab (anti-PD1) | Advanced Colorectal Cancer;<br>Unresectable Stage III or Stage IV Melanoma | NCT02991196<br><br>NCT02983006 |

**Glossary:**

**Type II transmembrane Proteins**

Type II transmembrane proteins are defined by a single transmembrane domain, an N-terminus facing the cytosol and an extracellular C-terminus.

**Glycocalyx**

Most cells are covered in a dense coat of glycoproteins extending into the extracellular space refereed to as the glycocalyx.

**Extrinsic Apoptosis**

Apoptosis is a type of programmed cell death involving the activation of caspases. Extrinsic apoptosis and caspase activation is triggered by death ligands binding to cell surface death receptors.

**Apoptosome**

The apoptosome refers to a heptameric multiprotein complex, which aids caspase activation during extrinsic and intrinsic apoptosis induction following mitochondrial outer membrane permeabilisation.

**Microprocessor Complex**

The microprocessor complex is a protein complex , which mediates maturation of small regulatory RNAs termed micro RNAs (miRNAs) in the nucleus.

**Nanobody**

Nanobodies are therapeutic proteins characterised by high affinity variable domains of the heavy chain antibodies (VHH) derived from a camelid.

**Anti-drug Antibody (ADA) Response**

Anti-drug antibody (ADA) response is an adverse immune response to a therapeutic protein that can interfere with the drug pharmacokinetics, pharmacodynamics, safety and efficacy.

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**Highlighted references and summary:**

- [9,10] References 8 and 9 describe the discovery of TRAIL as an apoptosis-inducing ligand belonging to the TNF superfamily.
- [11,12] References 11 and 12 provided the first in-vivo evidence of the anti-tumour activity of TRAIL and its safe administration.
- [27] This paper reports on the discovery of the first receptor for TRAIL capable of transducing apoptosis.
- [28-34] References 28 to 34 identify and characterise TRAIL-R2. This receptor later turned out to be the TRAIL-R with the highest affinity for TRAIL.
- [33-36] References 33 to 36 report on the discovery of TRAIL-R3, a TRAIL-R without an intracellular domain.
- [37,38] In these papers TRAIL-R4 is identified as another receptor for TRAIL, which cannot induce apoptosis as it only contains a truncated death domain.

- [39] This paper identifies OPG, a soluble receptor for RANKL, as the last of the human TRAIL-Rs discovered.
- [21,49] References 21 and 49 provided the first evidence of a strong and surprising synergy between an agonistic TRAIL-R2-specific antibody (AMG-655) and TRAIL in killing cancer cells.
- [57] In this paper, a mouse homologue of human TRAIL-R1 and -R2 that induces apoptosis is identified.
- [58] Here, murine TRAIL-receptors lacking an intracellular domain are discovered.
- [59] This study documents species cross reactivity of murine and human TNF-superfamily ligands. These data are of high importance when planning in vivo xenograft experiments.
- [60,61] References 60 and 61 report on the seminal discovery of a death-inducing signalling complex and its components in TRAIL-induced apoptosis.
- [77] This study was the first to describe a secondary cytosolic complex formed after TRAIL stimulation. It also showed that this complex initiates gene-activating signalling cascades resulting in cytokine production.
- [81] This study demonstrated that TRAIL-R2 can translocate to the nucleus upon activation where it inhibits maturation of let-7 and promotes proliferation in pancreatic cancer cells.
- [85] Here, the authors demonstrated that oncogenic KRAS prevents apoptosis induction by TRAIL and instead favours a pro-migration signal in colorectal cancer cells. These data highlighted for the first time potential adverse effects of treatment with TRAIL-R agonists in patients with *KRAS*-mutated cancers.
- [86] This study was the first to provide genetic proof that constitutive endogenous TRAIL–TRAIL-R2 stimulation promotes *KRAS*-mutated pancreatic and lung cancers.
- [96-99] These studies were the first to describe a role for TRAIL in immune surveillance against primary tumours and metastasis.
- [105] This study shows that survival of MDSCs in the tumour microenvironment is limited by TRAIL-R2-mediated apoptosis. Thereby, this study indirectly demonstrates that tumour growth and metastasis data obtained from *Trail-r* knockout mice might be influenced by the presence of a more stable population of MDSCs in the tumour microenvironment.
- [108]This study shows that endogenous TRAIL-R signalling in cancer cells induces the production of a FADD- and caspase-8-dependent secretome which promotes the presence of tumour-supportive type 2 myeloid cells in the cancer microenvironment via host cell-expressed CCR2.

- [109]This study shows that TRAIL-induced cytokine production depends on FADD and the scaffold function of Caspase-8, coining the term “FADDosome” for the secondary complex responsible for this signalling output.
- [130] This phase I clinical study reports that acute toxicity of the novel TRAIL-R2-targeting nanobody TAS266 was caused by immunogenicity of its camel nanobody-derived backbone.
- [162] This early study provided first in-vivo evidence of a cancer-promoting adverse effect of therapeutic TRAIL treatment.
- [187] This study provides genetic proof that XIAP is decisive in discriminating between type I and type II extrinsic apoptosis.

**Online only information**

Henning Walczak chairs the Department of Cancer Biology at the UCL Cancer Institute (London, UK) where he heads the Centre for Cell Death, Inflammation and Cancer. The research of his group focusses on two main topics: the biology of death receptor systems, including the TRAIL–TRAIL-R system, in cancer and the role of linear ubiquitin in balancing gene activation, cell death and inflammation. After working on the CD95 system during his PhD at the German Cancer Research Centre (DKFZ) in Heidelberg (Germany), he joined Immunex Corporation in Seattle (WA, USA) where he embarked on his work on TRAIL. Returning to the DKFZ as a junior group leader, he started his work on linear ubiquitin before moving to Imperial College London as Chair of Tumour Immunology from where he assumed his current position.

Silvia von Karstedt is currently working as a postdoctoral researcher at the Francis Crick Institute (London, UK). She previously worked in the laboratory of Henning Walczak, where she investigated the role of cancer cell-expressed TRAIL-Rs in KRAS-driven cancer models. Her current research interests focus on the interaction of KRAS-mutated tumours with the immune system.

Antonella Montinaro received her PhD from the University of Salerno, Italy. She then joined the laboratory of Henning Walczak UCL as a postdoctoral researcher. She investigates the therapeutic potential of novel, highly active TRAIL-comprising combination therapies for lung cancer. She is particularly interested in examining the effects of these therapies on both, cancer cells and the tumour microenvironment.

**Key points summary**

- Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) is a molecule belonging to the TNF superfamily. In humans, two TRAIL-receptors (TRAIL-R1 and TRAIL-R2) and in mice one receptor (mTRAIL-R) can signal apoptosis upon binding of TRAIL.
- Induction of apoptosis is enabled through recruitment of the death-inducing signalling complex (DISC). This complex leads to activation of a caspase cascade.
- TRAIL–TRAIL-R binding can also induce non-apoptotic signalling including via activation of NF-κB, p38, ERK, SRC and RAC1.
- Mice deficient in TRAIL or mTRAIL-R are more susceptible to various cancers whereas conditional deletion of mTRAIL-R in KRAS-driven cancers ameliorates disease progression. TRAIL–TRAIL-R blockade might therefore be a therapeutic option for patients with KRAS-mutated cancers.
- TRAIL–TRAIL-R interaction on myeloid-derived suppressor cells in the tumour microenvironment can limit their lifespan and thereby tip the balance towards an anti-tumour immune environment. Hence, patients with an immunosuppressive cancer microenvironment might benefit from TRAIL-R agonistic therapy.

- Clinical trials testing an untagged form of recombinant TRAIL, agonistic TRAIL-R1 or –R2-specific antibodies or antibody derivatives with higher crosslinking capacity have not led to anticipated therapeutic benefit.
- Pre-clinical approaches utilising therapeutic combinations comprising optimised TRAIL-R agonists and recently discovered powerful apoptosis sensitisers are, however, promising. Therefore, such novel pro-apoptotic combination therapies should be tested in clinical trials.
- Several drugs, some of which are FDA-approved, induce extrinsic apoptosis through autocrine engagement of the TRAIL–TRAIL-R pathway. Therefore, expression of TRAIL apoptosis pathway components, as well as lack of expression of inhibitors thereof, might be useful for patient selection in clinical trials testing the efficacy of such drugs.

**Competing interests statement**

H.W. is a scientific advisor, co-founder, and shareholder of Apogenix AG. The other authors declare no competing interests.

**Subject categories**

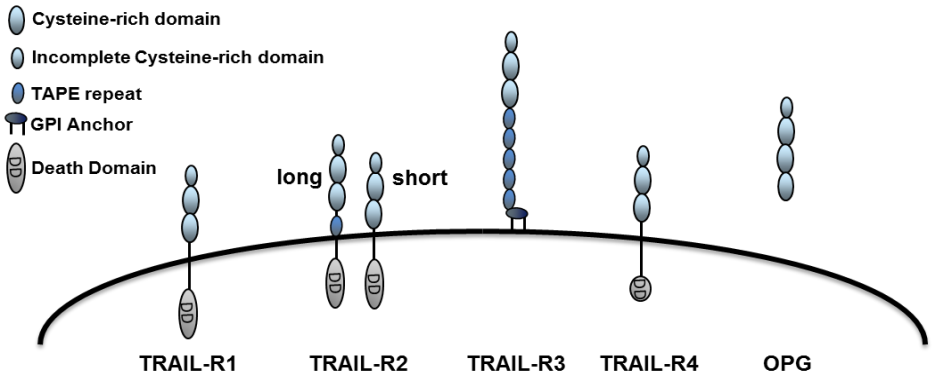
Biological sciences / Cell biology / Cell death / Apoptosis
[URI /631/80/82/23]
Biological sciences / Cancer / Tumour immunology
[URI /631/67/580]
Biological sciences / Cancer / Cancer therapy / Targeted therapies
[URI /631/67/1059/602]
Biological sciences / Cancer / Cancer therapy / Cancer therapeutic resistance
[URI /631/67/1059/2326]
Biological sciences / Cancer / Tumour biomarkers
[URI /631/67/1857]

**Table of contents summary**

Clinical trials testing activators of tumour necrosis factor-related apoptosis-inducing ligand-receptor (TRAIL-R) signalling in cancer have not met expectations. This Review discusses new insights that might explain clinical failure, but also provide the basis for harnessing TRAIL-Rs for cancer therapy.

# FIGURE 1

**a**


Cysteine-rich domain
Incomplete Cysteine-rich domain
TAPE repeat
GPI Anchor
Death Domain
DD
long
short
TRAIL-R1
TRAIL-R2
TRAIL-R3
TRAIL-R4
OPG

**b**

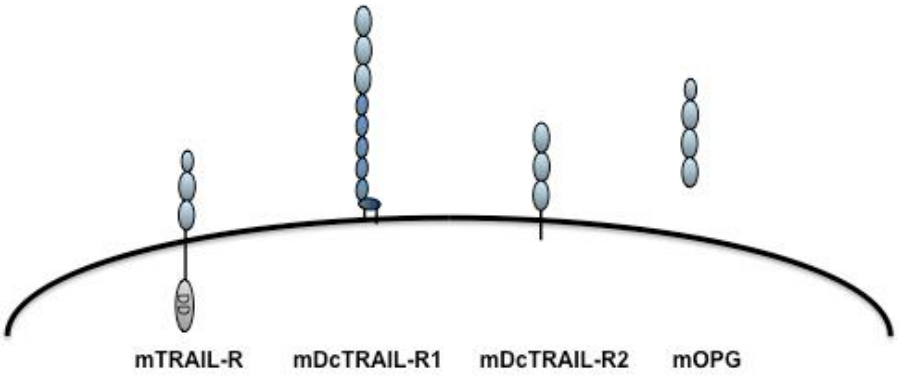
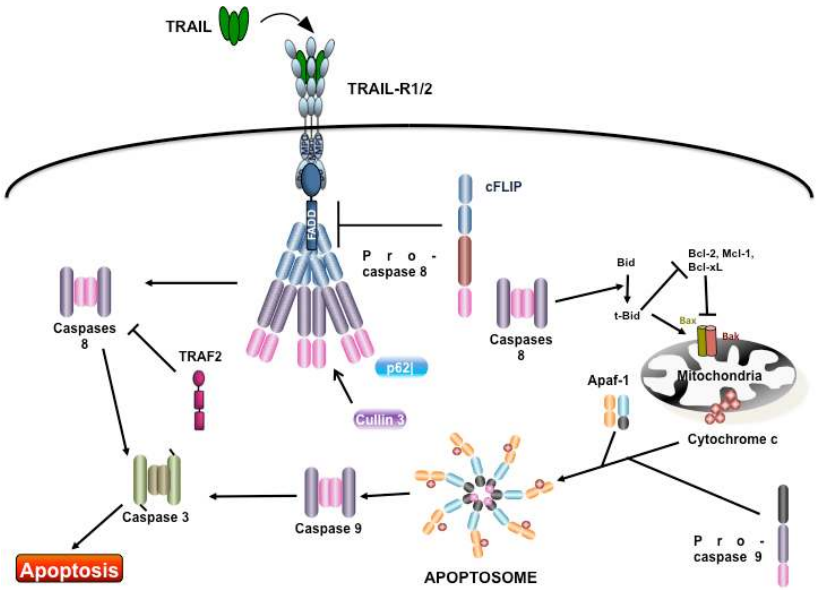
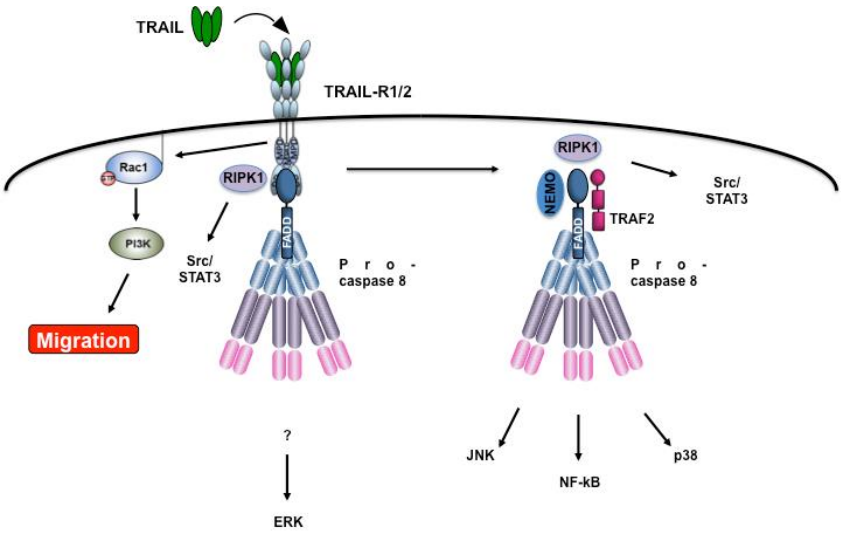

DD
mTRAIL-R
mDcTRAIL-R1
mDcTRAIL-R2
mOPG

**FIGURE 2**

**a**


TRAIL
TRAIL-R1/2
cFLIP
FADD
Pro-caspase 8
Caspases 8
TRAF2
p62
Cullin 3
Caspases 8
Bid
t-Bid
Bcl-2, Mcl-1, Bcl-xL
Bax
Bak
Mitochondria
Apaf-1
Cytochrome c
Caspase 3
Caspase 9
Apoptosis
APOPTOSOME
Pro-caspase 9

**b**


TRAIL
TRAIL-R1/2
Rac1
PI3K
Migration
RIPK1
Src/ STAT3
FADD
Pro-caspase 8
?
ERK
RIPK1
NEMO
TRAF2
FADD
Src/ STAT3
Pro-caspase 8
JNK
NF-kB
p38

# FIGURE 3

**a. Anti-tumourigenic role of the TRAIL–TRAIL-R system**

**b. Pro-tumourigenic role of the TRAIL–TRAIL-R system**

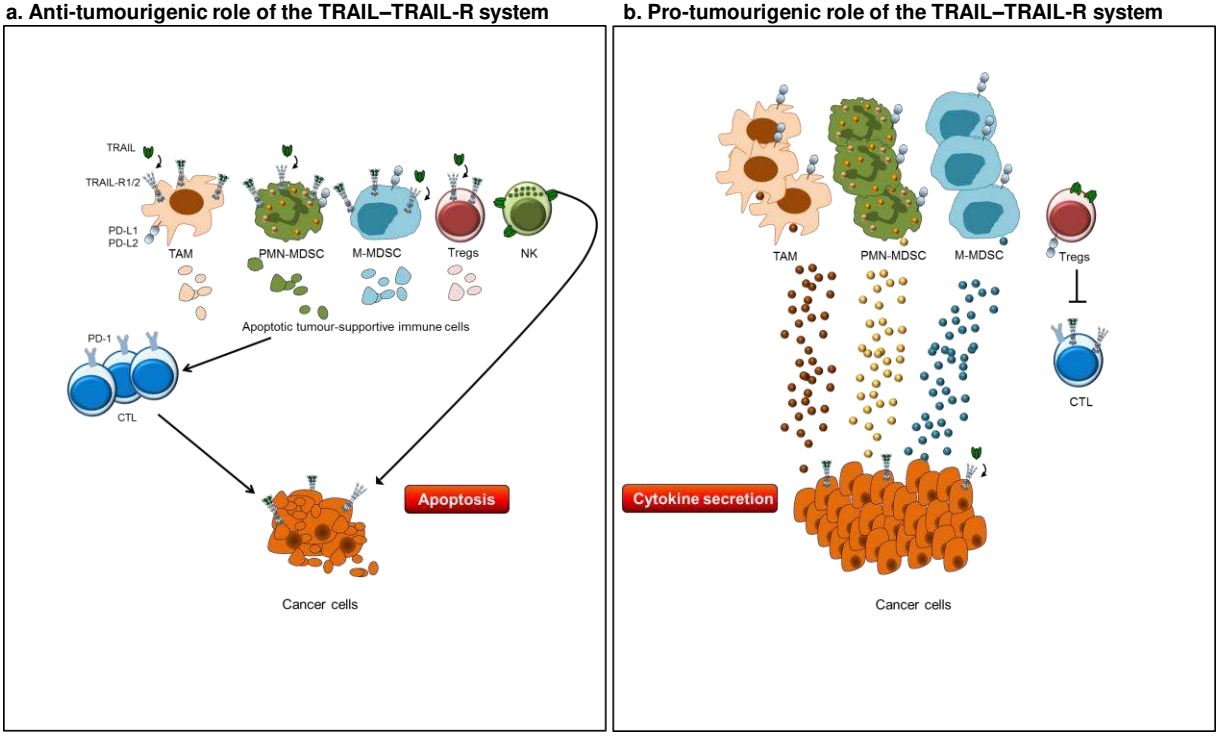
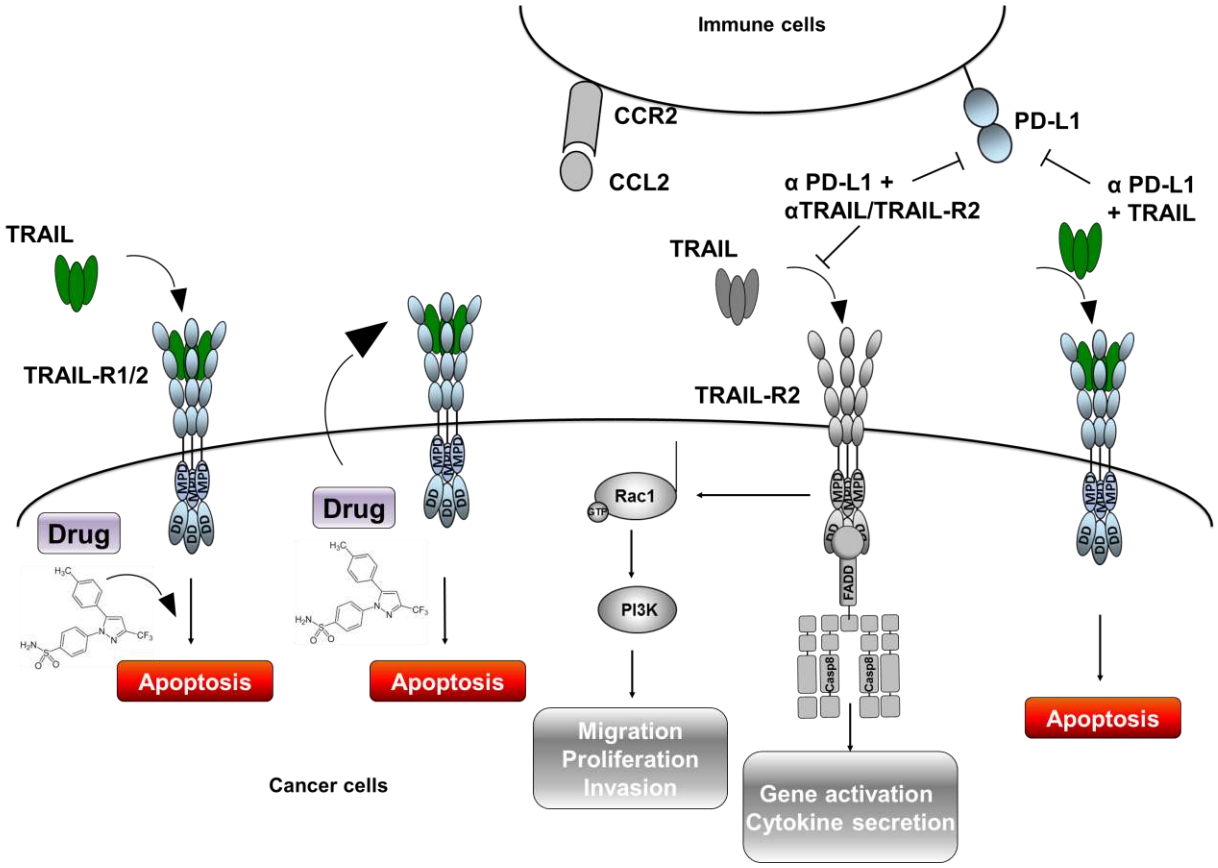


**FIGURE 4**


Immune cells
CCR2
CCL2
PD-L1
α PD-L1 +
αTRAIL/TRAIL-R2
α PD-L1
+ TRAIL
TRAIL
TRAIL-R1/2
TRAIL
TRAIL-R2
Drug
Drug
Apoptosis
Apoptosis
Rac1
PI3K
FADD
Casp8
Casp8
Migration
Proliferation
Invasion
Gene activation
Cytokine secretion
Apoptosis
Cancer cells