The multifaceted role of TRAIL signaling in cancer and immunity

Running title: Role of TRAIL in cancer and immunity

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List of commonly used abbreviations

AICD	activation-induced cell death
Bcl-2	B cell lymphoma 2
c-FLIP	cellular FLICE (FADD-like IL-1b-converting enzyme)-inhibitory protein
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DcR1	decoy receptor 1
DcR2	decoy receptor 2
DISC	death-inducing signaling complex
DR4	death receptor 4
DR5	death receptor 5
EAE	experimental autoimmune encephalomyelitis
EMT	epithelial to mesenchymal transition
ERK	extracellular signal-regulated kinase
FADD	Fas-associated death domain
GZMB	granzyme B
IKK	IkB kinase
JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAPK	mitogen-activated protein kinase
MCMV	murine cytomegalovirus
MDSC	myeloid-derived suppressor cells
MOG	myelin oligodendrocyte glycoprotein
NF-κB	factor nuclear kappa B
NK	natural killer
NSCLC	non-small cell lung cancer
PDAC	pancreatic adenocarcinoma cells

PI3K	phosphoinositide 3-kinase
PKC	protein kinase C
RIPK1	receptor-interacting serine/threonine-protein kinase 1
SCLC	small cell lung cancer
TCR	T-cell receptor
TNF	Tumor necrosis factor
TRAF2	TNF receptor-associated factor 2
TRAIL	TNF-related apoptosis-inducing ligand
TRAIL-R	TNF-related apoptosis-inducing ligand receptor
TRAs	TRAIL or TRAIL-R agonists
uPA	urokinase-type plasminogen activator
VSMCs	vascular smooth muscle cells

Abstract

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that can lead to the induction of apoptosis in tumor or infected cells. However, activation of TRAIL signaling may also trigger non-apoptotic pathways in cancer and in non-transformed cells, i.e. immune cells. Here, we review the current knowledge on non-canonical TRAIL signaling. The biological outcomes of TRAIL signaling in immune and malignant cells is presented and explained, with a focus on the role of TRAIL for natural killer (NK) cell function. Furthermore, we highlight the technical difficulties in dissecting the precise molecular mechanisms involved in the switch between apoptotic and non-apoptotic TRAIL signaling. Finally, we discuss the consequences thereof for a therapeutic manipulation of TRAIL in cancer and possible approaches to bypass these difficulties.

Introduction

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL/Apo-2L) is a member of TNF family proteins first described for its ability to induce apoptosis by binding to its cognate receptors on target cells. TRAIL is a type II transmembrane protein that was identified and cloned based on the sequence homology of its C-terminal extracellular domain with CD95L (FasL) and TNF [1, 2]. The extracellular domain of TRAIL can be proteolytically cleaved from the cell surface. The aspartic proteinase cathepsin E was found to induce the release of a soluble form of TRAIL, whose function was associated in this study with impaired tumor growth and metastasis [3]. However, the capacity to induce apoptosis of the soluble form of TRAIL is significantly lower than the membrane-bound form [4, 5].

The human TRAIL interacts with two agonistic TRAIL receptors, TRAIL-R1 (DR4, encoded by *TNFRSF10A*) and TRAIL-R2 (DR5, encoded by *TNFRSF10B*), which contain a conserved death domain motif that allows recruitment of apoptosis signaling molecules to induce cell death [6-12]. In contrast to humans, mice express only one functional TRAIL agonistic receptor (mTRAIL-R) [13]. TRAIL also binds to two other membrane receptors that do not transduce apoptotic signals, which are therefore considered to act as decoy receptors i.e., TRAIL-R3 (DcR1, encoded by *TNFRSF10C*) and TRAIL-R4 (DcR2, encoded by *TNFRSF10D*) [14, 15]. In addition, TRAIL binds with lower affinity to a soluble receptor called osteoprotegerin (OPG), but the physiological role of this interaction is still unknown [16].

TRAIL is expressed in a wide range of tissues and cell types, but it is found to be mainly expressed on the cell surface of immune cells, where it plays a critical role in inducing apoptosis of target cells [17, 18]. For instance, natural killer (NK) cells and cytotoxic T cells (CTLs) elicit apoptosis of target cells utilizing either soluble factors, through exocytosis of cytolytic granules containing perforin and granzymes, or by the engagement of death receptor ligands, like FasL and TRAIL [19-21]. TRAIL on NK and T cells was described to play a crucial role for the control of virus infections and tumor immune surveillance [22-28]. Besides its function for the clearance of pathogen, apoptosis is also involved in the T cell repertoire selection, therefore participating in the maintenance of

peripheral tolerance through a process called activation induced cell-death (AICD). Although FasL/Fas pathway is the main contributor to AICD/apoptosis in peripheral T cells, TRAIL may be also involved in AICD of $CD8^+$ T cells and subsets of T-helper cells [29-35]. Conflicting data were reported regarding the role of TRAIL in thymocyte negative selection, with one study suggesting that TRAIL is critical for negative selection of autoreactive thymocytes, while other reports indicating that TRAIL is not required for this process [36-39].

In contrast to TNF and FasL, TRAIL was described as a promising agent for cancer therapy, due to its ability to mediate apoptosis in transformed cells, with no or minimal effect on normal cells [40], and to the fact that TRAIL-R1 and TRAIL-R2 are often upregulated on cancer cells [41-43]. Despite encouraging early safety outcomes, several studies with TRAIL or TRAIL-R agonists (TRAs) proved to be disappointing, showing little antitumor efficacy [40, 44-46]. Failure to translate preclinical results to the clinic can be attributed, at least in part, to the resistance or reduced sensitivity to TRAIL-induced apoptosis of certain malignant cells [47, 48], as well as the poor efficacy of first and second generation of TRAs. Third generation of TRAs, engineered for higher valency and displaying stronger pro-apoptotic potential are being assessed in the clinic to overcome these limitations (reviewed in [49]).

Less studied but notwithstanding important are the noncanonical signaling capabilities of TRAIL exerted on normal or malignant cells. As a matter of fact, TRAIL is also able to induce non-apoptotic pathways, some of which may even drive pro-tumorigenic effects in resistant tumor cells by promoting receptor-induced kinase activation, thereby triggering survival, proliferation and/or metastasis [50-52], which will be discussed later in more detail.

Molecular basis of TRAIL/TRAIL-R signaling

TRAIL triggers apoptosis following binding to one of its cognate transmembrane death receptors, TRAIL-R1 or TRAIL-R2. Like other TNF death receptors, TRAIL-R1 and TRAIL-R2 contain an intracellular death domain that has the propensity to associate with other such domains upon the ligation of homotrimeric cognate ligand [53]. This association enables the recruitment of the adaptor protein FADD (Fas-associated death domain) and the formation of the death-inducing signaling complex (DISC) [9, 54]. Thereafter, initiator procaspase-8 and/or procaspase-10 are recruited to the DISC and are activated through proteolysis, before being released to further activate effector caspases, including caspase -3, -6 and -7 [54-57]. Activated effector caspases cleave in turn vital cellular proteins, which develops in a series of molecular processes resulting in the morphological and biochemical hallmarks of apoptosis [58]. This type of TRAIL-induced apoptosis pathway that starts at the cell membrane and directly leads to apoptosis is referred to as the "extrinsic apoptosis pathway". In type I cells, the extrinsic pathway is sufficient to induce apoptosis. However, apoptosis of type II cells requires an amplification of the signal via the mitochondrial apoptotic pathway (i.e. via the "intrinsic apoptosis pathway") [59, 60]. Triggering of the extrinsic apoptosis pathway in type II cells only results in limited activation of caspase 8 in the DISC (death-inducing signaling complex), which is unable to further activate the caspase amplification cascade. Instead, caspase 8 must first cleave the pro-apoptotic Bcl-2 (B-cell lymphoma 2) homolog BID. The carboxyl-terminal fragment of BID (tBID) then translocates to the mitochondria, where it mediates the release of cytochrome c and other pro-apoptotic molecules [61]. Subsequently, the mitochondria membrane potential is affected by the translocation of tBID, Bax and Bak to the mitochondria outer membrane, resulting in the release of cytochrome c and Smac/DIABLO into the cytosol. [62]. The apoptotic signal culminates in the activation of effector caspases-3, -6 and -7, which is mediated by the apoptosome [59, 62-65].

TRAIL-induced apoptosis pathway is tightly regulated at multiple levels to avoid uncontrolled cell death in normal cells. Tumor cells use these control mechanisms to escape from TRAIL-induced apoptosis, thereby developing TRAIL resistance. At the cell membrane level, selective regulation of TRAIL-induced apoptosis is mediated by TRAIL-

R3 and TRAIL-R4, two antagonistics receptors that either sequester the ligand from its functional death agonistic receptors or restrain caspase-8 recruitment and activation within the TRAIL DISC [66-68]. In a somewhat less selective manner, the cellular FLICE-like inhibitory protein (c-FLIP), known as a non-functional procaspase-8 homolog, inhibits the activation of caspase-8 by competing for binding to FADD [69, 70]. Further downstream, intracellular apoptosis inhibitors, such as X-linked inhibitor of apoptosis (XIAP) and anti-apoptotic Bcl-2 proteins, respectively, are able to control caspase activity and mitochondrial activation to prevent death induced by TRAIL [71-73].

Besides inducing apoptosis, TRAIL may also trigger non-apoptotic (i.e. non-canonical) signaling through the activation of pro-inflammatory pathways, including NF-κB (factor nuclear kappa B), PI3K/Akt (phosphoinositide 3-kinases /protein kinase B) and MAPK (mitogen-activated protein kinase) such as JNK (c-Jun N-terminal kinase), ERK (extracellular signal-regulated kinase) and p38 [9, 74-81], (reviewed in [51]). Results from *in vivo* studies in preclinical models evidenced that apoptosis-resistant cancer cells evade from TRAIL-induced apoptosis by activation of these non-canonical pathways. For instance, TRAIL monotherapy induced unwanted effects, namely survival, proliferation and migration of different tumor types [50, 52]. Activation of TRAIL-induced non-apoptotic pathway is thought to involve the formation of a secondary signaling complex that consists of FADD, caspase-8, RIPK1 (receptor-interacting serine/threonine-protein kinase 1), TRAF2 (TNF receptor-associated factor 2) and NEMO/IKK (NF-κB essential modulator) [82-84]. In this complex, caspase-8 has recently been described as a scaffold protein, enabling the assembly of the pro-inflammatory signaling during non-canonical TRAIL pathway, regardless of its enzymatic activity [85]. Yet, the caspase-8 enzymatic activity impairs NF-κB activation in response to TRAIL treatment [86]. Likewise, while in TRAIL-sensitive lymphoma cells, caspase-8 mediated RIP1 cleavage, results in impaired IκB kinase (IKK) recruitment and NF-κB activation, due to the loss of RIP kinase domain [86], in apoptosis-resistant lymphoma cells, expressing high levels of cFLIP, restricted caspase-8 activity was associated with higher NF-κB activation after TRAIL stimulation [86]. TRAIL-induced NF-κB activation can not only be mediated by TRAIL-R1 and TRAIL-R2 [9, 80], but also by TRAIL-R4 [74]. Since TRAIL-R4 is devoid of functional death domain, it will be needed to define whether the non-apoptotic signaling machinery

triggering NF-κB requires caspase-8 or not, regardless of TRAIL-R4's ability to interact with TRAIL-R1 or TRAIL-R2 after TRAIL stimulation [67, 87]. Despite the growing number of studies reporting the pleiotropic functions of TRAIL, the molecular mechanisms governing signal transduction of TRAIL non-apoptotic signaling remain elusive. Accordingly, it has only been demonstrated recently that TRAIL-R1 and TRAIL-R2 exert differential signaling capabilities. Likewise, TRAIL-R1 displays stronger TRAIL-mediated pro-apoptotic signal transduction capabilities than TRAIL-R2, but is unable, contrary to TRAIL-R2, to induce a pro-motile signaling pathway, associated with early calcium cytosolic flux changes [88].

Apoptosis-inducing agents, including TRAIL, trigger a heterogenous response in sensitive cancer cells, leading to the death of part of the clonal population, while a fraction survives and develops resistance, a process named "fractional survival". This variability in TRAIL-induced apoptosis has been linked to cell-intrinsic mechanisms, such as the naturally occurring differences in the levels or the activity status of pro and anti-apoptotic proteins apoptosis [89, 90]. Furthermore, the lack of sufficient TRAIL signal may also lead to the development of resistant clones. Yet, more recent findings suggest that TRAIL binding to TRAIL-R2 may lead to the formation of a dynamic composite signaling platform that can simultaneously activate pro-apoptotic and pro-survival pathways [91]. In this study, the authors proposed two different mechanisms possibly implicated in the decision between cell death or survival after TRAIL ligation. Firstly, and also in line with previous findings [92], the location of this platform within the plasma membrane may determine the signaling outcome. Platform formation inside lipid rafts, also called membrane rafts, leads to efficient caspase activation and apoptosis, whereas assembly of the TRAIL-R2 complex outside lipid rafts promotes non-apoptotic signaling. Second, the pro-survival platform becomes stabilized and activated when there is an excess of apoptosis inhibitors, i.e. cFlip, TRAF2, TRAIL-R4 [92, 93]. It should be stressed, here that the requirement of DISC formation in lipid rafts to induce apoptosis is controversial. Likewise, it has been demonstrated in a panel of cell lines, including hematopoietic cells, that the TRAIL-TRAIL-R2/TRAIL-R1-DISC complexes were mainly found in the soluble cellular fraction rather than in lipid rafts [67, 94]. TRAIL complexes composition and location, apart from the well-known pro-apoptotic complex, remain, to date largely unknown.

Nonetheless and interestingly, TRAIL-R1 and TRAIL-R2 are constantly expressed at high levels in many cancers, and throughout disease progression, which suggests that cell-intrinsic expression of these receptors may provide an advantage for tumorigenesis [50, 66, 95-97]. Therefore, there is a high need to investigate the function of the endogenous TRAIL/TRAIL-R system in cancer and normal cells, in order to understand the implications of this pathway for tumor biology, and immunity.

Effect of TRAIL/TRAIL-R signaling on tumor cells

TRAIL/TRAIL-R signaling may have different functions in tumor cells. The first *in vivo* evidence of the antitumor activity of recombinant TRAIL came from experiments done in mice bearing xenograft tumors. Two groups independently showed that treatment with different forms of recombinant soluble TRAIL resulted in tumor regression in immunodeficient mice challenged with human colon carcinoma cells or human mammary adenocarcinoma cells [40, 98]. Shortly, after these studies, came the first evidence that endogenous TRAIL, also plays a physiological role in the organism to control tumor growth [26, 99, 100]. Likewise, *Trail*-deficient mice were found to develop spontaneously tumors [26, 101] and to be more susceptible to A20 B cell lymphoma, with uncontrolled tumor progression and increased lymphoma nodules in the liver [100]. *In vivo* experiments using a renal cancer cell line and *Trail*-deficient mice indicated that TRAIL signaling is important to control tumor metastasis [102]. Similarly, mice lacking TRAIL-R showed an increase in lymph node metastasis, without any effect on primary tumor growth [103].

However, further studies revealed that TRAIL/TRAIL-R signaling in transformed cells is much more complex and may have outcomes different from apoptosis. Indeed, cancers cells may not only resist and survive TRAIL treatment, they can also benefit from TRAIL signaling by undergoing proliferation, migration, invasion or attract immune cells in the tumor microenvironment through secretion of cytokines or chemokines. These aspects are presented in further details below and in Figure 1.

Survival/Apoptosis resistance

With the exception of glioblastoma cells [104], activation of non-canonical TRAIL signaling, including NF-κB, has been reported to inhibit apoptosis induced by TRAIL, leading to resistance and thus survival in various cancerous cells. Stimulation of TRAIL-resistant human pancreatic adenocarcinoma (PDAC) cells with recombinant TRAIL induced the activation of protein kinase C (PKC) and NF-κB, while inhibition of PKC or NF-κB sensitized these cells to TRAIL-induced apoptosis [105]. Similarly, selective inhibition of the NF-κB pathway enhanced TRAIL-mediated apoptosis in neuroblastoma cells and mantle cell lymphoma (MCL) B cells [106, 107]. In lung cancer cells, TRAIL-induced apoptosis resistance occurs via NF-κB-dependent up-regulation of microRNAs, which in turn target caspase-8 (*CASP8*) and -3 (*CASP3*), *TRAF7* and *FOXO3* [108]. In the mouse system, the resistance of B16F10 murine melanoma cells to TRAIL-induced apoptosis [22, 109], was linked to activation of the NF-κB pathway after mTRAIL-R engagement [110]. In the same vein, constitutive or CD40-mediated activation of NF-κB in B-cell lymphoma, induce the up-regulation of c-FLIP in these cells and protect them from apoptosis induced by TRAIL, whereas selective inhibition of NF-κB restores their sensitivity [107, 111].

Besides NF-κB, the PI3K/AKT/mTOR pathway also plays a role in resistance to TRAIL-induced apoptosis in various types of malignant cells. In a panel of breast and ovarian cancer cells, AKT/mTOR pathway is activated in TRAIL-resistant cells, while AKT inhibition led to sensitization of these cells [112]. TRAIL signaling induces this pathway to promote resistance to apoptosis by decreasing BID protein levels [113]. Interestingly, the decoy receptor TRAIL-R4 can promote the survival of cervical carcinoma HeLa cells via triggering of the PI3K/AKT/mTOR axis [114].

TRAIL signaling may also support apoptosis resistance by activating the MAPK proteins ERK, JNK and/or p38 in pancreatic tumor cells, HeLa cells, and small cell lung cancer (SCLC) cells [77, 115, 116]. Accordingly, inhibition of JNK or p38 sensitized hepatocellular cells, respectively breast carcinoma cells to TRAIL-induced apoptosis [117, 118].

Proliferation

TRAIL-induced NF-κB activation was initially thought to solely induce resistance to apoptosis. However, several *in vitro* studies demonstrated that TRAIL may also trigger NF-κB to promote the proliferation of resistant Jurkat cells [52] or B16F10 murine melanoma cells [110].

TRAIL-induced ERK activation may exert a similar pro-proliferative effect on tumors. In caspase-8-deficient SCLC cells, TRAIL induces cell proliferation through the activation of ERK1/2, in a TRAIL-R2-dependent manner [116]. In addition, TRAIL-induced ERK1/2 activation and proliferation of human glioma cells was found to be dependent on the expression of c-FLIP [119].

It should be noted, however, that cell proliferation in these studies is measured mainly through indirect assays, for instance by measuring metabolic activity. Therefore, the reported effects of TRAIL in inducing cell proliferation have to be carefully evaluated.

Migration/Invasion and tumor microenvironment

NF-κB pathway

Asides from its role for survival and proliferation, the NF-κB pathway was also found to participate in the promotion of cancer cell migration and metastasis upon TRAIL signaling activation. Specifically, TRAIL-induced NF-κB has been reported to trigger the migration and invasion of B16F10 murine melanoma cells and to increase lung metastasis of cholangiocarcinoma cells *[110, 120]*

Other intracellular signaling pathways

In addition to NF-κB, several other signaling pathways have been also involved in the TRAIL-dependent migration of cancer cells. A comparison of kinase activation between apoptosis-resistant versus -sensitive non-small lung cancer (NSCLC) cells demonstrated that TRAIL may trigger migration in a RIPK1-, SRC- and STAT3-dependent manner [121]. Another study showed that TRAIL increases the invasive properties of colorectal cancer (CRC) cells – and their survival – via K-RAS [122]. Along these lines, activation of TRAIL signaling increased ERK phosphorylation only in lung adenocarcinoma cells

lines with mutant KRAS, and this activation was associated with enhanced cell migration [123]. Moreover, genetic depletion of mTRAIL-R in KRAS-driven models of PDAC and NSCLC reduced tumor growth and impaired metastasis [50]. The same effect was observed in human PDAC cells, where TRAIL-R2 ablation reduced cell proliferation, migration and invasiveness. Of note, the membrane-proximal domain (MPD) of TRAIL-R2 was sufficient to induce the migration of NSCLC cell lines through the activation of RAC1/PI3K signaling [50].

Epithelial-to-mesenchymal transition

Initiation of metastasis requires migration and invasion of primary cancer cells, which is enabled by epithelial-to-mesenchymal transition (EMT) [124]. Analysis of EMT markers demonstrated that acquired TRAIL resistance induced EMT in resistant NSCLC cells [108]. Moreover, in breast cancer cells, TRAIL induced EMT by suppressing PTEN expression via miR-221 [125].

Inflammatory mediators with direct effect on cancer cells

Endogenous TRAIL/TRAIL-R signaling may also promote the secretion of pro-inflammatory molecules, including chemokines, that participate in (cancer) cell migration and modulate tumorigenesis. For instance, TRAIL strongly activated NF-κB and MAPK in human PDAC cells, resulting in the induction of pro-inflammatory IL-8 and CCL-2 and in the enhancement of invasive properties via upregulation of MMP-7 and -9 and uPA (urokinase-type plasminogen activator). Consequently, TRAIL administration in an orthotopic xenotransplantation model of human PDAC increased primary tumor growth and the formation of distant metastases [126, 127]. Interestingly, activation by FasL had similar effects on the invasiveness of PDAC cells [128]. In MDA-MB-231 breast cancer cells, deletion of TRAIL-R2 downregulated the chemokine receptor CXCR4, thereby impairing the ability of these cells to disseminate to the bones [96]. In contrast, TRAIL was found to induce miR-146a in MDA-MB-231 cells thereby inhibiting their CXCR4-dependent migration [129].

Indirect effect on cancer via regulation of immune cells in the tumor microenvironment

TRAIL-induced inflammatory molecules may also have an indirect effect on cancer cells. While PDAC cells activated by TRAIL produce CCL20 in an NF-κB-dependent manner, this chemokine had no effect on PDAC cell death or migration. Rather, CCL20- were found to exert a paracrine action by recruiting immune cells, which in turn promoted TRAIL resistance in the malignant cells [130]. Among the cytokines released following TRAIL binding, the C-C motif chemokine ligand 2 (CCL2) was identified as the most important one for the recruitment of myeloid-derived suppressor cells (MDSCs) and M-2 like macrophage promoting tumor growth. Interestingly, unlike TNF-mediated cytokine secretion, TRAIL and FasL signaling required FADD and caspase-8 for cytokine induction in this study [131].

Lastly, TRAIL may also have an indirect effect on tumor growth, via modulation of the tumor-microenvironment. This may for instance occur by inducing apoptosis in MDSCs [132], by promoting angiogenesis in the tumor tissue [133], or by repolarizing tumor-associated macrophages (TAM) to an M1-like phenotype that support cytotoxic effects in the malignant cells [134].

Taken together, these findings from *in vitro* experiments and *in vivo* studies in mice demonstrate the dichotomous effect of TRAIL-TRAIL-R signaling for cancer biology. They also imply that further research is required to elucidate the molecular mechanisms regulating non-canonical TRAIL signaling.

TRAIL/TRAIL-R signaling in non-malignant and in immune cells

Function of TRAIL/TRAIL-R in normal, non-transformed cells

Like most of the mice with defects in molecules of the TNFR superfamily, *Trail*-deficient mice develop normally until adulthood and are fertile, indicating functional complementarity between these molecules in mammals [26, 100, 135-139]. Yet, *Trail*-deficient mice have increased susceptibility to autoimmune diseases and to tumor initiation and metastasis [26, 38, 99-102]. As mentioned above, TRAIL was initially described to mediate apoptosis of transformed cells, with no or minimal effect on normal cells [40]. However, TRAIL may also participate in thymocyte negative selection [36, 38] – although

this is a controversial issue [37, 140] – and play an active role in inducing apoptosis in normal non-immune cells, i.e. primary esophageal, salivary and prostate epithelial cells [141-143], possibly because some of these cells express less (anti-apoptotic) TRAIL decoy receptors.

Interestingly, there are few reports indicating that TRAIL can also induce apoptosis-independent effects in normal cells, such as proliferation, migration, differentiation and inflammation. For instance, TRAIL has been shown to induce the proliferation of fibroblasts isolated from synovial tissue, vascular smooth muscle cells (VSMCs), primary human endothelial cells and proximal tubular epithelial cells, through the activation of NF-κB, ERK1/2, p38 and PI3K-AKT pathways [78, 144-148]. TRAIL also promotes the migration of VSMCs through induction of the ERK1/2 pathway [144], and triggers NF-κB and inflammatory gene expression in human endothelial cells [149].

Moreover, TRAIL stimulates the differentiation of human keratinocytes in a caspase-dependent manner [150], while it supports osteoclast differentiation from mononuclear phagocyte precursors via NF-κB, ERK, p38 and TRAF6 signaling [151, 152]. However, conflicting studies also indicated an inhibitory role of TRAIL in osteoclast differentiation [153, 154]. The reasons for these discrepancies may be related to difference in the concentration of recombinant TRAIL used in the assays and the presence of other TNF family members, such as RANKL (receptor activator of nuclear factor kappa-B ligand).

In summary, these studies demonstrate that TRAIL also exert a pleiotropic role in normal cells, suggesting that the molecular circuitry is conserved from normal to neoplastic cells.

Function of pro-apoptotic TRAIL/TRAIL-R in immunity

The finding that TRAIL gets preferentially upregulated on immune cells during inflammation suggested its central involvement in immunity and immunoregulation. In fact, TRAIL expression is dependent on immune cell activation. For instance type I IFN (IFNα and IFNβ) stimulation leads to TRAIL upregulation on neutrophils, monocytes, macrophages, dendritic cells (DCs), plasmacytoid dendritic cells (pDCs), NK cells, T and

B cells [155-169]. Inducers of type I IFN, such as viruses and Toll-like receptors (TLRs) ligands also trigger TRAIL expression on DCs, pDCs, NK cells and B cells [163, 170-173], while IFNγ promotes it on neutrophils and NK cells [27, 174-177]. Importantly, as opposed to resting cells, activated immune cells, such as NK or $CD8^+$ T cells, often co-express in addition to TRAIL, the antagonistic receptors TRAIL-R3 or TRAIL-R4, as well as the caspase-8 inhibitor c-FLIP to protect themselves from TRAIL-induced apoptosis [178].

On the contrary, TRAIL agonistic receptors are often upregulated in infected cells (or certain types of infection-activated cells), thereby rendering these cells susceptible to apoptosis mediated by TRAIL-expressing cytotoxic cells [23, 179-183]. As a matter of fact, apoptosis induced by immune cells via TRAIL is an important mechanism to eliminate pathogen-infected cells. For instance, TRAIL expression on neutrophils has been implicated in the apoptosis of alveolar macrophages that phagocytosed *Streptococcus pneumoniae*, and clearance of this bacterial pathogen is therefore compromised in *Trail*-deficient mice *[184]*. $CD8^+$ T cells utilize TRAIL to eliminate virus-infected alveolar epithelia cells during influenza infection, and to clear West Nile virus (WNV) infection from neurons in the central nervous system [23, 185], while TRAIL-mediated killing by NK cells is crucial to limit *in vivo* replication of encephalomyocarditis virus and to control hepatitis C virus-replicating (HCV) hepatoma cells [164-166]. Interestingly, pathogens like cytomegaloviruses (CMV) were found to inhibit TRAIL receptor expression on infected cells as a mechanism to evade NK cells killing [186, 187], thus further highlighting the relevance of TRAIL-induced cytotoxicity for pathogen elimination.

Yet, TRAIL expression on immune cells not only contributes to contain infections, but it also plays a crucial role in the control of tumor growth (Figure 2A). Monocytes expressing TRAIL after IFNγ activation exhibited tumoricidal activity *in vitro [158]*. In FasL-resistant tumor cells, TRAIL mediated the apoptosis of melanoma cells through $CD4^+$ T cells [24]. NK cells are involved in the recognition and subsequent elimination of transformed cells [188], and membrane-bound TRAIL on NK cells is a central effector mechanism used by NK cells to suppress tumor growth and to protect mice against tumor metastases [22, 176, 189]. Of note, apoptosis triggered by TRAIL expressed on NK cells has been associated with transplant rejection [190]. Indeed, immunosuppressive therapy with cyclosporin A administration after renal transplantation may inhibit solid graft

rejection partially by downregulating the expression of TRAIL and FasL on NK cells [191]. However, TRAIL on hepatic NK cells might also have a protective role during liver transplantation, through the elimination of activated T cells [192] (Figure 2A).

Taken together, apoptosis elicited by TRAIL expressed on immune cells is an important mechanism for the control of pathogens and tumors (or non-self cells).

Pro- and non-apoptotic immunoregulatory function of TRAIL/TRAIL-R

Apart from its role in supporting effector function for the elimination of malignant or infected cells, apoptosis triggered by TRAIL has also been implicated in the control of the immune response. Such immunoregulatory function is particular important for the resolution phase of an immune response, as effector cell removal is essential to limit tissue damage and the possible autoimmune reactions. Activated or senescent neutrophils become sensitive to TRAIL-induced apoptosis, and their removal promotes the resolution of inflammation [193-196]. After *in vitro* activation, $CD4^+$ T cells eliminate antigen-presenting macrophages via TRAIL, which self-regulates their expansion by limiting the pro-stimulatory signal they can receive via their TCR [197]. NK cells engage TRAIL signaling to eliminate hepatitis B virus (HBV)-specific $CD8^+$ T cells or murine cytomegalovirus (MCMV)-induced $CD4^+$ T cells [179, 180]. TRAIL expressing pDCs isolated from HIV-infected patients triggered the apoptosis of activated autologous $CD4^+$ T cells [198]. Helpless $CD8^+$ T cells that are primed in the absence of $CD4^+$ T cells undergo apoptosis by AICD upon secondary stimulation, and this killing is mediated by TRAIL [199]. Last, deletion of TRAIL exacerbates mouse lymphoproliferative disease associated with the loss of FasL [35], suggesting that TRAIL might contribute to FasL-mediated AICD of $CD3^+CD4^-CD8^-B220^+$ T cells, $CD4^+$ and $CD8^+$ T cells, as well as follicular B cells. All these studies illustrate the role of apoptotic TRAIL as a negatively regulator of immune responses.

TRAIL/TRAIL-R signaling is not only involved in infection-related inflammation but also appears to be important in preventing inflammatory disease, including autoimmune disorders. Although TRAIL ligand- and receptor- deficient mice do not develop spontaneous autoimmunity, they show disease exacerbation in different experimental

models. Genetic deletion or sTRAIL-R2-mediated blockade of TRAIL worsen type I diabetes development. TRAIL inhibited the proliferation of autoreactive T cells by blocking cell cycle progression, through the upregulation of cyclin-dependent kinase inhibitor $p27^{kip1}$, leading to defective IL-2 transcription in nonobese diabetic (NOD) mice [200, 201]. Non-apoptotic TRAIL/TRAIL-R signaling inhibits the activation of colitogenic T cells and the development of gut inflammation in an adoptive transfer-induced colitis model [202]. In different mouse models of rheumatoid arthritis (RA), blockade of TRAIL aggravated the disease. TRAIL had no effect on apoptosis of inflammatory cells in these RA models, but it instead prevented cell cycle progression of lymphocytes, inhibited T cell proliferation and suppressed cytokine production [203, 204]. Inhibition of TRAIL signaling also exacerbated experimental autoimmune encephalomyelitis (EAE) in mice. In these studies, blockade of TRAIL pathway led to enhanced autoreactive T cell response [205-207].

Taken together, these studies unveil an intriguing immunoregulatory role of TRAIL, which may involve or be independent of TRAIL pro-apoptotic activity.

Role of non-canonical TRAIL/TRAIL-R signaling in immune cells

NK cells

NK cells are important effector immune cells involved in the defense against viral infections and in the control of malignant cells through their ability to induce cell death. NK cells can also exert an immunoregulatory function [208-210]. As mentioned above, NK cells may elicit apoptosis through the engagement of TRAIL into its cognate receptor on the target cell [20, 21]. Yet, our group recently described a novel, non-apoptotic role of TRAIL as an immune modulator of NK cell activity during virus infection [211]. In *Trail*-deficient mice infected with lymphocytic choriomeningitis virus (LCMV), we found that NK cells showed reduced granzyme B (GZMB) expression. This was associated with impaired NK cell-mediated killing of activated $CD8^+$ T cells, which in turn resulted in better LCMV clearance. Further dissection of the underlying mechanisms revealed that TRAIL promotes GZMB production in NK cells by supporting an IL-15 receptor-PI3K-AKT-mTOR-GZMB signaling axis. In line with these data, TRAIL blockade reduced the

signaling downstream of IL-2/IL-15 receptor in human NK cells [211] (Figure 2B). In contrast, hepatic *Trail*$^{-/-}$ NK cells isolated after ischemia-reperfusion injury expressed high levels of the degranulation marker LAMP-1/CD107a, which translated into increased cytotoxicity towards TRAIL-resistant YAC-1 cells *in vitro* [212]. Yet, it is not clear whether this was due to a direct effect of TRAIL signaling on NK cells.

Trail$^{-/-}$ NK cells also showed increased IFNγ secretion upon stimulation with an NK1.1 cross-linking antibody. Therefore, engagement of the TRAIL pathway in NK cells may promote or inhibit their (TRAIL-independent) cytotoxicity capacity, while reducing their cytokine-secreting function [211, 212] (Figure 2B). Lastly, gene expression analysis revealed that pathways related to leukocyte migration were differently affected in *Trail*$^{-/-}$ compared to wild-type NK cells isolated from LCMV-infected mice [211], which may affect their spatiotemporal distribution and thereby further impact on their *in vivo* function.

Notably, extensive analysis of naïve NK cells indicated no major impact of *Trail* deficiency on NK cell development. Specifically, *Trail*$^{-/-}$ NK cells and wild-type counterparts showed similar i) constitutive granzyme B expression; ii) expression of T-bet (*Tbx21*) and eomesodermin (*Eomes*), two transcriptional regulators of NK cells development; iii) proportion of NK cell maturation subsets (defined by CD11b and CD27 expression); and iv) expression of several activating and inhibiting NK cell markers – except Ly49H that was slightly downregulated on *Trail*$^{-/-}$ NK cells [211]. Taken together, this suggests that non-apoptotic TRAIL receptor signaling only affects NK cell function during LCMV infection.

Of note, another study reported a non-apoptotic role of TRAIL in modulating $CD8^+$ T cell response. Specifically, TRAIL expressed on NK cells induced arginase-1 mRNA expression in DCs, resulting in reduced generation of MHC-class I-antigen peptide complexes and eventually impaired cross-priming [213] (Figure 2C).

<u>*Eosinophils*</u>

TRAIL plays mostly a pro-inflammatory role in allergic airways disease. TRAIL expression is increased in bronchoalveolar lavage (BAL) of asthmatic patients, which is correlated with eosinophil accumulation. TRAIL did not trigger apoptosis of BAL eosinophils but rather induced their survival [214]. In a mouse model of eosinophilic

esophagitis (EoE) and during rhinovirus infection, E3 ubiquitin-ligase midline 1 (MID1) expression is upregulated mainly in bronchial epithelial cells in a TLR4- and TRAIL-dependent manner. MID1 decreases the activation of protein phosphatase 2Ac (PP2A) through association with its catalytic subunit, leading to NF-κB activation, pro-inflammatory chemokines and cytokines secretion and to a marked increase in the accumulation of eosinophils [215, 216] (Figure 3, panel A). However, conflicting studies indicated a protective role of TRAIL when administered intranasally as a pretreatment of airway inflammation [217], and also during the resolution phase of allergic asthma [218].

Monocytes and Macrophages

In myeloid cells, TRAIL signaling has been shown to trigger different processes, including differentiation, migration, cytokine production and lipid uptake and transport. In primary myeloid cells derived from $CD34^+$ hematopoietic stem cells TRAIL/TRAIL-R1 interaction was found to promote monocytic maturation in a caspase-dependent manner [219]. Moreover, TRAIL induces through the activation of TRAIL-R1 the chemotactic migration of the THP-1 monocytic cell line and of LPS-primed primary monocytes. Mechanistically, TRAIL signaling promoted migration of these cells by engaging PI3K, Rho GTPase and its downstream effectors MLC and PAK1 [220]. Similarly, TRAIL promotes pro-inflammatory cytokine secretion and macrophage migration via NF-κB activation after ischemia/reperfusion injury [221].

There are contradiction data on the role of TRAIL for cytokine production in macrophages. Peritoneal macrophage isolated from TRAIL-$R2^{-/-}$ mice stimulated with Bacillus Calmette-Guérin (BCG) or TLR agonists display increased TNF-α and IL-12 production [222]. In contrast, another study reports that murine peritoneal macrophages treated with TRAIL upregulated pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α. Accordingly, tumor-associated macrophages (TAM) isolated from mice treated with soluble TRAIL displayed an increased expression of pro-inflammatory cytokine and displayed anti-tumorigenic phenotype [134]. A possible reason for this discrepancy might be the type of stimuli, with one study working with naïve macrophages stimulated solely with recombinant TRAIL [134], while the other used TRAIL-$R2^{-/-}$ macrophages stimulated with LPS or BCG [222].

To further complicate this picture, early administration of recombinant TRAIL was shown to inhibit macrophage recruitment *in vivo*, which was associated with reduced mucosal inflammation and disease mitigation in the setting of chemically-induced colitis or colitis-associated carcinogenesis. *In vitro* mechanistic studies using a macrophage cell line suggested that TRAIL induced these phenotypes by curbing cytokine secretion while it promoted expression of the scavenger receptor CD36 and efferocytosis [223].

There also conflicting data on the role of TRAIL signaling for macrophages during atherosclerosis, a condition in which these cells play a key contribution by accumulating lipids in atherosclerotic plaques [224]. In a macrophage cell line, TRAIL was suggested to promote the upregulation of the scavenger receptor SR-AI/MSR1 through p38 pathway activation, in an apoptosis-independent fashion, resulting in an increase in lipid uptake and foam cell formation [225]. Yet, a recent study reported no difference in scavenger receptor expression and phagocytosis capacity of *Trail*$^{-/-}$ *Apoe*$^{-/-}$ (apolipoprotein E) primary murine macrophages [226]. Instead, a lack of TRAIL signaling in *Apoe*$^{-/-}$ macrophages was associated in this study with impaired cholesterol export capacity resulting in intracellular cholesterol accumulation, a hallmark feature of atherosclerosis [226]. Taken together, the current literature generally supports the notion of a protective role of TRAIL for atherosclerosis in mice [226-229] (Figure 3, panel B).

Dendritic cells

Studies reporting a role for TRAIL, regardless of its pro-apoptotic activity, in modulating DCs are scarce. Yet, it has been found that Trail receptor-deficient DCs isolated from mice infected with MCMV or activated *in vitro* with lipopolysaccharide (LPS) showed increased IL-12 production [222]. Moreover, binding of TRAIL-R2 on DCs by TRAIL-expressing murine NK cells induce arginase-1 mRNA expression. This leads to reduced generation of MHC-class I-antigen peptide complexes and thus impaired $CD8^+$ T cell cross-priming by DCs [213]. While these studies suggest that TRAIL inhibits DC function in mice, another study reported that TRAIL stimulation promotes the functional maturation of human monocyte-derived DCs, including up-regulation of co-stimulatory molecules and, when combined with LPS, cytokine production [230]. Taken together,

TRAIL non-apoptotic signal transduction in DCs likely requires further investigation to understand its effect on these cells (Figure 3, panel C).

T cells

Several studies described apoptosis-independent effects of TRAIL on T cells activation and proliferation. Human T cell lines and primary cells stimulated with TRAIL were found to display reduced proliferation and cytokine production. Several studies highlighted a possible role for TRAIL in mediating suppression of T cell proliferation through inhibition of calcium influx and cell cycle arrest [231-233]. Similarly, other reports mentioned that TRAIL blockade enhances the proliferation and cytokine secretion of encephalitogenic T cells and the degree of EAE symptoms [205, 206]. This is in contrast to other findings suggesting that disruption of the TRAIL/TRAIL-R interaction on T cells isolated from mice with either type I diabetes or autoimmune arthritis also suppressed T cell proliferation by inhibiting cell cycle progression [201, 203]. The reason for such discrepancies in these different studies are unclear.

Nevertheless, the effect of TRAIL on T cell response seems to be dependent on the T cell subtype. For instance, while TRAIL signaling on $CD8^+$ T cells rather inhibits cell proliferation, it promotes $CD4^+$ T cell function and expansion [234]. In a model of induced murine lupus, TRAIL expression on effector $CD4^+$ T cells sustained their proliferation, thus supporting the production of autoantibodies by autoreactive B cells. At the same time, TRAIL to a lesser degree negatively affected $CD8^+$ T cells cytotoxicity. Yet, the mechanisms involved in this dichotomy were not elucidated [235]. Furthermore, EAE mice treated with DCs expressing simultaneously MOG (myelin oligodendrocyte glycoprotein) peptide and TRAIL showed reduced disease severity and MOG-specific T cells response [236]. Moreover, T cells *in vitro* activated with DC-MOG-TRAIL showed decreased proliferative response. In another study, the same authors reported that EAE inhibition in DC-MOG-TRAIL-treated animals was mediated via an increase in the number of $CD4^+$ $CD25^+$ regulatory T cells [237]. In line with these findings, TRAIL treatment inhibited autoimmune thyroiditis by inducing $CD4^+$ $CD25^+$ regulatory T cell proliferation in mice [238].

Apart from the canonical TRAIL signaling pathways triggered through cognate TRAIL receptors, a reverse signaling mediated by the cross-linking of plate-bound TRAIL-R and T cell-expressing TRAIL has been described. In these experimental conditions, costimulation of T cells with plate-bound TRAIL-R1 and anti-CD3 resulted in enhanced T cell proliferation and activation. In addition, TRAIL activated p38 MAPK to increase IL-2, IL-4 and IFNγ secretion by T cells [239, 240]. Additional studies revealed that TRAIL costimulation induces phosphorylation of the tyrosine kinases LCK and ZAP70, which are involved in transduction of the T-cell receptor (TCR) signaling pathway, resulting in NF-κB activation and T cell proliferation [241]. However, engagement of TRAIL-R during T cell activation prevents the recruitment of TCR-associated signaling molecules to lipid rafts, resulting in inhibition of T cell activation and proliferation [202, 204, 207, 242]. Therefore, TRAIL might either enhance or inhibit TCR-induced T cell proliferation depending on whether the signaling to the T cells is mediated through TRAIL itself or TRAIL-R, respectively. Although, other members of the TNF family ligands had been described to elicit reverse signaling, the existence of TRAIL reverse signaling is controversial due to the very short cytoplasmic moiety of TRAIL [243] (Figure 3, panel D).

B cells

Few studies showed that antibody production might be affected by TRAIL signaling on B cells, which was only reported for the IgG class. Administration of soluble TRAIL in autoimmune thyroiditis or TRAIL blockade using soluble TRAIL-R2 in autoimmune arthritis either inhibited or promoted IgG2a production, respectively [203, 244]. Accordingly, antibody-mediated neutralization of TRAIL increased serum auto-antibody levels, particularly IgG1 antibody, in autoimmune-prone FasL deficient (*gld/gld*) mice. Yet, these different works did not test whether these responses were due to a direct effect of TRAIL signaling on B cells. [245] (Figure 3, panel E).

Conclusion and Discussion

The initial observation that TRAIL preferentially triggers apoptosis of cancer cells led to a great number of studies, which sought to dissect the mechanisms involved in the sensitivity and resistance to apoptosis of cancer cells and healthy cells, respectively. In spite of substantial advance in the field, TRAIL signaling revealed to be much more complex than first thought, and a fair number of questions are still unsolved. The lack of knowledge on the precise molecular events triggered by TRAIL signaling in different cells and conditions is a major drawback to fully understand the physiological roles of the TRAIL pathway. This is due to the biological difficulties in dissect the cell-specific variations, the impossibility to avoid the crosstalk between pathways and the influence of the external stimuli to the cells. Another important unknown aspect is how the different pathways triggered upon TRAIL-R activation are regulated intracellularly. For instance, it is unclear whether there are, downstream of TRAIL-R, specific proteins or a precise mechanism responsible for the switch between apoptotic and non-apoptotic pathway in cancer cells or between pro-inflammatory and anti-inflammatory signaling in immune cells. Much similar to TRAIL, FasL/Fas signaling also exhibits pleiotropic signaling properties on healthy and cancer cells. Recently, an evolution-guided analysis suggested that the outcome of Fas signaling could be dependent on the phosphorylation status of the receptor death domain and the position of the phosphorylation. According to this study, the degree and site of the phosphorylation may mediate the switch between apoptotic and pro-survival signal in cancer cells [246]. Additional post-translational regulations, such as glycosylation, or selective engagement of any of the four TRAIL receptor or the ligand itself may also be at work to dictate which signaling pathway will be activated [247]. The TRAIL system gathers the most complex set of receptors amongst TNF members, yet most studies fail to explore, in an exhaustive manner, the role of each of these receptors, often focusing on either TRAIL-R1 or TRAIL-R2. Clearly, a better understanding of the molecular events involved in regulating apoptotic and non-apoptotic TRAIL signaling is needed, not only to understand TRAIL biology but also to envision the development of successful therapies relying on TRAIL or its derivatives.

TRAIL-based therapy – an outlook

Several aspects have to be considered before the use of recombinant TRAIL or TRAIL receptor agonists for clinical applications. Indeed, the development in cancer cells of TRAIL resistance is a major limitation of TRAIL therapy. To overcome this, the combination of TRAIL along with novel TRAIL-sensitizing agents may represent a clinical option to enhance TRAIL-mediated apoptotic effect [248-251]. However, several of these combined therapies may potentially also cause *in vivo* toxicity effects. Moreover, the identification of biomarkers that can predict the type of cancer that will respond to TRAIL-induced apoptosis is another important aspect to consider. As a matter of fact, certain factors have been described to correlate with resistance or sensitivity to TRAIL-induced apoptosis in cancer cells. For instance, high expression of the mRNA that encodes for uPA was shown to be associated with resistance to TRAIL-induced cell death [252]. As a consequence, its depletion in different cancer cell lines decreased basal ERK1/2 pro-survival signaling and reduced recruitment of TRAIL-R4 to the DISC upon TRAIL stimulation [252]. Elevated expression of GALNT14 enzyme – which is involved in the O-glycosylation of TRAIL receptor in cancer cells - was correlated with sensitivity to TRAIL. Such post-translation modification of the TRAIL-R promotes receptor clustering upon TRAIL binding, resulting in effective DISC formation and caspase-8 activation [253]. In addition, elevated expression of the homeobox protein SIX1 leads to TRAIL-apoptosis resistance in ovarian carcinoma cell [254]. Silencing of *SIX1* increased levels of apoptosis-related proteins, such as truncated BID, caspase-8 and -3, and bypassed TRAIL-resistance in ovarian cancer cells [255]. Recently, the expression patterns of the regulators involved in TRAIL pathway were used to develop a list of 11 markers that can predict, with 80-100% accuracy, the sensitivity of a melanoma cancer cell towards a combined therapy with TRAIL-R agonist and IAP antagonist. [256]. Thus, a successful future therapy comprises a combination of TRAIL signaling activator with strategies to prevent induction of resistance.

Therapies should also consider the quality of the intracellular signal induced by TRAIL. Even though TRAIL-R1 and TRAIL-R2 activate the same downstream pathways, cell death may be induced preferentially through one of these receptors depending on the cancer cell. For instance, in pancreatic and chronic lymphocytic leukemia cells, apoptosis is

mainly triggered through TRAIL-R1 [257, 258], while TRAIL-R2 is more active in glioblastoma and breast cancer cells [259, 260]. Thus, a targeted therapy using TRAIL receptor-specific agonist might be a better choice than the use of recombinant TRAIL.

Systemic delivery of TRAIL therapy should be careful evaluated for cancer treatment. As mentioned above, TRAIL may also direct impact the function of immune cells in many ways. It is well-established that the immune system is essential for treatment response and tumor development [261]. The ratio of cytotoxic $CD8^+$T cells to $CD4^+$ regulatory T cells present in the tumor microenvironment is important for the outcome of the immune response against cancer cells [262]. Interestingly, in the context of autoimmune diseases, TRAIL signaling was shown to inhibit $CD8^+$ T cells proliferation, while enhancing the activity and expansion of $CD4^+$ $CD25^+$ regulatory T cells [234]. Thus, it is important to investigate how the different T cells populations in the tumor microenvironment may be affected by TRAIL treatment in order to facilitate the development of an anti-tumor immune response.

Discrepancies and unresolved issues

As outlined in this review, many published studies generated conflicting findings. These discrepancies may be explained through differences in the type of cells that were investigated, the activation status of these cells, distinct disease contexts, and whether the TRAIL (or TRAIL-R) was analyzed for its function at the physiologic, endogenous level versus overexpressed or added as recombinant protein. Although *in vitro* tumor studies have made substantial contribution to our understanding of TRAIL signaling, it remains difficult to translate these findings to the complex dynamics found in *in vivo* tumor models, not to mention that mice only harbor one agonistic receptor, while humans express two TRAIL-Rs, namely TRAIL-R1 and TRAIL-R2. Therefore, not all aspects of the TRAIL signaling can be studied in mouse models [13]. Thus, it might be advantageous to develop more complex systems to study the activity of TRAIL *in vitro* – for instance by using 3D cell co-culture and organoids – in order to facilitate the translation into *in vivo* applications. Alternatively, the development of a humanized mouse expressing human TRAIL/TRAIL-R system could be a possible approach to dissect the role of each of these receptors in cancer cells and immune cells.

In conclusion, despite great advance in the knowledge of the function of TRAIL or TRAIL-R, many relevant aspects of TRAIL signaling in cancer and immune cells remain to be further elucidated.

Author contributions

LCA and PK wrote the manuscript and made the figures. NC and OM revised the text and provided insightful comments.

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Disclosure of conflict of interest

The authors declare that no conflict of interest exists.

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Figure legends

Figure 1

Contribution of non-canonical TRAIL to tumorigenesis. TRAIL/TRAIL-R signaling in transformed cells may lead to different outcomes. In apoptosis-resistant cancer cells, TRAIL signaling activates various pathways, i.e. NF-κB, PI3K-AKT-mTOR, MAPKs and PKC, resulting in apoptosis resistance, cell survival and proliferation. Furthermore, TRAIL may also trigger migration and invasion of cancer cells through activation of NF-κB, MAPKs, PI3K and SRC-STAT3 pathways. Furthermore, TRAIL-induced cytokines produced by the tumor can promote the recruitment and polarization of myeloid-derived suppressor cells (MDSCs), which in turn support cancer cells proliferation and migration. Figure adapted from stock images provided by Servier (https://smart.servier.com/smart_image/).

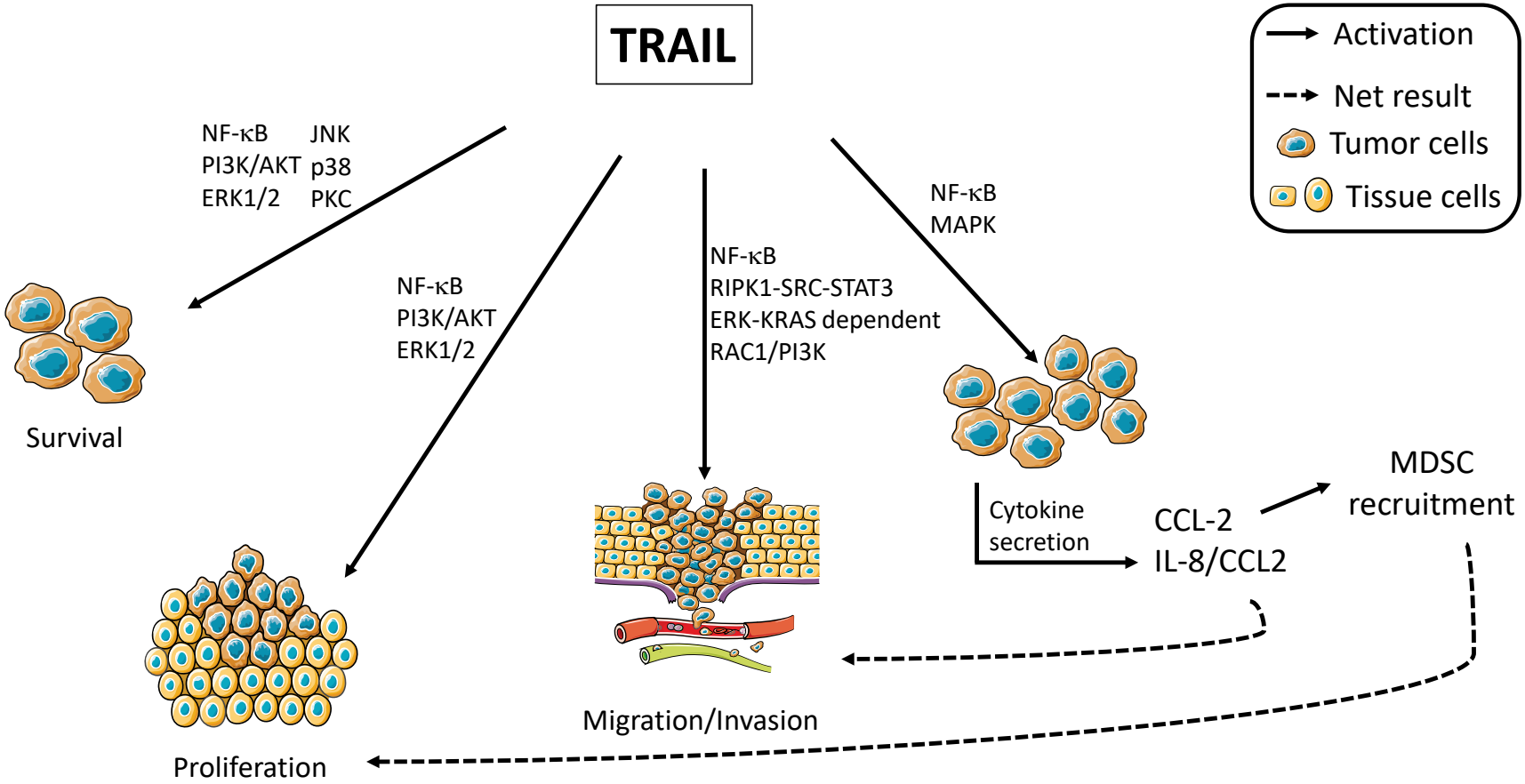

TRAIL
Activation
Net result
Tumor cells
Tissue cells
NF-κB
PI3K/AKT
ERK1/2
JNK
p38
PKC
NF-κB
PI3K/AKT
ERK1/2
NF-κB
RIPK1-SRC-STAT3
ERK-KRAS dependent
RAC1/PI3K
NF-κB
MAPK
Survival
Cytokine
secretion
CCL-2
IL-8/CCL2
MDSC
recruitment
Migration/Invasion
Proliferation

Figure 2

TRAIL/TRAIL-R signaling and NK cells. (A) TRAIL on NK cells engages its cognate receptor to elicit apoptosis of target cells (i.e. pathogen-infected cells, activated $CD4^+$ and $CD8^+$ T cells and tumor cells) following recognition of stress molecules by activating receptors or a reduction of MHC class I expression by inhibitory receptors on NK cells. TRAIL/TRAIL-R engagement on DCs induce arginase-1 mRNA expression, resulting in reduced MHC-class I-antigen peptide complexes and reduced activation of $CD8^+$ T cells. **(B)** In NK cells, TRAIL signaling promotes GZMB production by supporting the PI3K-AKT-mTOR pathway, thereby promoting NK cell cytotoxicity toward antigen-specific T cells. In addition, TRAIL inhibits IFNγ secretion by NK cells stimulated through the NK1.1 receptor. **(C)** NK cells engage TRAIL signaling in DCs to promote arginase-1 expression, thereby reducing the generation of MHC-class I-antigen peptide complexes and impairing $CD8^+$ T cell cross-priming. Figure adapted from stock images provided by Servier (https://smart.servier.com/smart_image/) and from Cardoso Alves *et al*. [211].

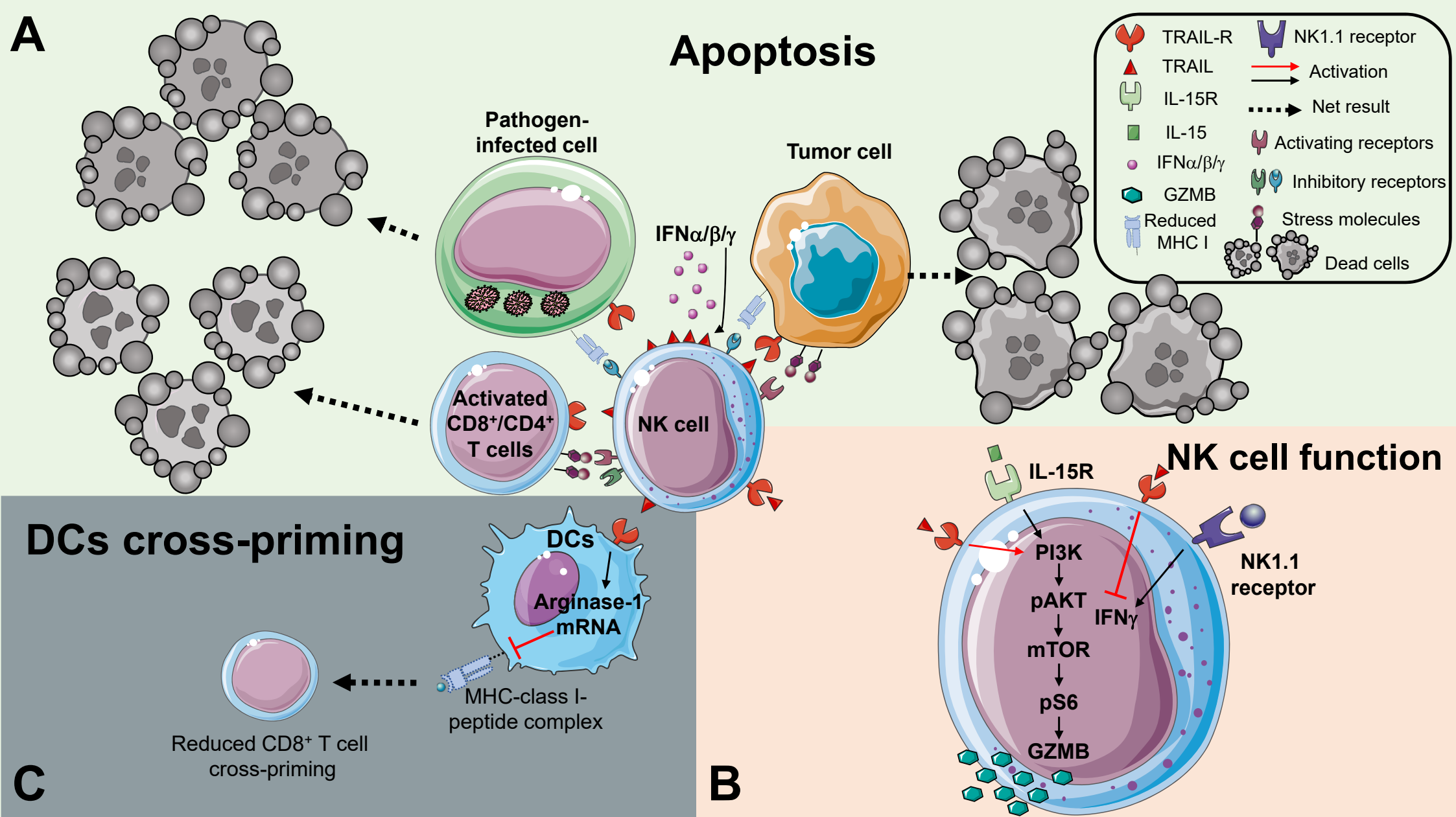
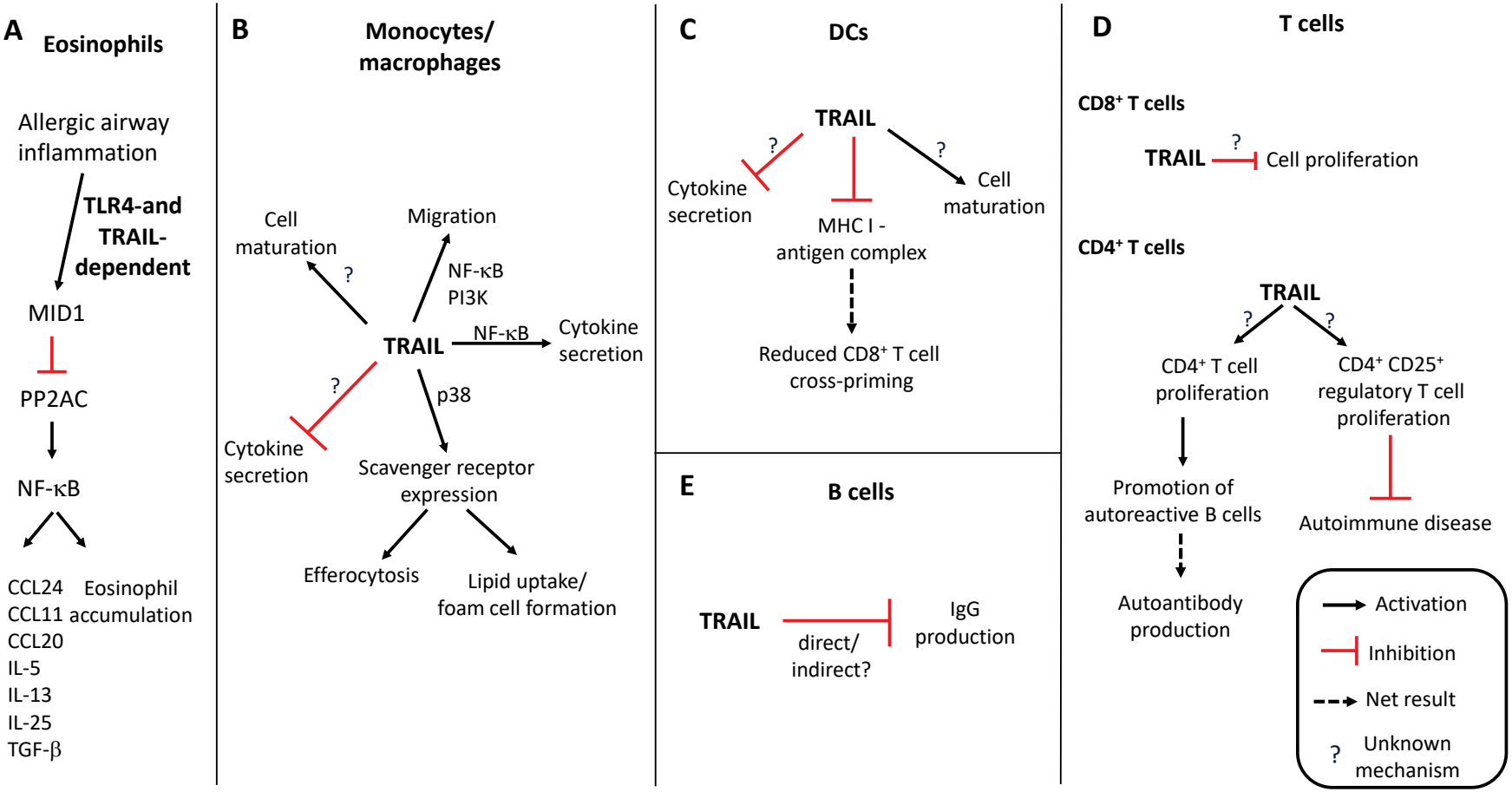

A
Apoptosis
Pathogen-infected cell
Tumor cell
IFNα/β/γ
Activated CD8+/CD4+ T cells
NK cell
TRAIL-R
TRAIL
IL-15R
IL-15
IFNα/β/γ
GZMB
Reduced MHC I
NK1.1 receptor
Activation
Net result
Activating receptors
Inhibitory receptors
Stress molecules
Dead cells
DCs cross-priming
DCs
Arginase-1 mRNA
MHC-class I-peptide complex
Reduced CD8+ T cell cross-priming
C
NK cell function
IL-15R
PI3K
pAKT
mTOR
pS6
GZMB
IFNγ
NK1.1 receptor
B

Figure 3

Non canonical TRAIL-mediated regulation of immune cell function. **(A)** During allergic inflammation, TRAIL and TLR4 promote the upregulation of MID1 in the airway wall, which in turn deactivates PP2AC, thereby resulting in NF-κB activation, pro-inflammatory cytokines and chemokines production and to an increase in the eosinophils accumulation. **(B)** In macrophages, TRAIL supports cell migration and cytokine secretion through activation of the NF-κB and PI3K pathways. However, in certain contexts, TRAIL may also inhibit the cytokine secretion in macrophages. In addition, TRAIL promotes cell maturation and the expression of scavenger receptor through activation of the p38 pathway, which leads to an increase in efferocytosis and lipid uptake. **(C)** In dendritic cells (DCs), TRAIL inhibits cytokine secretion and the generation of MHC-class I-antigen peptide complexes, which in turn impairs $CD8^+$ T cell cross-priming. Furthermore, TRAIL promotes DC maturation by an unknown mechanism. **(D)** In NK cells, TRAIL promotes GZMB production by supporting the PI3K-AKT-mTOR pathway, thereby leading to higher NK cell cytotoxicity and subsequently a reduced $CD8^+$ T cells response. In contrast, in certain conditions, TRAIL may impact NK cell cytotoxicity by curbing NK cell degranulation. TRAIL inhibits IFNγ secretion by NK cells that have been stimulated with an NK1.1 cross-linking antibody. **(E)** The effect of TRAIL on T cell response is T cell subtype dependent. TRAIL signaling inhibits $CD8^+$ T cells proliferation while it promotes $CD4^+$ T cell function and expansion. Although increased numbers of TRAIL-stimulated conventional $CD4^+$ T cells promote autoantibody production by autoreactive B cells, higher proliferation of TRAIL-stimulated $CD4^+$ regulatory T cells inhibits autoimmune disease **(F).** Through an unknown mechanism, TRAIL suppresses autoantibody production by autoreactive B cells.


A
Eosinophils
Allergic airway inflammation
TLR4-and TRAIL-dependent
MID1
PP2AC
NF-κB
CCL24
CCL11
CCL20
IL-5
IL-13
IL-25
TGF-β
Eosinophil accumulation
B
Monocytes/ macrophages
Cell maturation
?
Migration
NF-κB PI3K
TRAIL
NF-κB
Cytokine secretion
?
Cytokine secretion
p38
Scavenger receptor expression
Efferocytosis
Lipid uptake/ foam cell formation
C
DCs
TRAIL
?
Cytokine secretion
MHC I - antigen complex
?
Cell maturation
Reduced CD8+ T cell cross-priming
E
B cells
TRAIL
direct/ indirect?
IgG production
D
T cells
CD8+ T cells
TRAIL
?
Cell proliferation
CD4+ T cells
TRAIL
?
?
CD4+ T cell proliferation
CD4+ CD25+ regulatory T cell proliferation
Promotion of autoreactive B cells
Autoimmune disease
Autoantibody production
Activation
Inhibition
Net result
? Unknown mechanism