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Review

Non-canonical kinase signaling by the death ligand TRAIL in cancer cells: discord in the death receptor family

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-based therapy is currently evaluated in clinical studies as a tumor cell selective pro-apoptotic approach. However, besides activating canonical caspase-dependent apoptosis by binding to TRAIL-specific death receptors, the TRAIL ligand can activate non-canonical cell survival or proliferation pathways in resistant tumor cells through the same death receptors, which is counterproductive for therapy. Even more, recent studies indicate metastases-promoting activity of TRAIL. In this review, the remarkable dichotomy in TRAIL signaling is highlighted. An overview of the currently known mechanisms involved in non-canonical TRAIL signaling and the subsequent activation of various kinases is provided. These kinases include RIP1, IκB/ NF-κB, MAPK p38, JNK, ERK1/2, MAP3K TAK1, PKC, PI3K/Akt and Src. The functional consequences of their activation, often being stimulation of tumor cell survival and in some cases enhancement of their invasive behavior, are discussed. Interestingly, the non-canonical responses triggered by TRAIL in resistant tumor cells resemble that of TRAIL-induced signals in non-transformed cells. Better knowledge of the mechanism underlying the dichotomy in TRAIL receptor signaling may provide markers for selecting patients who will likely benefit from TRAIL-based therapy and could provide a rationalized basis for combination therapies with TRAIL death receptor-targeting drugs.
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Facts

- Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors are attractive targets for anticancer therapy due to their selective ability to mediate caspase-dependent apoptosis in tumor cells upon ligand binding without harming healthy tissues.
- Different TRAIL receptor-targeted agents have been developed showing promising antitumor activity in preclinical models, and several receptor agonists are being evaluated for activity in clinical studies.
- Resistance to the apoptosis-inducing effect of TRAIL receptor agonists is frequently encountered in tumor cells and can often be bypassed by combined treatments with radiotherapy and/or chemotherapy.
- Contrasting its apoptotic activity, in TRAIL-resistant tumor cells as well as in normal non-transformed tissue cells, TRAIL can activate non-apoptotic (non-canonical) signals, resulting in the activation of various kinases that can enhance the proliferation, survival, migration/invasion and angiogenic properties in a cell type-dependent manner.

Open Questions

- What is/are the molecular mechanism(s) underlying the dichotomy in TRAIL signaling in sensitive *versus* resistant tumor cells?
- What is the molecular basis of the apparent differences between TRAIL-R1- and TRAIL-R2-dependent signaling?
- Which factors produced by the tumor microenvironment determine the outcome of TRAIL signaling and through what mechanism(s)?

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Abbreviations: ADAM, a disintegrin and metalloproteinase; c-Cbl, Casitas B-lineage lymphoma; cFLIP, cellular flice-like inhibitory protein; DISC, death-inducing signaling complex; ECs, endothelial cells; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular regulated kinases; FADD, Fas-associated protein with death domain; HER, human epidermal receptor; IGF1R, insulin-like growth factor type 1 receptor; JNK, Jun NH_2 terminal kinases; MAPK, Mitogen-activated protein kinases; MMP, Matrix metalloproteinase; Mst1, mammalian sterile 20-like kinase 1; mTOR, mammalian target of rapamycin; NEMO, NF-κB essential modulator; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; OPG, osteoprotegerin; PI3K, Phosphatidylinositide 3-kinases; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTEN, phosphatase and tensin homolog deleted on chromosome ten; RIP1, receptor-interacting protein kinase 1; RIP3, receptor-interacting protein kinase 3; ROCK, Rho kinase; ROS, reactive oxygen species; SEK1, stress-activated protein/ERK kinase 1; SFK, Src family kinases; SMAC, second mitochondria-derived activator of caspase; Src, Rous sarcoma oncogene cellular homolog; TAK1, transforming growth factor-β (TGF-β)-activated kinase 1; TGF-β, transforming growth factor-β; TRADD, TNF-receptor-associated death domain protein; TRAF2, TNF receptor-associated factor 2; TRAIL, Tumor necrosis factor-related apoptosis-inducing ligand; uPA, urokinase-type plasminogen activator; VSMCs, vascular smooth muscle cells; XIAP, X-linked inhibitor of apoptosis protein.

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- Can possible identified mechanism(s)/proteins that function as an apoptotic switch in the TRAIL pathway be used as a target for developing therapeutic strategies for sensitizing tumor cells?
- Can TRAIL apoptosis sensitivity in tumor cells be predicted by not yet identified biomarkers, allowing the preselection of patients eligible for TRAIL receptor agonistic therapy?

The death ligand TRAIL induces apoptosis in a wide variety of tumors without harming normal cells.[1–3] Moreover, its killing effect is irrespective of the proliferation status or tumor suppressor p53 status of cancer cells. These properties make TRAIL receptor-targeted therapy a very attractive anticancer approach.

The mechanisms by which TRAIL induces programmed cell death or apoptosis in cancer have been intensively investigated. TRAIL activates apoptosis via two membrane receptors, designated TRAIL-R1 (DR4) and TRAIL-R2 (DR5), whereas TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and circulating osteoprotegerin (OPG) have been proposed to function as decoy receptors that sequester TRAIL, causing the suppression of apoptosis.[4,5] However, TRAIL-R4 was found to be co-recruited with TRAIL-R2 to the death-inducing signaling complex (DISC) were it prevented initiation of caspase activation, which can be considered as an alternative regulatory mechanism of TRAIL signaling by these receptors.[6] Overall, the more precise role and function of these decoy receptors in TRAIL resistance is currently not fully understood. A number of TRAIL receptor-targeting agents have been developed, including preparations of recombinant human soluble TRAIL (rhTRAIL) and derived variants, and agonistic monoclonal antibodies selective for either TRAIL-R1 or TRAIL-R2.[1,7–10] High affinity and selective binding of either TRAIL-R1 or TRAIL-R2 by these receptor-selective agents together with reduced binding to decoy receptors is expected to enhance antitumor activity. However, currently it is unclear whether it will be more beneficial to target either TRAIL-R1 or TRAIL-R2 for optimal treatment, which may also vary in a tumor cell-specific way. For example, TRAIL signals its cell death function through TRAIL-R1 in pancreatic cancer cells[11] and chronic lymphocytic leukemia,[12] whereas in glioblastoma,[13] colon and breast cancer cell lines,[14] apoptosis induced by TRAIL goes via TRAIL-R2.

TRAIL apoptotic signaling is initiated following ligand binding to TRAIL receptors and subsequent recruitment of the adapter protein Fas-associated protein with death domain (FADD) and the cystein-aspartic protease procaspase-8, leading to the formation of a complex named the DISC, which promotes caspase-8 activation and further downstream caspase-3 activity, ultimately leading to cell death[1,4,5,15] (see Figure 1). Cellular flice-like inhibitory protein (cFLIP), a non-functional procaspase-8 homolog, can compete with procaspase-8 for FADD binding leading to apoptosis suppression. Full activation of this so-called extrinsic apoptosis pathway often requires the cross-activation of intrinsic or mitochondrial apoptosis that is mediated by caspase-8-dependent cleavage of pro-apoptotic Bcl-2 family member Bid and subsequent mitochondrial disruption.[16] Cells in which TRAIL-induced apoptosis depends on activation of the mitochondrial pathway have been named type II cells, contrasting type I cells where caspase-8 activation is sufficient to directly activate effector caspases and apoptosis.[16] The inhibitor of apoptosis protein (IAPs) family comprises proteins that can bind and inactivate caspases. For example, X-linked IAP (XIAP) inhibits caspases-3 and -9, and its anti-apoptotic activity is neutralized by the release of second mitochondria-derived activator of caspase (SMAC) from mitochondria.[17] More recently, death receptors have been discovered to trigger another way to die, named necroptosis. This caspase-independent form of regulated necrotic cell death has been mostly studied in TNF receptor signaling, and appears important for the regulation of immunity and inflammation.[18] TNF-induced necroptosis depends on the activation of receptor-interacting protein 1 (RIP1; also known as RIPK1), and RIP3 (also known as RIPK3), in a complex consisting of TRADD (TNF-receptor-associated death domain protein), FADD and caspase-8. Recently, TRAIL was found to activate necroptosis in tumor cells under acidic extracellular pH conditions, involving RIP1 and RIP3.[19] Whether TRAIL can also induce the formation of a similar, so-called 'necroptosome' complex, consisting of, among others, RIP1/RIP3, remains to be shown.

In preclinical studies, approximately half of the tumor cells show resistance towards TRAIL-induced apoptosis, but combined treatment with various standard or experimental agents can re-sensitize these cells (reviewed in[20,21]). Intrinsic TRAIL resistance in tumor cells involves blockades at different levels in the pathway, such as high levels of decoy receptors, limitations in DISC formation due to cFLIP-mediated inhibition, post-translational modifications of DISC proteins and high expression of anti-apoptotic Bcl-2 proteins that inhibit mitochondrial apoptosis. Combined therapy with standard chemo- and radiotherapy as well as a number of targeted agents can overcome these apoptotic blockades. Commonly, these agents act by upregulation of TRAIL receptors and downregulation of anti-apoptotic proteins, resulting in enhanced DISC formation and mitochondria-dependent apoptosis.[21,22]

The promising antitumor activity in preclinical models has spurred early clinical studies with TRAIL receptor agonists in various tumor types.[23–26] However, as described below, recent preclinical findings indicate pro-survival, proliferation and even metastatic activity of TRAIL, suggesting more caution when applying these TRAIL receptor agonists, particularly as single agents. In this review, we will give an update on the non-canonical (non-apoptotic) signaling activity of the TRAIL pathway in tumor and non-transformed cells. The different kinase cascades involved are described and the consequences for TRAIL-based therapy are discussed.

TRAIL Receptor Signaling Complexes and Kinase Activation

The early molecular events leading to non-canonical TRAIL signaling are complex and not well-understood. Co-immunoprecipitation experiments have indicated the formation of a so-called secondary signaling complex subsequent to the assembly of the primary DISC.[27] This secondary complex was found to contain RIP1, TNF receptor-associated factor 2

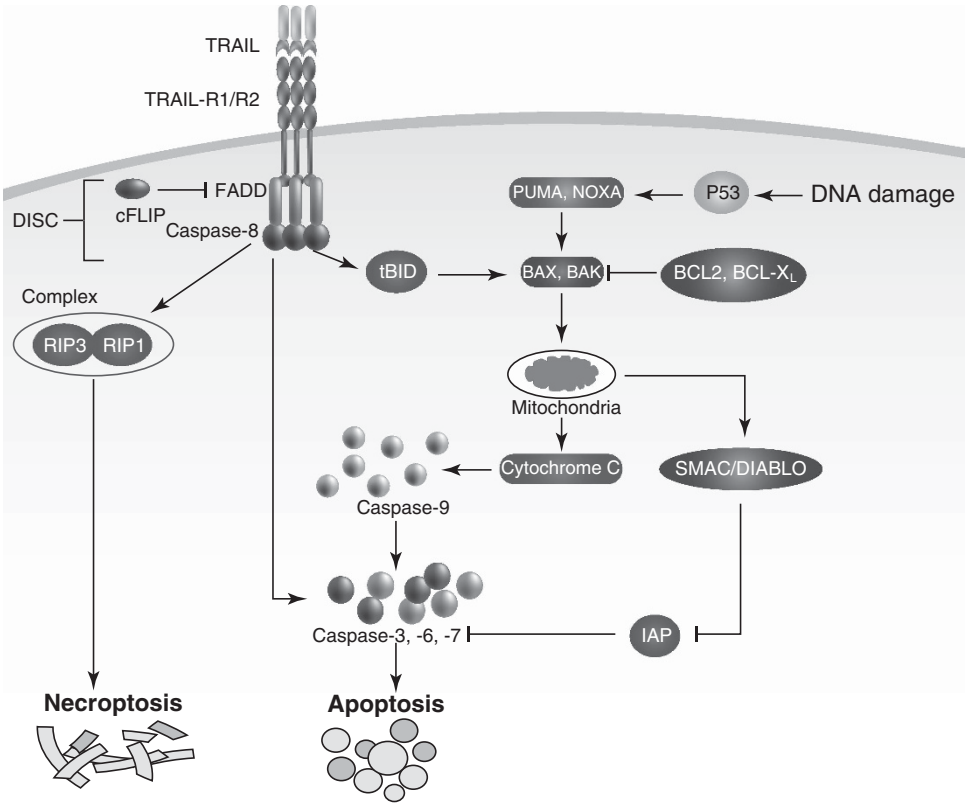

Figure 1 The TRAIL apoptotic pathway. Simplified and schematic representation of the pathway. Binding of TRAIL or generated agonistic agents to the TRAIL receptors results in the recruitment of FADD and procaspase-8, also known as the DISC. Subsequently, caspase-8 cleavage in the DISC can activate downstream caspase-3, leading to the induction of apoptosis (extrinsic pathway). The caspase-8 analog cFLIP can compete for FADD binding and inhibit DISC formation. Caspase-8 can also cleave the Bcl-2 family member Bid into tBid engaging the intrinsic pathway by binding to Bax, causing mitochondrial membrane permeabilization and the release of apoptogenic factors such as cytochrome *c*. Pro-survival Bcl-2 family members can prevent mitochondrial permeabilization. IAPs, such as XIAP, can bind to caspases and inhibit apoptosis. Necroptosis, a regulated form of caspase-independent necrotic cell death, can also be activated through TRAIL receptors under specific conditions. This involves the activation of RIP1 and RIP3 in a complex that has not been precisely clarified as yet. See text for more details

(TRAF2) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) essential modulator (NEMO)/IKKγ, in addition to FADD and active caspase-8. In this context, the localization of the TRAIL receptors in the cell membrane also appears to have a role in complex formation. In particular, TRAIL receptor localization in lipid rafts enables apoptosis activation.[28] Lipid rafts are plasma membrane domains enriched in cholesterol and glycolsphingolipids, and function as signaling platforms that serve colocalization of requisite components. In the same study, TRAIL receptors' complex assembly outside the rafts was associated with non-canonical signaling, mediated by RIP1 and cFLIP.[28] Aggregation of the TRAIL-R1 and -R2 in lipid rafts also occurred only in TRAIL-sensitive non-small lung cancer (NSCLC) H460 cells and not in the resistant A549 cells.[29] Whether lipid raft localization of TRAIL receptors is a consequence or a cause of DISC formation remains to be proven.

Additional layers of regulation are provided by the expression of DISC inhibitors, such as cFLIP, and the phosphorylation and/or ubiquitination of several TRAIL receptor-interacting proteins, including caspase-8 and RIP1, which has been more extensively reviewed elsewhere.[15,30,31] Regardless of the more precise composition and regulation of the complexes involved, non-canonical TRAIL receptor-induced kinase activation has been reported in various cell- and tumor types. These cascades were demonstrated to result in pro-inflammatory, proliferative, survival and migratory responses, and are in more detail discussed below.

IκB/NF-κB Survival Signaling

Activation of the inflammatory and cell survival pathways controlled by the transcription factor NF-κB was one of the first reported non-canonical signals elicited by TRAIL (see Figure 2 and Table 1). Both TRAIL-R1 and -R2 were found to activate NF-κB in a TRADD- and RIP1-dependent way.[32–34] NEMO (IKK-γ) was found to be part of the secondary complex, able to recruit IKKα/β to the signaling complex causing the phosphorylation and subsequent proteasomal degradation of the inhibitor of kβ (IκB), leading to release and accumulation of NF-κB.[27] NF-κB then translocates to the nucleus where it can activate the transcription of, among others, the anti-apoptotic genes *cFLIP*, *Bcl-x$_L$*, *Mcl-1* and *cIAP*s.[35–38] In preclinical tumor models such as (primary) leukemia,[39] neuroblastoma,[40] pancreatic cancer,[41] mantle cell lymphoma cells[42] and NSCLC,[43] inhibition by over-expression of an IκB dominant-negative version or by selective chemical inhibitors enhanced TRAIL apoptosis.

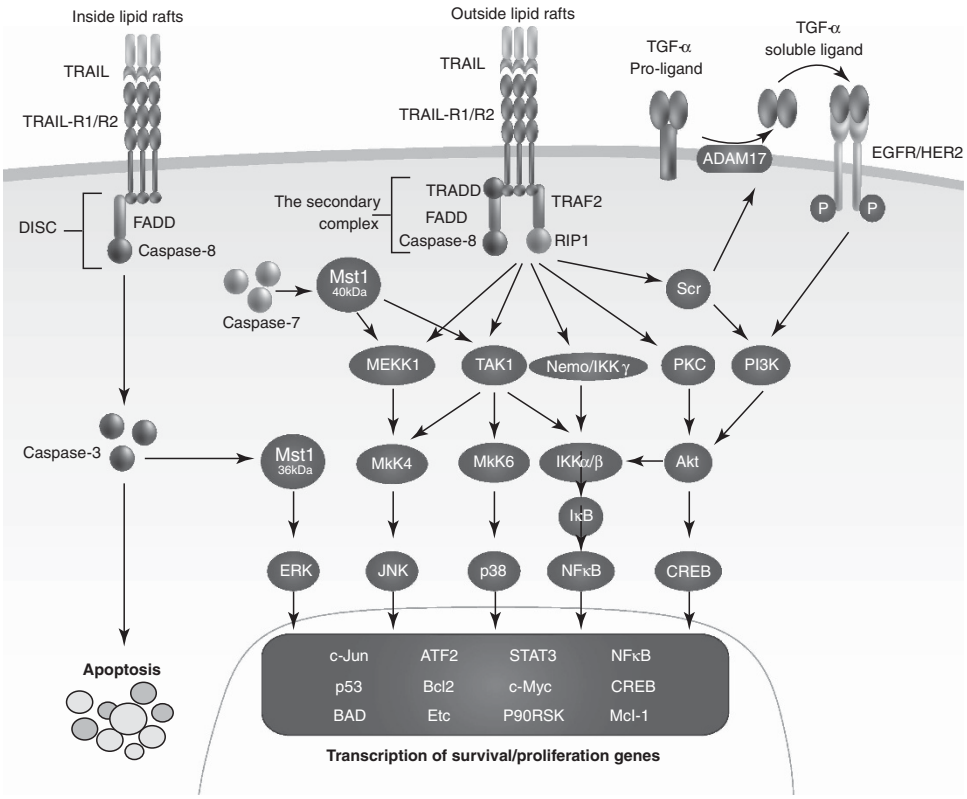

Figure 2 Canonical and non-canonical TRAIL signaling in cancer cells. Schematic overview of apoptotic and proliferation/pro-survival signals elicited by the activation of TRAIL receptors. Following binding of TRAIL receptor agonists to their death receptors, the DISC can be formed, resulting in apoptosis. A secondary complex can also be formed after TRAIL receptor activation, leading to the activation of various kinases and the induction of direct or indirect non-apoptotic responses as indicated. The more precise molecular events required for secondary complex formation are subject of investigations. The complex could be associated with TRAIL receptors at the membrane or formed in the cytoplasm. See text for more details

Interestingly, NF-κB can have dual activity, as it was found to mediate the upregulation of TRAIL receptors on one hand, and enhance Bcl-x_L expression and apoptosis resistance on the other.[38] More recently, a pro-apoptotic role for NF-κB was demonstrated in glioma cell lines, where inhibition of the NF-κB pathway reduced TRAIL-induced apoptosis via an as yet elusive mechanism.[44]

Mitogen-Activated Protein Kinases (MAPKs) in TRAIL Signaling

MAPKs are enzymes that control important physiological processes, such as gene expression, motility, metabolism, mitosis and programmed cell death. In mammals, six distinct groups of MAPKs have been characterized, extracellular regulated kinases (ERK1/2), Jun NH2 terminal kinases (JNK1/2/3), p38 (p38 $\alpha/\beta/\gamma/\delta$), ERK7/8, ERK3/4 and ERK5.[45] TRAIL can activate c-Jun N-terminal kinase (JNK), p38 and ERK1/2 in several cancer cell lines with dual effects, but mostly contributing to cell proliferation and pro-survival signaling, as outlined further below.

c-Jun N-terminal kinases. The JNKs are stress-activated members of the MAP kinase family that can be activated by TRAIL via both caspase-dependent and -independent mechanisms in a cell type-specific way.[46] FADD appeared to be dispensable for JNK activation. In lymphoid cells, JNK pathway activation by TRAIL contributed to apoptosis activation.[47] JNK activation involved a TRAF2-MEKK1-MKK4-dependent signaling pathway in human embryonic kidney 293 cells[48] and required RIP1 in prostate cancer cells.[49] Both TRAF2 and RIP1 were detected in the secondary complex and were required for JNK activation in fibrosarcoma cells.[27] JNK activation results in phosphorylation of its well-known target, the transcription factor c-Jun/ AP1, but can also directly phosphorylate the pro-apoptotic Bcl-2 family member Bim in hepatocytes, thereby stabilizing the protein and facilitating mitochondrial apoptosis.[50] A role for JNK and Bim has also been found in the activation of a lysosmal death pathway induced by TRAIL in cholangiocarcinoma cells.[51] Another cell death route was also connected to TRAIL receptor-dependent activation of JNK; JNK appeared to phosphorylate a key autophagy regulator, Beclin-1, leading to autophagic cell death in HCT116 cells.[52] Synergistic apoptosis activation by chemotherapy and TRAIL receptor agonists was shown to involve MKK4-dependent JNK activation. Mitogen-activated protein kinase (MAPK) p38 was also activated and suppression of the activation of these kinases by the antioxidant N-acetyl cysteine prevented synergistic effects.[53] However, in contrast, direct inhibition of JNK by RNA interference or chemical inhibition augmented TRAIL-induced apoptosis in

Table 1 Summary of the kinases activated by TRAIL in different tumor types

Kinase	Tumor type	Response	Reference
IκB/NF-κB	Leukemia, neuroblastoma, pancreatic, mantle cell lymphoma, NSCLC	Pro-survival	39–43
	Glioma	Pro-apoptotic	38
JNK	Lymphoid, cholangiocarcinoma	Pro-apoptotic	47,51
	Hepatocellular carcinoma	Pro-survival	54
Short JNK1	Colon	Pro-survival	56
Long JNK1	Colon	Pro-apoptotic	56
p38	Ovarian	Pro-apoptotic	57
	Prostate, breast	Pro-survival	59,60
ERK	Colon, SCLC, melanoma, glioma	Proliferative	62–65
TAK1	Prostate, ovarian	Pro-survival	59,68
PKC	Pancreatic	Pro-survival	70
δ, ε, η	Melanoma, breast	Pro-survival	71,72
α, β, γ	NSCLC	Pro-apoptotic	75
PI3K/Akt	Leukemia, ovarian, breast, NSCLC, prostate	Pro-survival	77,81–83
Src	Prostate, NSCLC, hepatic, breast	Migratory/invasive and pro-survival	80,82,86,87

Abbreviations: ERK, extracellular regulated kinases; IκB, inhibitor of kβ; JNK, c-Jun NH_2 terminal kinases; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NSCLC, non-small cell lung cancer; PI3K, Phosphatidylinositide 3-kinases; PKC, protein kinase; SCLC, small cell lung cancer; Src, Rous sarcoma oncogene cellular homolog; TAK1, transforming growth factor-β (TGF-β)-activated kinase 1
The functional consequences of activation being either pro-apoptotic, pro-survival, proliferative or migratory are indicated.

hepatocellular carcinoma cells.[54] This indicates that JNK can have opposite effects in TRAIL signaling that may be dependent on the cellular context. These dual functions can in part be due to the duration or magnitude of the activation of the pathway. For example, it was found that prolonged activation of JNK by TNF induces apoptosis, whereas transient activation of JNK promotes cell survival.[55] Furthermore, a recent study has shown that in colon cancer cell lines, the short JNK1 isoforms (JNK1α1 and JNK1β1) transmit an anti-apoptotic signal, whereas the long isoforms (JNK1α2 and JNK1β2) are pro-apoptotic upon activation of TRAIL.[56] This may also provide an explanation for the dual role of JNK.

P38 MAPK. Several reports have shown that TRAIL can activate p38 MAPK. P38 activation can occur through the formation of the secondary complex consisting of FADD, caspase-8, RIP1 and TRAF2.[27] Lee *et al.*[57] found TRAIL-induced p38 activation in HeLa cells to be responsible for caspase activation and apoptosis. Elevation of reactive oxygen species (ROS) by TRAIL appeared instrumental for p38 activation. P38 activation by TRAIL was also observed in DLD1 colon cancer cells, however, in these cells inhibition of p38 did not affect TRAIL-mediated cell death.[58] In yet other tumor cells, p38 was found to suppress apoptosis. In prostate cancer cells, TRAIL induced p38 phosphorylation causing the transcriptional upregulation of anti-apoptotic Bcl-2 family member Mcl-1, thus rescuing the prostate cancer cells from apoptosis. Inhibition of p38 with chemical inhibitor, SB203580, increased the level of cell death by TRAIL in these cells.[59] Breast carcinoma cells could also be sensitized for TRAIL after inhibition of p38, indicating that this kinase contributes to cell survival in these cells.[60] Thus, p38 can either suppress or enhance the apoptotic effect of TRAIL in a cell type-specific way.

ERKs. The activation of ERKs by TRAIL has also been reported in a number of reports. In neuroblastoma cells, TRAIL-induced ERK1/2 phosphorylation[61] and the inhibition of ERK1/2 could enhance TRAIL-dependent death in colon cancer cells.[62] Even more, in small cell lung cancer (SCLC) cells lacking caspase-8, TRAIL caused cell proliferation in some but not all SCLC cell lines tested and was mediated by TRAIL-R2.[63] TRAIL-induced proliferation could be prevented by chemical inhibition or siRNA-mediated knockdown of ERK1/2, identifying this pathway as a critical proliferation mediator.[63] Also in a panel of human melanoma cell lines with variable sensitivities for TRAIL, ERK1/2 phosphorylation was detected within 30 min of TRAIL treatment.[64] ERK1/2 inhibition resulted in downregulation of Bcl-2, Bcl-X_L and Mcl-1 expression, providing an explanation for enhanced TRAIL-induced mitochondrial apoptosis in these cells. TRAIL-resistant human glioma cells also demonstrated enhanced cell proliferation upon TRAIL treatment that could be linked to ERK1/2 activation.[65] ERK inhibition suppressed stimulation of proliferation but did not sensitize for TRAIL. Knockdown of cFLIP on the other hand prevented ERK activation and resulted in partial sensitization for TRAIL-dependent apoptosis.[65] Thus, the activation of ERK by TRAIL generally has been implicated in stimulation of cell survival and proliferation of tumor cells.

As mentioned, the secondary complex and active caspase-8 have been implicated in MAPK activation.[27] However, an alternative caspase-8-requiring mechanism was reported by Song and Lee[66] in DU-145 prostate cancer cells. They found that mammalian sterile 20-like kinase 1 (Mst1), a ubiquitously expressed serine/threonine kinase and caspase substrate, could activate p38 and JNK through a caspase-7 cleavage-generated 40 kDa form of Mst1. A caspase-3-generated 36 kDa form of Mst1 could activate ERK.[66] The same group reported MAP3K (MEKK1) and stress-activated protein/ERK kinase 1 (SEK1) to mediate TRAIL-induced JNK/p38 phosphorylation, also requiring active caspase-8.[67]

TGF-β-activated kinase 1 (TAK1). TAK1 is a member of the MAP3K family and is activated by various cytokines, such as the TGF-β, TNF-α, interleukin-1 (IL-1) and ligands of the Toll-like receptors. It is a key regulator of the NF-κB subunit p65/RelA and MAPK activity. TRAIL activated TAK1 in HeLa cells resulting in p65, JNK and p38 activation; siRNA knockdown or chemical inhibition of TAK1 enhanced TRAIL-induced apoptosis.[68] The earlier mentioned activation of p38 by TRAIL in prostate cancer cells was preceded and dependent on activation of TAK1, leading to transcriptional upregulation of Mcl-1 and suppression of apoptosis.[59] Inhibition of TAK1 is an effective approach to increase TRAIL sensitivity as was reported by Morioka *et al.*[69], although in their study, direct phosphorylation of TAK1 after TRAIL was not demonstrated. In fibroblasts and keratinocytes derived from $TAK^{-/-}$ knockout mice, as well as in tumor cell lines Saos2 and HeLa cells with silenced TAK1 expression, sensitization to TRAIL killing was observed

independent of NF-κB activity. In the absence of TAK1, TRAIL exposure resulted in ROS accumulation and subsequent degradation of cIAP, leading to caspase-3 activation and apoptosis. Overall, TAK1 activation by TRAIL appears to be associated with apoptosis resistance through either affecting NF-κB signaling and/or JNK, p38 activation.

Dual Effects on Apoptosis by Protein Kinase C (PKC)

A number of studies have reported on the TRAIL receptor-dependent activation of PKC and its isoforms. Activation of PKC by TRAIL in resistant pancreatic adenocarcinoma cells had anti-apoptotic effects, and PKC inhibition with Gö6983 sensitized for apoptosis.[70] Furthermore, TRAIL-sensitive cells could be made resistant following PKC activation with phorbol 12-myristate 13-acetate (PMA). In apoptosis-sensitive melanoma cells, TRAIL stimulated the phosphorylation of PKCδ and PKCε, resulting in apoptosis-protecting effects.[71] In the same study, activation of PKC by PMA suppressed apoptosis by preventing the translocation and activation of pro-apoptotic Bax to the mitochondria and subsequent apoptosis. Other studies also reported extracellular stimuli-dependent activation of PKC, in particular of the isotypes PKCε and PKCη. For example, in breast cancer cells, PKCε caused TRAIL resistance by activating Akt, followed by Hdm2 phosphorylation. Hdm2, on its turn, reduced p53 expression, leading to the downregulation of Bid and suppression of mitochondrial pathway activation.[72] Activation of PKC also inhibited the recruitment of FADD and caspase-8, resulting in disruption of DISC formation and a decrease in apoptosis.[73]

In contrast to the mentioned anti-apoptotic effects of TRAIL- and extracellular-induced PKC activation, the activation of conventional PKC isoforms (α, β, γ) by PMA or bryostatin-1 was reported to increase the expression of pro-apoptotic Bad and the TRAIL receptors, leading to sensitization for apoptosis by TRAIL.[74] In NSCLC, PKCα and PKCβ increased TRAIL-R2 expression, and sensitized for TRAIL-induced apoptosis.[75] Thus, it appears that activation of PKCδ, PKCε and PKCη leads to apoptosis inhibition, whereas activation of PKC isoforms α, β and γ enhances apoptosis induced by TRAIL, which is cell type-dependent.

Phosphatidylinositide 3-Kinases (PI3K)/Akt Signaling Counteracts Apoptosis

Akt or protein kinase B (PKB) is one of the most critical kinases in the regulation of cell survival. Enhanced activity of the PI3K/Akt pathway is found in many malignancies and is associated with the stimulation of cell growth and cell survival.[76] In leukemic T Jurkat cells, TRAIL phosphorylated PI3K and Akt within 30 min, and inhibition of PI3K with the pharmacological inhibitor LY294002 sensitized cells for TRAIL-induced apoptosis.[77] Sensitization was associated with reduced nuclear translocation of NF-κB p65, reflecting an earlier-found direct ability of PI3K/Akt to phosphorylate and transactivate p65 and NF-κB signaling upon TNF treatment.[78,79] However, NF-κB inhibition did not sensitize for TRAIL apoptosis but enhanced necrotic cell death. More recently, cross-talk between Akt and NF-κB was found to involve direct phosphorylation of IKKβ by Akt, leading to nuclear translocation of NF-κB and increased anti-apoptotic gene expression causing resistance for TRAIL.[80]

TRAIL-induced Akt phosphorylation has been reported in various other tumor cells. In TRAIL-resistant ovarian- and breast cancer cell lines, TRAIL exposure induced phosphorylation of Akt and its substrate, the serine/threonine kinase mTOR (mammalian target of rapamycin), within hours of treatment.[81] The cells could be sensitized for TRAIL by adding the PI3K inhibitor LY294002 or by restoring the expression of the tumor suppressor gene PTEN (phosphatase and tensin homolog deleted on chromosome ten) in PTEN-negative tumor cells. PTEN inhibits the PI3K/Akt pathway by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate (PIP3), a cofactor for Akt activation. Recently, also TRAIL-dependent phosphorylation of Akt was reported in TRAIL-resistant NSCLC cells, and inhibition of Akt resulted in sensitization for TRAIL apoptosis.[82] Akt activation has also been described in TRAIL-sensitive prostate adenocarcinoma DU-145 cells.[83] Akt inhibition enhanced apoptosis activation by TRAIL, and interestingly, roles for the Rous sarcoma oncogene cellular homolog (Src) and Casitas B-lineage lymphoma (c-Cbl) kinases were found upstream of Akt in this signaling route. Based on work in the same prostate cancer cell line, Song *et al.* [84]proposed TRAIL-induced Akt activation to be in part responsible for the development of acquired TRAIL resistance. In this model, Akt phosphorylates its substrate Bad, another Bcl-2 family protein, leading to inactivation of the pro-apoptotic function of this BH3-only protein and suppression of mitochondrial apoptosis. Moreover, the same group identified another TRAIL-dependent route leading to Akt activation, involving a cascade of p38 and subsequent HSP27 phosphorylation that was required for catalytic Akt activation.[67] An interesting but mechanistically poorly understood phenomenon is the recent finding that ectopic expression of TRAIL-R4/DcR2 in HeLa cells enhanced cell growth *in vitro*, and as xenograft transplants in mice in a ligand-independent way.[85] In these cells, constitutive Akt activation was detected and inhibition of Akt suppressed tumor growth. Overall, the activation of the PI3K/Akt pathway by TRAIL has been demonstrated in several tumor models, showing that inhibition of this route sensitizes for apoptosis.

Direct and Indirect Activation of Src Suppresses Apoptosis

As mentioned earlier, in DU-145 prostate cancer cells, TRAIL-induced Akt activation was mediated by the non-receptor tyrosine kinase Src and inhibition of Src sensitized cells for apoptosis.[83] In line with this, the inhibition of Src could restore TRAIL sensitivity in resistant hepatic carcinoma cells by facilitating caspase-8 cleavage.[86] Interesting in this respect is the finding that survival of metastatic breast cancer cells in the bone of mice was dependent on Src.[87] Intriguingly, Src activation amongst other pathways was associated with TRAIL resistance, allowing the survival of disseminated breast cancer cells in the bone marrow by rendering them resistant to TRAIL produced by the microenvironment. Mechanistically, Src has been directly linked with apoptosis resistance. The epidermal growth factor (EGF) was found to promote Src-mediated phosphorylation of caspase-8 at

Tyrosine 380, thus impairing death receptor/caspase-8-dependent apoptosis.[88] Another link between TRAIL-induced Src activation and resistance to apoptosis has been identified, which involves the indirect activation of the EGF receptor (EGFR).[89] TRAIL could stimulate signaling by human epidermal receptor (HER) family members, EGFR and HER2, in colorectal cancer cells by activating the Src family kinases (SFK), which in turn activates a disintegrin and metalloproteinase (ADAM) family member ADAM-17, leading to the shedding of TGF-α. Subsequently, TGF-α, a ligand of EGFR, activated the EGFR/HER2 pro-survival signaling in an autocrine and paracrine manner.[89] Taken together, Src activation has been consistently associated with TRAIL signaling and apoptosis resistance.

Mechanisms of TRAIL-Induced Migration and Invasion

When considering the physiological function of TRAIL, which is thought to include innate immune surveillance against tumor development by suppressing tumor initiation and metastasis,[90,91] the more recently found proinvasive effects of TRAIL on tumor cells were surprising (see also Figure 3). Ishimura *et al.*[92] reported that TRAIL administration stimulates cell migration and invasion in apoptosis-resistant cholangiocarcinoma cells in a NF-κB-dependent manner. In another study, the administration of aggregated TRAIL enhanced primary tumor growth and also stimulated the formation of distant metastases in an orthotopic xeno-transplantation model of human pancreatic ductal adenocarcinoma cells overexpressing Bcl-x_L (Colo357/Bcl-x_L).[93] In these cells, TRAIL stimulated the expression of pro-inflammatory cytokines, such as IL-8, urokinase-type plasminogen activator (uPA) and the matrix metalloproteinases (MMP)-7 and -9, which were linked to invasive behavior.[93,94] In colon cancer HCT116 cells, ectopic expression of mutated PI3K catalytic subunit alpha (PIK3CA) blocked TRAIL-induced apoptosis downstream of caspase-8 cleavage, leading to induction of proinflammation and cell invasion responses.[95] The oncogenic proteins, K-RAS and Raf-1 were mentioned as critical factors in switching the pro-apoptotic function of death receptors into a proinvasive function in these colon cancer cells. Evidence was provided that these oncogenic kinases convert death receptors into invasion-inducing receptors by suppressing the Rho kinase (ROCK)/LIM kinase-mediated phosphorylation of the actin-severing protein cofilin, which is a regulator of actin dynamics during cell invasion.[96] However, whether the suggested critical role of K-RAS and RAF-1 in switching the function of death receptors to invasive receptors is of more general relevance will need further confirmation in a broader panel of tumor cells. More recently, the RIP1/Src/STAT3 axis was identified to mediate TRAIL-dependent migration and invasion of TRAIL-resistant NSCLC cells.[82] Using TRAIL receptor-selective TRAIL variants, TRAIL-R2 was found to be the main mediator of invasion. In this model, inhibition of Src as well as STAT3 prevented invasion but did not notably sensitize for TRAIL.

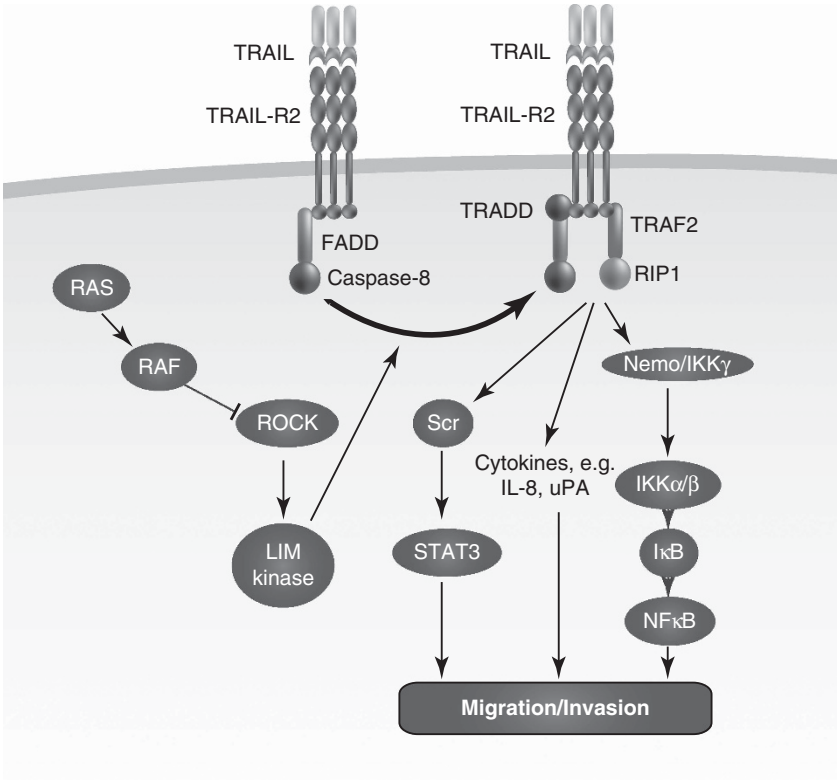

Figure 3 TRAIL-dependent routes that can induce migration and invasion of tumor cells. Schematic representations of the mechanism identified that mediate TRAIL-induced tumor cell invasion. The composition of the different protein complexes induced by TRAIL and the mechanisms controlling this remain to be further elucidated. See text for more details

Overall, it appears that tumor cells have altered the antimetastatic activity of TRAIL into an invasion stimulatory signal, thus using this death pathway to their malignant benefit.

Non-Apoptotic TRAIL Signaling in Non-Transformed Cells

Non-canonical kinase activation by TRAIL is also known to occur in normal healthy tissue cells that are resistant to the killing effect of TRAIL receptor agonists.[97] TRAIL-dependent pro-inflammatory, pro-survival, proliferation and cell migratory responses have been observed in different cell types. For example, TRAIL activated Akt-dependent survival and ERK1/2-dependent proliferation in human endothelial cells (ECs).[98] In ECs, TRAIL was also reported to induce NF-κB signaling leading to enhanced expression of adhesion molecules.[99] Furthermore, ECs exposed to TRAIL displayed enhanced migration and vessel tube formation ability suggestive of proangiogenic activity of TRAIL.[100] However, a recent study by Wilson *et al.*[101] showed that activation of murine TRAIL-R disrupts tumor – and not normal vasculature – in tumor-bearing mice models, suggesting differences in TRAIL signaling networks between normal and tumor stromal ECs. In vascular smooth muscle cells (VSMCs), TRAIL stimulated the production of the pro-inflammatory cytokines TNFα, IL-1β and INFγ, leading to apoptosis protection and induction of proliferation and migration in an ERK1/2-dependent manner.[102] In addition, several other mechanisms have been reported to contribute to these effects of TRAIL, such as activation of NF-κB, insulin-like growth factor type 1 receptor (IGF1R) and SP-1.[103,104] Furthermore, TRAIL could stimulate p38-dependent INFγ secretion and proliferation in T cells,[105] enhance survival signaling in eosinophils of asthma patients,[106] and stimulate ERK1/2 and Akt-dependent survival of fibroblast-like synoviocytes derived from rheumatoid arthritis patients.[107]

Interestingly, also a role for TRAIL signaling has been found in the differentiation of different cell types. In intestinal mucosa cells, TRAIL acted as a growth-arrest mediator via activation of cyclin-dependent kinases p21 and p27 thought to stabilize the differentiated phenotype.[108] In human keratinocytes, TRAIL induced both caspase-dependent differentiation and apoptosis.[109] Furthermore, TRAIL induced differentiation of macrophage lineage precursors into osteoclasts by stimulating pro-inflammatory cytokine production via NF-κB, ERK and p38 activation, independently of caspase cleavage.[110] The molecular mechanisms causing the activation of these alternative TRAIL signaling routes in normal cells remain largely elusive. Some light on this was shed recently by the observation that in mouse embryonal fibroblasts derived from TAK1 and TRADD knockout mice, apoptosis sensitivity to TRAIL was detected. TRADD and TAK1 could be linked to the activation of NF-κB-dependent pro-survival signals, such as upregulation of cFLIP, thus causing resistance.[111,112] Another study proposed that recruitment of TRADD to the TRAIL receptor reduces the levels of FADD in the complex while stimulating RIP1 interactions leading to pro-survival signals.[113] Thus, it appears that similar non-canonical signals can be activated by TRAIL in non-transformed normal cells as in TRAIL-resistant tumor cells.

CD95/FAS

The above discussed activation of non-canonical signaling by TRAIL receptors is not unprecedented in the death receptor family. The well-studied death receptor CD95 (FAS/Apo1) and CD95 ligand (FAS ligand/Apo1 ligand) are not only known for their apoptosis-activating properties but also for their ability to activate non-apoptotic signaling cascades, leading to increased cell proliferation, survival, inflammation, differentiation, migration and invasion (reviewed in[114]). For example, CD95 induces apoptosis in the liver. However, in a damaged liver, it stimulates liver regeneration by enhancing proliferation of hepatocytes.[115] CD95 can also stimulate MAPK activation in neural progenitor cells and was found to regulate neural branching in the developing mouse brain.[116,117] Non-apoptotic signaling in apoptosis-resistant tumor cells has also been reported, including tumor-promoting activity in a lung cancer mouse model,[118] and CD95L-dependent NF-κB and ERK1/2 activation leading to enhanced invasiveness in various tumor cell types.[119] In glioblastoma, CD95-dependent tumor cell invasion was found to be mediated by the Src family member Yes and PI3K, leading to enhanced MMP expression.[120] On first glance, CD95 and TRAIL appear to have similar mechanisms of non-canonical signaling, although this remains to be further examined. Overall, a picture emerges in which both non-apoptotic and apoptotic signaling by death receptors is important for their physiological functions.

Concluding Remarks

In pursue of novel potent tumor-selective apoptosis-inducing strategies, the TRAIL receptors provide excellent targets on first sight. Indeed, their favorable property of triggering tumor-selective apoptosis has been demonstrated in many preclinical studies. However, it is becoming clear that signaling via the TRAIL receptors is much more complex than initially thought and can have different functional outcomes as is illustrated here. TRAIL receptor activation can have antitumor effects by inducing caspase-dependent apoptosis in TRAIL-sensitive cells, whereas in TRAIL-resistant tumor cells, the activation can lead to protumorigenic effects, such as enhanced proliferation, survival and invasion. Whether TRAIL-induced necroptosis contributes to antitumor activity remains to be demonstrated. The effects of TRAIL receptor activation in resistant tumor cells resemble that observed in non-transformed apoptosis-resistant (normal) cells. This suggests that the protumorigenic mechanisms elicited by TRAIL in resistant tumor cells are actually part of normal physiological signaling in non-transformed cells. However, an important difference is that combination therapies that sensitize cancer cells for TRAIL-induced apoptosis are in most cases not harmful for normal cells, indicating specific changes in tumor cells that allow the activation of caspase-dependent apoptosis.

The precise kinase cascades triggered by TRAIL remain to be further identified, which is complicated due to cross-activation between kinases, cell type-dependent variation and effects of external stimuli produced by the tumor microenvironment. A number of studies have attempted to unravel the molecular mechanisms responsible for the dichotomy in

TRAIL signaling, but the underlying mechanisms remain poorly understood. Thus far, the DISC (FADD, caspase-8) and the signaling complex (FADD, caspase-8, RIP1, TRAF2 and NEMO) have been identified[27] as main mediators of apoptosis and non-apoptotic signals, respectively. RIP1-dependent signaling can stimulate cell survival and even tumor cell invasion. In addition, TRAIL has been shown to induce RIP1-dependent necroptosis under specific conditions. The more precise mechanisms by which RIP1 can transmit these signals remain to be determined. Clearly, more research is required to unravel the molecular mechanism regulating non-canonical TRAIL signaling and to identify molecular switches that may be used for setting the system into the 'apoptosis position'. This will provide new clues for developing better strategies to use TRAIL receptor agonists for the treatment of cancer.

Conflict of Interest

The authors declare no conflict of interest.

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