Molecular and functional determinants of the TRAIL-induced tumour-supportive secretome

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DECLARATION

I, Torsten Hartwig, hereby declare that the work presented in this thesis is original and my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is known for specifically killing a wide range of cancer cells in vivo, without toxicity to non-transformed cells. The initial enthusiasm about the therapeutic potential of TRAIL was however dampened in recent years, as it has become apparent that many human primary tumour cells are resistant to TRAIL-mediated apoptosis. In certain resistant cells, TRAIL-mediated non-apoptotic signals can even promote tumour progression migration and invasion which, as recently demonstrated by our laboratory, can involve cancer-cell autonomous mechanisms mediated by the endogenous TRAIL/TRAIL-R system independently of FADD. Interestingly, TRAIL has recently also been shown to induce the secretion of cytokines. Cytokines have been characterized as central orchestrators of the tumour microenvironment and can modulate its composition to either promote or inhibit tumour growth. However, it was not known whether and to which extent TRAIL/TRAIL-R signalling in cancer cells can affect the immune-microenvironment. Based on these findings, the aim of this dissertation was to study the role of the TRAIL-induced secretome in tumour biology. This thesis demonstrates that TRAIL-induced cytokine production from TRAIL-resistant cancer cells is FADD-dependent, and identifies the TRAIL-induced secretome to drive monocyte polarisation to M2-like myeloid derived suppressor cells (MDSC) and tumour-associated macrophages (TAM), two alternatively activated myeloid cell subsets. Strikingly, TRAIL-R suppression in tumour cells impaired CCL2 production and diminished both, MDSC accumulation in lungs of tumour-bearing mice and tumour growth. In accordance, the receptor of CCL2, CCR2, was required to facilitate increased MDSC recruitment and tumour growth. Finally, TRAIL and CCL2 are co-expressed with M2-like markers in lung adenocarcinoma patients. Collectively, endogenous TRAIL/TRAIL-R-mediated CCL2 secretion promotes the accumulation of alternatively activated myeloid cells in the cancer microenvironment, in favour of tumour growth. Hence, this dissertation reveals a novel tumour-supportive immune-modulatory role of the TRAIL/TRAIL-R system in cancer biology.

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1 Introduction

1.1 The TNF/TNF-receptor superfamily

Tumour Necrosis Factor (TNF) is a central molecule in tumour immunology and pro-inflammatory signalling. Its isolation and cloning by Aggarwal et al. 25 years ago laid the foundation for the discovery of 19 ligands which comprise the TNF superfamily (TNFSF) and 29 receptors, constituting the TNF-receptor superfamily (TNFR-SF) (Aggarwal, 2003, Aggarwal et al., 2012). TNFSF signalling results in a variety of different cellular responses which include the induction of cell death by apoptosis and necroptosis, proliferation, downregulation of the immune response as well as immunostimulatory, pro-inflammatory functions. In light of this vast array of functions, the most central role of TNF is its ability to induce cytokines and chemokines as part of the inflammatory cascade (Walczak, 2013).

The majority of the TNFSF proteins are expressed by immune cells such as monocytes, dendritic cells, B cells, T cells and natural killer (NK) cells and act as central regulators of the immune system (Aggarwal et al., 2012). Most TNFSF ligands are type II transmembrane proteins, with the exception of lymphotoxin alpha (LTα) and vascular endothelial growth inhibitor (VEGI) which are soluble and contain a carboxy-terminal TNF homology domain (THD), allowing the formation of active homotrimers (Warzocha et al., 1995). Since there are more receptors than ligands, some ligands bind to multiple receptors, allowing for an additional level of complexity in their regulation. TNFR-SF family members are type I transmembrane proteins which bind their respective ligands with a characteristic extracellular cysteine rich domain (CRD) (Warzocha et al., 1995). Six members also contain a conserved intracellular domain called the death domain (DD). Since this domain was first associated with enabling the transmission of cell death, this subgroup of TNFR-SF is also referred to as death receptors (DR). Intriguingly, some of these DRs have also been associated with the induction of non-apoptotic, pro-survival and pro-inflammatory responses as well as tumour promoting capacities, further highlighting the versatility of TNFSF members (Aggarwal, 2003). The most extensively studied death domain-containing TNFSF/TNFR-SF members showing this intriguing dipartite response are the TNF/TNF-R1, CD95L/CD95 and TRAIL/TRAILR systems (Walczak, 2013).

Although originally receiving its name for “tumour-necrotizing abilities”, the induction of cell death via TNF/TNF-R1 is only initiated as a secondary signal when its gene-activatory arm is obstructed. Therefore, TNF-R1 is now well known for its capacity to enable gene-activatory signalling via the mitogen activated protein kinase (MAPK) pathway and activation of the transcription factor nuclear factor kappa B (NF-κB) (Wajant et al., 2003). In fact, TNF-mediated NF-κB activation can facilitate tumour progression in liver, skin,

pancreatic, colorectal and lung cancer mouse models (Scott et al., 2003, Pikarsky et al., 2004, Egberts et al., 2008, Popivanova et al., 2008, Takahashi et al., 2010).

TNF binding to TNF-R1 promotes receptor trimerization, facilitating the recruitment of the DD-containing protein TNF-receptor associated death domain protein (TRADD) (Hsu et al., 1995) and receptor-interacting serine/threonine-protein kinase-1 (RIP1) to initiate the formation of the TNF-R1 signalling complex (TNF-RSC). TRADD recruits the ubiquitin ligase TNF-receptor associated factor-2 (TRAF2) which serves as a scaffold for cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2) recruitment (Hsu et al., 1996, Shu et al., 1996, Tsao et al., 2000). These two E3 ligases in turn ubiquitylate RIP1, which enables the binding of TAB2/TAB3 that subsequently promote the recruitment of transforming growth factor-*β* activated kinase-1 (TAK1). Next, IκB kinase (IKK) complex, composed of NEMO/IKKα/IKKβ is recruited and consequently activated by TAK1-mediated phosphorylation. The gene-activatory effector output of TNF-R1 is subsequently mediated by NF-κB and mitogen activated protein kinase (MAPK) activation (O'Donnell et al., 2007, Walczak et al., 2012). Our laboratory has recently uncovered that the TNF-RSC recruits the linear ubiquitin chain assembly complex (LUBAC) via cIAP's catalytic activity. LUBAC is composed of three subunits: SHANK-associated RH domain interacting protein (SHARPIN), heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1) and HOIL-1 interacting protein (HOIP) (Ikeda et al., 2011, Haas et al., 2009, Kirisako et al., 2006, Tokunaga et al., 2011). Its presence and linear ubiquitination activity, mediated by the catalytically active component HOIP, are required to mediate TNF-R1's gene-activatory output to its full, and physiologically required, extent (Haas et al., 2009). Thereby, presence of LUBAC tips the balance towards gene-activation and prevents TNF-mediated apoptotic cell death and auto-immune inflammation in mice (Gerlach et al., 2011).

Similar to TNF/TNF-R1, the CD95/CD95L system is also capable of inducing apoptotic and gene-activatory signalling, although induction of apoptosis is the default signalling output for CD95L. CD95's potent apoptotic effects were first identified in 1989 by the groups of Peter Krammer and Shin Yonehara in 1989, which generated the anti-APO-1 and anti-Fas antibodies respectively, and found them to potently induce cell death in patient derived leukemic cells and a rhabdomyosarcoma cell line (Trauth et al., 1989, Yonehara et al., 1989). Precipitation of the intracellular complex revealed the presence of a death-inducing signalling complex, in which the foremost components were shown to be Fas-associated death domain-containing protein (FADD) and caspase-8 (Boldin et al., 1996, Muzio et al., 1996).

TRAIL is the most recently discovered of the three death ligands and was first identified in 1995, based on high sequence homology with TNF and CD95L (Wiley et al., 1995, Pitti et al., 1996). As suggested by their high homology, TRAIL and CD95L signalling are also

mechanistically very similar, with both systems forming a death-inducing signalling complex (DISC) of the same composition. In accord, TRAIL was also found to potently induce cell death in cancer cells, which will be further discussed in the following chapters (Walczak et al., 1999). One decisive difference between TRAIL and CD95L was their applicability in cancer treatment. Although appealing due to its high potency in killing cancer cells, initial optimism about CD95L vanished when agonistic antibodies resulted in acute hepatotoxicity (Ogasawara et al., 1993). In contrast, TRAIL was shown to specifically kill tumour cells without harming non-transformed cells (Walczak et al., 1999, Ashkenazi et al., 1999). Ever since, vast efforts have been undertaken to harness TRAIL's potential for cancer therapy leading to novel, highly active, TRAIL agonists still under development today (de Miguel et al., 2016). It is therefore not surprising that in the field of cancer therapeutics, TRAIL has gained more attention than any other TNFSF member (Wajant et al., 2002).

1.1.1 The TRAIL/TRAIL-R system in humans and mice

TRAIL is a 35 kDa type II transmembrane protein which shares 65% identity between humans and mice and was first identified by screening an expressed sequence tag (EST) database (Pitti et al., 1996). Aside from being present as a membrane bound version, TRAIL is also found to be present in blood plasma at around 100 pg/ml under physiological conditions (Gibellini et al., 2007). It can be cleaved from the cell surface by to date unidentified proteases, while retaining its pro-apoptotic activity as a soluble ligand (Wiley et al., 1995). The death ligand is composed of four parts: a small cytoplasmic domain, a transmembrane helix, an extracellular stalk, and an extracellular TNF-like domain. The C-terminus contains the receptor binding region (Manzo et al., 2009). Within this region, the death ligand contains an unpaired cysteine residue (Cys230) which enables interaction with a zinc ion. This residue is crucial for TRAIL's function because it enables formation and stabilization into homotrimeric TRAIL complexes. Importantly, only the trimeric form of TRAIL is active in killing. This was shown by mutation of Cys230, which diminished trimerization, rendering the resulting monomeric forms of TRAIL inactive (Bodmer et al., 2000). TRAIL is expressed in various tissues including spleen, thymus, prostate and lung and particularly also by cells of the immune system. (Wiley et al., 1995).

In humans TRAIL is capable of interacting with five receptors, TRAIL-R1-4 and osteoprotegerin (OPG) (Figure 1). Since all five receptors are located on the same chromosomal locus, 8p21-22, it is feasible that they have emerged via gene duplication events. Only TRAIL-R1 (DR4) and -R2 (DR5) contain a full DD enabling pro-apoptotic signal transduction via these two death receptors (Walczak et al., 1997, Pan et al., 1997). The other three receptors, TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) (Degli-Esposti et al.,

1997b, Degli-Esposti et al., 1997a) and OPG (Simonet et al., 1997) are incapable of inducing an apoptotic signal.

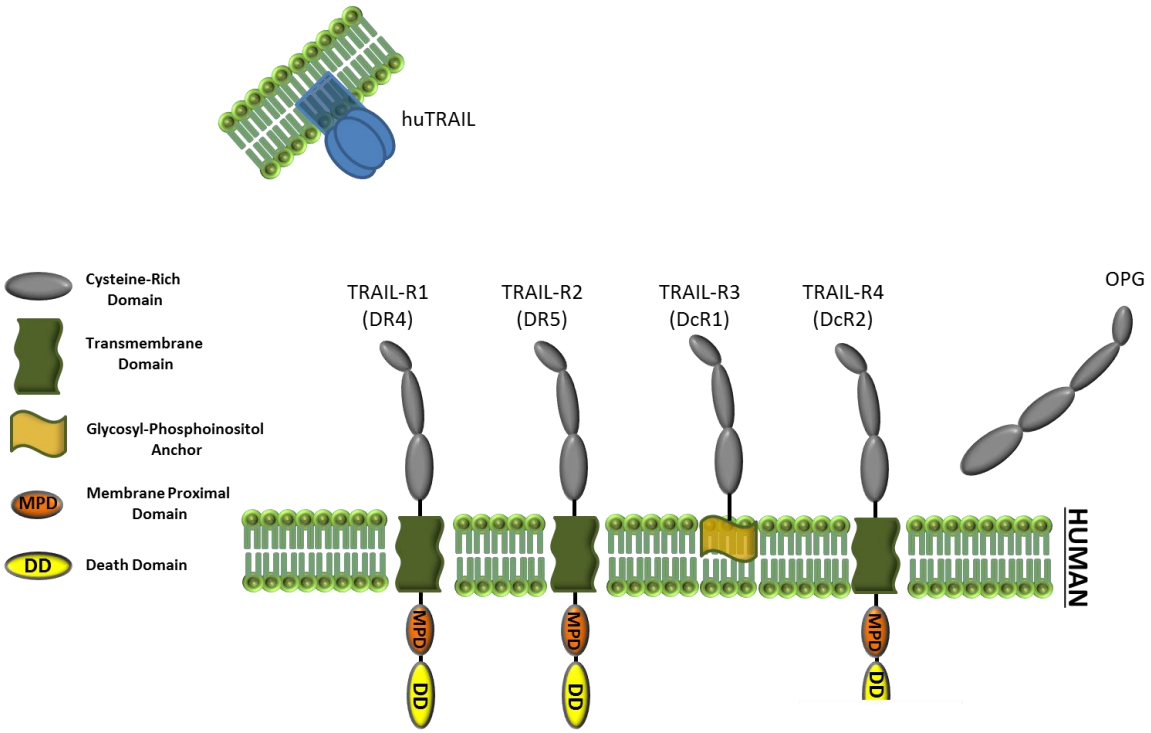


Figure 1. The TRAIL/TRAIL-R systems in humans and mice

TRAIL is a homotrimeric ligand with high homology among humans and mice. In humans, TRAIL can engage five closely related members of the TNFRSF. TRAIL-R1 and -R2 contain a death domain, enabling induction of apoptosis, and the membrane-proximal domain (MPD). The functions of TRAIL-R3 and -R4 have not been clearly defined, although it has been proposed that they can function as “decoy receptors”, as they are capable of inhibiting TRAIL-induced apoptosis upon overexpression. The mouse system only contains one death receptor, mTRAIL-R with equal homology to TRAIL-R1 and -R2. Mouse mDCTRAIL-R2 has two soluble splice variants mDcTRAIL-R2S and a transmembrane form mDcTRAIL-R2L.

TRAIL-R2 exists in two splice variants which differ in length by 23 amino acids and share overall high sequence identity with TRAIL-R1. However, the two receptors do differ significantly in their membrane proximal domain (MPD), a short, ten amino-acid-long stretch close to the plasma membrane. TRAIL was shown to bind TRAIL-R2 with the highest affinity at 37°C compared to other membrane-bound TRAIL-Rs, indicating that interaction of TRAIL with TRAIL-R2 might be favoured over interaction with any other TRAIL-R (Truneh et al., 2000). Regarding cancer progression, it is interesting to note that our laboratory has recently shown that TRAIL-R2 but not TRAIL-R1 is capable of mediating cancer cell-autonomous cancer progression via a MPD-Ras-related C3 botulinum toxin substrate 1 (Rac1) axis. It is likely that the differences in the MPD enable TRAIL-R2, but not TRAIL-R1 to promote cancer progression (von Karstedt et al., 2015).

The two death receptors are also posttranslationally modified by O-glycosylation, shown to regulate initiating events of TRAIL DR signalling. A study of expression profiles from 119 cancer patients revealed that GALNT14, an O-glycosylation initiating enzyme, was frequently overexpressed and correlated with TRAIL sensitivity in pancreatic carcinoma, non-small cell lung cancer (NSCLC) and malignant melanoma cell lines. Therefore, GALNT14 was suggested as a potentially interesting bio-marker for TRAIL sensitivity (Wagner et al., 2007). The sensitization was shown to be independent of death receptor expression but instead caused by GALNT14's ability to facilitate clustering of death receptors and subsequent DISC formation (Wagner et al., 2007).

TRAIL-R3 and TRAIL-R4 share high identity with the extracellular domains of the death receptors. Other than the death receptors, TRAIL-R3 completely lacks an intracellular DD while TRAIL-R4 only contains a truncated version thereof, rendering TRAIL-R3 and TRAIL-R4 unable to induce apoptosis (Degli-Esposti, 1999) (Figure 1). Instead, the two receptors have previously been proposed to act as "decoy receptors" (DcR1 and DcR2) by sequestering TRAIL and thereby preventing it from binding to the apoptosis-inducing receptors. Moreover, TRAIL-R3 and TRAIL-R4-expressing cells would show increased apoptosis resistance by decreasing the likelihood of TRAIL binding to TRAIL-R1 and TRAIL-R2. TRAIL-R4 has further been proposed to inhibit apoptosis induction by forming heterotrimeric complexes with TRAIL-R2 (Merino et al., 2006). Herein, the truncated DD of TRAIL-R4 was suggested to render the DISC non-functional by preventing cleavage of procaspase-8. However, this death-inhibitory effect was only demonstrated by strong overexpression of the two receptors. Therefore, the role of endogenous TRAIL-R3 and TRAIL-R4 remains rather controversial.

In addition to these four membrane spanning receptors, OPG was described to act as a soluble TRAIL receptor (Emery et al., 1998). OPG's physiological function arises from acting as a decoy for receptor activator of nuclear factor kappa-B ligand (RANKL) during osteoclastogenesis. Upon binding to its natural receptor, receptor activator of nuclear factor kappa-B (RANK), RANKL induces NF-κB to promote osteoclast-mediated bone resorption (Simonet et al., 1997). Thus, binding of OPG to TRAIL might prevent OPG from sequestering RANKL, thereby RANKL-RANK binding might be favoured, increasing bone resorption (Vitovski et al., 2007). However, TRAIL knock out (KO) mice did not show any defects in bone formation or homeostasis, indicating no involvement of TRAIL in these processes under physiological conditions (Cretney et al., 2002, Newsom-Davis et al., 2009).

Unlike humans, mice only possess one DD-containing TRAIL receptor, mTRAIL-R (mouse DR5, murine killer) (Figure 1 lower panel), which shares similar sequence identity with human TRAIL-R1 and -R2 and is thus capable of inducing apoptosis (Wu et al., 1999,

Walczak et al., 1997). Additionally, two non-death receptors which lack an intracellular DD, mDcTRAIL-R1 (mDcR1) and mDcTRAIL-R2 as well as the soluble mOPG have been identified in mice. Due to alternative splicing, mDcTRAIL-R2 is expressed as a secreted (mDcTRAIL-R2S) and a transmembrane form (mDcTRAIL-R2L). The murine non-death receptors were incapable of inducing cell death or NF-κB activation upon overexpression and their functions are still unclear (Schneider et al., 2003).

1.1.2 Molecular determinants of TRAIL-induced apoptotic signalling

Binding of TRAIL to its death receptors initiates receptor trimerization. TRAIL-R1 and TRAIL-R2 can form both homo- as well as heterotrimeric complexes capable of inducing apoptosis via the same pathway components. The receptor trimer initially recruits the intracellular adapter molecule FADD via its death domain. FADD enables the recruitment of the protease procaspase-8 to its death effector domain (DED) resulting in the formation of the DISC (Kischkel et al., 1995, Sprick et al., 2000). Employing quantitative mass spectrometry, Marion MacFarlane's group recently demonstrated that one FADD molecule is bound to trimeric TRAIL-Rs and surprisingly to 9 caspase-8 molecules (Dickens et al., 2012). Upon recruitment to FADD, procaspase-8 undergoes autocatalytic activation and is released into the cytosol, where it subsequently cleaves the effector caspase-3 and -7 (Peter et al., 2007). These in turn activate a multitude of substrates ultimately leading to the key characteristics of apoptosis such as DNA fragmentation, cell shrinkage and ultimately cell death (Wang and El-Deiry, 2003). The DISC has also been shown to contain additional factors such as the initiator caspase-10 and cellular FLICE inhibitory protein (cFLIP) (Thome and Tschopp, 2001, Sprick et al., 2002).

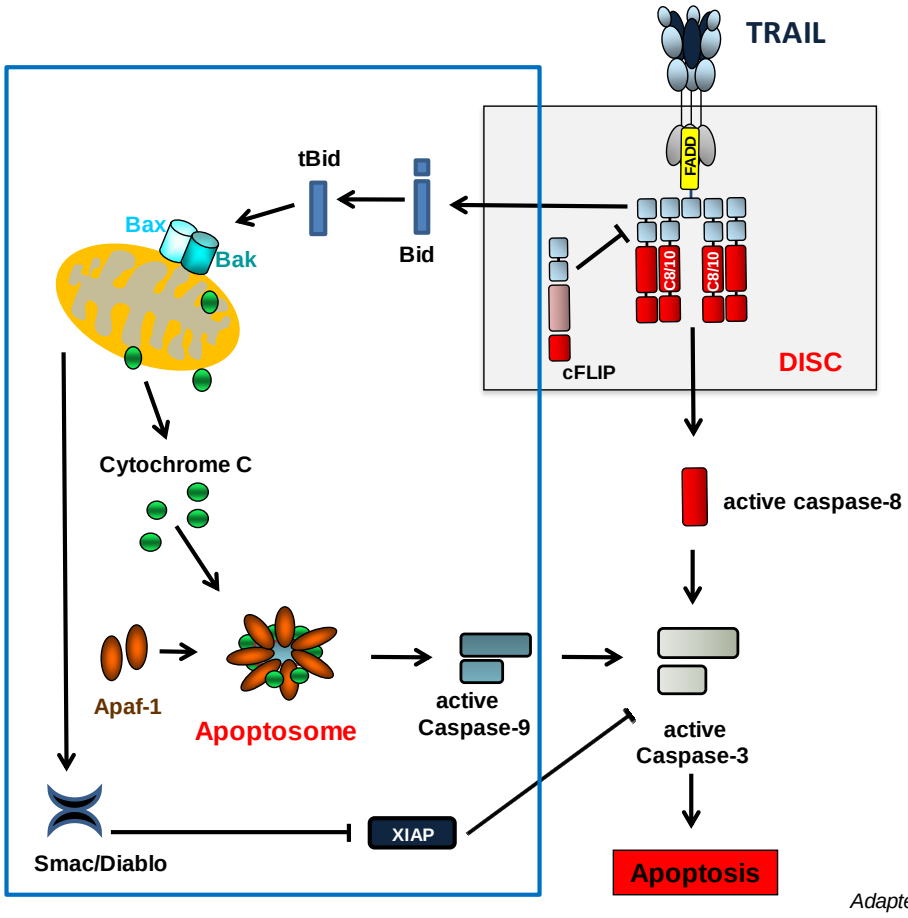
Caspase-10 is an initiator caspase which shares a homologous DED with caspase-8 and is therefore capable of binding FADD to form part of the TRAIL DISC (Sprick et al., 2002). Although it undergoes similar cleavage kinetics as caspase-8, caspase-10 has been shown to be unable of substituting for caspase-8 mediated events. Moreover, the ability to induce apoptosis in caspase-8 deficient Burkitt's lymphoma cells, which natively do not express caspase-10, was not rescued by stably expressing caspase-10 (Sprick et al., 2002). On the other hand, reports with T cells indicate the opposite. In this system, caspase-8 deficient cells were indeed sensitized upon transient expression of caspase-10 constructs (Kischkel et al., 2001). Since the caspase-10 expression systems differed along with the levels of caspase-10 expression and different cell lines were employed, it is feasible that the conflicting results are due to over-expression related artefacts or of contextual nature.

cFLIP is a molecule highly homologous to caspase-8 but lacks enzymatic activity. It exists in three main splice variants $cFLIP_L$, $cFLIP_S$ and $cFLIP_R$ all of which share high N-terminal sequence identity with both caspase-8 and -10 and also contain a FADD-binding DED

(Irmler et al., 1997) (Scaffidi et al., 1999) (Golks et al., 2005). The C-terminus of $cFLIP_L$ consists of two catalytically inactive caspase-like domains (p20 and p12), whereas the short C-terminus of $cFLIP_S$ is not homologous to procaspases. Unlike the two initiator caspases, cFLIP can inhibit apoptotic signalling by preventing the activation of initiator caspases, hindering apoptotic triggering (Irmler et al., 1997). Although $cFLIP_R$ is not well characterized, it is suggested to elicit its anti-apoptotic effects via a similar mechanism as $cFLIP_S$ (Golks et al., 2005). $cFLIP_L$ and $cFLIP_S$ can both bind to the DISC by interacting with caspase-8 via their DEDs, yet they differ in their functions and mechanism. When both $cFLIP_S$ and $cFLIP_L$ are absent, the DISC facilitates the association of two procaspase-8 molecules, which promotes their autocatalytic cleavage to induce the release of the catalytic caspase-8 subunits p10 and p20 (Micheau et al., 2002). If $cFLIP_S$ is present, it can form heterodimeric complexes with procaspase-8, which prevents autocatalytic cleavage and thereby renders caspase-8 inactive. $cFLIP_L$'s functions on the other hand are more controversial (Micheau et al., 2002). Some reports indicate that $cFLIP_L$ can function analogously to $cFLIP_S$ (Irmler et al., 1997), whereas others describe pro-apoptotic functions of $cFLIP_L$ (Micheau et al., 2002). Although its two C-terminal domains do not appear to possess any catalytic functionality, $cFLIP_L$ was demonstrated to increase procaspase-8 recruitment to the DISC and allow for the first cleavage of procaspase-8 to occur (Hughes et al., 2016, Micheau et al., 2002). In line with potential pro-apoptotic functions, cFLIP KO mice exhibit heart failure at embryonic day 10.5, which is the same phenotype seen in mice deficient in caspase-8 and FADD (Varfolomeev et al., 1998, Zhang et al., 1998). Whilst the cell death in the cFLIP KO could be attributed to apoptosis, the observed cell death in the caspase-8 and FADD KO mice was later attributed to necroptosis a recently discovered form of non-apoptotic cell death, covered in the next chapter (Bonnet et al., 2011, Kaiser et al., 2011, Kaiser et al., 2013, Welz et al., 2011, Dillon et al.).

Depending on the cell type (Type I or Type II), receptor stimulation activates apoptosis through two complementary pathways; the extrinsic (Type I cells) or intrinsic (Type II cells) pathway. Type I cells are characterized by high levels of DISC formation with proportionally high levels of active caspase-8. Therefore, extrinsic death ligand stimulation is sufficient to directly trigger apoptosis in these cells (Fulda and Debatin, 2002, Fulda et al., 2002a). Type II cells have weaker DISCs and also exhibit higher expression of the caspase-8 inhibitors X-linked inhibitor of apoptosis (XIAP) and cFLIP (Jost et al., 2009). Therefore, caspase-8 activation is often insufficient and requires further cell-intrinsic amplification to activate effector caspases and trigger apoptosis (Fulda et al., 2002a). This is accomplished by caspase-8 mediated cleavage of BCL-2 Interacting Domain (Bid) to truncated Bid (tBid) which interacts with BCL2-associated X protein (BAX) and BCL2 antagonist/killer 1 (BAK). BAX/BAK subsequently oligomerize to form pores in the

mitochondrial membrane which trigger mitochondrial outer membrane permeabilization (MOMP). Mitochondrial membrane permeabilization subsequently leads to the release of multiple pro-apoptotic factors such as cytochrome c and second mitochondria-derived activator of caspases/direct inhibitor of apoptosis protein-binding protein with low pI (SMAC/DIABLO). SMAC/DIABLO then counteracts XIAP, while cytochrome c induces the formation of a caspase-activating multi-protein complex, the apoptosome, thus allowing full activation of the effector caspase-3 and -7 and induction of apoptosis (Shi, 2004). Mitochondrial apoptosis can also be triggered by cellular stressors such as DNA damage or hypoxia. A brief model of apoptotic signalling is summarized in Figure 2.



Adapted from Kantari et al.,2014

Figure 2. Molecular determinants of TRAIL-induced apoptotic signalling

The extrinsic and intrinsic (blue box) pathways share several factors in promoting the execution of apoptosis. TRAIL-mediated death receptor trimerization promotes the recruitment of FADD which enables the recruitment of caspase-8 to the form the DISC. In type I cells, caspase-8 is activated and directly activates caspease-3 to trigger apoptosis by the extrinsic pathway. Type II cells have higher levels of anti-apoptotic proteins such as cFLIP and XIAP and therefore require further amplification of the extrinsic pathway via MOMP. Caspase-8 mediated activation of tBid triggers BAX/BAK to induce MOMP. In turn, cytochrome c and Apaf-1 are released to form the Apoptosome which activates caspase-9, to enable amplification of caspase-3 activation and apoptosis.

1.1.3 Apoptotic cell clearance

Apoptotic death is part of the everyday homeostatic turnover of cells and therefore rather immunologically silent. To achieve this, apoptotic cells do not permeabilize the membrane but instead undergo an internal dismantling of cellular components via caspase mediated cleavage (Green et al., 2009). Thereby, the release of highly immunogenic proteins, endogenous danger associated molecular patterns (DAMPs), is prevented to minimize immunogenicity and tissue stress (Green et al., 2009, Kono and Rock, 2008). Apoptotic cells and the debris remaining post-death still need to be cleared from the tissue, which is achieved by eliciting what has been termed “find me” and “eat me” signals. These signals, released by dying cells, enable the recruitment and engulfment by resident phagocytic cells, respectively (Cullen et al., 2013, Ravichandran, 2010). Examples of previously identified find me signals include fractalkine and sphingosine 1 phosphate, which are only released at very low concentrations and quickly degraded (Truman et al., 2008). Therefore, chemoattractive gradients by find me signals have been postulated to only have a very short range and are only capable of attracting nearby, resident phagocytes. However, some recent reports indicate that apoptotic death, caused by CD95L engagement can be associated with the release of inflammatory chemo-/cytokines (Cullen et al., 2013). These events were however not directly dependent on apoptosis and could be uncoupled from death via caspase inhibition. Since chemokines are more stable than previously identified find me signals and therefore have a longer range, chemokine release may enable apoptotic cells to chemoattract immune cells outside the bounds of the tissue. This hypothesis however still requires further validation, particularly regarding to its role *in vivo*. Other than find me signals, eat me signals promote the engulfment by phagocytic cells and include phosphatidylcholine (Fadok et al., 1992, Fadok et al., 2000). This lipid is normally contained in the inner leaflet of the plasma membrane but gets exposed on the outer side during the membrane permebilization stages of apoptosis. Although this process is deemed to be caspase-dependent, its molecular mechanism still remains elusive. It is however known that phosphatidylcholine can activate phagocytes to engulf the late stage apoptotic cell as well as facilitate the release of anti-inflammatory cytokines by macrophages (Savill et al., 2002). One such example is transforming growth factor-beta (TGFβ) which prevents further release of pro-inflammatory factors from the activated macrophages (Chen et al., 2001). Overall, previous work indicates that apoptosis is indeed of non-immunogenic or rather tolerogenic nature (Green et al., 2009).

1.1.4 Necroptosis – a non-apoptotic form of cell death induced by TRAIL

Necroptosis is a caspase-independent, programmed form of non-apoptotic cell death, involved in the regulation of immunity and inflammation. Other than apoptosis, necroptosis involves bursting of the cell membrane, leading to the release of DAMPs such as IL-33 and HMGB. Their release elicits strong inflammatory responses alerting the immune

system of imminent danger, such as invading pathogens (Kono and Rock, 2008). Necroptosis is mediated by the action of RIP1 and RIP3, which form the core of the necrosome complex, as well as the executer mixed linkage kinase domain like pseudokinase (MLKL), and was initially described to be induced by TNF (Degterev et al., 2005, Vercammen et al., 1998, Murphy et al., 2013, Vandenabeele et al., 2010). Mechanistically, association within the TNF-RSC promotes RIP1 activation that leads to release of RIP1 into the cytoplasm. Next, RIP1 associates with RIP3 via RHIM domain-mediated interaction which promotes RIP3-mediated phosphorylation of MLKL. MLKL in turn undergoes oligomerization, promoting its translocation to the plasma membrane via exposure of its positively charged N-terminal helix bundle, which exhibits low affinity for to polar phospholipid head groups (Dondelinger et al., 2014). Upon recruitment to the membrane, further conformational changes in MLKL lead to the exposure of additional phospholipid binding sites promoting the integration of MLKL oligomers into the membrane (Quarato et al., 2016). Thereby MLKL oligomers decrease membrane integrity to initiate necroptosis. Recent evidence further demonstrates that MLKL mediated disruption of membrane integrity and necroptosis induction is counteracted by the ESCRT-III machinery. In line with this, transient knockdown of ESCRT-III components significantly induced MLKL-dependent necroptosis of L929 cells (Gong et al., 2017, Yoon et al., 2017). It has been postulated that the ESCRT-III machinery antagonizes necroptosis by facilitating the secretion of MLKL, via extra cellular vesicles. Since active caspase-8 dimers cleave RIP1 and RIP3, and FADD is required for caspase-8 activation, caspase-8 and/or FADD KO facilitate necroptotic cell death. Accordingly, the lethal phenotype of caspase-8 or FADD knockout (KO) mice, which succumb to enhanced necroptosis, is rescued by co-deletion of RIP3 (Bonnet et al., 2011, Kaiser et al., 2011, Kaiser et al., 2013, Welz et al., 2011). cFLIP KO mice on the other hand were not rescued by co-deletion of RIP3, but instead require an additional FADD KO to be rescued. The fact that FADD KO can rescue cFLIP RIP3 double KO (DKO) mice indicates that it is likely to be increased apoptotic death which leads to heart failure and embryonic death in the DKO mice (Dillon et al., 2012).

First evidence from the CD95/CD95L system, mechanistically very similar to TRAIL signalling, indicated that other death receptor systems may also induce necroptosis. Thus, CD95L-induced cell death in L929 cells could not be entirely rescued by caspase inhibition (Vercammen et al., 1998). Studies on activation induced cell death (AICD) finally revealed that that both CD95L as well as TRAIL were able to induce RIP1-dependent necroptotic cell death in Jurkat-T cells (Holler et al.2000). It was further shown that particularly the kinase activity of RIP1 was required for mediating necroptosis via identification of the specific RIP1 inhibitor necrostatin-1, capable of blocking necroptotic cell death (Degterev et al., 2005). It is currently under investigation whether TRAIL forms a similar necrosome

complex as TNF and whether TRAIL is indeed capable of eliciting pro-inflammatory outputs similar to those induced by TNF-mediated necroptosis (Azijli et al., 2013). Since the detailed aspects of necroptosis are not part of the scope of this thesis, they will not be discussed further but have been reviewed in detail elsewhere (Vandenabeele et al., 2010).

1.1.5 Molecular determinants of TRAIL-induced non-apoptotic signalling

TRAIL's ability to specifically kill tumour cells is well-established. Given their apparent sensitivity to TRAIL-induced cell death, it seems paradoxical that cancer cells would still retain TRAIL-R expression. Interestingly, data from stage III non-small cell lung cancer patients (NSCLC) indicated high TRAIL-R expression. Furthermore, in renal cell cancer both TRAIL-R1 and -R2 expression negatively correlated with patient survival (Spierings et al., 2003, Macher-Goeppinger et al., 2009). These observations suggest relevance for non-apoptotic, cancer-promoting functions of the TRAIL/TRAIL-R system. Non-apoptotic functions of TRAIL-R signalling are mediated by two signalling arms requiring the Membrane proximal domain (MPD) or the DD of TRAIL-R (Figure 2.1) to promote cell invasion, survival and proliferative pathways (Ehrhardt et al., 2003) (von Karstedt et al., 2015, Hartwig et al., 2017). MPD-mediated non-apoptotic signalling occurs via activation of Rac1, a GTPase of the RAS superfamily of small GTP-binding proteins, and will be further covered in section 1.2.2. DD-mediated signalling can promote the activation of transcription factors such as NF-κB and members of the MAPK families which have also been linked to pro-inflammatory signalling in the context of TNF (Schneider et al., 1997, Lawrence, 2009).

NF-κB, a central transcription factor in mediating expression of pro-inflammatory cytokines, has been shown to be activated by TRAIL binding to TRAIL-R1, -R2 and -R4 (Degli-Esposti et al., 1997a, Schneider et al., 1997). In line with these findings, the receptor interacting protein RIP1, which is involved in TNF mediated NF-κB activation, has also been identified in the TRAIL DISC (Li and Lin, 2008, Varfolomeev et al., 2005). By employing RIP1 or IKK-negative cells, TRAIL-signalling was demonstrated to require these factors to mediate NF-κB activation and thereby promote survival and proliferation of TRAIL-resistant Jurkat cells (Ehrhardt et al., 2003). Although the exact mechanistic details of how TRAIL induces NF-κB activation remain elusive, a secondary intracellular complex similar to the DISC has been deemed responsible (Varfolomeev et al., 2005). This complex, recently named the 'FADDosome', was shown to contain FADD, caspase-8, RIP1, TRAF2 and NEMO, whereas other studies have also indicated a role of TRADD (Henry and Martin, 2017, Jin and El-Deiry, 2006). Within this complex, NEMO was suggested to recruit IKKα/β, thereby facilitating phosphorylation and consequent proteasomal degradation of the inhibitor of κB (IκB), promoting release of NF-κB (Varfolomeev et al., 2005). NF-κB then translocates to the nucleus where it can activate transcription of pro-inflammatory cytokines such as

CCL2, IL-8, CXCL1, CXCL5 as well as various anti-apoptotic genes such as cFLIP and Mcl-1, explaining previous observations in Jurkat cells (Henson et al., 2003). Early studies in leukemic and non-leukemic cancer cells showed that non-apoptotic TRAIL-induced NF-κB promotes proliferation independently of FADD and caspase-8, two core components of the DISC (Ehrhardt et al., 2003). However, another report claims that caspase-8 and FADD are required for TRAIL-mediated NF-κB activation, rendering the underlying mechanism elusive (Varfolomeev et al., 2005).

Next to NF-κB, TRAIL is also implicated in the activation of the MAPK member c-Jun N-terminal kinase (JNK), which controls central physiological processes such as gene expression, cell proliferation and inflammation. JNK activation was previously shown to require the TRAF2-MEKK1-MKK4-signalling axis in HEK293 cells, and additionally requiring RIP1 in prostate cancer cells (Hu et al., 1999, Lin et al., 2000). This observation is further supported by TRAF2 and RIP1 presence and requirement in secondary complex-mediated JNK activation in fibrosarcoma cells (Varfolomeev et al., 2005). JNK mediates its effects by phosphorylating and activating the transcription factor AP1 which has also been linked with promoting the induction of various cytokines such as IL-8, CXCL1 and CXCL2 (Karin, 1995). Interestingly, direct inhibition of JNK via siRNA sensitized hepatocellular carcinoma (HCC) cells to TRAIL. This indicates that JNK can mediate TRAIL resistance, whilst a role of JNK in TRAIL-mediated cytokine induction was not investigated (Mucha et al., 2009).

NF-κB as well as MAPK activation is regulated by the TGFβ activated kinase (TAK1) (Sakurai et al., 1999). The MAP3K family member is activated upon interaction with TAK1 binding protein (TAB1) enabling phosphorylation of IKK and subsequent initiation of NF-κB signalling as detailed above. Interestingly, TAK1 has previously been suggested to mediate TRAIL resistance in Hela cells via NF-κB and JNK activation, as transient knockdown of TAK1 decreased gene activation and sensitized to TRAIL-induced apoptosis (Choo et al., 2006). TAK1 therefore appears to be an important factor in TRAIL's non-apoptotic signalling machinery, although evidence regarding its role in TRAIL-induced cytokine induction is lacking.

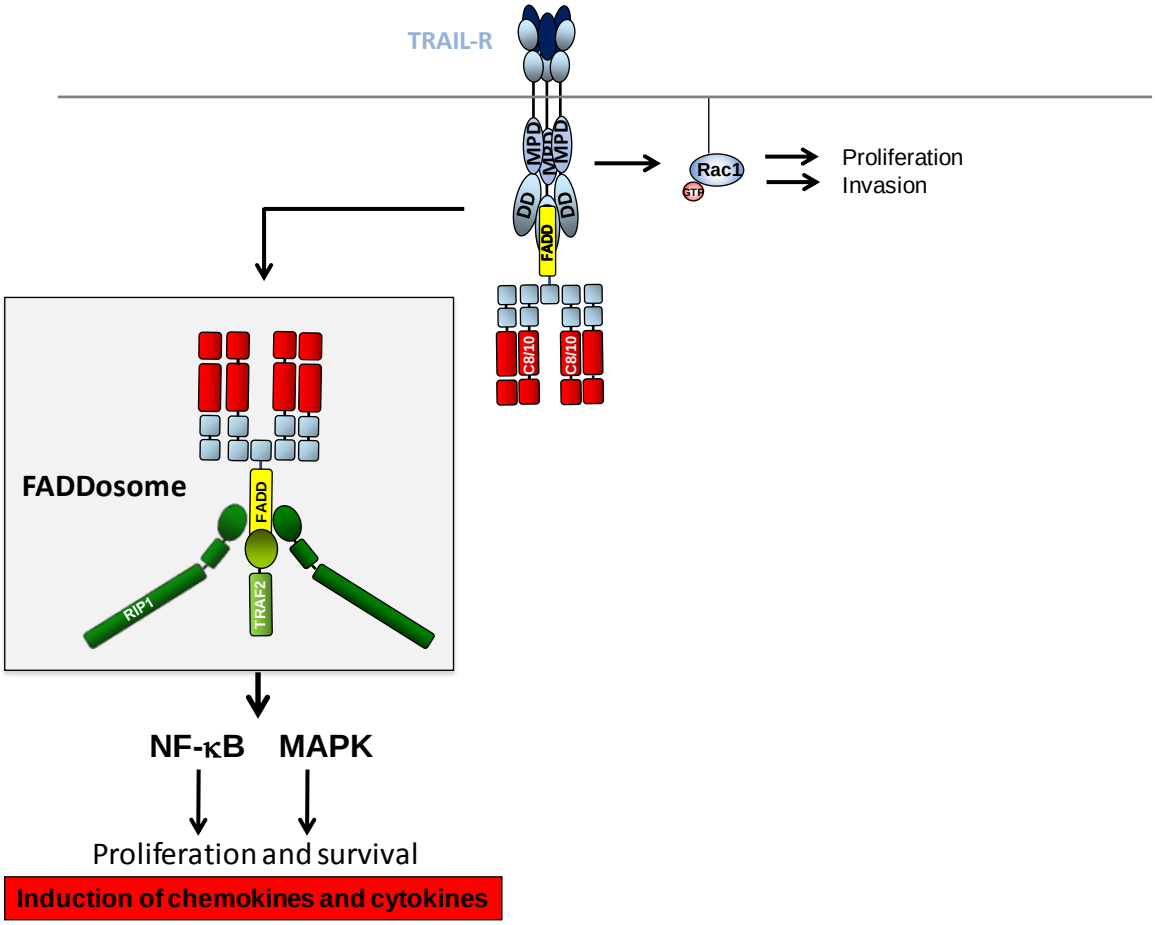


Figure 2.1 Molecular determinants of TRAIL-induced non-apoptotic signalling

TRAIL-mediated non-apoptotic signalling occurs via the Membrane Proximal Domain (MPD) or the DD of TRAIL-R. MPD-dependent signalling is mediated via Rac1 activation to elicit proliferation and invasion in *KRAS*-mutated cells (see section 1.2.2). DD-dependent signalling can occur upon formation of a secondary intracellular complex similar to the DISC (Varfolomeev et al., 2005). This complex, recently named the 'FADDosome', facilitates induction of gene activatory pathways which can elicit cell proliferation, survival and the induction of chemokines and cytokines.

1.1.6 Mutational landscape in TRAIL/TRAIL-R signalling

Mutations in TRAIL-R1 and -R2 have been identified in several human cancers including HNSCC, Lymphoma and NSCLC (Lee et al., 1999, Fisher et al., 2001, Lee et al., 2001, Shin et al., 2001). TRAIL-R2 and in particular its death domain are prominently affected by mutations as evidenced by a study demonstrating that 10% of NSCLC patients harboured mutations in the DD, which included eight missense alterations (Lee et al., 1999). Interestingly, some of these mutations have been found repeatedly, suggesting that they are selected for during cancer progression and may thus have tumour-promoting effects. Using expression constructs for the mutated versions of TRAIL-R2, McDonald et al. demonstrated that DD point mutants exhibit decreased induction of apoptosis; thereby explaining the possible positive selection for these mutants in cancer (McDonald et al., 2001). In light of the potential tumour supportive effects of TRAIL/TRAIL-R signalling, it

would be interesting to determine to which extent non-apoptotic TRAIL-signalling was impacted by the mutations in TRAIL-R2.

Regarding expression levels, patient derived melanoma and lung tumour tissues exhibited upregulated TRAIL-R1 and -R2 expression as determined by antibody staining (Daniels et al., 2005). Furthermore, samples from stage III unresectable *non-small cell lung cancer* (NSCLC) patients, analyzed by Spierings et al., were strongly positive for TRAIL-R1 and/or -R2, whilst high TRAIL-R2 expression correlated with increased risk of death (Spierings et al., 2003). In line with this, high TRAIL-R2/TRAIL expression negatively correlates with disease free survival in renal cell cancer (Macher-Goeppinger et al., 2009) and TRAIL-R2/R4 expression in breast cancer (Ganten et al., 2009). TRAIL-R1 and –R2 expression is also upregulated in pancreatic cancer samples as compared to normal control tissue (Ozawa et al., 2001). In line with this, chronic pancreatitis, which is known to be a risk factor for pancreatic cancer, presents with elevated TRAIL expression in sites of numerous inflammatory infiltrates (Hasel et al., 2003). Interestingly, studies have revealed that TRAIL-R expression is particularly high at the invasive edge of cancers (Strater et al., 2002). Even though it is possible that TRAIL-Rs are just paving the way at the invasive edge via apoptosis, it is also possible that the previously introduced non-apoptotic, geneactivatory TRAIL-signalling enhances tumour progression; thereby explaining the upregulation of TRAIL-Rs.

Downstream of the TRAIL-Rs, somatic mutations have also been observed in FADD and Caspase-8, albeit to a lesser extent that in the receptors. Only one somatic mutation of FADD was found in a study analysing 116 stomach and 98 colon cancers, whilst 4 out of 80 NSCLC patients exhibited mutations in FADD (Shin et al., 2002, Soung et al., 2004). These missense mutations were detected in the DD and DED of FADD and possibly decrease FADD's ability to bind to TRAIL-R1/2 and recruit Caspase-8 respectively. In line with this, overexpression of the FADD mutants decreased apoptosis induction as compared with wildtype FADD. Regarding FADD expression levels, FADD protein was also strongly decreased in murine thyroid adenoma/adenocarcinoma (Tourneur et al., 2003). Furthermore, samples from acute myeloid leukaemia patients exhibited low expression or complete absence of FADD, which correlated with poor clinical outcome (Tourneur et al., 2004). On the other hand, more recent studies show that FADDs locus on chromosome 11q13.3 is frequently amplified in human squamous cell carcinoma of the head and neck (HNSCC) resulting in increased FADD abundance (Gibcus et al., 2007). Interestingly, increased FADD levels correlate with aggressive disease and poor prognosis in HNSCC and NSCLC, possibly implicating FADD with tumour supportive roles herein (Gibcus et al., 2007, Chen et al., 2005). The discrepancies in FADD expression levels in different cancers might be related to cell-type specific differences in the signaling output

elicited by FADD. It is possible that changes in FADD expression could result in either loss of apoptosis, or gain of the previously described non-apoptotic, tumour supportive functions. Thus, understanding the mechanisms that modulate the nature of FADDs signaling output could potentially reveal molecular switches which shift the non-apoptotic arm towards the apoptotic arm.

A large scale study on caspase-8 by Soung et al. screened 162 gastric carcinoma patients, 93 breast carcinoma patients, 185 NSCLC patients and 88 leukaemia patients for mutations in caspase-8 using PCR-single-strand conformation polymorphism (SSCP) (Soung et al., 2005). Herein, caspase-8 mutations were mainly detected in gastric cancers and were particularly pronounced in 13 out of 122 advanced gastric cancers. The caspase-8 mutants reduced apoptosis induction as compared to wildtype caspase-8. Although 3 missense and one in frame deletion mutation occurred in caspase-8's DED, the caspase was still capable of interacting with FADD and readily co-precipitated with the adaptor. However, mutated caspase-8 proteins are frequently unable to promote apoptosis whilst geneactivatory signalling remains intact (Ando et al., 2013). Therefore, caspase-8 mutations tend to shift TRAIL-signalling towards the non-apoptotic pathway and might drive TRAIL/TRAIL-R-mediated tumour supportive functions.

Frequent mutation and upregulation, particularly of the TRAIL receptors in various cancers, indicates that TRAIL signalling can contribute to cancer progression. Although several apoptosis inactivating mutations and changes in expression levels have been found for core components of the TRAIL pathway, the effect of these alterations on non-apoptotic signalling requires further investigation. Since non-apoptotic signalling can potentially promote cancer progression, it would be interesting to determine whether the mutational landscape of TRAIL signalling not only functions to benefit cancer by inhibiting apoptosis, but possibly also by tumour supportive non-apoptotic signalling.

1.2 The physiological role of the TRAIL/TRAIL-R system

In order to determine the physiological role of TRAIL, Cretney et al. and Sedger et al. developed TRAIL knockout mice in 2002 (Cretney et al., 2002, Sedger et al., 2002). Following the development of these mice, TRAIL-R KO mice were generated by us and others (Diehl et al., 2004, Finnberg et al., 2005, Grosse-Wilde et al., 2008). Surprisingly, neither of these mice exhibited any obvious phenotype. They were viable, fertile and contained normal bone density, contradicting any role of TRAIL-R in osteoclastogenesis. TRAIL and TRAIL-R KO mice also did not show any enhanced susceptibility towards spontaneous tumour formation in an otherwise wildtype background. However in the lymphoma-prone Eμ-myc genetic background, loss of TRAIL-R reduced lymphoma free survival, whilst enhanced metastatic ability of squamous cell carcinoma was observed in

DMBA/TPA-treated TRAIL-R KO mice, indicating an immune surveillance role of TRAIL (Grosse-Wilde et al., 2008, Finnberg et al., 2008). Although several lines of evidence indicate an immune-regulatory role for TRAIL upon pathological challenge, it is feasible that lack of a gross phenotype is due to redundancy in TRAIL's function (Schaefer et al., 2007).

1.2.1 The role of TRAIL/TRAIL-R in the immune system

Although TRAIL mRNA is expressed constitutively, TRAIL protein is mainly expressed on the surface of innate and adaptive immune cells, indicative of a potential role in the immune system (Falschlehner et al., 2009). Herein, TRAIL expression is regulated in a stimulation-dependent manner, with type I and type II interferons (IFN) playing a particularly prominent role. As such, IFN-γ can increase TRAIL surface expression on monocytes, dendritic cells (DC) and NK cells (Fanger et al., 1999).

In mice, NK cells highly express TRAIL already during early development in the liver, which is under control of autocrine IFN-γ (Takeda et al., 2005). It was shown that TRAIL expression enables NK cells to kill immature DCs (Hayakawa et al., 2004). In fact, surface-expressed TRAIL has been demonstrated to be one of the effector mechanisms of NK cells, promoting their cytolytic, antiviral and antitumour activity (Sato et al., 2001, Kayagaki et al., 1999). In line with this, NK cell ability to eliminate virally infected cells was demonstrated to be dependent on TRAIL, as ezephalomyocarditis virus (ECMV)-infected mice had higher viral titers if TRAIL was blocked (Sato et al., 2001). In this study, TRAIL expression on NK cells was dependent on IFN-α and -β produced by virally infected cells. Apart from NK cells, $CD8^+$ T cells have also been shown to utilize surface TRAIL in killing (Brincks et al., 2008). Brincks et al. demonstrated that influenza virus-infected TRAIL KO mice have higher virus titers than their wildtype counterparts. Importantly, influenza virus mediated lethality could be rescued by adoptive transfer of $CD8^+$ T cells from wildtype but not from TRAIL KO mice confirming this finding. TRAIL-R has also been implicated in viral defense. In line with this, TRAIL-resistant fibroblasts infected with human cytomegalovirus (HCMV) were shown to upregulate TRAIL-R, thereby enabling their sensitization to TRAIL-mediated apoptosis (Sedger et al., 1999). To investigate the role of TRAIL-R in antiviral responses in mice, Diehl et al. infected TRAIL-R KO and wildtype mice with murine cytomegalovirus (MCMV). Surprisingly, TRAIL-R deficient mice were more resistant to MCMV than wildtype mice (Diehl et al., 2004). The mice also contained macrophages and DCs which express increased levels of IL-12 and TNF, thereby implicating TRAIL-R with a potential downregulation of the innate immune system. Although discrepancies remain, strong evidence does suggest that the TRAIL/TRAIL-R system is involved in regulation of the immune systems response to viral challenges.

Next to viral responses, TRAIL has also been associated with establishing immune-tolerance particularly regarding T cell functionality. A study by Gurung et al. using ovalbumin (OVA)-immunized mice demonstrated that TRAIL-expressing $CD8^+$ regulatory T cells can kill antigen-reactive T cell clones dependent on TCR expression (Gurung et al., 2010). Accordingly, neither TRAIL- nor TRAIL-R-deficient mice developed a tolerogenic response (Gurung et al., 2010). TRAIL can also regulate the homeostasis of a particular type of T cells, so called 'helpless T cells'. Their name derives from a lack of 'help' from $CD4^+$ T cells during priming, rendering 'helpless' T cells incapable of further clonal expansion upon antigen re-challenge (Janssen et al., 2005). However, TRAIL-deficient mice contain helpless $CD8^+$ T cells which, surprisingly, are still capable of a second round of clonal expansion. Therefore, TRAIL is likely to be involved in restricting clonal expansion upon antigen re-challenge (Janssen et al., 2005). Furthermore, TRAIL is also involved in regulating the balance between T helper 1 (Th1) and T helper 2 (Th2) cells. Upon CD3 stimulation of naïve T helper cells *in vitro*, the differentiated Th2 cells, which express surface TRAIL, upregulate the apoptosis inhibitory protein cFLIP (Zhang et al., 2003, Roberts et al., 2003). Th1 cells on the other hand remain TRAIL-sensitive and are killed by Th2 cell expressed TRAIL. Therefore, TRAIL-expression shifts the Th cell balance towards more immune-suppressive Th2 cell subsets (Falschlehner et al., 2009).

Furthermore, $CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) are not only crucial in the maintenance of immune homeostasis, but have also been implicated with mediating their potent immune-suppressive functions via TRAIL (Pillai et al., 2011). Tregs are dependent on IL-10/IL-35 expression, however, upon IL-10/IL-35-double deficiency, Tregs upregulate TRAIL production and become dependent on the death ligand for their regulatory function, both *in vitro* and *in vivo* (Workman et al., 2011). In line with this, upregulation of TRAIL on Tregs was responsible for their cytotoxicity towards effector T cells as demonstrated in an allogenic skin graft model (Ren et al., 2007). Herein, systemic TRAIL-R blockade inhibited Treg-mediated effector T cell death, thereby decreasing survival of allogenic skin grafts. Interestingly, costimulation of TRAIL-R1/R2 as well as the TCR blocks T cell activation (Lehnert et al., 2014) and prevents activation of autoreactive T cells derived from patients with Omenn syndrome, a rare autosomal recessive genetic disorder characterized by severe combined immunodeficiency (Zhang et al., 2011b).

1.2.2 TRAIL in tumourigenesis

TRAIL is well known for its ability to specifically kill tumour cells without harming non-transformed cells (Walczak et al., 1999). Therefore, TRAIL-based therapeutics such as recombinant TRAIL and anti-TRAIL-R antibodies have been extensively researched (Lemke et al., 2014b). However, the initial enthusiasm was dampened as several cancer cells were found to be resistant to TRAIL-induced apoptosis (de Miguel et al., 2016). Some

cancers, such as pancreatic ductal adenocarcinoma (PDAC) and NSCLC even upregulate TRAIL-R expression, indicating a potential benefit of TRAIL-signalling in tumour biology (Spierings et al., 2003, Ozawa et al., 2001). As such, the role of TRAIL in tumourigenesis remains conflicting and several lines of evidence exist for both anti- and pro-tumour functions of the TRAIL/TRAIL-R system.

After the initial discovery that TRAIL can selectively kill tumours in a xenograft, TRAIL-deficient mice were quickly sought to validate this finding. TRAIL KO mice exhibited enhanced tumour burden upon transplantation with A20 B cell lymphoma in comparison to wildtype counterparts, thus further recapitulating the initial finding and expanding the tumour suppressor role of TRAIL to a syngeneic system (Walczak et al., 1999, Sedger et al., 2002). In this model, surface expression of TRAIL on liver NK cells, previously associated with anti-viral defence, actually enables TRAIL-mediated anti-tumour immune surveillance; particularly regarding metastasis suppression. In accord, TRAIL-deficient mice were more susceptible to experimental liver metastasis (Sedger et al., 2002, Seki et al., 2003). In line with a role in liver tumourigenesis, TRAIL-R deficiency leads to enhanced development of macroscopic liver lesions in a model of diethylnitrosamine (DEN)-induced hepatocarcinogenesis (Finnberg et al., 2008). Eu-myc-driven lymphomagenesis as well as lung and liver metastases were also enhanced in this study in TRAIL-R-deficient mice. It has been demonstrated that high levels of surface-bound TRAIL on liver NK cells promote immune surveillance against liver metastases (Takeda et al., 2001, Smyth et al., 2001). Furthermore, the expression of IFN-γ was required for inducing the expression of TRAIL *in vivo* to prevent the formation of experimental metastases (Smyth et al., 2001). In line with a role in metastasis suppression, rather than mediating cell death of primary tumours, our laboratory has shown that detachment sensitizes tumour cells to TRAIL-induced apoptosis without affecting the growth rates of primary tumours (Grosse-Wilde et al., 2008).

Although TRAIL KO mice are not explicitly described as having increased susceptibility to spontaneous tumour formation, some cases of lymphoma development in older mice, aged over 500 days, have been described (Zerafa et al., 2005). Haematological pathologies are further exacerbated in mice with $p53^{-/+}$ haploinsufficiency. Here, combined $p53^{-/+}$- and TRAIL-deficient mice were found to be more susceptible to methylcholanthrene (MCA)-induced fibrosarcoma formation than $p53^{+/-}$ only (Zerafa et al., 2005). However, conflicting reports from Yue et al. did not find any difference in lymphoma formation between TRAIL KO and wildtype mice on a $p53^{-/-}$ background (Yue et al., 2005). The observed conflict may be due to the respective levels of p53 expression in the two models, different tumour induction protocols or tissue specific effects.

The role of TRAIL as a metastasis suppressor is well established, however, more recent evidence indicates that TRAIL can actually promote the formation of metastases in TRAIL-resistant cells, depending on oncogenic mutation status. Resistance mechanisms involve high expression of anti-apoptotic mediators such as cFLIP, XIAP or a specific balance of anti- and pro-apoptotic BCL-2 family members (Falschlehner et al., 2007). In a study from our laboratory, cancer cell endogenous mTRAIL-R expression was shown to promote progression, invasion and metastasis in autochthonous KRAS-driven murine pancreatic and lung cancer models (von Karstedt et al., 2015). The tumour-supportive effects were regulated cell-autonomously by TRAIL-R2-mediated activation of Rac1/PI3K signalling. Importantly, this effect was triggered by the membrane-proximal domain (MPD) of TRAIL-R2, independently of its DD and FADD. The observed cancer-promoting effect was specifically facilitated by oncogenic KRAS. Further evidence for a tumour-supportive role of TRAIL in pancreatic ductal adenocarcinomas derives from xenograft studies employing COLO357 cells, rendered resistant to TRAIL by BCL-XL overexpression. Here, TRAIL treatment induced a fourfold increase in the number of liver metastases (Trauzold et al., 2006). This pro-metastatic effect was attributed to TRAIL-mediated gene-activatory signalling promoting the induction of survival pathways and speculated to involve the upregulation of IL-8. However, the role of TRAIL-induced cytokines in the observed increase of metastases was not investigated *in vivo* and its relevance to tumour biology in physiological setting remained elusive.

In light of a role for TRAIL as a potential anti-tumour drug, the possible tumour-supportive nature of non-apoptotic pathways initiated by endogenous TRAIL, highlights potential risks of TRAIL-based cancer therapy. Therefore, it is of high importance to study the mechanism of non-apoptotic signalling, as well as its potential effects on the tumour microenvironment, to establish potential avenues of blocking TRAIL's non-apoptotic effects, or better yet, converting them to a pro-apoptotic outcome.

1.3 The immune-microenvironment in tumourigenesis

The first reported evidence for a role of immune cells in promoting tumourigenesis was made by Rudolf Virchow in 1863 who observed that cancers were frequently associated with inflammatory cell infiltrates. This led him to hypothesize that cancers originate from such inflammatory sites which are in favour of tumour progression (Virchow et al., 1863). Since his original discovery, the complex interactions between tumour cells and immune cells have been extensively investigated (Balkwill and Mantovani, 2001, Gajewski and Schumacher, 2013). It has become increasingly clear that the observed chronic or “smouldering” inflammation can trigger various types of cancer (Colotta et al., 2009). It is estimated that 15-20% of worldwide cancer deaths are linked to underlying infections and

inflammatory responses (Balkwill and Mantovani, 2001). However, today it has been widely accepted that nearly all cancers are characterized by an inflammatory microenvironment with myeloid and lymphoid immune cells along with a vast array of cytokines constituting the central modulators herein (Grivennikov et al., 2010). Therefore, it is not surprising that tumour-promoting inflammation has been added as one of the emerging hallmarks of cancer (Hussain and Harris, 2007, Hanahan and Weinberg, 2011). Due to its relevance in tumour progression, the next few chapters will hone in on core components within the microenvironment, followed by ways in which the TRAIL/TRAIL-R system might influence the immune cell compartment of the tumour microenvironment.

The tumour microenvironment is composed of cancer cells, stromal tissue, and the extracellular matrix where the immune system is a crucial determinant skewing the tumour-microenvironment towards either tumour progression or inhibition (Whiteside, 2008, Hagemann et al., 2007, Hanahan and Weinberg, 2011). In essence, the immune-microenvironment is in place to combat the foreign malignant cell and elicit tumour immune surveillance. As such, particularly the presence of $CD8^+$ T cells and NK cells within the tumour as well as in the tumour stroma, is beneficial for the patient (Gajewski et al., 2013). However, high mutation rates and stringent selection promote the evolution of tumour-immune evasive mechanisms, shielding the tumour from anti-tumour immunity and actually modulating the immune system towards outputs which are beneficial for tumour progression (Khong and Restifo, 2002, Kim et al., 2007).

1.3.1 Roles of myeloid cells in tumour progression

Although the activation of anti-tumour lymphocytic cell subsets has recently shown clear therapeutic benefit, the presence of myeloid cell subsets is gaining attention as the composition of the myeloid immune compartment can function in cancer initiation, progression, metastasis and resistance to therapy (Hanahan and Coussens, 2012). Therefore, it is also not surprising that specific myeloid cell subsets are frequently linked to altered patient survival (Engblom et al., 2016).

Myeloid cells were discovered by Elie Metchnikoff in 1884 in starfish larvae microscopy studies. He noted that infection sites are infiltrated by leukocytes which are capable of ingesting and destroying foreign material (Metchnikoff et al., 1884). Today, myeloid cells are accredited with central roles in the innate immune response as sustainers of tissue homeostasis and crucial defenders against infection (Arandjelovic and Ravichandran, 2015). They are primarily involved in the phagocytosis of pathogens and subsequent priming of lymphocytes to recognize and initiate the release of regulatory proteins for foreign cell destruction (Mosser and Zhang, 2008). Consistent with their protective function, syngeneic macrophages can inhibit tumour growth (Adelman et al., 1983). This effect was initially shown by Adelman et al. who demonstrated that syngeneic

macrophages from tumour bearing mice were capable of inhibiting tumour growth in nude mice, whilst control macrophages were not (Adelman et al., 1983) (Adelman et al., 1983). However, emerging evidence now indicates that myeloid cells can also promote cancer progression by direct myeloid-tumour cell interaction, as well as by modulating the tumour stroma towards tumour progression enhancing outputs (Gabrilovich et al., 2012). As such, data from mouse models and patients indicate that myeloid cells can promote tumour progression by enhancing tumour cell proliferation, neo-vascularization metastasis and immune-evasion (Ungefroren et al., 2011). In particular, breast and recently also lung cancers have been correlated with a poor prognosis when high densities of myeloid cells are present (Fujimoto et al., 2000, Bremnes et al., 2011). Accordingly, in a spontaneous tumour model using mouse mammary tumour virus- polyomavirus middle T antigen (MMTV-PyMT) mice, removal of monocytes by crossing to colony stimulating factor-1 (csf1) KO mice significantly reduced tumour metastasis (Lin et al., 2001). Furthermore, co-culture experiments of myeloid cells with tumour cells describe the release for tumour proliferation promoting substances from myeloid cells, indicating that the tumour actively modulates myeloid cells in favour of tumour growth (Komohara et al., 2012). The discrepancies in the observed effects of myeloid cells are likely due to the diversity of the myeloid compartment, and the specific nature of the respective myeloid tumour infiltrates. Therefore, the next sections will describe the major myeloid cell subsets associated with tumour effector functions; namely tumour associated macrophages (TAM) and myeloid derived suppressor cells (MDSC) (Solinas et al., 2009, Gabrilovich and Nagaraj, 2009).

As an important constituent of the myeloid immune compartment, fully differentiated tumour-associated macrophages (TAMs) constitute a major component of leukocytic tumour infiltrating and are considered critical effectors of tumour progression (Solinas et al., 2009). The tumour-immune environment hosts a vast spectrum of macrophage subtypes, at the opposite ends of which are classically activated type 1 macrophages (M1) and alternatively activated type 2 macrophages (M2) macrophage (Mantovani et al., 2002). M1 macrophages are differentiated upon encounter with toll-like-receptor (TLR) ligands and particular cytokines such as LPS and IFN-γ respectively (Wang et al., 2014). They defend the host against foreign material such as microorganisms and virally infected cells, but also against neoplastic cells. M1 cells elicit their function via eliminating foreign material by phagocytosis or via eliciting cytotoxic activities. They can also present antigens as well as release inflammatory cytokines which activate naïve memory CD8 and CD4 T cells (Solinas et al., 2009). In line with this, high M1 islet density in NSCLC patients correlated with favourable prognosis (Ohri et al., 2009). The associated anti-tumour effect was further connected to high levels of inducible nitric oxide synthase (iNOS) which can have cytotoxic effects. Accordingly, culturing monocytes with tumour microvesicles from pancreas, lung or colon cancer upregulated the production of monocyte-derived reactive

oxygen species. Furthermore, monocytes were also found to upregulate HLA-DR, a cell surface receptor central in antigen presentation (Baj-Krzyworzeka et al., 2007). Other than M1 macrophages, the alternatively activated M2 subset, promotes tumour progression by directly enhancing proliferation, neo-angiogenesis and quite opposite to their M1 counterparts by suppressing adaptive immunity. These cells originate from unpolarised monocytes which are recruited into the tumour microenvironment by cancer cell released chemokines, most notably CCL2 (Bottazzi et al., 1983). In accordance, M2 cell abundance positively correlated with CCL2 levels in ovarian, breast and pancreatic cancer (Balkwill, 2004). Moreover, differentiated M2 macrophages also secrete CCL2 themselves, thereby eliciting positive feedback, facilitating their accumulation in the tumour microenvironment (Mantovani et al., 2004). Next to CCL2, other cytokines such as CXCL8 (IL-8) and M-CSF are also involved in orchestrating the infiltration and expansion of M2 cells in tumours (Allavena et al., 2008). Once in the tumour environment, the tumour generated cytokine milieu predominantly facilitates the differentiation of recruited monocytes towards M2 type macrophages (Mantovani et al., 2004). Polarisation can occur via IL-4 although several chemokines such as CCL2 have not only been associated with the recruitment, but have also been shown to promote M2 polarisation and survival in prostate carcinoma (Roca et al., 2009). Although fully differentiated M2 macrophages are characterized by specific effector molecules, such as arginase-1, they also exhibit distinctive surface expression of HLA-DR, CD14 and elevated levels of mannose receptor C type 1 (CD206 or MRC1), a scavenger receptor and general marker for alternatively activated cells (Sica and Mantovani, 2012). CD206 is largely expressed intracellularly and although its exact function in M2-cell biology remains obscure, it has been associated with increased expression of effector molecules such as IL-10 and is considered a strong marker of alternative activation (Martinez et al., 2009). High levels of $CD206^{+}$ macrophages have been associated with poor prognosis and decreased survival in lung adenocarcinoma patients (Zhang et al., 2011a)

M2 cells characteristically accumulate in oxygen-poor, necrotic regions of tumours which has been attributed to presence of the transcription factor HIF-1α in these areas (Du et al., 2008). In line with this, Du et al. determined that the ablation of HIF-1α in glioblastoma impaired levels of tumour supportive, pro-angiogenic, $CD11b^{+}$ and $F4/80^{+}$ myeloid cells. Herein, elevated levels of the tumour released chemokine CXCL12, increased the motility of macrophages into the tumour microenvironment. Therefore, the transcriptional output of HIF-1α, particularly regarding chemokine and cytokine induction, can regulate the immune microenvironmental composition.

Within hypoxic tumour areas, M2 macrophages initiate a pro-angiogenic program as evidenced by a correlation of high levels of tumour-associated macrophages with high

tumour vascularisation (Leek et al., 1996). M2 macrophages mediate angiogenesis by the release of endothelial cell growth factors such as vascular endothelial growth factor (VEGF) as well as matrix metalloproteases (MMPs). MMPs facilitate neo-angiogenesis by degrading the extracellular matrix thereby increasing matrix turnover and restructuration of the vascular network (Hagemann et al., 2004). In accord, a study by Giraudo et al. demonstrated that cervical mouse tumours contain high levels of MMP-9 expressing macrophages. Upon targeting MMP-9, either by inhibition or genetic ablation, tumour vascularisation and tumour burden were significantly reduced (Giraudo et al., 2004). Mechanistically, decreased levels of MMP-9 were associated with increased mobilization and receptor binding of VEGF, although the direct association of MMP-9 and VEGF was not elucidated. Similar accounts from a Lewis lung carcinoma model (3LL) associate the presence of MMP-9-expressing macrophages in primary tumours with increased angiogenesis and enhanced metastasis (Hiratsuka et al., 2002). Using VEGFR KO mice it was indicated that MMP-9 may indeed be directly induced by VEGF, as MMP-9 levels in tumour bearing VEGFR KO lungs were drastically reduced. TAMs support cancer cell invasiveness by secreting MMP-2 and MMP-9 (Whiteside, 2007). By degrading the ECM, these pro-invasive factors enable the cancer cell to break away from the tumour and enter foreign tissues. M2 macrophages also enhance the motility of cancer cells by promoting epithelial-mesenchymal transition (EMT) (Liu et al., 2013). During EMT, the expression of E-cadherin is decreased, resulting in loss of cell-cell adhesion and increased migration motility of cancer cells. Accordingly, macrophage conditioned medium was recently shown to induce colon and pancreatic cancer cell migration *in vitro* (Jedinak et al., 2010). Next to MMPs and pro-angiogenic factors, M2 macrophages also release several chemokines and cytokines which promote neoangiogenesis, such as IL-8 and CXCL1 further covered in following chapters.

A central role of M2 macrophages in promoting tumour progression is their suppressive activity towards cytotoxic $CD8^+$ T cells (Noy and Pollard, 2014). This is enabled by the inhibition of T cell activation, as well as by the secretion of factors which favour the attraction of immunosuppressive Tregs (Mantovani et al., 2002). In line with this, M2-depletion in a human papilloma virus (HPV) mouse model, was demonstrated to increase HPV16 E7-specific $CD8^+$ T cells, which correlated with tumour growth inhibition (Lepique et al., 2009). M2 cells also exhibit characteristically low surface expression levels of costimulatory receptors such as CD86, but instead they are capable of expressing the immune checkpoint ligand PD-L1 (Kuang et al., 2009). Accordingly, Kuang et al. noted that high infiltration of PD-$L1^+$ monocytes correlated with disease stage and poor survival in HCC patients. The tumour-supportive effect of PD-$L1^+$ M2 cells, was confirmed by adoptively transferring tumour reactive T cells and specifically treated monocytes, isolated from HCC patients, into subcutaneous tumour bearing NOD/SCID mice. Tumour burden

was significantly decreased if the monocytes were pre-treated with anti-PD-L1 indicating that monocyte specific PD-L1 facilitates tumour burden by suppressing T cells (Kuang et al., 2009). M2 cells also secrete an array of immune-suppressive cytokines, such as IL-10 and TGFβ.

Next to M2 macrophages, another highly immunosuppressive myeloid cell subset which has gained considerable attention in the recent past, are myeloid derived suppressor cells (MDSC) (Marvel and Gabrilovich, 2015). MDSC are a highly heterogeneous cell subset which, unlike fully differentiated TAMs, is immature. Due to their alternative activation state, and suppressive activity this immune cell subset also acquire an 'M2-like' phenotype (Parker et al., 2015). The presence of MDSC negatively correlated with the presence of antigen reactive T cells as well as with survival in melanoma patients (Weide et al., 2014). MDSC are broadly subdivided into two classes, polymorphonuclear, or granulocytic MDSC (PMN-MDSC) and monocytic MDSC (M-MDSC). In mice PMN-MDSC are characterized by $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$ surface expression levels, whilst M-MDSC are $CD11b^{+}$ $Ly6G^{-}$ $Ly6C^{hi}$. MDSC can elicit their suppressive activities by abrogating antigen specific T cell activation. This is accomplished by decreasing the antigen recognition reactivity of the TCR, thus generating T cell tolerance. M-MDSC promote antigen-specific suppressive effects by releasing NO, which reacts with superoxide to generate peroxynitrite (PNT). PNT binds to T cell receptors and thereby decreases their sensitivity to tumour antigens (Nagaraj et al., 2007). MDSC further use two enzymes involved in the arginine metabolism to control the T cell response: inducible NOS2 and arginase-1, which deplete the milieu of L-arginine, causing peroxynitrite generation, as well as decreased CD3ζ chain expression and T cell apoptosis. In prostate cancer, selective antagonists of these two enzymes proved beneficial in restoring T cell-mediated cytotoxicity (Serafini et al., 2006). MDSC can not only suppress anti-tumour lymphoid effectors, but also induce the development of tumour supportive lymphoid Treg subsets *in vivo*, which has been suggested to be due to MDSC-mediated release of IL-10 (Huang et al., 2006, Hsu et al., 2015).

Similar to their fully differentiated M2 counterparts MDSC are also capable of eliciting tumour growth both indirectly via facilitating neovascularization but also by directly promoting cancer cell proliferation (Gabrilovich and Nagaraj, 2009, (Gorgun et al., 2013). In line with a role in neovascularization, Yang et al. observed greater microvessel density and vascular maturation when tumour cells were co-injected with MDSC (Yang et al., 2004). MDSC enable angiogenesis via the release of VEGF, BV8 and MMPs (Marvel and Gabrilovich, 2015). Co-culture experiments by Gorgun et al. who cultured patient-derived MDSC with multiple myeloma cells further indicate direct proliferation enhancing effects of MDSC in ^{3}H-thymidine incorporation assays (Gorgun et al., 2013).

Throughout tumour development, the frequency of MDSC in the blood was found to increase, whilst 3-4 weeks after tumour resection decreases in the levels of patient MDSC were noted. This development indicates that MDSC accumulation is mainly generated via factors released by the tumour itself (Talmadge and Gabrilovich, 2013, Jayaraman et al., 2012). The accumulation of MDSC within the tumour immune-environment is subject to three main signals which originate from the tumour and its stroma: generation/expansion, trafficking and activation. The generation/expansion of MDSC is mediated by mainly by tumour secreted VEGF, TNF and GM-CSF which elicit the development of immature myeloid cells in the bone marrow. Trafficking to the tumour is mainly regulated by the CCL2/CCR2 axis. In line with this, a prostate cancer transplant model revealed the CCL2-CCR2 interaction to be crucial in the recruitment of bone marrow-derived myeloid cells to the blood and into the tumour microenvironment (Izhak et al., 2012). Furthermore, M-MDSC infiltrating melanomas display CCR2-dependent immunosuppressive activities in presence of GM-CSF (Lesokhin et al., 2012). Upon arrival at the tumour site, MDSC become activated and capable of eliciting their immune suppressive functions. The activation is again mediated by the tumour generated cytokine milieu, and involves cytokines such as IL-4, TNF and IL1-β (Umansky et al., 2016).

1.3.2 Cytokines and chemokines orchestrate the composition of the tumour immune-microenvironment

Cytokines and chemokines are central modulators of the cancer microenvironment and are intimately involved in recruiting and polarizing immune cells (Hanahan and Weinberg, 2011). Cancer cells frequently modulate their cytokine milieu to alter the cellular composition of their microenvironment in favour of tumour progression (Hoesel and Schmid, 2013, Qian et al., 2011, Hanahan and Weinberg, 2011). As such, elevated levels of cytokines such as CCL2, IL-8, CXCL1 and CXCL5 have been associated with increased growth and progression of breast, prostate and ovarian cancer (Qian et al., 2011, Fader et al., 2010, Zhang et al., 2010, Singh and Lokeshwar, 2009, Begley et al., 2008, Dong et al., 2013). These cytokines can mediate tumour-supportive effects by paracrine recruitment and polarisation of cancer-promoting myeloid cell subsets (Fujita et al., 2011, Chun et al., 2015, Highfill et al., 2014).

Cytokine	Cancer	Source
CCL20	Breast, Colon	(Tang et al., 2009)
CXCL2	Breast, Colon	(Tang et al., 2009)
CCL2	Breast, Colon, Pancreas	(Varfolomeev et al., 2005, Levina et al., 2008)
CXCL8 (IL-8)	Breast, Colon, Pancreas, Lung	(Varfolomeev et al., 2005, Zhou et al., 2008, Leverkus et al., 2003)
CCL5	Breast, Lung	(Levina et al., 2008)

Table 1. Cytokines which have been shown to be induced by TRAIL in cancer cells

Cytokines are low-molecular-weight proteins that mediate cell-to-cell communication and are capable of triggering signalling cascades which activate key transcription factors such as AP-1, NF-κB, STAT3 or Notch (Grivennikov and Karin, 2010). They are synthesized by the tumour as well as by immune and stromal cells, such as fibroblasts and endothelial cells, and capable of regulating proliferation, cell survival, differentiation, immune cell activation. Chemokines, a specific subset of cytokines, are small (5-20kDa) molecules mainly involved in the recruitment of immune cells into sites of inflammation. They are categorized into four subfamilies based on the number and location of their cysteine motif: CXC, CC, C and CX3C. Depending on the context, cytokines can modulate an anti-tumoural response, but during chronic inflammation, they frequently induce cell transformation and malignancy, conditional on the balance of pro- and anti-inflammatory cytokines, their relative concentrations, cytokine receptor expression content, and the activation state of surrounding cells (Zamarron and Chen, 2011).

Interestingly, TRAIL has been shown to induce several cytokines in cancer cells (Table 1). Amongst these cytokines is CXCL-8 (IL-8), a multifunctional cytokine which can elicit paracrine chemoattractive functions to alter the immune microenvironmental composition, whilst also promoting migration independent, autocrine functions. IL-8 is a homologue of murine CXCL1 and was originally known as neutrophil-activating peptide-1 for its activity as a potent chemotactic agent for neutrophils in inflammatory diseases (Xie, 2001). It mediates its tumour pro-angiogenic, pro-mitotic, invasive and migratory functions via its cognate receptors C-X-C chemokine receptor 1 (CXCR1) and C-X-C chemokine receptor 2 (CXCR2) (Chuntharapai et al., 1994). As such, high IL-8 levels have been correlated with poor disease prognosis in breast, colon, ovarian, and prostate cancers, as well as in

melanoma (Xie, 2001). The recent finding that MDSC express CXCR1 and CXCR2, led to the identification of IL-8 as an important paracrine chemotactic factor in MDSC recruitment in a subcutaneous colorectal cancer graft model (Casilli et al., 2005, Alfaro et al., 2016). Accordingly, supernatants of HT-29 cells were capable of chemoattracting MDSC *in vitro*. Interestingly, IL-8 was shown to specifically target PMN-MDSC to release Neutrophil Extracellular Traps (NETs) which are associated with metastasis (Cools-Lartigue et al., 2013). Combined CXCR1/2 blockage using the antagonist Reparixin was capable of blocking the effects of IL-8 *in vivo* (Alfaro et al., 2016).

Apart from chemotactic functions, IL-8's direct, proangiogenic and mitogenic roles have been related to lung cancer progression, where high IL-8 expression levels were associated with increased cancer risk of 45%-86% (Pine et al., 2011). In line with this, an initial study by Arenberg et al. showed that NSCLC cells express high levels of IL-8. The authors were able to demonstrate that neutralization of IL-8 in a subcutaneous SCID mouse model reduced tumour growth by 40% (Arenberg et al., 1996). Mechanistically, IL-8 neutralization was correlated with decreased vessel density whilst corneas treated with homogenates of IL-8-neutralized tumours accordingly exhibited a decreased corneal neovascular response. Therefore, the authors concluded that tumour cell secreted IL-8 mitigated its protumour function by facilitating neovascularization (Arenberg et al., 1996).

Regarding its autocrine functionality, increased levels of IL-8 in ascites prompted Abdollahi et al. to investigate potential involvement of IL-8 in resistance mechanisms against cancer therapeutics (Abdollahi et al., 2003). Interestingly, they found IL-8 treatment to enhance resistance of ovarian carcinoma cells to TRAIL. They further related this effect to an IL-8-mediated decrease in surface expression levels on TRAIL-R1. In line with IL-8 as an autocrine TRAIL-resistance factor, Wilson et al. found similar effects in prostate cancer cells (Wilson et al., 2008). In the latter study, upregulation of cFLIP upon IL-8 treatment was proposed as the mechanism for the observed enhanced resistance to TRAIL treatment.

The chemokine CCL2, also known as monocyte chemotactic protein 1 (MCP1) is a central mediator between tumour and host cells and associated with tumour-supportive roles in prostate (Li et al., 2009), breast (Soria et al., 2008), and lung cancer (Cai et al., 2009). CCL2 affects tumour progression by affecting cancer cell proliferation, migration and survival and is particularly known for its paracrine chemotactic ability to modulate the composition of the tumour immune-microenvironment. Herein, CCL2 is capable of chemoattracting monocytes, memory T-lymphocytes and natural killer cells but is most potently associated with the recruitment of monocytic cells and has been considered as one of the core determinants of human tumour macrophage content (Balkwill, 2004, Charo and Taubman, 2004, Mantovani et al., 2002). It was first purified and cloned in 1989 from

human gliomas and myelomonocytic cells, dependent on its ability to chemoattract monocytes (Yoshimura et al., 1989). CCL2 elicits its effector functions by binding to its receptor, CCR2, which was verified using CCL2 and CCR2 KO mice (Kurihara et al., 1997). Neither CCL2 or CCR2 KO mice exhibit any overt phenotypes but have shown altered inflammatory responses and decreased ability to clear bacterial pathogens due to significantly reduced ability to recruit macrophages (Kurihara et al., 1997, Dewald et al., 2005).

The ability of CCL2 to enhance tumour growth rates is particularly attributed to its chemoattraction of TAM and MDSC (Li et al., 2013; Qian et al., 2011). As such, CCL2 and CCR2 KO mice exhibited decreased primary tumour burden which correlated with decreased proportions of tumour associated $CD11b^+$ and $CD11b^+GR1^+$ cells in orthotopic mammary tumour models (Li et al., 2013). Interestingly, the further use of neutralizing antibodies against CCL2 had a biphasic effect, where the overall final tumour burden was decreased, although initial early growth was actually enhanced as compared to control. This effect may be attributed to an immunosurveillance function of CCL2 in early tumour growth and the initial recruitment of anti-tumour immune cell subsets. In line with this, the authors also observed enhanced pulmonary metastases in CCR2 KO and CCL2 KO mice (Li et al., 2013). The latter effect is at odds with previously published data by Qian et al., who actually associated CCL2 neutralization with decreased metastatic tumour burden in ortotopic breast cancer mouse models (Qian et al., 2011). Although, the authors also associate CCL2 neutralization with a decrease in inflammatory $GR1^+$ cells, differences in the effect of CCL2 on metastatic tumour burden may stem from differences in the utilized mouse models. Moreover, CCR2 or CCL2 KO fully abrogates the CCL2/CCR2 axis whereas neutralization only elicits partial and temporary inhibition.

Interestingly, in addition to its potent effects on the chemoattraction of tumour-supportive myeloid cells, CCL2 has also been demonstrated to promote myeloid cell polarisation and survival (Roca et al., 2009). In this study, CCL2 stimulation of isolated human $CD11b^+$ cells induced the upregulation of anti-apoptotic proteins cFLIP as well as BCL-2 and BCL-XL (Roca et al., 2009). Accordingly, decreased caspase-8 cleavage and apoptotic cell death, was observed under stress from serum deprivation. Importantly, CCL2 treatment was also capable of promoting the upregulation of CD206, a surface marker which is associated with alternatively activated cells as previously indicated in this thesis.

Although the functional roles of cytokines in promoting tumour progression by modulating the composition of the immune-microenvironment are undisputed, the literature is rather obscure as to what initiates the cytokine secretion. Elucidation of potential triggers of a tumour-supportive cytokine milieu could alleviate the tumour mediated corruption of the immune microenvironment and is therefore of high relevance to cancer therapy.

1.3.3 The TRAIL/TRAIL-R system as a mediator of cytokine secretion

Although TRAIL has been shown to induce various cytokines in cancer cells (Table 1), the underlying functional role of TRAIL as a trigger for cytokine production in cancer cells has remained elusive, particularly regarding physiologically relevant *in vivo* settings. Studies by Tang et al. utilized TRAIL-R1 or TRAIL-R2 overexpression systems to elucidate the mechanistic regulation of TRAIL-induced cytokines. They implicate either TRAIL-R1 or TRAIL-R2 with an equal ability to induce IL-8, TNF, CCL20 and CXCL2 on the transcriptional level using transfected reporter constructs of the identified cytokines. Mechanistically, Tang et al. implicate NF-κB signalling to be involved in the transcriptional upregulation of cytokines via a TRADD-TRAF-NIK-IKKα/β axis (Tang et al., 2009). They also implicate a potential requirement of FADD in NF-κB activation although the adaptor's role in cytokine induction is not investigated. The mechanistic elucidation relied on co-overexpression systems of dominant negative versions of the investigated proteins, together with TRAIL-R1 or TRAIL-R2. Regarding TRAIL-R domain requirements, Tang et al. rule out a role of the extracellular domains using truncation mutants (Tang et al., 2009). The potential role of the intra-cellular domain regarding gene activation was addressed in earlier studies by Harper et al. (Harper et al., 2001). Utilizing a TRAIL-R2 truncation mutant (ΔSer324-369) and an NFκ-B reporter construct linked to a secretable alkaline phosphatise product, they demonstrate TRAIL-R2's DD to be required for NFκ-B activation. Other than Tang et al., Varfolomeev et al. were unable to detect TRADD in TRAIL's previously mentioned secondary complex, which they associated with gene-activatory signalling. Herein, the authors demonstrate the presence of FADD within this complex, but do not assay for FADD's role in cytokine production (Varfolomeev et al., 2005). Therefore, a potential involvement of FADD and TRADD in TRAIL-mediated cytokine induction would be interesting to investigate.

Immediately downstream of FADD, the role of caspase-8 in TRAIL-mediated cytokine induction is controversial. Studies by Zhou et al. indicate that TRAIL stimulation can induce the transcriptional upregulation of IL-8 and the matrix metalloproteases MMP-7 and MMP-9 (Zhou et al. 2008). They follow up to examine a role of caspase activity using the pan-caspase inhibitor zVAD, which blocked IL-8 induction in some cell lines, but actually upregulated it in others. Furthermore, Levina et al. indicate that pan-caspase inhibition blocks IL-8 induction in ovarian and breast cancer cells (Levina et al. 2008), whereas studies by Berg et al. suggest that caspase activity is dispensable for TRAIL-mediated NF-κB activation. It is possible that these discrepancies result from differential levels of cell death upon TRAIL stimulation, as the role of caspase-8 inhibition on cytokine induction was not investigated independently of caspase-8 requirements in apoptosis. Studies on CD95L signalling by Kreuz et al. lend further explanation to the discrepancies reported for the role of caspase-8 activity in cytokine induction. Utilizing CD95L sensitive wildype

HT1080 and resistant BCL-2 over expressing HT1080 cells with or without caspase inhibiton, their study indicates that caspase-8 activity does not affect CD95L-mediated cytokine induction independently of cell death (Kreuz et al., 2004). Moreover, caspase inhibition with zVAD increased CD95L-mediated cytokine production only in CD95L sensitive cells but had no effect in resistant, BCL-2 over expressing, cells. Instead, a non-enzymatic role of caspase-8 was indicated using Jurkat cells in which deficiency for caspase-8, abrogated CD95L-mediated gene activatory signalling. Due to the similarity of the CD95L and TRAIL signalling systems, it is likely that caspase-8 also has a non-enzymatic, scaffold-dependent role in TRAIL signalling.

The studies investigating functional effects of TRAIL-induced cytokines are scarce and at times controversial, but most importantly they do not investigate any functions *in vivo*. TRAIL-induced cytokines have been associated with the previously mentioned proliferative effects of TRAIL as suggested by studies from Levina et al. (Levina et al. 2008). Although they show the induction of IL-8, CCL5, CCL2 and bFGF, as measured by sandwich immunoassay, which is capable of giving absolute concentrations, they proceed with transforming their data to percent relative to control; thereby leaving open which cytokine is actually secreted at the highest level upon TRAIL. They do observe increases in proliferation using both directly stimulated cells as well as conditioned supernatants of TRAIL-treated cells and implicate IL-8 with facilitating autocrine proliferation of lung, ovarian and breast carcinomas. Studies by Trauzold et al. demonstrate TRAIL to be capable of inducing IL-8, CCL2 and urokinase-type plasminogen activator (uPA) at the transcriptional level. Although it is not a cytokine, uPA is frequently secreted by cancer cells and is associated with the activation of MMPs (Legrand et al., 2001). TRAIL-induced uPA was further demonstrated to increase the capacity of colorectal cancer cells to displace permeabilised fibroblasts. In line with this, the authors demonstrate increased metastasis of orthotopic colorectal tumours in SCID mice upon TRAIL treatment. It would have been interesting to determine whether TRAIL-induced uPA, or any other TRAIL-induced secreted factor, is involved in generating this metastatic phenotype *in vivo* (Trauzold et al., 2006). The observed effects could alternatively also be mediated by tumour promoting, cell-autonomous TRAIL-R signalling and/or TRAIL-mediated paracrine effects. Regarding paracrine effects of TRAIL-induced chemokines on immune cell subsets, the study by Varfolomeev et al. provides first insights. The authors demonstrate that TRAIL can induce the secretion of IL-8 and CCL2 from colorectal cancer cells. Interestingly, they follow up the noted chemokine induction and show increased chemoattraction of $CD11b^+$ cells towards conditioned media of TRAIL-treated colorectal cancer cells in a transwell migration assay *in vitro*. Since the conditioned media contained TRAIL as well as the TRAIL- induced cytokines, possible confounding effects of TRAIL cannot be ruled out. It would also be interesting to determine specifically which

chemokines were responsible for the observed effect, as only IL-8 and CCL2 were assayed for by ELISA, and neither chemokine was neutralized in the migration assay (Varfolomeev et al., 2005).

In sum, ample evidence supports the ability of TRAIL to induce cytokines, although insights regarding their molecular regulation are remain partially elusive. Functionally, some studies demonstrate potential tumour supportive functions of TRAIL, as well as paracrine functions of TRAIL-induced factors towards immune cells *in vitro*. Importantly, evidence for a role of TRAIL-induced cytokines *in vivo* is lacking and requires further investigation.

1.4 Rationale, Hypothesis and Aims: The TRAIL/TRAIL-R system in the modulation of the tumour immune-environment

The TRAIL/TRAIL-R system exhibits opposing functions in tumour biology. On the one hand, it can function as an effective killer of tumour cells (Walczak et al., 1999), yet on the other, several studies have proposed it to exert tumour-supportive functions (Trauzold et al., 2006; von Karstedt et al., 2015). Although both effects need to be considered in the design of optimal treatment strategies involving TRAIL, the non-apoptotic, tumour-supportive mechanisms and their *in vivo* functions are rarely studied (Newsom-Davis et al., 2009, Lemke et al., 2014b, Johnstone et al., 2008). The current indications regarding TRAIL's non-apoptotic tumour-supportive functions focus on cancer cell-autonomous signalling, lack *in vivo* validation, or, importantly, do not study the role of the endogenous TRAIL/TRAIL-R system with regards to involvement in the tumour microenvironment (von Karstedt et al., 2015; Trauzold et al., 2006; Varfolomev et al., 2005).

Tumour-secreted factors, and particularly cytokines and chemokines, are central orchestrators in the formation of a tumour-supportive microenvironment *in vivo* (Kim, Emi, & Tanabe, 2007; Landskorn et al. 2014). Since their modulatory function within the tumour microenvironment is undisputed, potential upstream regulators of cytokine secretion could function as promising drug targets. TRAIL can elicit the secretion of the very same cytokines which independent studies demonstrate to be crucial paracrine drivers within the tumour immune-environment (Mantovani et al., 2008, Balkwill and Mantovani, 2012). Hence, TRAIL might function as such a trigger of cytokines and, thereby, modulate the tumour immune microenvironment. However, the underlying molecular mechanism and potential functional roles of the TRAIL/TRAIL-R system in the tumour microenvironment *in vivo* have not been addressed. Therefore, this thesis aims to investigate whether the TRAIL/TRAIL-R system influences the tumour immune-microenvironment through cytokine induction and, if so, to which extent endogenous TRAIL/TRAIL-R may contribute to tumour growth *in vivo* by affecting the cancer microenvironment.

Aims:

1. *Establish a comprehensive expression profile of the TRAIL-induced secretome*
2. *Elucidate the molecular machinery involved in TRAIL-mediated cytokine secretion*
3. *Determine whether TRAIL-induced cytokines elicit autocrine effects on tumour cell migration, proliferation and resistance to apoptosis*
4. *Elucidate potential paracrine effector functions of TRAIL-induced cytokines on immune cell subsets*
5. *Establish syngeneic mouse models for the investigation of potential* in vivo *effector functions of TRAIL-induced cytokines*
6. *Determine whether TRAIL-induced cytokines can modulate the immune-microenvironment* in vivo
7. *Determine crucial immune-modulatory drivers within the TRAIL-induced secretome*

2 Material and Methods

2.1 Material

2.1.1 Chemicals and Reagents

Unless stated otherwise, all reagents employed in this work were obtained from the following vendors:

2.1.2 Common Buffers

Blocking Buffer	5 % milk powder 0.05 % Tween-20 in PBS
Freezing medium (for cells)	90 % FCS (v/v) 10 % DMSO (v/v)
FACS buffer	1 x PBS 5% FCS
HBS (2x, pH = 7.0)	50 mM HEPES 280 mM NaCl 1.5 mM Na_2HPO_4 x 2 H_2O
Isopropanol/ Acetic Acid	95% Isopropanol (v/v) 5% Acetic Acid (v/v)
Lysis Buffer (cell lysates and IP)	30 mM Tris-Base (pH 7.4) 120 mM NaCl 2 mM EDTA 2 mM KCl 10 % Glycerol (v/v) 1 % Triton X-100 (v/v) (IP-LB1) COMPLETE protease-inhibitor cocktail

MES running buffer	50 mM MES 50 mM Trizma Base 1 mM EDTA
MOPS Running Buffer (pH = 7.7)	50 mM MOPS 50 mM Tris-Base 3.5 mM SDS 1.0 mM EDTA
PBS (pH = 7.4)	137 mM NaCl 8.1 mM Na_2HPO_4 2.7 mM KCl 1.5 mM KH_2PO_4
PBS/Tween	137 mM NaCl 8.1 mM Na_2HPO_4 2.7 mM KCl 1.5 mM KH_2PO_4 0.05 % Tween-20 in PBS (v/v)
Stripping Buffer (pH = 2.3) (western blots)	50 mM Glycine in H_2O
Transfer Buffer (western blots)	192 mM Glycine 25 mM Tris-Base 0.01 % EDTA 10% Methanol (v/v)

2.1.3 Media for culturing bacteria

LB

10 g/l Tryptone

10 g/l NaCl

5 g/l Yeast extract

pH 7.4

2.1.4 Bacterial strains

STRAIN	PURPOSE	SUPPLIER
TOP10F	Propagation of plasmids	Invitrogen

2.1.5 Antibodies

Unconjugated Antibodies (Western Blot)			
REAGENT	ISOTYPE	SOURCE	IDENTIFIER
α-FADD	mouse IgG1	BD Bioscience	Cat#556402
α-β-Actin	mouse IgG1	Sigma	Cat#A1978
α-TRAIL-R2	rabbit	Cell Signalling	Cat#3696
α-Caspase-8	goat	Santa Cruz	sc6134
α-TRAIL-R1	rabbit	ProSci	Cat#PSC-1139-C100
α-IkBa	rabbit	Cell Signalling	9242
α-pIkBα (5A5)	mouse IgG1	Cell Signalling	Cat#9246
α-RIP-1	IgG2a	BD Bioscience	Cat#610458
α-JNK	rabbit	Cell Signalling	9258
α-pJNK	mouse IgG1	Cell Signalling	9255S
α-cFLIP	mouse IgG1	Adipogen	Cat#AG-20B-0056-C050
α-RIPK3	rabbit	Enzo Life Sciences	ADI-905-242

Unconjugated Antibodies (Flow cytometry)			
REAGENT	ISOTYPE	SOURCE	IDENTIFIER
α -TRAIL-R1 (HS101)	mouse IgG1	Adipogen	Cat#AG-20B-0022-C100
α -TRAIL-R2 (HS201)	mouse IgG1	Adipogen	Cat#AG-20B-0023-C100
α -TRAIL-R3 (HS301)	mouse IgG1	Adipogen	Cat#AG-20B-0024-C100
α -TRAIL-R4 (HS402)	mouse IgG1	Adipogen	Cat#AG-20B-0025-C100

Conjugated Antibodies (Flow cytometry)			
REAGENT	FLOUROPHORE	SOURCE	IDENTIFIER
α -CD45 (murine)	AF700	BioLegend	Cat#103127
α -CD11b (murine)	PerCP	BioLegend	Cat#101230
α -GR-1 (murine)	PE-Cy7	BioLegend	Cat#108416
α -CD274 (murine)	PE	eBioscience	Cat#12-5982-82
α -CD206 (murine)	FITC	BioLegend	Cat#141703
α -CD11c (murine)	BV711	BioLegend	Cat#117349
α -F4/80 (murine)	BV785	BioLegend	Cat#123141
α -Ly6C (murine)	BV450	BD Bioscience	Cat#560594
α -CD14 (human)	APC	BD Bioscience	Cat#555397
α -HLA-DR (human)	FITC	BD Bioscience	Cat#556643
α -HLA-DR (human)	APC-H7	BD Bioscience	Cat#641393
α -CD206 (human)	PerCP-Cy5.5	BioLegend	Cat#321121
α -CD11b (human)	PE	BD Bioscience	Cat#555388

2.1.6 Biological agents

AGENT	SOURCE
iz-TRAIL	Previously produced in *E. coli* and purified in our laboratory
iz-mTRAIL	Previously produced in *E. coli* and purified in our laboratory
CD95L-FC	Produced in HEK293T and purified in our laboratory
His-TNF	Previously produced in HEK293T and purified in our laboratory

2.1.7 Specific Inhibitors

INHIBITOR	MANUFACTURER
COMPLETE Protease Inhibitor Cocktail	Roche
Phosphatase Inhibitor Cocktail	Sigma/Aldrich
SM-83	P. Seneci and L. Manzoni
Actinomycin D	Sigma/Aldrich
QVD	R & D Systems
Rac1 Inhibitor (#553502)	Calbiochem
zVAD.fmk	R & D Systems

2.1.8 Cell culture media and supplements

Dulbecco's Modified Eagle Medium	Invitrogen
DPBS	Invitrogen
FCS	Sigma
Glutamine	Invitrogen
PBS	Invitrogen
Puromycin	Sigma
RPMI 1640	Invitrogen
Sodium Pyruvate	Invitrogen
Trypsin/EDTA solution	Invitrogen

2.1.9 Cell lines

NAME	TYPE	SPECIES	MEDIUM	PROVIDER
A549-luc	Non-small cell lung carcinoma	Human	RPMI-1640, 10% FCS	ATCC (purchased)
HEK 293T	SV40 Large T antigen embryonic kidney	Human	DMEM, 10% FCS	
COLO357	Lymph node metastasis from pancreatic primary tumour	Human	DMEM, 10% FCS	Dr. A.Trauzold
HCT116	Colorectal carcinoma	Human	DMEM, 10% FCS	Dr.B.Vogelstein
HCT 116 –DKO	Colorectal carcinoma	Human	DMEM, 10% FCS	Dr.B.Vogelstein
3LL	Lewis Lung Carcinoma	Mouse	DMEM, 10% FCS	Dr. S.A.Quezada

2.1.10 Ready-to-use kits and solutions

BCA Protein assay	Perkin Elmer
ECL Western Blotting Detection Plus	GE Healthcare
Chemoluminescent Substrate	GE Healthcare
SuperSignal West Femto	Pierce
E.Z.N.A. Plasmid Maxi Kit	OMEGA bio-tek
TrypanBlue	Serva
RevertAid cDNA synthesis kit	Thermo Scientific
RNeasy mini kit	Qiagen

2.1.10 Consumables

Cell Culture Petri dishes	TPP
Cell Culture Test Plates (6-, 12-, 24-well)	TPP
Cell Sieve (40 and 70 µm pore size)	Becton Dickinson
CIM 16 plates	Roche
Cryogenic vials	Nunc
Conical tubes (15 ml and 50 ml)	TPP
Glassware	Schott
Hybond ECL Nitrocellulose Membrane	GE Healthcare
Luciferin (*in vivo*)	Caliper Life Science
NuPAGE® 4-12% Bis-Tris Gels	Invitrogen
PCR Tubes	StarLab
Pipette tips (0.1-10, 1-200, 101-1000 µl)	StarLab
Plastic pipettes (5 ml, 10 ml and 15 ml)	Becton Dickinson

Polypropylene round bottom tube (5 ml)	Becton Dickinson
Round and flat bottom 96-well test plates	TPP
Safe-Lock Reaction Tubes (1,5ml, 2 ml)	Eppendorf
SeeBlue™ Plus2 Pre-Stained Standards	Invitrogen
SDS-Sample buffer	Invitrogen
Single-Use Syringe (5 ml, 30 ml, 50 ml)	Terumo
Single-Use Syringe (1 ml, 2 ml)	Becton Dickinson
Single-Use Scalpel	Feather
Single-Use Needles	Becton Dickinson
Sterile filter (0.22 µm and 0.45 µm pore size)	Millipore
Tissue Culture flasks (25, 75, 150 cm^2)	TPP
Whatman paper	GE Lifesciences
X-Ray film Hyperfilm™ ECL	GE Lifesciences

2.1.11 Instruments

Multiskan Ascent	Thermo Labsystems
Varioskan Ascent	Thermo Scientific
Mithras LB 940	Berthold Technologies
Hyper Processor X-Ray film Developer	Amersham Bioscience
Blotting equipment X cell II™	Novex
NanoDrop Spectrophotometer ND-1000	NanoDrop Technologies
Steri-Cult Cell culture bench	HERA Safe
Incubator Stericult 2000	Forma Scientific
Millipore Super-Q water installation	Millipore
Microwave	AEG
Table Centrifuge Biofuge	Heraeus
Multifuge 3S-R	Heraeus
Biofuge Stratos	Heraeus
Centrifuge 5810R	Eppendorf
Light Microscope	Zeiss
Vortex	VWR
Cryo 1°C Freezing container	Nalgene Labware
pH Meter	VWR

Thermomixer compact	Eppendorf
Freezer -20° C	Liebherr
Freezer -80° C	Forma Scientific
Pipettes (10 µl, 100 µl, 200 µl, 1 ml)	Eppendorf
Pipetboy	Starlab
Multichannel pipettes	Eppendorf
xCELLigence RTCA DP	Roche
IVIS Lumina	Caliper Life Science

2.1.12 Software

Microsoft® Excel 2010	Microsoft
Microsoft® Powerpoint 2010	Microsoft
Microsoft® Word 2010	Microsoft
GaphPad Prism 7	GraphPad
Sequencing	GATC Biotech
ND1000 V3.3.0	NanoDrop Technology
Ascent Software Version 2.6	Thermo Labsystems
MikroWin 2000	Berthold Technologies
Ivis Spectrum software	Caliper Life Science
xCELLigence software	Roche
EndNote	Thomson Reuters
FACSDiva	BD Biosciences
FlowJo 7.6.5	TriStar
ApE	Wayne Davis (University of Utah)

2.2 Cell biological methods

2.2.1 Cell culturing

The cell lines were cultured in the above indicated medium without antibiotics. Culture conditions were 37ºC with 96% relative humidity and 5% CO_2 in either RPMI1640 or DMEM. Cells were passaged before reaching confluence. To do so, cells were washed with PBS and detached using 1 x trypsin/EDTA solution and diluted either 1/10 or 1/15 depending on the cell lines growth rate.

2.2.2 Freezing and storage of cell lines

For freezing, cells were centrifuged at 1500 rpm for 3 min and resuspended in FCS with 10% DMSO at 1-2 x 10^6 cells /ml. DMSO addition prevented the formation of ice crystals during freezing. The cell suspension was then transferred into cryo-tubes, placed at -80ºC for short term storage and 24-48 h later transferred liquid nitrogen (-196 ºC) for long term storage.

For thawing, cells were resuspended in 10 ml of pre-warmed media, supplemented with 10% FCS and immediately centrifuged at 1500 rpm for 3 min. To remove the residual DMSO, the supernatant was decanted and the cell pellet resuspended in 1 ml pre-warmed media. The suspension was then transferred into a T-75 cell culture flask containing 10 ml of the appropriate media.

2.2.3 Reverse transfection of siRNA

1,5 ul Dharmafect transfection reagent and 200 µl medium without FCS were mixed and incubated for 5 min at room temperature (RT), followed by addition of 2,2 µl siRNA smart-pool [20 µM] obtained from Dharmacon. After 30 min of incubation, the transfection mix was added into the well of a 6 well plate and 1-2 x 10^5 cells were added. The cells were incubated with the siRNA for 48 h, followed by re-seeding into a 96 well plate for subsequent treatments.

2.2.4 Generation of FADD KO cells

FADD KO cells were generated via clustered regularly interspaced short palindromic repeats CRISPR/Cas9 technology by targeting exon 2 of murine FADD. sgRNAs were generated using MIT's CRISPR design tool (http://crispr.mit.edu/) and ligated into the MSCV-IRES-GFP Vector. Cells were seeded at 70% confluency and transfected using 1.25 ug of plasmid via Lipofectamine 2000 according to manufacturer instructions. Limiting dilution was employed to achieve single cell cloning. Three weeks later colonies from single cell clones were harvested and KO was validated via western blot.

2.2.5 Retroviral transduction of FADD KO cells

Wildtype FADD was ligated into the retroviral MSCV vector, followed by an internal ribosome entry site (IRES) and the open reading frame of EGFP. Lipofectamine 2000 was used to transfect the vector constructs into Phoenix-Ampho HEK2937T cells. The medium was replaced 24 h post-transfection followed by collection of viral supernatants after 72 h. The viral supernatants were then filtered using a 0.45 µm filter and added to the cells in presence of polybrene (6 µg/ml) followed by spinfection (2500 rpm, 45 min, 30°C). The medium was replaced after 48 h and the cells were left to grow until confluence. To achieve single cell cloning, EGFP positive single cells were then sorted into 96 well plates using the BDAria.

2.2.6 Generation of recombinant lentiviruses for shTRAIL-R

HEK293T cells were seeded at 2 x10^6 cells in a 10 cm dish. The next day, cells were transfected using 20 µg plasmid DNA (pLKO.1 empty vector or shRNA sequence for murine TRAIL-R containing vector) 6 µg of envelope pMD2G (VSVG), 15µg packaging vector pCMV-dR8.91(HIV_Gag, HIV_Rev) mixed with 50 µl 2M CaCl2 and topped off to a final volume of 500 µl with sterile H_2O. The solution was then mixed with 500 µl 2x HBS and incubated at room temperature for 30 min. Next, the solution was added to the cells in 10 cm dishes as single drops and the dishes were transferred to 37ºC. 48 h later, the lentivirus-containing supernatants were collected, centrifuged and filtered through 0,2 µm pore filters and directly used for cell infection. Remaining supernatants were aliquoted and frozen at 80ºC for later use.

2.2.7 Production of stably transfected shTRAIL-R knockdown cells

3LL cells were seeded to obtain 90% confluence; 24 h later, the supernatant was removed and replaced with a 1:2 dilution of lentiviral particles in medium. After 48 h, the letntiviral medium was replaced with medium containing 12 ug/ml puromycin. The cells were selected for 2 weeks, whilst selection medium was replaced every 3 days. The optimal puromycin concentration, which was just high enough to kill the cell, was titrated apriori.

2.2.8 Quantification of chemo-/cytokines via enzyme-linked immunosorbent assay (ELISA)

The cells were seeded to 60% confluence in a 96 well plate. The next day, they were stimulated with iz-TRAIL [100 ng/ml] (human or mouse) in medium containing 0.5% FCS. After 24 h, the cells were centrifuged at 1500 rpm for 3 min followed by removal of supernatants. Chemo-/cytokine levels in the cell supernatants were determined via ELISAs obtained from R&D according to the manufacturer's instructions.

2.2.9 Quantification of cell viability via CellTiter-Glo

CellTiter-Glo solution enables measurement of viability by quantifying ATP, which is indicative of metabolically active cells. The solution was purchased from Promega and used at a dilution of 1:4 in PBS. The cells were centrifuged at 1500 rpm for 3 min and supernatants were removed; 100 µl of the diluted CellTiter-Glo solution was subsequently added to the cells. After 12 min of incubation, 90 µl were transferred to a whitewall plate to enhance measurement of luminescence. The absolute bioluminescence was measured at 0.1s intervals using the Mithras plate reader. The percentage viability was then calculated as relative to untreated control.

2.2.10 Cell proliferation via bromodeoxyuridine (BrdU) incorporation

BrdU is a thymidine analog which is incorporated only into newly synthesized DNA of actively dividing cells. The amount of incorporated BrdU can be immunochemically detected to give a colorimetric signal which is representative of the number of dividing cells. To conduct the assay, 2 x 10^3 cells were seeded per well; 24 h later the cells were treated as indicated. 18 h prior to the final timepoint (48h or 72h) the BrdU reagent was added to the wells at 1:1000 dilution. At the endpoint, the cells were denatured and fixed using the supplied solution and stored at 4ºC. The next day, the cells were washed and incubated with BrdU detection antibody for 1 hour. Next the peroxidase conjugated secondary antibody was added and incubated for 30 min. After a final water wash step the TBM peroxidase solution was added. After an additional 30 min, 2N H_2SO_4 was added to stop the reaction and the absorbance was read at 450 nm. The raw optical density values were used for quantification of BrdU incorporation.

2.2.11 Fluorescent associated cell sorting (FACS)

To stain lung immune cell infiltrates, murine lungs were manually dissociated, washed in PBS and centrifuged at 1.5x10^3 rpm. The pellet was resuspended in 5 ml RBC lysis buffer (BioLegend) at RT and incubated for 5 min; the reaction was stopped by addition of 30 ml 1xPBS followed by centrifugation. Cells were then labelled with fixable viability dye eFluor780 (eBioscience) for 30 min in the dark at RT, followed by addition of Fc block (BD Biosciences). Antibodies used were against CD45-AF700, CD11b-PercpCy5.5, CCR2-AlexaFluor647, GR-1-PE-Cy7, CD274-PE, CD206-FITC, CD11c-BV711, F4-80-BV785, MHCII-BV510, Ly6C-BV450 (detailed in 2.1.5). Human monocyte-derived macrophages and MDSC were stained with antibodies against CD14-APC, HLA-DR-FITC, HLA-DR-APC-H7, CD206-PE-Cy5, CD11b-PE (detailed in 2.1.5). Intracellular staining was performed using an Intracellular Fixation and Permeabilization Buffer Set (eBioscience) according to the manufacturer's instructions. Fluorescence minus one (FMO) controls were used to distinguish between positively and negatively stained cells. Flow cytometric analysis was performed with a LSRFORTESSA X-20 (BD) or Accuri (BD); data analysis was conducted using FlowJo software.

2.2.12 Immune cell quantification by flow cytometry

Flow cytometric reference beads (Invitrogen) were resuspended and added to the samples before analysis. The absolute number (Abs) of cells was calculated using the following formula: Abs= (count of gated cells/count of beads)/number of beads added to the sample.

2.2.13 *In vitro* bioluminescent luciferase activity assay

The cells were filtered using 40 µm cell strainers and seeded in a seven step ½ serial dilution starting from 100000cells/well. The next day, the cells were washed with PBS and

incubated with 30 µl passive lysis buffer (Promega) and incubated on the shaker for 15 min. Next, 20 µl of the lysate was transferred to a white walled plate and 100 µl Luciferase assay reagent was added and incubated for 10 min. Bioluminescence was the determined using the Mithras plate reader set to 0.3s reading time.

2.2.14 *In vitro* monocyte differentiation

Conditioned media was generated by stimulating A549 with iz-TRAIL in RPMI1640 for 2 h, followed by 2 washes with PBS and centrifugation at 5000 rpm for 5 min. PBMCs from healthy adult donors were isolated via Ficoll density gradient centrifugation; anti-CD14 coated microbeads (Milteny Biotec) were used for $CD14^+$ monocyte isolation, followed by purity analysis via flow cytometry. Monocytes were then cultured at $1x10^6$ cells/ml in A549 conditioned media, supplemented with 10% human serum. Cell differentiation state was determined via flow cytometry 48 h later.

2.3 Biochemical methods

2.3.1 Cell lysate preparation

The cells were trypsinized as previously described, washed with PBS and resuspended in 30 µl - 60 µl lysis buffer containing 1x Complete™ protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (cocktail I and II SIGMA). The cell suspension was then incubated on ice for 30-40 min followed by centrifugation for 30 min at $13x10^3$ rpm at 4°C. The lysate was then transferred to a fresh tube without disturbing the pellet and stored at -20°C.

2.3.2 Determination of lysate protein concentration

The lysate concentration was determined via Bicinchoninic acid (BCA)-containing protein assay (Pierce, Rockford, IL). To this end, 1 µl of lysate was incubated at 37°C with 100 µl BCA Reagent for 30 min. Protein levels were then determined by measuring absorbance at 560 nm via a Multiskan Ascent plate-reader (Thermo Labsystems). The lysates were subsequently equilibrated to the least concentrated sample.

2.3.3 Western blot procedure

The cell lysates were separated via SDS polyacrylamide gel electrophoreses (SDS-PAGE). To this end, the cell lysates were incubated with 1x sodium dodecyl sulfate (SDS) sample buffer at 90°C for 10 min. Next, the lysates were separated on 4-12% Bis-Tris-NuPAGE gradient gels (Novex, San Diego, CA) in MOPS buffer by first applying 80V for 10 min followed by 180V for 50 min. The transfer was conducted using the NOVEX mini-cell chamber (Invitrogen). The gel was positioned on top of a ECL Hybond nitrocellulose membrane (GE Healthcare), on the cathode, on top of three Whatman blotting papers

(Sigma) which were pre-wet with transfer buffer. After adding an additional three Whatman papers on top, the cathode and containing gel were placed into the anode chamber which contained three sponges soaked in transfer buffer. The transfer was then conducted at 30 V for 2 h. After transfer completion, the membranes were stained with Ponceau to confirm successful protein transfer. The membranes were then blocked with 2.5% Milk in PBS containing 0.05% Tween-20 (PBS/Tween) for 1 h, briefly washed in PBS and subjected to immunoprobing with primary antibody overnight at 4°C. Next day, the membranes were washed with 3x with PBS/Tween for 10 min and incubated with the respective secondary HRP-conjugated antibody for 45 min followed by 3 additional 10 min PBS/Tween wash steps. Antibody binding was determined by developing with enhanced chemiluminescence (ECL) substrate and X-Ray film (Scientific laboratory supplies).

2.3.4 Immunoprecipitation of the DISC and post-DISC complex

For TRAIL DISC-IP, A549 cells were stimulated with FLAG-iz-TRAIL as indicated. Cells were lysed in IP-lysis buffer (30 mM Tris-HCl, pH 7.4, 120 mM NaCl, 2 mM EDTA, 2 mM KCl, 10% Glycerol, 1% Triton X-100, 1x COMPLETE protease-inhibitor cocktail and 1x PhosSTOP (Roche)) at 4°C for 30 min. Lysates were cleared by centrifugation at 13,500 rpm for 30 min. FLAG-iz-TRAIL (200 ng) was added to the non-stimulated samples before all samples were pre-cleared using Sepharose beads (Sigma) for 1 hour at 4°C. 15 µL of M2 beads (Sigma) were then added to the samples and incubated overnight at 4°C. To analyse the post-DISC complex, the DISC-depleted lysates were collected and incubated overnight at 4°C with 15 µL protein G beads pre-blocked with 1% BSA and coupled with 3 µg anti-caspase-8 antibody (Santa Cruz Biotechnology).

2.3.5 Stripping of blotting membranes

Before adding a different primary antibody, the membranes were incubated with the acidic stripping buffer for 12 min at room temperature. Subsequently the membranes were washed with PBS/Tween and re-blocked as described in 2.3.3.

2.4 Molecular biological methods

2.4.1 Transformation of competent *E.coli*

A 50 µl aliquot of One Shot® TOP10 Chemically Competent E.coli (Invitrogen) was incubated with 2 µl of plasmid DNA on ice for 20 min. To enhance plasmid uptake, the mixture exposed to a heat shock in a 42°C water bath for 45 seconds. Next, the bacteria were incubated on ice for 3 min followed by addition of 200 µl SOC recovery medium. The bacteria were then plated on a previously prepared agar plate containing the respective antibiotic and placed at 37°C overnight. The next day colonies were picked and incubated in lysogeny broth (LB) for further amplification.

2.4.2 Isolation of plasmid DNA

To obtain sufficient amount of plasmid, the bacteria were grown overnight in 1L LB Media followed by centrifugation at 4500 rpm for 30 min to precipitate the bacteria. Plasmid isolation was conducted using E.Z.N.A. Plasmid Maxi kit (OMEGA bio-tek) according to the manufacturer's instructions.

2.4.3 Isolation of RNA

For isolation of RNA from lungs, the tissue was snap frozen in liquid nitrogen. Total RNA was extracted from cells or tissues using the RNeasy mini spin kit (Quiagen) according to manufacturer's instructions. The concentration and purity of RNA was determined with a ND-1000 (NanoDrop).

2.4.4 Generation of cDNA

Upon determining the concentration of RNA via the NanoDrop Spectrophotometer, 500ng -1 µg were mixed with 1 µl oligo (dt), filled to 12 µl with dH_2O and incubated at 65°C for 5 min. Next, RNase Inhibitor, dNTP's, reverse transcriptase were added in the appropriate buffer and incubated at 42°C for 60 min. The reverse transcriptase was then inactivated by incubating at 70°C for 5 min.

2.4.5 qPCR

qPCR synthesis reactions were performed in 96 well plates with 50 ng of cDNA on a Realplex Mastercycler (Eppendorf) using the Fast Start ROX mix (Roche). Roche's Probe finder software was used to design gene-specific primers and choose respective probes. cDNA amplification was carried out by incubating at 95°C for 10 min followed by a two step amplification cycle at 95°C for 15 seconds and 60°C for 1 min cycled 40 times. Relative mRNA levels were calculated using the ΔΔCt method by normalizing to GAPDH or HPRT mRNA. The following primer pairs were used:

Species	Gene	Fwd.	Rev.
Homo sapiens	IL-8	agacagcagagcacacaagc	atggttccttccggtggt
	CCL2	agtctctgccgcccttct	gtgactggggcattgattg
Mus musculus	CCL2	catccacgtgttggctca	gatcatcttgctggtgaatgagt

2.5 Mouse work

2.5.1 Induction of A549 xenograft lung tumours

6-8 week-old female Fox Chase® SCID Beige Mice were obtained from Charles River (UK) and employed for the experiment prior to reaching the age of 11 weeks. A549 wildtype or FADD KO cells were expanded without reaching confluence and the media was replaced 24 h prior to injection; splitting was coordinated to reach 50% - 70% cell confluence on injection day. The cells were then washed 3 times in DPBS, filtered via a 40 µM cell strainer, counted and diluted to 10 x 10^6 cells/ml.

Mice were warmed to 37°C in a heating chamber prior to injection. When tail veins were clearly visible, 2 x 10^6 cells were slowly injected into the lateral tail vein in 200 µl DPBS using 27 gauge needles and 1 ml syringes. Mice were maintained according to the institutional guidelines (University College London) and under a UK Home Office project license. The required risk assessment was obtained for this study.

2.5.2 *In vivo* bioluminescence imaging

Approximately 3 h after cell injection, equality in bioluminescence between different cell lines was confirmed. Subsequently, all mice were imaged weekly for bioluminescence. 1 gram D-luciferin was purchased from GoldBio and dissolved in DPBS at a final concentration of 30 mg/ml and syringe filtered at 0,2 µm. Next, luciferin was aliquoted (750 µl/aliquot) and frozen at -80 °C. To avoid variability, all luciferin used in this study was only frozen once. Prior to imaging, each mouse was anaesthetised by 4% Isofluorane gas and received 100 µl subcutaneous injection of 3 mg luciferin per 20 g mouse. To maintain anaesthesia, the Isofluorane dose was reduced to 1,5%. Bioluminescence images were acquired 10 min after luciferin injection using the IVIS Lumina® at 1 min exposure time (Caliper Life Science). Afterwards, mice recovered from anaesthesia in a 37°C heating chamber. Photons per second were quantified using the IVIS software.

2.5.3 Induction of 3LL syngeneic lung tumours

C57BL/6 mice were obtained from Charles River (UK) and employed for the experiment prior to reaching the age of 12 weeks. For experiments with CCR2 KO mice, the obtained C57BL/6 control mice were age and gender matched to the available CCR2 KO mice; the age cut off was maintained as above. 3LL (wildtype, FADD KO, pLKO.1, shTRAIL-R) were expanded without reaching confluence and media was replaced 24 h prior to cell injection. Mice were injected with 5 x 10^5 3LL cells as described in 2.5.1. Immediately post-injection, a 400 µl aliquot of each injected cell line was placed in culture and confirmed for equal confluence after 24 h. Mice were maintained according to the institutional guidelines (University College London) and under a UK Home Office project license. The required risk assessment was obtained for this study.

2.5.4 Histological examination and quantification of tumour burden

Mice were sacrificed 28-32 days after cell injection according to schedule 1. The left lung lobe was removed and fixed in 10% Formalin (Sigma) for 48 h. Paraffin embedding, cutting into 5µm sections and H&E-staining was performed by the histological staining service of Lorraine Lawrence at the Imperial College London Research Histology Facility. Tumour burden quantification was determined as % lung covered by tumour tissue; performed by the experienced pathologist Dr. Mona A. El-Bahrawy in a blinded manner.

2.6 Mass spectrometry

2.6.1 Sample preparation for mass spectrometry

A549 cells were stimulated with the caspase inhibitor Quinoline-Val-Asp-Difluorophenoxymethyl Ketone (QVD) or QVD + TRAIL [100 ng/ml] for 24 h followed by removal of supernatants. Next, the supernatants were centrifuged at 4000 g for 15 min to remove cellular debris. Next, supernatants were filtered with a 0.2µm pore size filter. Further processing of samples for mass spectrometry was conducted by Dr. Silvia Surinova. Proteins were denatured with 6M urea in 50mM ammonium bicarbonate (AB). Denatured proteins were reduced using 4mM dithiothreitol (DTT) in 50mM AB at 56°C for 25 min, cooled to room temperature followed by alkylation with 8mM iodoacetamide in 50 mM AB at room temperature in the dark for 30 min. Excess of iodoacetamide was then removed with an additional 4mM DTT in 50mM AB. Next, Urea diluted to 1.5 M with 50 mM AB. Proteins were subsequently digested with sequencing grade trypsin (Promega) at a protease to protein ratio of 1:50 at 37ºC for 15 h. Digestion was stopped with 1% trifluoroacetic acid (TFA). Peptide digests were desalted with microspin columns filled with SEM SS18V silica (The Nest Group), eluted with 50% acetonitrile 0.1% TFA, evaporated to dryness at 30ºC, and resolubilised in 20 µl of 10% formic acid in water. 1-2 µL of peptides was used for nLC-MS analysis.

2.6.2 Mass spectrometry conditions

nLC-MS/MS was performed on a Q Exactive Orbitrap interfaced to a NANOSPRAY FLEX ion source and coupled to an Easy-nLC 1000 (Thermo Scientific). Peptides separation was conducted via a 20 cm fused silica emitter, 75µm diameter, packed in-house with Reprosil-Pur 200 C18-AQ, 2.4 µm resin (Dr.Maisch) using a linear gradient from 5% to 30% acetonitrile/ 0.1% formic acid over 4 h, at a flow rate of 300 nL/min. Precursor ions were measured in a data-dependent mode in the orbitrap analyzer at a resolution of 70,000 and a target value of 1×10^6 ions. The ten most intense ions from each MS1 scan were isolated, fragmented in the HCD cell, and measured in the orbitrap at a resolution of 17,500. The bicinchoninic acid (BCA) assay was used to determine total protein content

of the concentrated secretome. An equal protein load, in the range of 10-100 ug between replicate experiments, was used across conditions.

2.6.3 Mass spectrometric data analysis

Mass spectrometric data analysis was conducted by Dr.Silvia Surinova. Raw data were analyzed with MaxQuant version 1.4.1.2 and searched against the human UniProt database (http://www.uniprot.org/, downloaded 03/04/2013) using default settings. Carbamidomethylation of cysteines was set as fixed modification, and oxidation of methionines and acetylation at N-termini were set as variable modifications. Enzyme specificity was set to trypsin with allowing maximally 2 missed cleavages. PSMs, peptides, and proteins were filtered at a less than 1% false discovery rate (FDR), to ensure high confidence identifications. The label-free quantification workflow was employed with a match time window of 4 min, an alignment time window of 20 min, and a match between selected runs. Statistical protein analysis was performed with MSstats.daily_2.3.4. MaxQuant output was converted to the format required by MSstats using a converter script from the MSstats website (http://msstats.org/). P-values were adjusted to control for the false discovery rate using the Benjamini-Hochberg procedure. Quantified proteins were annotated with cellular compartments as reported in UniProt and predicted with the transmembrane topology and signal peptide predictor Phobius (http://phobius.sbc.su.se/). Identified intracellular contaminants were removed, and secreted as well as membrane proteins were retained to comprise the secretome. Secretome proteins upregulated by at least twofold upon TRAIL treatment were submitted to DAVID Bioinformatics Resources 6.7 for enrichment analysis of gene ontology molecular function terms. The quantified proteins associated with the top three enriched molecular functions (chemokine activity, chemokine receptor binding, cytokine activity) were visually inspected and in cases where noisy peptide features were present (standard error>0.3) or where peptide features failed to be extracted in all replicates by MaxQuant, the peptide with the highest signal to noise ratio for that protein was reanalysed in Skyline via targeted data quantification. For the comparisons of means, a student’s paired t-test with a one-tail distribution was used.

2.7 TCGA expression analysis

TCGA Bioinformatics analysis was performed by Ankur Chakravarthy. RNAseq V2 level 3 data were downloaded for 489 LUAD samples from the TCGA data portal and parsed via a custom R function. The RSEM expression values were transformed to log2 counts per million using the voom function from the limma R package. High and Low composite groups for TRAIL & CCL2, or TRAIL & CCR2, were defined using overlapping samples for both genes of a pair in the top and bottom 50% of expression values. Differentially expressed genes were determined using limma-trend at a BH-adjusted P-value of 0.01

and a twofold change in expression between the composite high and low groups and were filtered to a curated list of immune factors for visualisation on heatmaps.

3 Results

3.1 Characterization of the TRAIL-induced secretome in resistant cancer cells

Tumour-secreted factors are highly relevant to cancer biology and TRAIL has shown the ability to enhance or disable tumour growth depending on the oncogenic context. It is, however, uncharacterized what the TRAIL-induced secretome is constituted of in its entirety. It therefore also remains unexplored what its biological function in cancer might be and, importantly, whether it may contain factors of particular relevance to the described pro-tumourigenic properties of TRAIL. Therefore, appropriate conditions to assay for TRAIL-mediated cytokine secretion were established, followed by a comprehensive analysis of TRAIL's secretome and the cells responsible for its generation.

3.1.1 Determination of TRAIL-resistant and sensitive cell lines

To study TRAIL's tumour secretome, suitable cell lines and conditions needed to be determined. Criteria for cell line choice included resistance at high TRAIL concentrations and high TRAIL-R1 and TRAIL-R2 expression levels. Since recent literature indicates that dying cells may also be involved in the release of cytokines, a TRAIL-sensitive cell line was also identified (Cullen et al., 2013).

Initially, a cell line was defined as resistant if it retained greater than 80% viability after treatment with isoleucine-zipper tagged TRAIL (iz-TRAIL). The iz-motif stably links the TRAIL ligand into a trimer, thereby contributing to the stability and potency of the death ligand (Ganten et al., 2006). After titrating several cell lines with iz-TRAIL, A549 (NSCLC) and HCT116 BAX/BAK KO (colorectal carcinoma) were considered as resistant (Figure 3A) at 100 ng/ml iz-TRAIL; HCT116 wildtype cells, isogenic to the HCT116 BAX/BAK KO, were classified as sensitive. Since 100 ng/ml iz-TRAIL was the highest concentration at which resistance was observed for both A549 and HCT116 BAX/BAK KO, this concentration was chosen for subsequent analyses of TRAIL-induced non-apoptotic signalling. A549 cells also expressed high levels of surface TRAIL receptor and were therefore selected for further analysis of TRAIL-induced cytokines *in vitro* (Figure 3B).

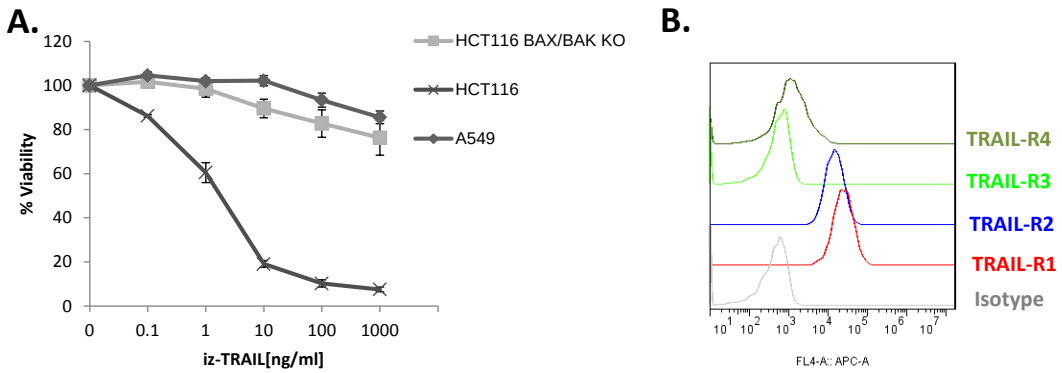


Figure 3. Determination of experimental cell line models

A. A549, HCT116 and HCT116 BAX/BAK KO were stimulated with indicated concentrations of iz-TRAIL for 24 h; cell viability was determined by CellTiter-Glo. **B.** TRAIL receptor surface staining in A549 cells via FACS.

3.1.2 TRAIL induces IL-8 in A549

Next, the optimal conditions to assay for TRAIL-induced cytokines secretion were established. To determine the optimal timepoint for assaying TRAIL-mediated cytokine induction, A549 were stimulated for different times over a total period of 24h, using the previously determined iz-TRAIL concentration of 100 ng/ml (Figure 4A). The kinetic indicated that TRAIL was able to significantly induce the secretion of IL-8 after 6h. Although A549 appeared to secrete low levels of basal IL-8 in the untreated control, the most significant induction was observed at 24h (Figure 4A). Therefore, this timepoint was maintained in all experiments assaying for the induction of TRAIL-induced cytokine production.

To determine whether IL-8 induction was dosage dependent and thus a direct consequence of iz-TRAIL concentration, A549 cells were subsequently titrated with iz-TRAIL (Figure 4B). iz-TRAIL induced IL-8 in a dose-dependent manner, as higher concentrations of iz-TRAIL also induced higher concentrations of IL-8 (Figure 4B). To exclude the involvement of any LPS contaminants, which could lead to confounding variables regarding cytokine secretion, the iz-TRAIL batch (TRAIL) used for stimulations throughout this thesis was tested for LPS presence and, importantly, tested negative (data not shown).

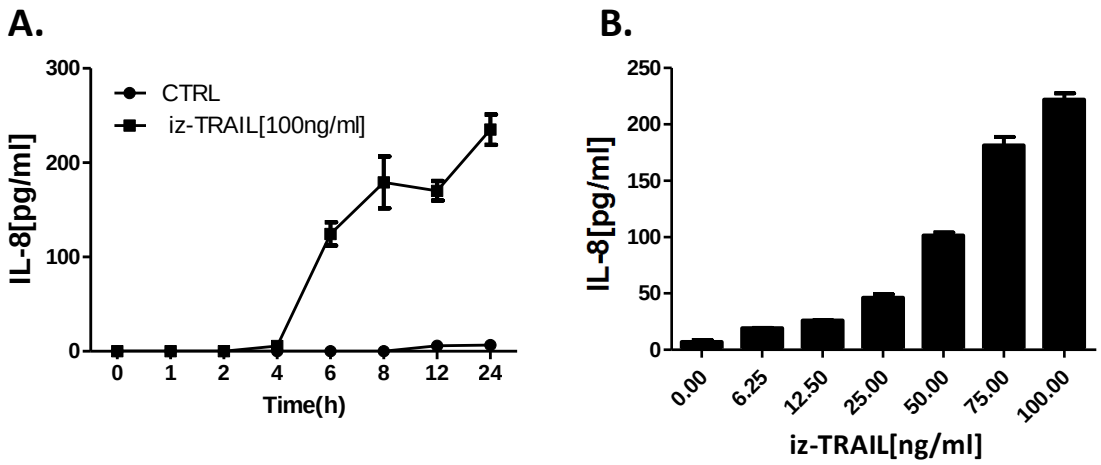


Figure 4. TRAIL induces IL-8 in A549 in a time- and dose-dependent manner

A. A549 cells were stimulated with iz-TRAIL [100 ng/ml] or medium (CTRL) for the indicated timepoints; next, the supernatant was removed and assayed for IL-8 via ELISA. **B.** A549 were stimulated with iz-TRAIL at the indicated concentrations for 24h; the supernatants were then analyzed by ELISA for IL-8 concentration.

3.1.3 Unbiased and comprehensive analysis of the TRAIL-induced secretome in resistant A549 cells

After identifying optimal cell lines, conditions, controlling for the integrity of the protocol and determining a positive control cytokine (Table 1), the next step required an unbiased approach. It is uncharacterized what the TRAIL-induced secretome is constituted of in its entirety. Therefore, it also remains unexplored what its biological function in cancer might be and, importantly, whether it may contain factors of particular relevance to the described pro-tumourigenic properties of TRAIL. To comprehensively elucidate TRAIL's secretome, an unbiased proteomic analysis of the TRAIL-induced cancer secretome was conducted by mass spectrometry. Herein, the goal was to detect factors that are both, secreted upon TRAIL stimulation and functionally enriched according to gene ontology (GO) functional enrichment analysis (Huang da et al., 2009).

Using a label-free quantitative workflow (Figure 5A), 1723 proteins could be detected, quantified and used for statistical testing of differential abundance between TRAIL stimulated and unstimulated samples. Among these 1723 proteins, 720 proteins were designated as the 'secretome', since they were classified as secreted or membrane proteins according to the UniProt database and/or predicted by the Phobius transmembrane topology and signal peptide predictor. A pool of 35 proteins, which were upregulated more than 2-fold (Figure 5B) by TRAIL [100 ng/mL] treatment was then searched via the functional annotation tool DAVID, for the most significantly enriched molecular functions as defined by gene ontology (GO) terms. GO molecular function analysis revealed that and NAMPT were functionally enriched (Figure 5C). Moreover, IL-

8, CXCL5, CXCL1 and CCL2 were highly induced as determined by label-free quantification and the only constituents discovered in the secretome which continuously clustered together in three separate GO molecular functions, indicating a high potential for functional significance (Figure 5B, C). Therefore, subsequent studies focused on the cytokine components within the TRAIL-induced secretome.

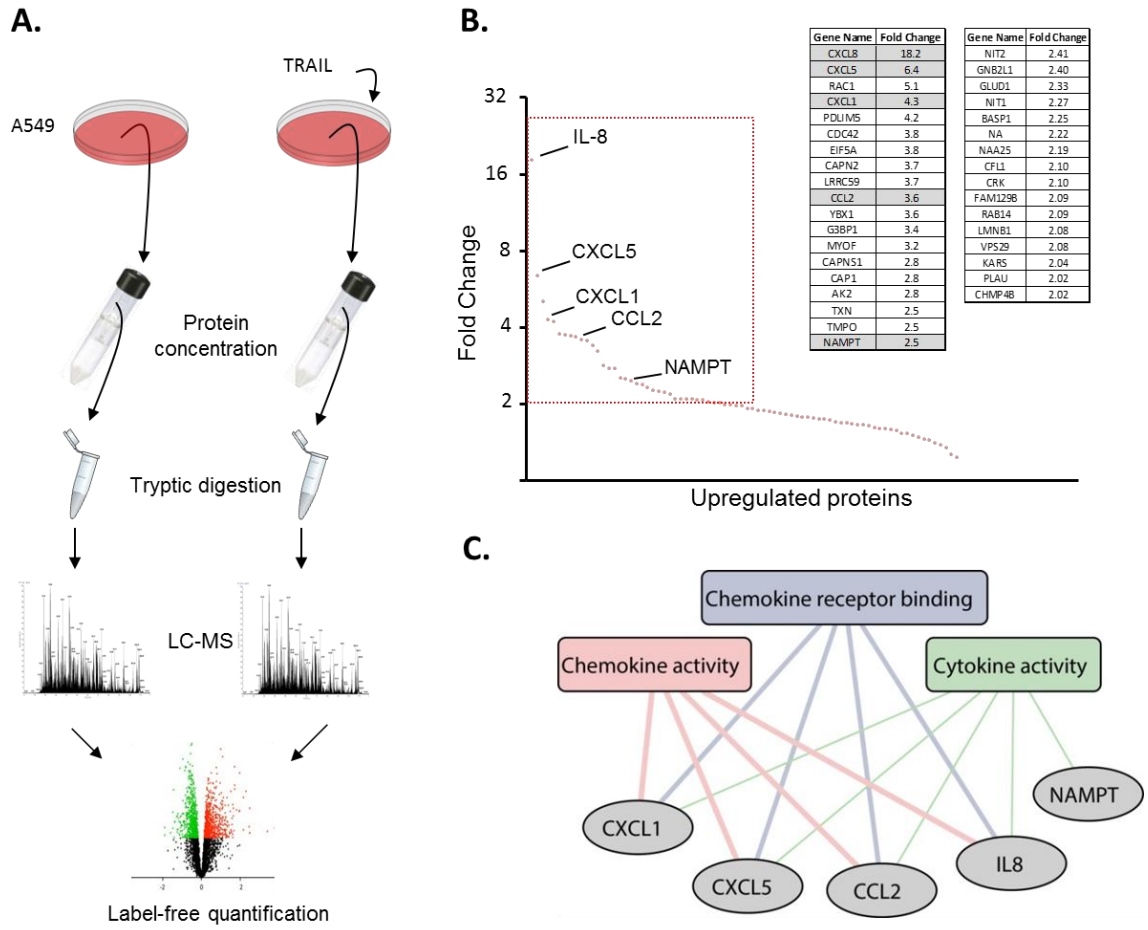


Figure 5. A TRAIL-induced cancer secretome

A. Schematic overview of quantitative, label-free secretome profiling approach. **B.** Proteomic quantification of TRAIL induced cytokines expressed as fold change from unstimulated control. **C.** Pool of all secreted proteins that were upregulated by iz-TRAIL [100 ng/ml] (> 2-fold induction as cut-off) was searched via the Gene Ontology database (GO) for the most significantly enriched molecular functions. Sample processing for mass spectrometry and mass spectrometric analysis was conducted by Dr.Silvia Surinova.

3.1.4 Validation of the identified cytokines from the secretome analysis by ELISA

In order to validate the induction of IL-8, CXCL5, CXCL1 and CCL2 identified by mass spectrometry, respective ELISAs were conducted on supernatants of A549 and COLO357, a TRAIL-resistant pancreatic cancer cell line (Figure 6). Both A549 and COLO357 cell lines exhibited significant induction of these factors upon TRAIL stimulation. Therefore, the cytokines identified by mass spectrometry were confirmed, whilst their TRAIL-mediated

secretion is not just lung cancer-specific, but constitutes a broader phenomenon in resistant cancer cells.

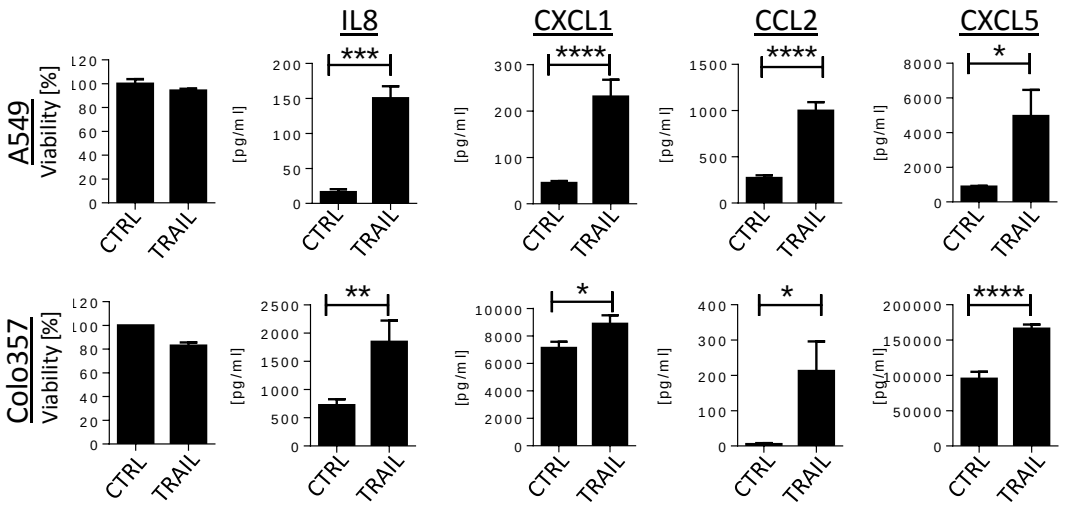


Figure 6. TRAIL induces IL-8, CXCL1, CCL2 and CXCL5 in resistant cancer cell lines

A549 or COLO357 were stimulated with iz-TRAIL [100 ng/ml]. After 24h, cell viability was determined by CellTiter-Glo and cytokine concentrations in the cell supernatants were measured via ELISA. Error bars represent the mean +/- SEM of experiments performed in triplicate.

3.1.5 Surviving cancer cells are the source of cytokines produced upon TRAIL stimulation

Recent *in vitro* studies by Cullen et al. showed that CD95L can induce cytokine release from dying cancer cells as a means of generating “find me” signals, to assure rapid removal of dead cells (Cullen et al., 2013). Therefore, it was next determined whether surviving or dying cells would produce cytokines upon TRAIL treatment. To this end, TRAIL-sensitive HCT116 were compared to the TRAIL-resistant isogenic cell line HCT116 BAX/BAK KO (Figure 7).

Cytotoxic doses of TRAIL [100 ng/ml] only induced low levels of IL-8 and CXCL1 in sensitive HCT116 WT cells (Figure 7B). However, TRAIL treatment of surviving HCT116 BAX/BAK KO cells strongly induced IL-8 and CXCL1 secretion, indicating that TRAIL-induced cytokine secretion is mediated by surviving cells (Figure 7A). Accordingly, blocking of cell death with the pan-caspase inhibitor Q-VD-OPh (QVD) significantly rescued cytokine induction in WT HCT116 and HeLa cells, whilst caspase activity did not affect cytokine induction independently of its effect on cell death in resistant A549 (Figure 7B and 19A).

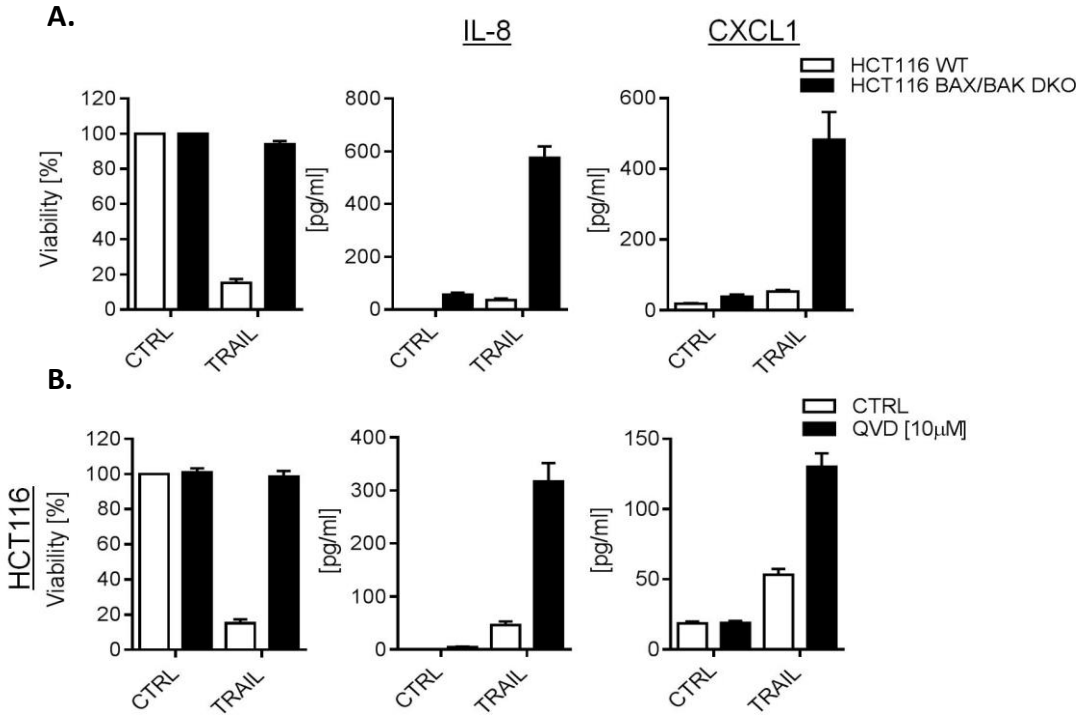


Figure 7. Surviving colorectal cancer cells secrete cytokines upon TRAIL stimulation

A. HCT116 or HCT116 BAX/BAK DKO cells were stimulated with iz-TRAIL [100 ng/ml] for 24h; cell viability was determined by CellTiter-Glo and chemo-/cytokine concentrations in the cell supernatants were measured via ELISA. **B.** HCT116 cells were pre-incubated with QVD [10 µM] or DMSO for 30 min followed by addition of iz-TRAIL (100 ng/ml) for 24h.

To address the cellular source of cytokines from a different angle, resistant A549 were sensitized to TRAIL using the small molecule inhibitor PIK-75 which sensitizes to apoptosis by downregulation of Mcl-1 and cFLIP (Lemke et al., 2014a). PIK-75 significantly decreased TRAIL-mediated IL-8 induction, which could be rescued by addition of QVD (Figure 8). Therefore, TRAIL induces the release of cytokines in cells surviving TRAIL stimulation.

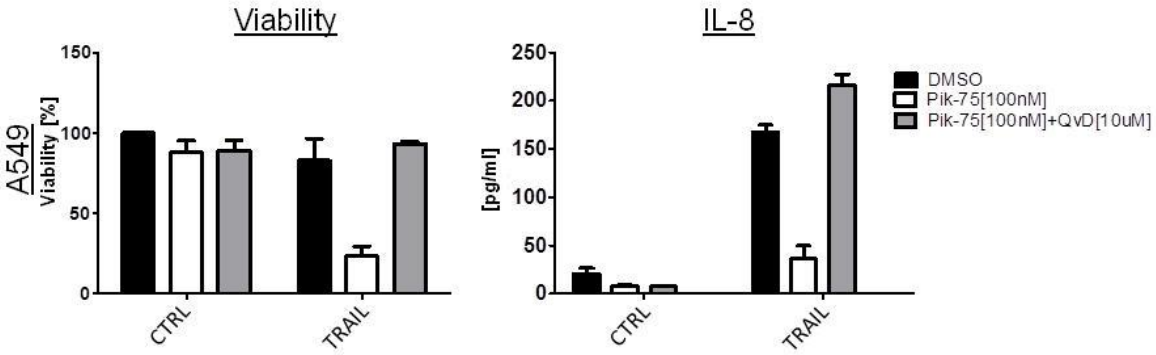


Figure 8. Surviving NSCLC secrete chemo-/cytokines upon TRAIL stimulation

A549 cells were pre-incubated with Pik-75 [100 nM] or QVD [10 µM] + Pik-75 [100 nM] for 30 min followed by addition of iz-TRAIL(100 ng/ml); cell viability and IL-8 concentration were determined as above.

3.2 Regulation of TRAIL-mediated cytokine production

In the previous chapter, it was shown that cytokines are functionally enriched in the TRAIL-induced secretome of surviving cancer cells. Due to the apparent importance of cytokines in the secretome, as justified by their functional enrichment and high levels of induction upon TRAIL stimulation, it was next assessed how cytokine production is regulated in regard to the overall mechanism, as well as regarding the molecular determinants involved.

3.2.1 TRAIL-induced cytokine production is mechanistically distinct from the tumour-supportive MPD/Rac1 axis

Our laboratory has recently shown that murine TRAIL-R and human TRAIL-R2 can activate a Rac1/PI3K signalling axis to increase migration and invasion of *KRAS*-mutated cells resulting in the promotion of KRAS-driven lung and pancreatic cancer progression and metastasis *in vivo* (von Karstedt et al., 2015). These cancer cell-intrinsic effects are mediated by the MPD of TRAIL-R2. Since TRAIL induced various cytokines in resistant, *KRAS*-mutated cancer cells, cancer cell secreted factors might mediate the previously observed tumour-supportive pro-migratory effects. However, this was not the case, as incubation with cancer cell-conditioned supernatants was incapable of rescuing the impaired migration of A549 cells silenced for TRAIL-R2 (Figure 9). Therefore, TRAIL-R2-mediated pro-migratory effects are independent of cancer cell-secreted factors.

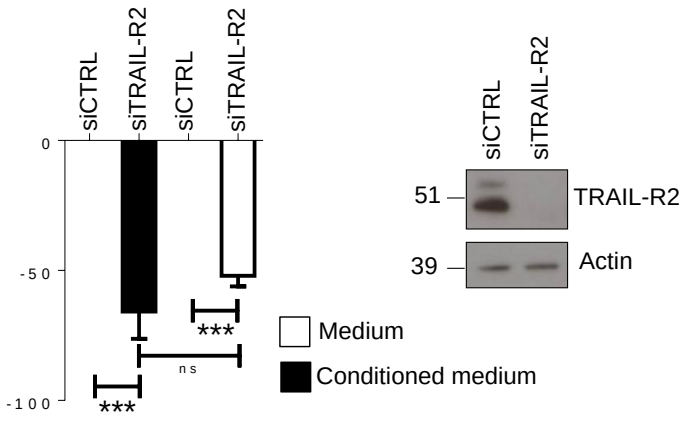


Figure 9. Cancer cell-secreted factors do not affect TRAIL-R2-mediated migration

A549 cells were transfected with siRNAs silencing TRAIL-R2 and subjected to migration assays in the presence of either control medium or A549-conditioned medium in the upper transwell chamber (experiment conducted by S. von Karstedt). Migration was normalized to control transfected cells in each of the cell lines.

To determine whether TRAIL-mediated cytokine secretion involved the TRAIL-R2/Rac1 signalling axis, Rac1 was inhibited, using inhibitor NSC23766, followed by TRAIL

treatment and subsequent analysis of cytokine secretion. NSC23766 specifically inhibits Rac1 activity by binding to the surface groove of Rac1, known to be critical for GEF interaction (Gao et al., 2004). Other than in TRAIL/TRAIL-R-mediated pro-migratory signalling, Rac1 inhibition increased TRAIL-mediated cytokine secretion, indicating that Rac1 activity is inhibitory to TRAIL-mediated cytokine secretion. Moreover, Rac1 activity does not elicit an activatory outcome as in cell autonomous signalling, thereby indicating that the Rac1-mediated pro-migratory effects are mechanistically distinct from TRAIL-mediated cytokine secretion (Figure 10A). To validate that migration and cytokine induction are two distinct mechanisms, it was next determined whether the MPD domain, crucial in TRAIL-R-mediated cell-autonomous migration, could be ruled out as promoter of cytokine secretion. To this end, A549 cells were transfected with full length or TRAIL-R2 mutant constructs lacking either the DD (ΔDD), the DD and CTD (ΔDD/CTD) or the entire intracellular domain (ΔICD) and assayed for CXCL1 secretion (Figure 10B). Expression of full-length TR2 was able to induce CXCL1, whilst induction was abrogated with all tested mutants. Since the ΔDD/CTD mutant, which only contained the MPD, failed to show induction, this domain on its own cannot be involved in TRAIL-mediated gene activation. The ΔDD construct was also incapable of rescuing cytokine secretion. Hence, TRAIL-R2 requires the DD, but not the MPD, to induce cytokine secretion. Overall, this demonstrates that TRAIL/TRAIL-R2-mediated cytokine production is mechanistically distinct from cell-autonomous Rac1-mediated pro-migratory effects.

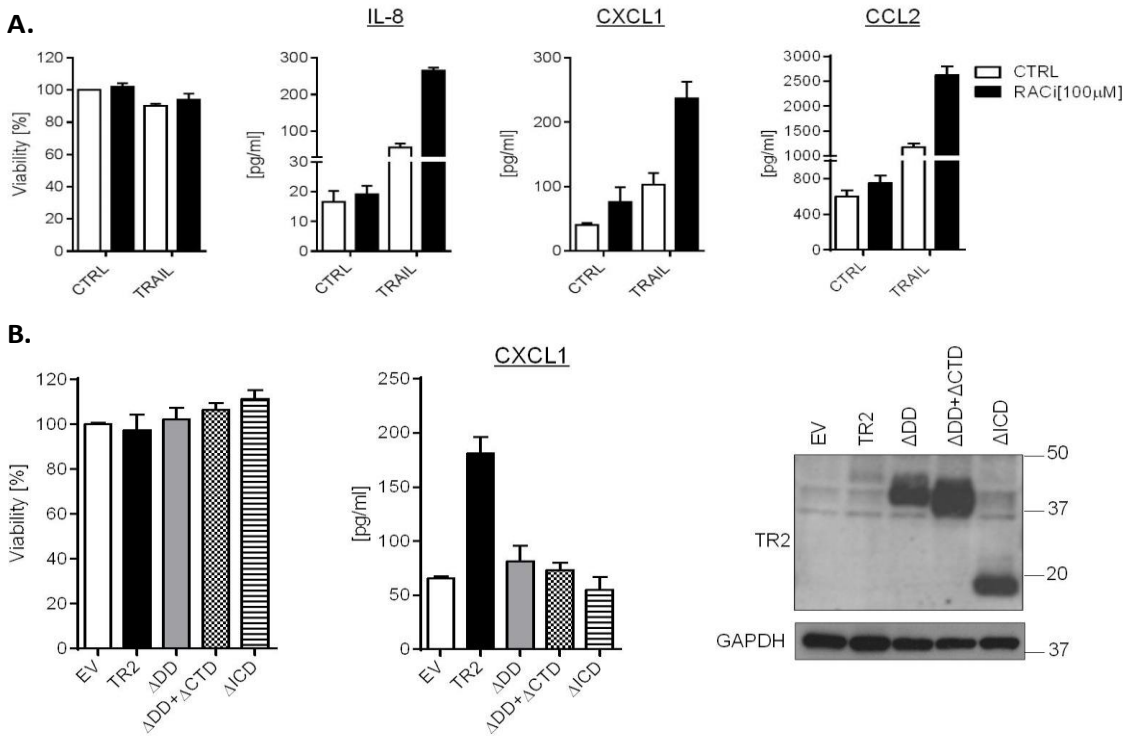


Figure 10. TRAIL induces cytokines independently of the TRAIL-R2-MPD/Rac1 axis

A. A549 cells were treated with iz-TRAIL [100 ng/ml] for 24h in the presence or absence of Rac1 inhibitor (NSC23766 [100 µM]); cell viability was determined by CellTiter-Glo and the indicated cytokines were quantified from supernatants by ELISA. **B.** A549 cells were transiently transfected with either empty vector (EV) or with vector containing the indicated TRAIL-R2 mutant and subsequently subjected to CellTiter-Glo; supernatants from these cells were subjected to CXCL1 ELISA.

3.2.2 TRAIL-induced cytokines are transcriptionally regulated

The secretion of cytokines can occur via two main mechanisms, regulated secretion or constitutive secretion (Stanley and Lacy, 2010). Regulated secretion occurs via release of pre-synthesized cytokines contained in secretory vesicles or granules, whilst constitutive secretion requires *de novo* cytokine synthesis and thus transcription. TRAIL-independent studies show that the expression of CCL2, CXCL1, IL-8 and CXCL5 are transcriptionally regulated by NF-κB (Kunsch and Rosen, 1993, Smith et al., 2002, Ohmori et al., 1995, Deng et al., 2013)

However, several of the identified, TRAIL-induced cytokines such as IL-8, CXCL1 and CCL2 have been shown to exist in preformed vesicles in endothelial cells and thus can follow the regulated secretory pathway in certain contexts (Oynebraten et al., 2005). To address whether TRAIL-induced chemo-/cytokine production involves *de novo* synthesis, it was first determined whether the indicated cytokines were upregulated transcriptionally upon TRAIL stimulation (Figure 11).

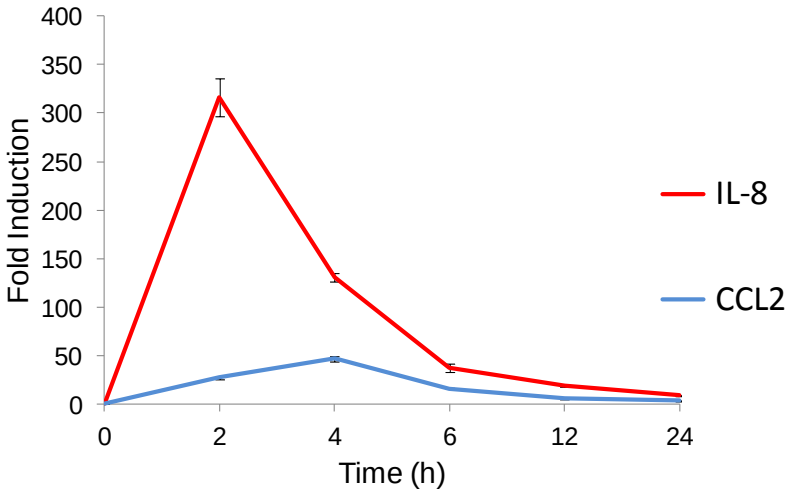


Figure 11. TRAIL induces expression of IL-8 and CCL2 transcripts

A549 cells were stimulated with iz-TRAIL [100 ng/ml] for the indicated timepoints, followed by RNA isolation and determination of transcript levels via qPCR.

In line with data previously obtained by ELISA (Figure 6), TRAIL stimulation also significantly induced transcript levels of IL-8 and CCL2, indicative of transcriptional regulation of these cytokines. IL-8 was strongly induced after 2 h whilst CCL2 transcription peaked at 4h.

To validate whether cytokines were indeed transcriptionally regulated, transcription was blocked by incubating A549 with actinomycin D prior to TRAIL treatment. Actinomycin D potently blocks all transcriptional activity by binding DNA and preventing transcriptional elongation. As seen in Figure 11, actinomycin D sensitized A549 to cell death, which was likely due to the transcriptional dependence of anti-apoptotic proteins such as Mcl-1 and cFLIP (Lemke et al., 2014a). In order to specifically assay for the role of transcription in TRAIL-mediated cytokine induction, independently of potential conflicting effects of cell death, the A549 cells were also co-incubated with QVD. Whilst QVD rescued actinomycin D-mediated TRAIL-induced cell death, IL-8 induction was not detected in this condition. Therefore, TRAIL-mediated IL-8 production is induced at the transcriptional level.

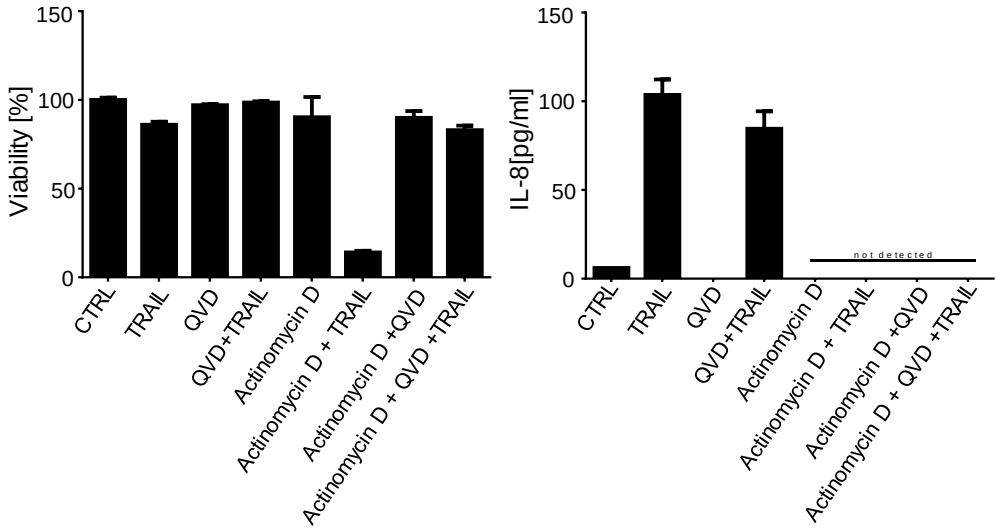


Figure 12. Actinomycin D blocks TRAIL-induced chemo-/cytokine secretion

A549 cells were incubated with actinomycin D and QVD [10 µM] followed by treatment with iz-TRAIL [100 ng/ml] for 24h; cell viability was determined by CellTiter-Glo and chemo-/cytokine concentrations in the cell supernatants were measured via ELISA.

Since NF-κB is a key regulator of IL-8 production it was next assessed whether TRAIL mediates NF-κB activation in A549 cells. As shown in Figure 13A, TRAIL was indeed capable of inducing IkB phosphorylation and consequent degradation after 0.5h and 1h respectively. Superimposing the gene activation, with the qPCR kinetic indicates that NF-κB is likely driving IL-8 and CCL2 transcription as both cytokines are already strongly induced after 2h during the peak of IkB phosphorylation. The requirement of NF-κB signalling in TRAIL mediated cytokine induction was verified using the small molecule IKKα/β inhibitor TPCA-1, which prevents phosphorylation and degradation of IkBα and nuclear translocation of the NF-κB subunit p65 (Figure 13A). TPCA-1 completely abrogated TRAIL-mediated production of IL-8, CXCL1 and CCL2 thereby indicating that NF-κB is a key transcription factor in TRAIL-mediated cytokine production (Figure 13B).

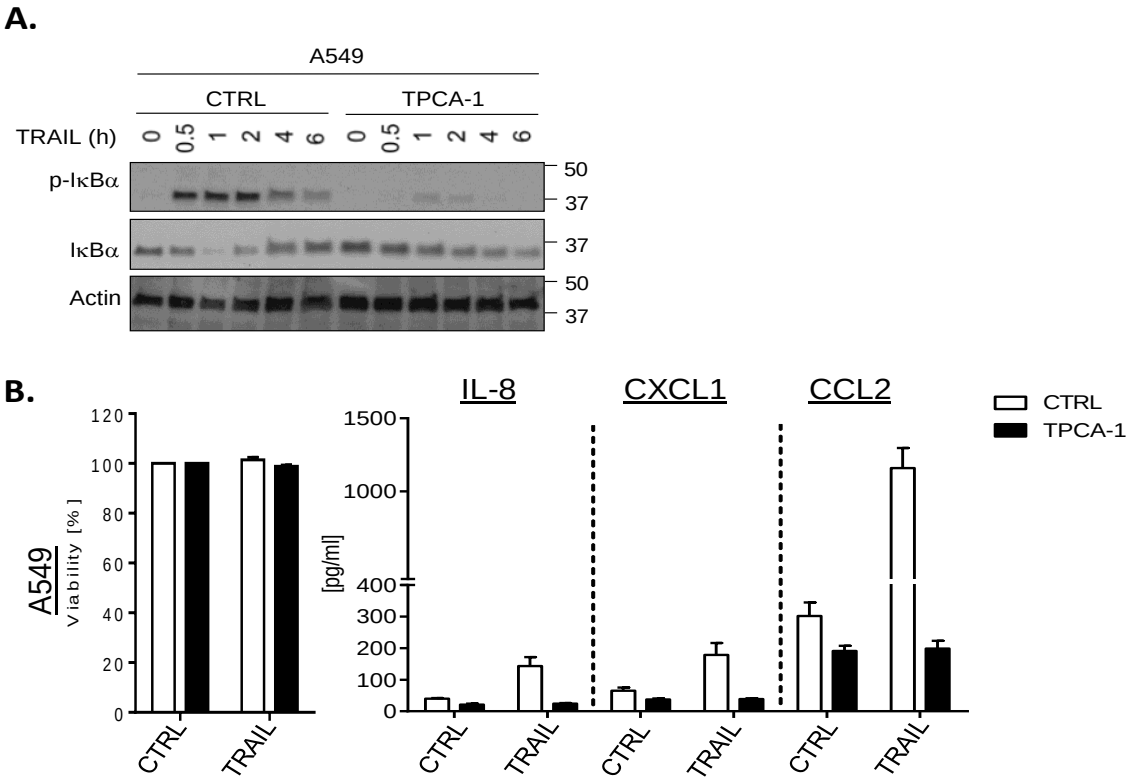


Figure 13. TRAIL induces NF-kB-dependent cytokines

A. A549 cells were treated with CTRL, TRAIL [100 ng/ml] or TPCA-1 [5 µM] for the indicated times followed by immunoblotting for the indicated proteins. **B.** A549 cells were treated as above for 24h followed by determination of the indicated cytokines by ELISA. All treatments were conducted in presence QVD [10 µM].

The specific requirement of the NF-κB subunit p65 in cytokine production was further verified, as transient knockdown of p65 significantly abrogated TRAIL-mediated cytokine production (Figure 14).

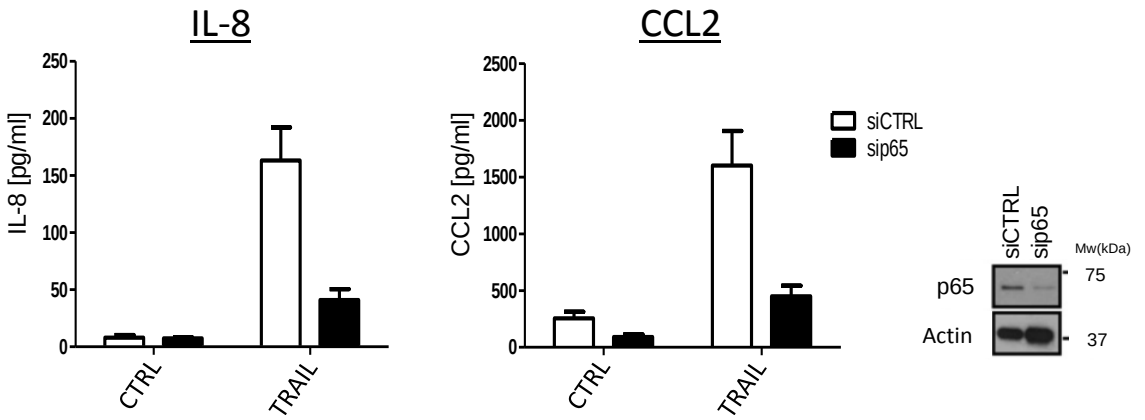


Figure 14. TRAIL-mediated cytokine induction is specifically dependent on p65

A549 cells were transiently transfected with siRNA targeting p65; 24h later the cells were stimulated with QVD [10 µM] (CTRL) or QVD [10 µM] +TRAIL [100 ng/ml] cytokine concentrations were determined by ELISA. A knockdown control western blot is shown.

3.2.3 The presence of FADD is required for TRAIL-mediated gene activation and cytokine induction

The adaptor molecule FADD is well known for its requirement in TRAIL/TRAILR-mediated recruitment of caspase-8 to the death-inducing signalling complex (DISC), thereby initiating the intrinsic apoptosis pathway (Chinnaiyan et al., 1995). However, the role of FADD in TRAIL-mediated, gene-activatory, signalling has remained contradictory (Ehrhardt et al., 2003; Varfolomeev et al., 2005). To investigate the involvement of FADD in gene activation and cytokine induction, A549 FADD KO cells were generated via zinc-finger nuclease (ZFN) technology in our laboratory.

As shown in Figure 15A, TRAIL was capable of inducing the activation of NF-κB and JNK in A549 WT cells. Interestingly, in contrast to A549 WT, gene activation was entirely abrogated in FADD KO cells, demonstrating that FADD presence is essential for TRAIL-mediated NF-κB and JNK pathway activation. Accordingly, TRAIL-induced cytokine secretion was abrogated in FADD KO cells as compared to wildtype A549, whilst cell viability was not affected (Figure 15B and data not shown).

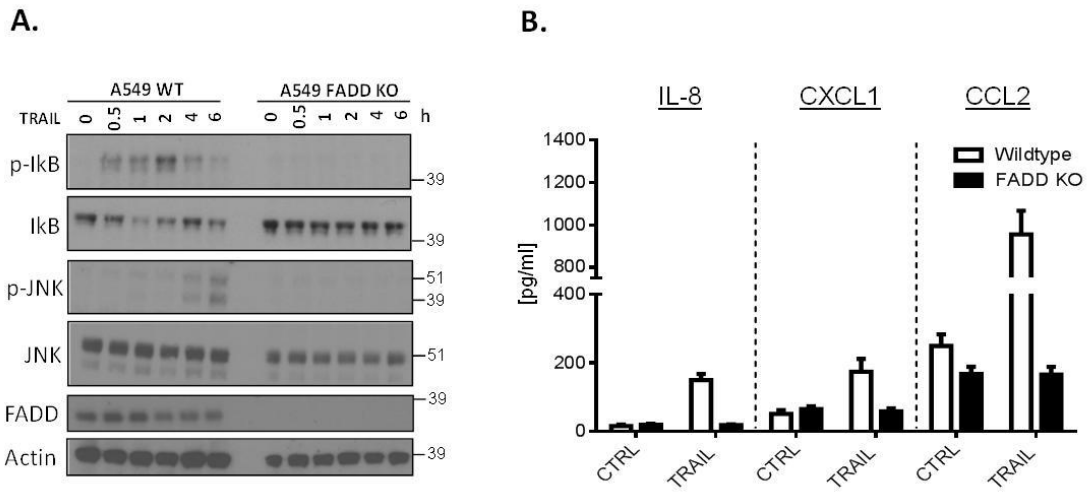


Figure 15. The presence of FADD is required for TRAIL-induced gene activation and cytokine induction

A. A549 WT or FADD KO cells were stimulated with iz-TRAIL [100 ng/ml] for the indicated timepoints followed by immunoblotting for the listed proteins. **B.** A549 FADD KO cells were generated via transfection of ZFN-constructs targeting exon1 of FADD, followed by sub-cloning and KO confirmation via western blot. A549 WT or FADD KO cells were stimulated with iz-TRAIL [100 ng/ml] for 24h, followed by determination of cell viability and cytokine concentrations by ELISA.

FADD deficiency might sensitize to other cell death pathways, most importantly RIP3 mediated necroptosis (Bonnet et al., 2011). A potential increase in necroptosis, upon FADD-deficiency, could result in selection for a cell line that is also deficient in the expression of factors required for necroptosis. To exclude that A549 FADD KO cells lack cytokine induction due to selection for the loss of necroptosis components, their RIP3

levels and ability to undergo necroptosis were determined. Since RIP3 was not detectable, FADD deficiency was unlikely to enable necroptosis-based selection (Figure 16A). In line with this, SMAC and zVAD combination treatment was unable to sensitize wildtype or FADD KO cells to TRAIL-induced necroptosis whereas necroptosis sensitive, RIP3-expressing cells died (Figure 16B). The inability of A549 cells to undergo necroptosis rules out any potential confounding effects on cytokine production from necroptosis-based selection and indicates that specifically the loss of FADD is indeed responsible for the abrogation of cytokine secretion.

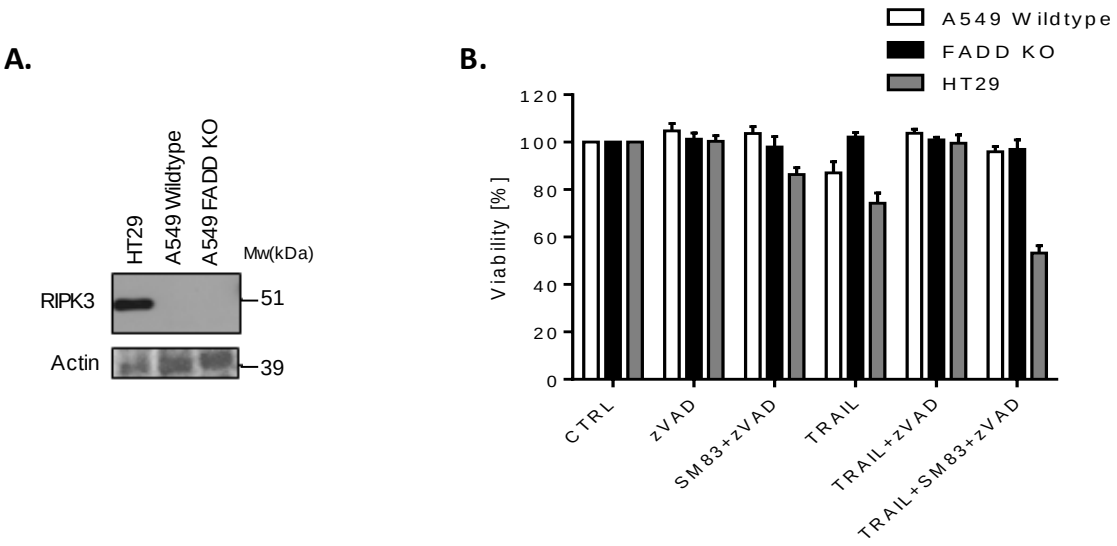


Figure 16. A549 cells are incapable of undergoing necroptosis

A. The indicated cell lines were probed for RIP3 expression by Western Blot. **B.** A549 Wildtype or FADD KO clones, in comparison to HT29 were treated with zVAD [10 µM], SM-083 [100 nM] or the combination of both for 24h and subjected to viability assays via CellTiter-Glo.

In order to further verify a specific requirement of FADD for cytokine induction, A549 FADD KO cells were reconstituted with wildtype FADD or empty vector (EV) containing constructs. FADD reconstitution fully reinstated cytokine production as well as IkBα phosphorylation (Figure 17A and B). Therefore, specifically the loss of FADD was responsible for the abrogation of gene activation and cytokine production.

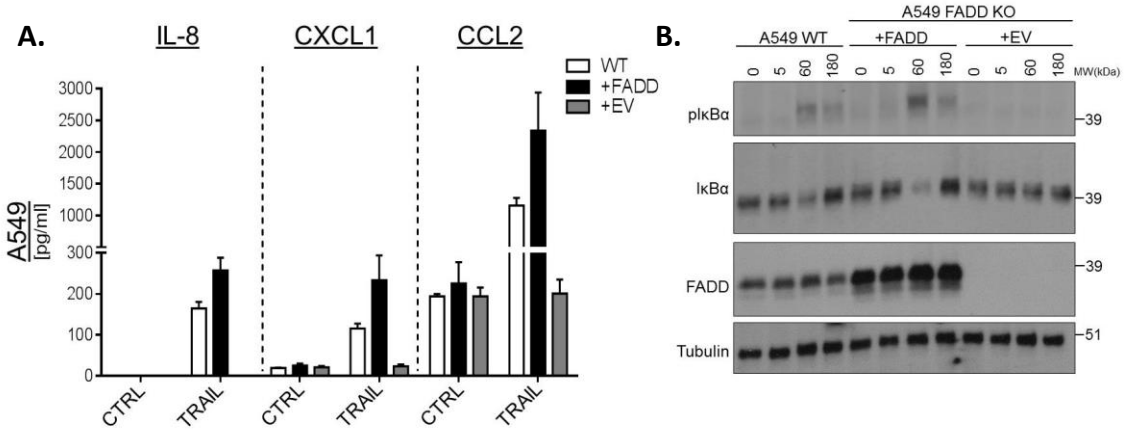


Figure 17. The reconstitution of FADD rescues cytokine production and gene activation

A. A549 FADD KO were reconstituted with either wildtype FADD (+FADD) or empty vector (+EV) and stimulated with [100 ng/ml] iz-TRAIL for 24h, followed by collection of supernatants and ELISA for the indicated cytokines. **B.** Cells as in (A) were stimulated with iz-TRAIL [100 ng/ml] for the indicated times, lysed and analyzed for the indicated proteins by western blot.

3.2.4 FADD is required for TRAIL-mediated DISC and complex II formation

TRAIL-induced gene activation has been associated with the formation of a secondary intracellular complex (complex II) retaining caspase-8 and FADD (Varfolomeev et al., 2005). Since FADD was found to be essential for TRAIL-mediated cytokine induction and gene activation, the role of FADD in secondary complex formation was investigated next. To this end, a two-step immunoprecipitation was conducted by first precipitating the membrane-associated DISC from lysates, followed by immunoprecipitation of cytosolic caspase-8. To specifically assay for a role of FADD, A549 WT or FADD KO cells reconstituted with either FADD or EV were utilized for this purpose (Figure 18A and B). Initial immunoprecipitation of the DISC revealed the presence of caspase-8, FADD, RIP1, $cFLIP_L$ and $cFLIP_S$ starting at 5 min, in A549 WT and FADD-reconstituted KO cells but not in EV reconstituted FADD KO cells. As expected, TRAIL-R2 was absent in the cytosolic complex, thereby verifying complete depletion of the DISC in the first immunoprecipitation (Figure 18B). The secondary complex contained the same components observed in the DISC in A549 WT and FADD-reconstituted A549 cells after 1 hour of stimulation, and therefore at a later stage than in the DISC, indicating that the TRAIL complex II possibly forms sequentially after the DISC. TRADD could not be detected in either complex (data not shown). Importantly, FADD KO cells did not form complex II, demonstrating that FADD is essential in both DISC but also complex II formation in TRAIL signalling. Therefore, FADD's requirement in TRAIL-mediated cytokine induction might be attributed to its role as an essential platform in TRAIL-induced complex formation.

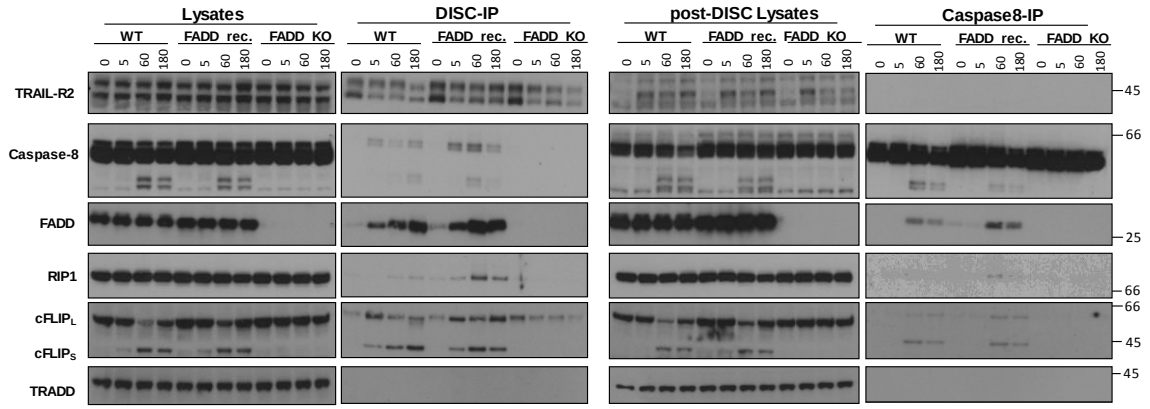


Figure 18. FADD is required for TRAIL-mediated DISC and post-DISC complex formation

A. Analysis of complex I (DISC) or complex II (post-DISC complex) in the presence of zVAD [20 µM] for A549 wildtype, A549 FADD KO reconstituted with FADD (FADD rec.), or A549 FADD KO cells reconstituted with empty vector (FADD KO). The cells were incubated with FLAG-TRAIL [500 ng/ml] for the indicated times and lysates were prepared. TRAIL-R2 was then immunoprecipitated from the cell lysates via anti-FLAG M2 beads (DISC-IP). **B.** Subsequently, caspase-8 was precipitated from DISC-depleted lysates using an anti-caspase-8 antibody (caspase-8-IP).

3.2.5 The presence of caspase-8 but not its activity is required for TRAIL-mediated cytokine induction

Since TRAIL's secondary complex contained caspase-8, it was hypothesized that the initiator caspase and/or its activity may be involved in TRAIL-mediated cytokine induction. To test this hypothesis, A549 cells were pre-treated with the caspase inhibitor QVD and subsequently stimulated with TRAIL. Interestingly, QVD failed to significantly block cytokine induction, even though it was capable of rescuing cell death and is therefore active as demonstrated previously (Figure 19A and 7B). Together, these results show that caspase activity is not required for TRAIL mediated cytokine induction. To specifically investigate a role for the presence of caspase-8 in TRAIL-mediated cytokine induction, A549 were transiently transfected with siRNA targeting caspase-8 and subsequently stimulated with TRAIL. Surprisingly, TRAIL-mediated cytokine induction was stunted, indicating that caspase-8 presence is required for TRAIL-mediated cytokine induction. Therefore, whilst its activity is dispensable, the presence of caspase-8 may serve a crucial scaffold function required in the non-apoptotic, cytokine-inducing arm of TRAIL-R signalling.

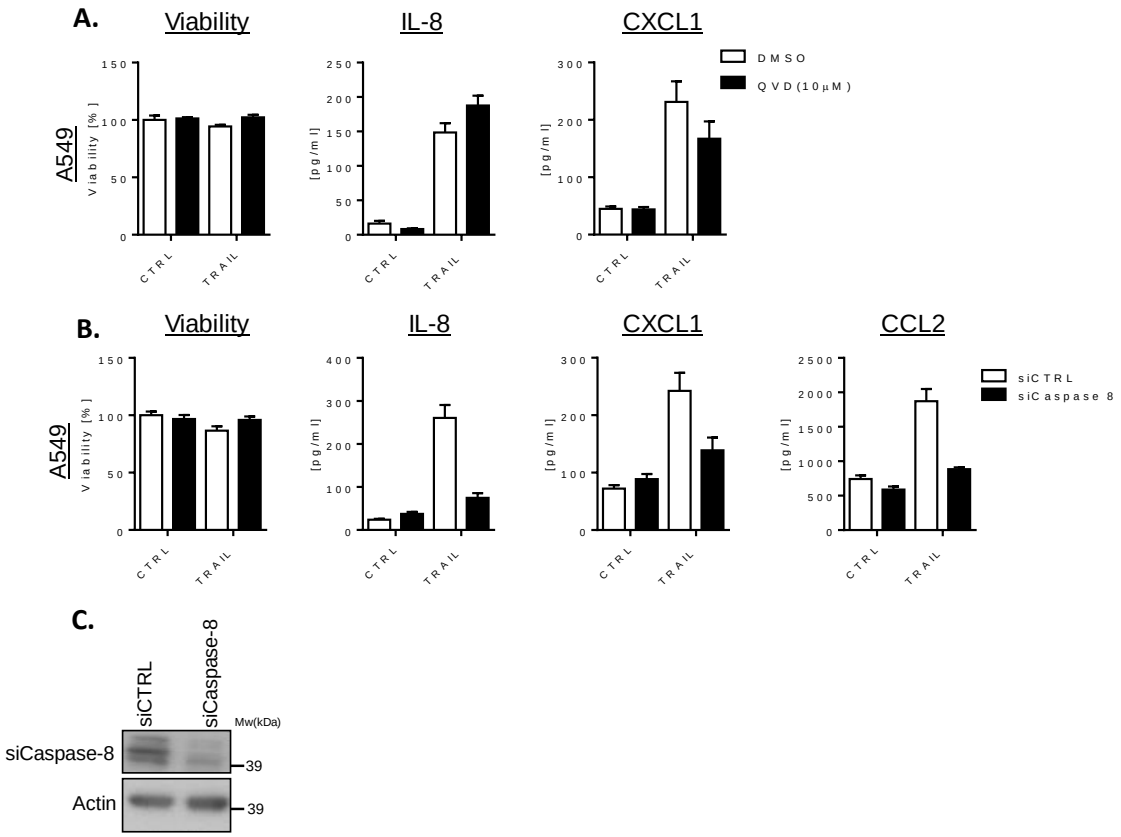


Figure 19. Caspase-8 presence, but not its activity, is required for TRAIL-mediated cytokine induction

A. A549 cells were pre-incubated with QVD [10 uM] followed by stimulation with iz-TRAIL [100 ng/ml]; after 24h supernatants were collected and cell viability was determined by CellTiter-Glo; cytokine concentrations were determined by ELISA. **B**. A549 were transiently transfected with siRNA targeting caspase-8; 24h later the cells were stimulated with iz-TRAIL [100 ng/ml]; cytokine concentrations and cell viability were determined as above. **C.** Western blot showing knockdown control.

3.2.6 TRADD promotes TRAIL-mediated cytokine production

Although it was not detected in either complex of TRAIL/TRAIL-R signalling, TRADD plays a crucial upstream adaptor role in TNF-mediated gene activation (Pobezinskaya et al., 2008). However, its role in TRAIL-induced gene activation has remained conflicting and has not previously been addressed specifically regarding TRAIL-mediated cytokine secretion (Varfolomeev et al., 2005; Tang et al., 2009). To explore the potential role of TRADD, possibly as a transiently binding adaptor within the secondary complex, TRADD was silenced followed by determination of TRAIL-mediated cytokine secretion. Interestingly, cytokine production was significantly decreased although not blunted in TRADD-silenced A549 cells, identifying an involvement of TRADD in this TRAIL signalling output (Figure 20). Therefore, subsequent analyses examined how expression ablation of several other factors, which are also involved in TNFR1 signalling affected TRAIL-induced cytokine secretion (Mahoney et al., 2008).

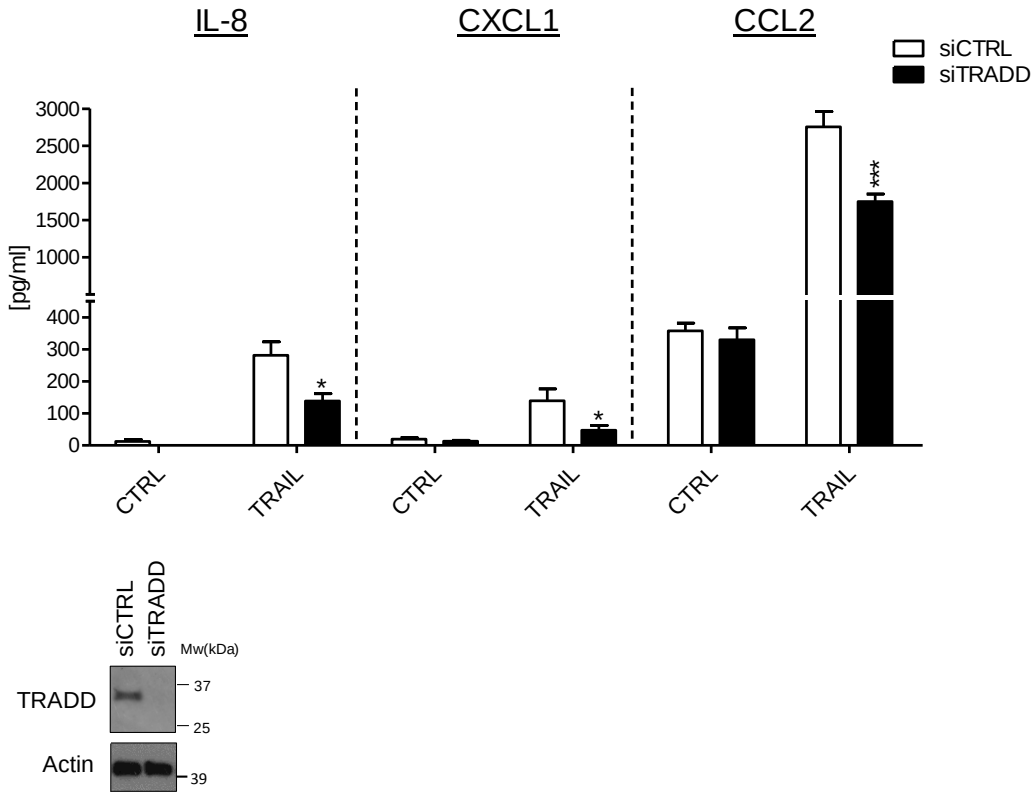


Figure 20.TRADD promotes TRAIL-induced cytokine production

A549 cells were transiently transfected with siRNA targeting caspase-8; 24h later the cells were stimulated with QVD [10 µM] (CTRL) or QVD [10 µM] + TRAIL [100 ng/ml]; cytokine concentrations were determined by ELISA. A representative knockdown control blot is shown.

3.2.7 TRAF2 presence supports TRAIL-mediated cytokine induction

TRAF2 was not clearly visible in the complex analysis, yet it is well known for its requirement in TNF-mediated gene activation and directly interacts with TRADD, which was previously identified as a mediator of TRAIL-R's cytokine-inducing arm (Figure 20). In fact, earlier data suggest that TRAF2 may play a similar role in TRAIL signalling. However previous work on this topic employed overexpression systems and relied on gene expression data rather than absolute cytokine quantification via ELISA (Tang et al., 2009). To address the role of TRAF2 in cytokine induction, A549 were transiently transfected with siRNA targeting TRAF2. As seen in Figure 21, TRAF2 knockdown significantly sensitized A549 to TRAIL-induced cell death, which is in line with recently published data (Karl et al., 2014). Since the differential cell death would conflict with readout of TRAF2's role in cytokine induction, the cells were pre-treated with QVD. Although some residual sensitization remained, even with QVD pre-treatment, TRAIL-mediated cytokine induction was severely stunted upon knockdown of TRAF2, therefore indicating that TRAF2 supports TRAIL-mediated cytokine induction.

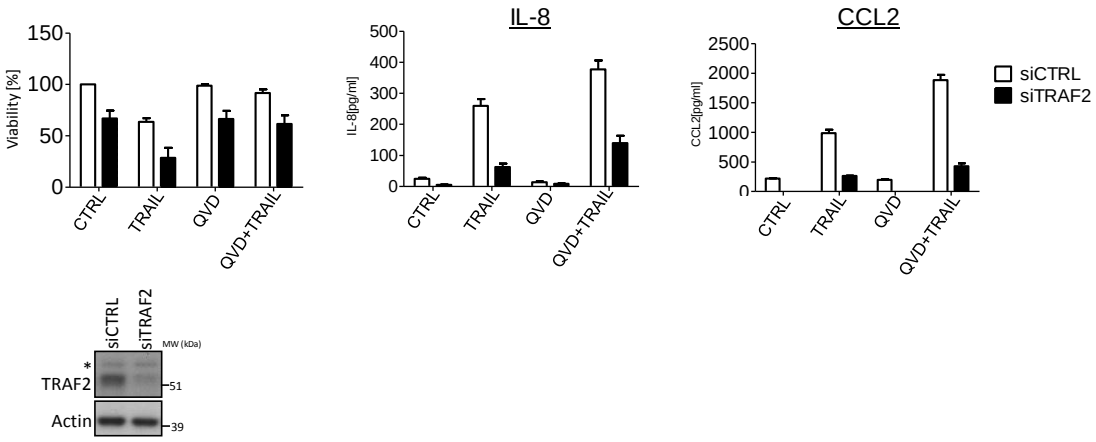


Figure 21. TRAF2 presence supports TRAIL-mediated cytokine induction

A549 were transiently transfected with siRNA targeting TRAF2; 24h later cells were stimulated with iz-TRAIL [100 ng/ml] in presence or absence of QVD [10 µM]. IL-8 concentrations and cell viability were determined as above.

3.2.8 cIAP1 and 2 support TRAIL-mediated cytokine secretion

In TNF-mediated gene activation, TRAF2 elicits its gene-activatory effects via recruitment of cIAP1/2 (Blackwell et al., 2009). Since TRAF2 was involved in TRAIL-mediated cytokine induction, it was hypothesized that cIAP1/2 were also involved herein. As cIAP1 and 2 can perform redundant functions in TNF-mediated gene activation, both were knocked down simultaneously to assess their role in TRAIL-mediated cytokine induction. Using specific siRNA, diminishing of cIAP1 and 2 significantly reduced TRAIL-mediated IL-8, CXCL1 and CCL2 secretion (Figure 22A). Furthermore, degradation of cIAP1/2 using the SMAC mimetic compound SM-83, blunted TRAIL-mediated cytokine production, verifying the result from the knockdown (Figure 22B) (Lecis et al., 2013). These data demonstrate that cIAP1/2 promote TRAIL-mediated cytokine secretion.

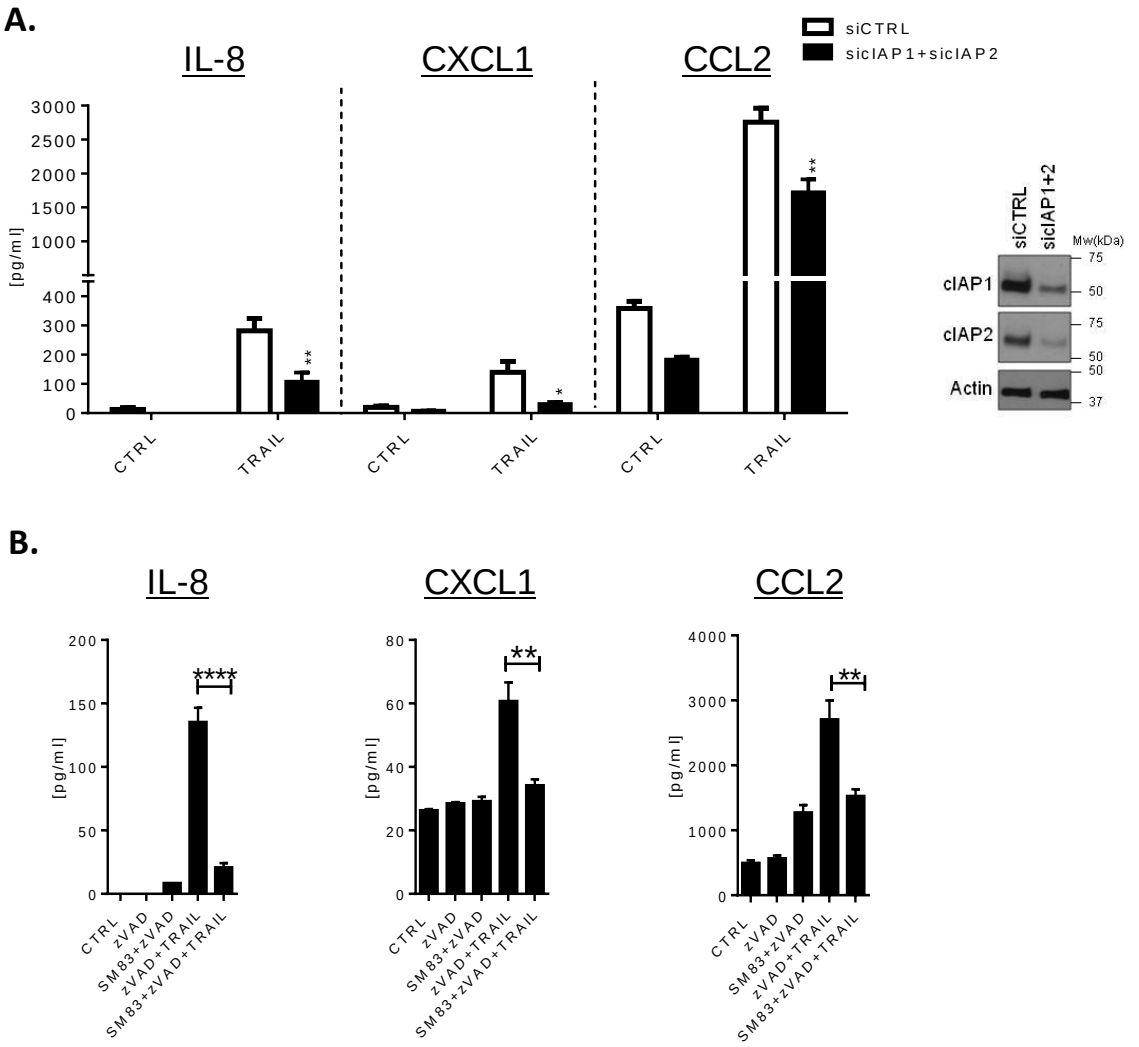


Figure 22. cIAP1 and 2 are required for TRAIL-mediated cytokine production

A. A549 cells were pre-incubated with QVD [10 µM] followed by stimulation with iz-TRAIL [100 ng/ml]; after 24h supernatants were collected and cell viability was determined by CellTiter-Glo; cytokine concentrations were determined by ELISA. **B.** A549 cells were treated with zVAD [10 µM], SMAC mimetic SM-83 [100 nM] with or without TRAIL [100 ng/ml] for 24h; cytokines were determined as before.

3.2.9 The presence of HOIP and its activity promote TRAIL-mediated cytokine induction

Our laboratory has previously shown that HOIP, a crucial component of LUBAC, and in particular its linear chain forming capability promote TNF-mediated gene-activation (Haas et al., 2009). Herein, HOIP is recruited via cIAP1/2-mediated ubiquitylation. Since cIAP1/2 were required for cytokine induction, it was hypothesized that HOIP may also be involved in TRAIL-mediated cytokine induction. To test this hypothesis, HOIP was knocked out in A549 using CRISPR/Cas9 technology. The KO cells were subsequently reconstituted with either EV, a WT or a mutated version of the E3 ligase (C885S). The C885S version of HOIP contained a point mutation at residue 885 (C to S), rendering the protein

enzymatically inactive, thereby enabling the determination of a specific role for HOIP's linear ubiquitin generating activity in TRAIL-mediated cytokine production.

In order to validate these cells regarding their suitability for the evaluation of HOIP mediated gene-activatory signalling they were initially stimulated with TNF (Figure 23A). In line with the previously mentioned requirement of HOIP activity in TNF-mediated cytokine production, both empty vector as well as the enzymatically inactive reconstituted A549 HOIP KO were stunted in their ability to induce IL-8 and CCL2 (Figure 23A). To prevent differential cell death between wildtype, C885S and EV reconstituted cells and from conflicting with and obscuring a potential direct role of HOIP in cytokine production, A549 cells were pretreated with QVD (Lafont et al., 2017). Upon TRAIL stimulation, both HOIP KO cells as well as HOIP C885S cells exhibited significantly decreased cytokine induction, whilst low levels of cytokine induction were still observed in the C885S reconstituted HOIP KO cells (Figure 23B). Therefore, HOIP presence and activity, promote TRAIL-mediated cytokine induction.

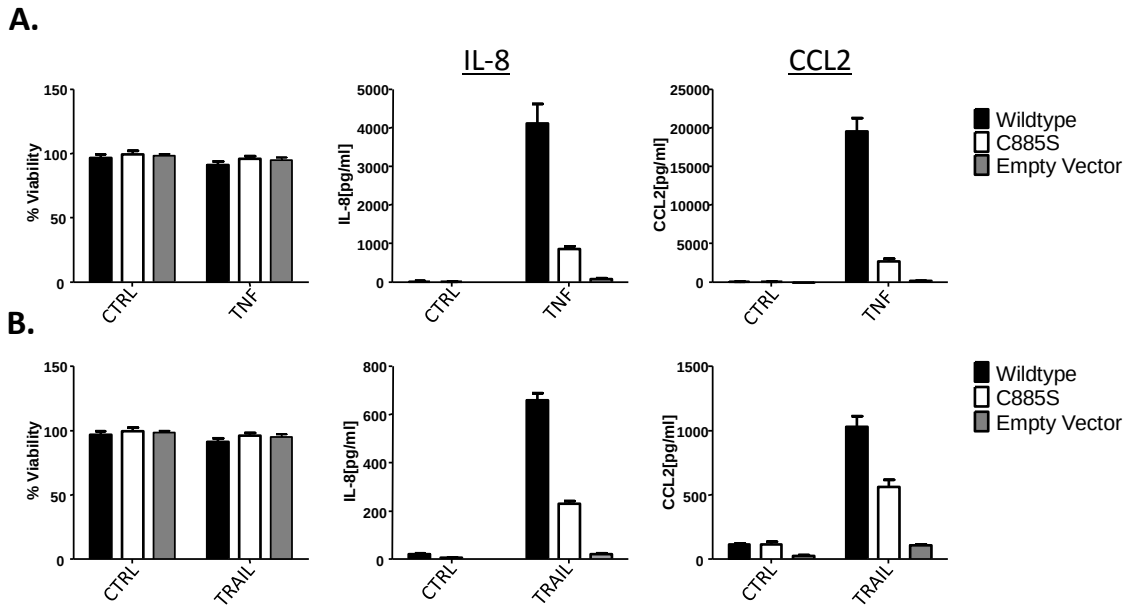


Figure 23. The presence of HOIP and its activity promote TRAIL- and TNF-mediated cytokine induction

A. A549 HOIP KO cells reconstituted with Empty Vector, C885S or Wildtype HOIP were stimulated with QVD [10 µM] (CTRL) or QVD [10 µM] + TNF [50 ng/ml] (TNF); after 24h supernatants were collected and cell viability was determined by CellTiter-Glo**;** cytokine concentrations in supernatants were determined by ELISA. **B.** A549 HOIP KO cells reconstituted with Empty Vector, C885S mutated or Wildtype HOIP were stimulated with QVD [10 µM] (CTRL) or QVD [10 µM] + TRAIL [100 ng/ml]; cell viability and cytokine concentrations were determined as above.

3.2.10 TAK1 promotes TRAIL-mediated cytokine induction

The linear chain forming ability of HOIP enables the recruitment of TAK1 into the TNF and IL-1α signalling complexes (Haas et al., 2009; Zhang et al., 2014), TAK1 in turn catalyses

the activation of IKK-β to promote NF-κB signalling (Zhang et al., 2014). Since HOIP promoted TRAIL-mediated gene activation, it was hypothesized that the E3 ligase also mediates gene activation via TAK1 in TRAIL signalling. To this end, A549 cells were transiently transfected with siRNA targeting TAK1 and stimulated with TRAIL. The loss of TAK1 has previously been shown to sensitize cells to TRAIL-induced apoptosis (Morioka et al., 2009). To exclude any confounding effects from differential viability, caspase activity was inhibited by addition of QVD. TAK1 knockdown significantly decreased TRAIL-mediated IL-8 and CCL2 secretion, demonstrating that TAK1 promotes cytokine induction (Figure 24).

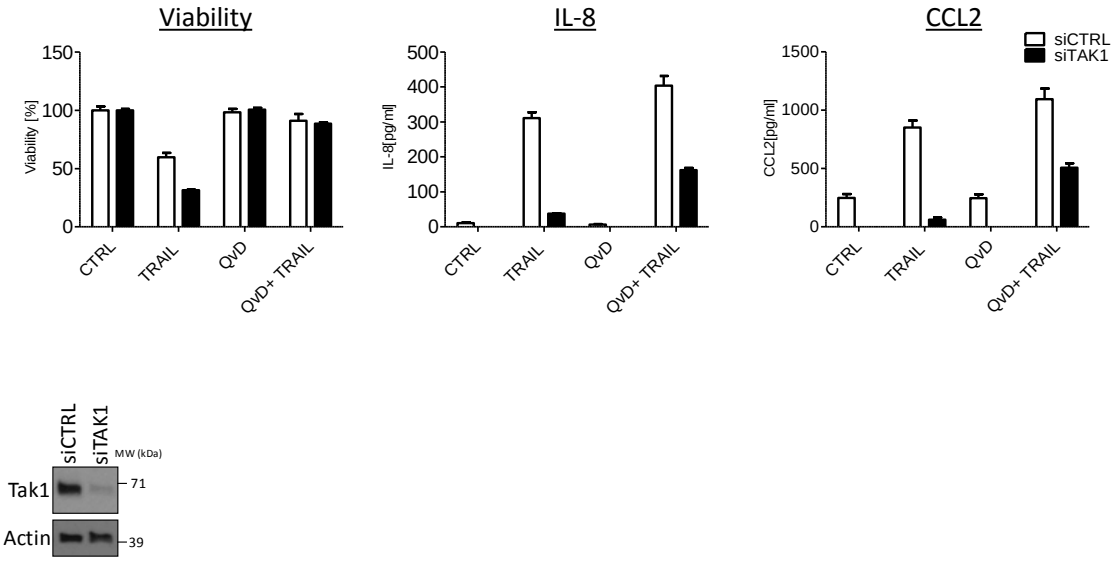


Figure 24. TAK1 promotes TRAIL-mediated cytokine induction

A549 cells were pre-incubated with medium (CTRL) or QVD [10 µM], followed by stimulation with iz-TRAIL [100 ng/ml]; after 24h supernatants were collected and cell viability was determined by CellTiter-Glo; cytokine concentrations in supernatants were determined by ELISA. A knockdown control western blot is shown.

To validate a role of TAK1 in TRAIL-mediated cytokine secretion, particularly in regards to TAK1 activity, the irreversible TAK1 protease and kinase inhibitor 7-Oxozeanol, was used. As seen in Figure 25, incubation with 7-Oxozeanol abrogated TRAIL-mediated cytokine secretion (A) and decreased the activation of NF-κB (B). Therefore, TAK1 mediates cytokine production by means of its activity.

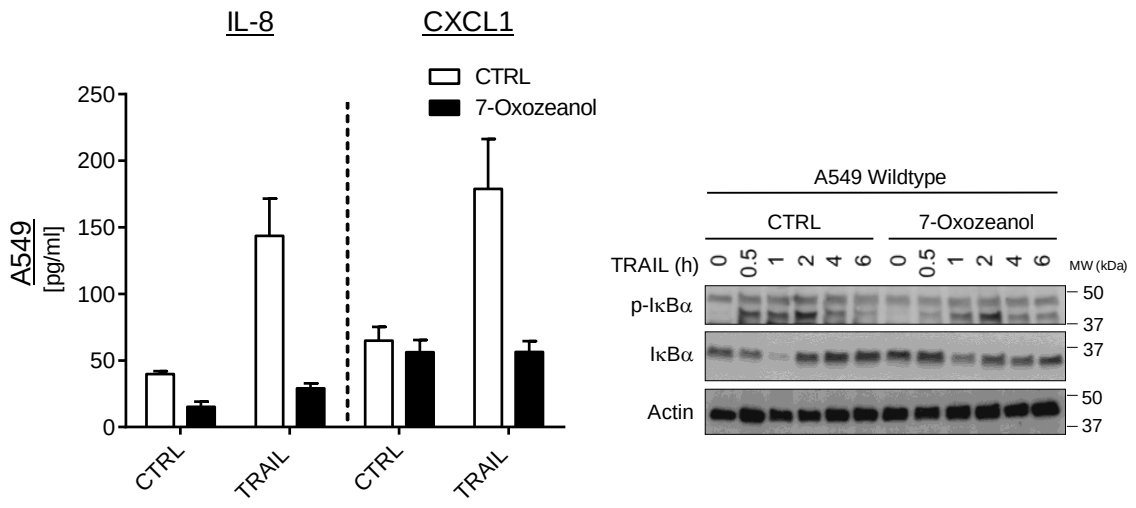


Figure 25. TAK1 activity is required for cytokine induction and activation of NF-κB

A. A549 cells were treated with 7-Oxozeanol [1 µM] and/or TRAIL [100 ng/ml] for 24h and indicated cytokines were quantified by ELISA. **B.** Cells were stimulated accordingly for the indicated times and lysates were analysed for the listed proteins by western blot.

3.2.11 Both TRAIL-R1 and TRAIL-R2 contribute to TRAIL-induced cytokine production

Previous studies provide conflicting evidence regarding the contribution of TRAIL-R1 and TRAIL-R2 in gene activation. TRAIL-R1 has been demonstrated as the central inducer of cytokine production, as TRAIL-R1 specific blocking antibodies elicited robust abrogation of TRAIL-mediated IL-8 production. TRAIL-R2 blocking antibodies only had marginal blocking effects herein (Leverkus et al., 2003). However, other studies indicate that both receptors can equally mediate the TRAIL's cytokine output (Tang et al., 2009). Only TRAIL-R1 and TRAIL-R2 contain a death domain and are thereby capable of binding to FADD. As previously shown in this thesis, FADD is essential for TRAIL-mediated gene activation and cytokine production. Therefore, it was hypothesized that both DD-containing receptors are capable of promoting cytokine production. To test this hypothesis, A549 cells containing a control vector (pLKO.1) or stable knockdown vectors for either TRAIL-R1 (shTR1) or TRAIL-R2 (shTR2) were stimulated with TRAIL and analysed for cytokine secretion. As shown in Figure 26B, cytokine induction was severely stunted in both shTR1 and shTR2 cells as compared to pLKO.1, whilst cell viability was not significantly impacted (data not shown). The decrease in induction held true for all cytokines tested, whilst IL-8 showed the strongest decrease when either TRAIL-R1 or TRAIL-R2 was downregulated. Although the shTR-1 cells showed a slight trend towards a stronger decrease in induction, this was not significant and therefore supports the hypothesis that both receptors are involved in TRAIL-mediated cytokine induction.

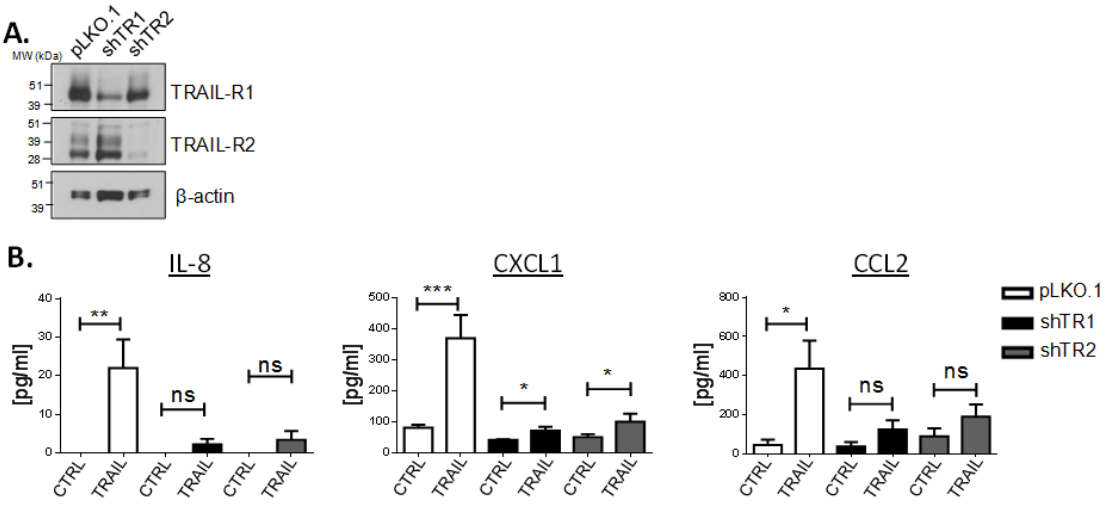


Figure 26. Both TRAIL-R1 and TRAIL-R2 promote cytokine induction

A. Lysates from A549 cells stably transfected with either empty vector (pLKO.1), shTRAIL-R1 (shTR1) or shTRAIL-R2 were prepared, run on western and blotted for the indicated proteins. **B.** A549 cells from A. were stimulated with iz-TRAIL [100 ng/ml]; 24h later supernatants were collected and analysed for cytokine levels via ELISA.

3.3 Functional characterization of the TRAIL-induced secretome

The previous two chapters have comprehensively elucidated TRAIL's secretome and provided insight into the regulation of its expression. The tumour's chemo-/cytokine milieu is an important cancer driver in tumour progression by eliciting both autocrine and paracrine effects (Landskron et al., 2014). However, the functional role of TRAIL-induced cytokines is only poorly understood. Therefore, this chapter investigates whether, and to what extent, TRAIL-induced chemo-/cytokines impact the cancer cell itself as well as the immune microenvironment to facilitate tumour growth.

3.3.1 TRAIL-induced cytokines do not induce autocrine proliferation

Earlier in this thesis it was shown that TRAIL can induce IL-8 and CXCL1. Both cytokines have been associated with promoting tumour cell growth in NSCLC or epithelial ovarian cancer cells via direct stimulation or overexpression, respectively (Luppi et al., 2007, Bolitho et al., 2010). To determine whether the TRAIL-induced secretome was capable of eliciting a proliferative response, A549 cells were incubated with serum-deprived medium (0.5% FCS) or stimulated with non-cytotoxic concentrations of either 10 ng/ml or 100 ng/ml TRAIL (0.5% FCS) and incubated for five days. As a positive control, the cells were separately stimulated with medium supplemented with 10% FCS. BrdU incorporation was chosen as the readout for cell proliferation, as it detects actively dividing cells and therefore enables to specifically quantify proliferating cells. As seen in Figure 27A, TRAIL stimulation did not increase BrdU incorporation in A549 and murine Lewis lung carcinoma cells (3LL)

at any concentration (Figure 27A and B). 3LL were resistant to TRAIL (Figure 32B). The positive control showed a marked increase in both cases. Therefore, TRAIL and its induced secretome do not promote proliferation in these systems.

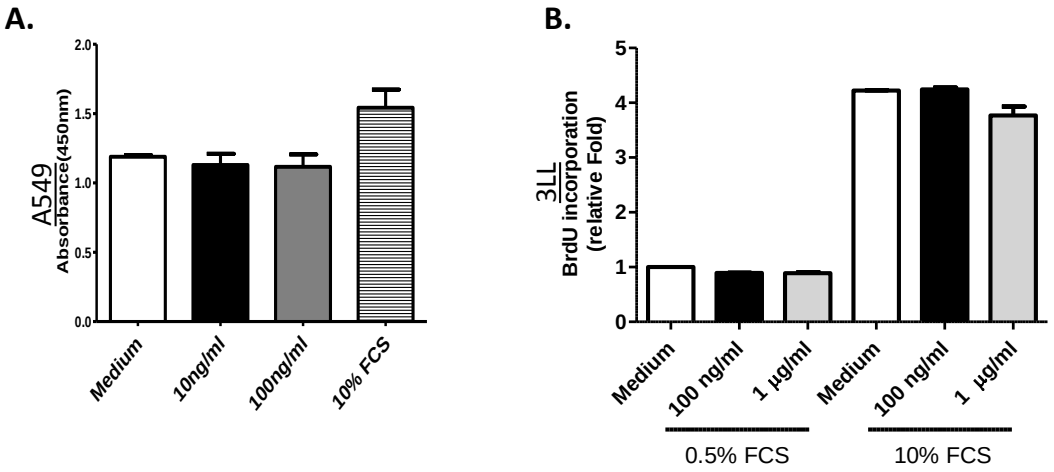


Figure 27. TRAIL induced chemo-/cytokines do not promote autocrine proliferation

A. A549 cells were stimulated with medium (0.5% FCS), positive control (10% FCS) or iz-TRAIL (in 0.5%FCS) at the indicated concentrations for 72h. Cell proliferation was determined by BrdU incorporation. **B.** 3LL cells were incubated with iz-mTRAIL in either 0.5% or 10% FCS as indicated for 72h. Proliferation was determined as above.

3.3.2 TRAIL-induced cytokines do not promote resistance to TRAIL

IL-8 can promote the development of resistance to TRAIL, by decreasing surface TRAIL-R1 levels (Abdollahi et al., 2003). CXCR2, a cognate receptor for IL-8, is expressed on HCT116 cells (Ning et al., 2012). Since this thesis has shown the induction of IL-8 in HCT116 wildtype and HCT116 BAX/BAK KO cells upon TRAIL treatment, it was hypothesized that TRAIL-induced IL-8 may lead to the development of resistance to TRAIL in HCT116 wildtype cells. To test this hypothesis, TRAIL-resistant HCT116 BAX/BAK KO cells were stimulated with different doses of TRAIL to generate supernatants containing IL-8. As previously shown in this thesis, HCT116 BAX/BAK KO cells basally produce cytokines under the tested conditions. To control for effects thereof and specifically investigate the potential resistance caused by TRAIL-induced IL-8, supernatants from medium treated HCT116 BAX/BAK KO were generated as well. To probe for the development of resistance, HCT116 cells were incubated with the conditioned supernatants or fresh media (0.5% FCS) for 24h and subsequently stimulated with TRAIL. In order to prevent the sensitive HCT116 cells from succumbing to residual TRAIL in the KO supernatant, the death ligand was removed after 2h of stimulation by washing, which did not significantly impact cytokine induction (data not shown). As a positive control, HCT116 cells were also incubated with different doses of recombinant IL-8 (Figure 28B). Although a trend towards resistance was observed at 3.7 ng/ml TRAIL, the HCT116 cells

failed to develop strong resistance as no significant difference in viability between CTRL (NT SUPE) incubated and TRAIL conditioned media incubated HCT116 was detected (Figure 28A). A lack of resistance was also observed in the HCT116 incubated with recombinant IL-8 (Figure 28B). Interestingly, a trend towards increased resistance was also visible when comparing the viability of fresh medium incubated HCT116 with any condition involving incubations with HCT116 BAX/BAK supernatants. Overall, these results indicate that HCT116 cells may secrete factors, other than IL-8, which contribute to resistance, yet these are not induced by stimulation with TRAIL.

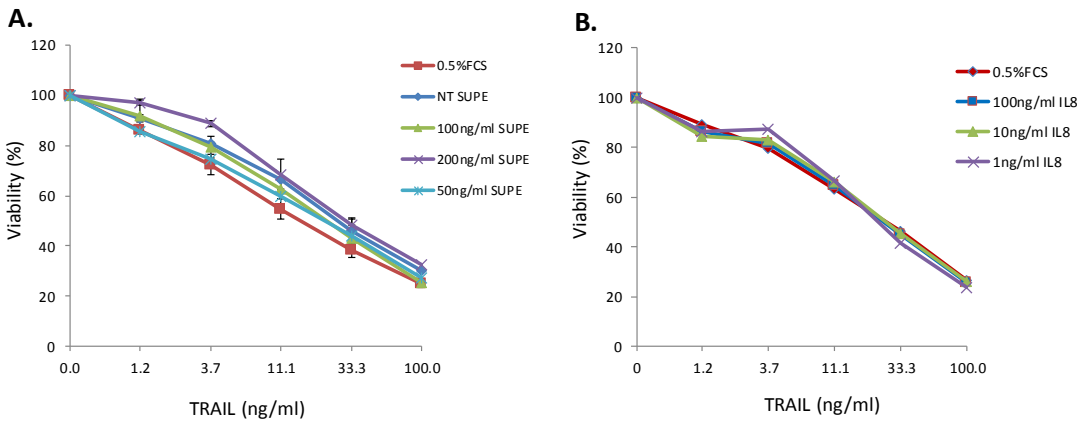


Figure 28. TRAIL-induced cytokines do not promote resistance to TRAIL

A.HCT116 BAX/BAK KO cells were stimulated with iz-TRAIL [100 ng/ml] or incubated with media for 2h, then washed with PBS and incubated with fresh media for 24h. Next, supernatants were removed and sterile filtered, followed by supernatant transfer to HCT116 WT cells. After 24h, iz-TRAIL was added at the indicated concentration; 24h later, cell viability was determined by CellTiter-Glo. **B.** HCT116 WT cells were pre-incubated with the indicated concentrations of IL-8 for 24h, followed by the addition of indicated concentrations of iz-TRAIL; 24h later cell viability was determined as above.

3.3.3 FADD is required for TRAIL-induced secretion of factors which polarise monocytes to EMT-promoting MDSC

Although previous results in this thesis indicate the induction of various cytokines by TRAIL, the TRAIL-induced secretome failed to elicit any substantial autocrine effects regarding the enhancement of proliferation or TRAIL-resistance. However, interestingly, secretion of the NF-κB-induced cytokines, CCL2 and IL-8, by cancer cells is frequently associated with promoting the formation of a tumour-supporting microenvironment (Hoesel et al., 2013; Qian et al., 2011). In fact, secretion of CCL2 or IL-8 by the tumour can facilitate myeloid cell migration and promote their polarisation towards tumour-supportive $GR1^{+}CD11b^{+}$ MDSC and fully differentiated M2-like macrophages (Qian et al., 2011; Fujita et al., 2011; Chun et al., 2015; Highfill et al., 2014). Therefore, it was hypothesized that TRAIL-induced chemo-/cytokines could act in a similar, paracrine fashion, to influence myeloid cell polarisation. Since the requirement of FADD for cytokine secretion was

previously demonstrated, it was further hypothesized that any polarising effects would require the presence of FADD in cancer cells. To investigate this, conditioned media (CM) from A549 WT or FADD KO cells were tested for their ability to polarise $CD14^+$ cells from healthy donors towards an alternatively activated myeloid cell phenotype *in vitro* (Figure 29A). Analysis of the myeloid cell polarisation state revealed that the supernatants of TRAIL-treated WT A549 cells were indeed capable of promoting the polarisation of $CD14^+$ cells towards a HLA-$DR^{lo/neg}$ phenotype, the human equivalent immune cell population to murine $CD11b^+GR1^+$ cells (Sevko and Umansky, 2013). Furthermore, fully differentiated HLA-DR^+ cells were also detected in this condition (Figure 29D). Interestingly, HLA-$DR^{lo/neg}$ as well as HLA-DR^+ cells displayed increased levels of CD206 expression, indicating polarisation towards MDSC and fully differentiated M2-like macrophages (Figure 29C and D). These alternatively activated myeloid cell subsets are frequently associated with eliciting tumour-supportive effects via immuno-suppression of anti-tumour T cells as well as by directly promoting tumour growth (Gabrilovich and Nagaraj, 2009; Lesokhin et al., 2012; Mantovani and Sica, 2010; Toh et al., 2011). Importantly, CM from TRAIL-treated FADD KO cells was not able to elicit this polarisation. In line with a tumour-promoting phenotype of the myeloid cells polarised by TRAIL CM, myeloid-A549 cell co-culture promoted E-cadherin loss in A549, which is indicative of EMT. As expected, myeloid cells previously incubated with CM from TRAIL-treated FADD KO cells were unable to elicit EMT in co-culture (Figure 29E). Therefore, TRAIL induces the FADD-dependent secretion of factors, which can polarise myeloid cells towards EMT-promoting, alternatively activated cells.

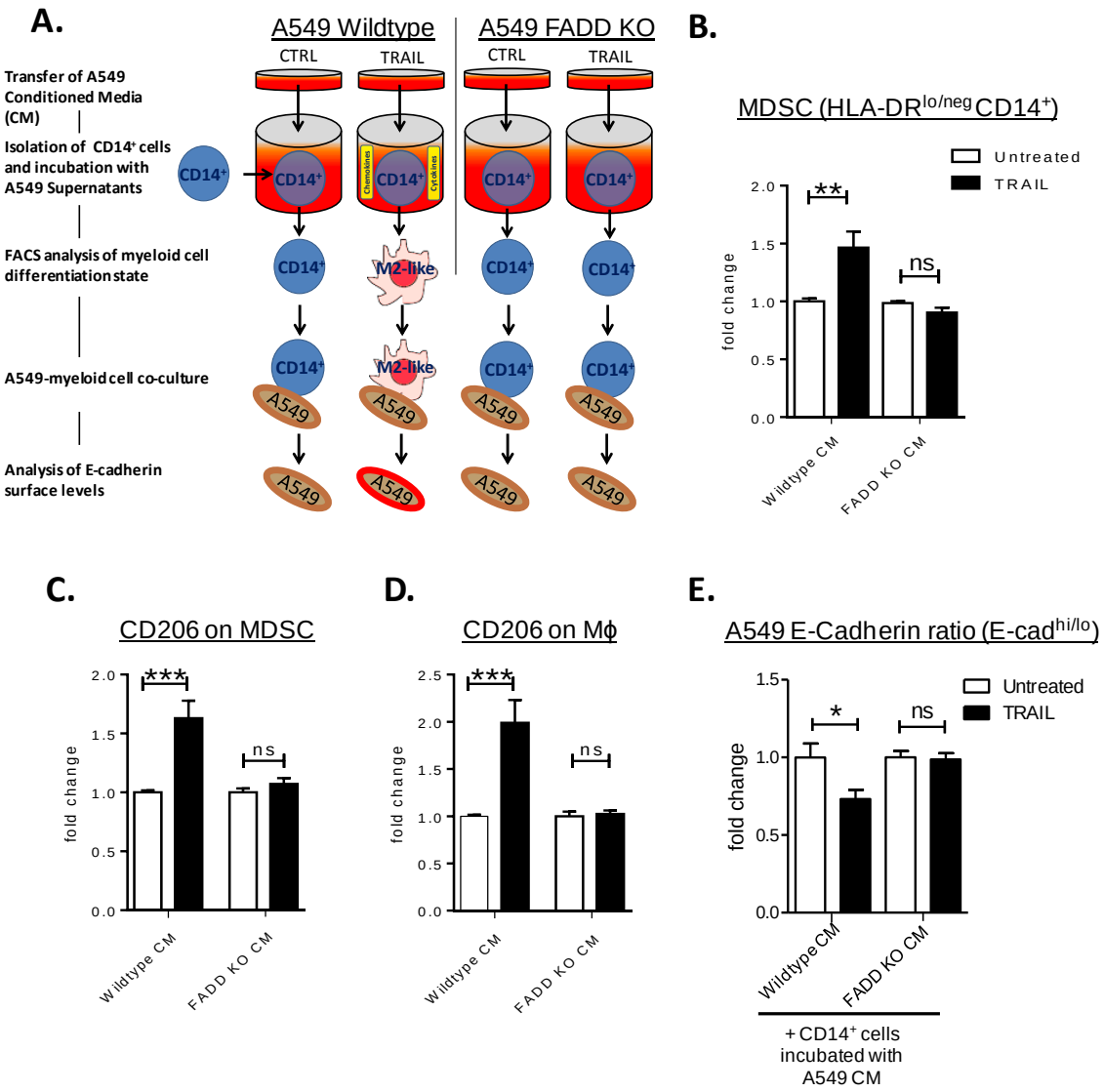


Figure 29. FADD is required for TRAIL-induced secretion of factors which polarise monocytes to EMT-promoting MDSC

A. Schematic of monocyte polarisation protocol. **B-D.** $CD14^+$ cells were isolated from healthy donor PBMCs via magnetic $CD14^+$ microbeads and incubated with conditioned media (CM) from untreated or iz-TRAIL [100 ng/ml] treated wildtype or FADD KO A549. After 48h, the conditioned myeloid cells were stained with fluorochrome-labeled antibodies against HLA-DR, CD14 and CD206 and analysed by flow cytometry. Data is represented as fold-change in HLA-$DR^{lo/neg}$ $CD14^+$ cells (B.) or HLA-$DR^{lo/neg}$, $CD14^+$, $CD206^+$ cells (C.) or HLA-$DR^+CD14^+CD206^+$ (D.) **E.** $CD14^+$ cells, previously incubated as above, were co-cultured with A549 wildtype cells for 72h and subsequently stained with fluorochrome-labeled antibodies against E-Cadherin. Analysis was conducted as above.

3.3.4 Validation of FADD KO cells for *in vivo* suitability

Previous results in this dissertation indicated that FADD is required to enable TRAIL-mediated cytokine induction and that the TRAIL-induced secretome can polarise myeloid cells *in vitro*. Therefore, it was tested whether presence of FADD would enable the induction of chemo-/cytokines and consequently modulate the myeloid microenvironment in a tumour-supportive manner *in vivo*. To exclude cell-autonomous proliferation rates as

a driver of differential *in vivo* growth, wildtype and FADD KO A549 cell proliferation rates were compared *in vitro*. Importantly, no significant differences in proliferation were observed at either 48 or 72h post-seeding (Figure 30A). In addition to a knockout, the cells also contained a stably transfected luciferase construct, which enables visualization and quantification of tumour burden via live cell imaging *in vivo*. The FADD KO generation did not impact luciferase expression levels, therefore enabling reliable comparison of tumour burden quantification via photon flux *in vivo* (Figure 30B). TRAIL-R surface expression levels were also comparable between WT, CTRL and FADD KO A549 cells (Figure 30C).

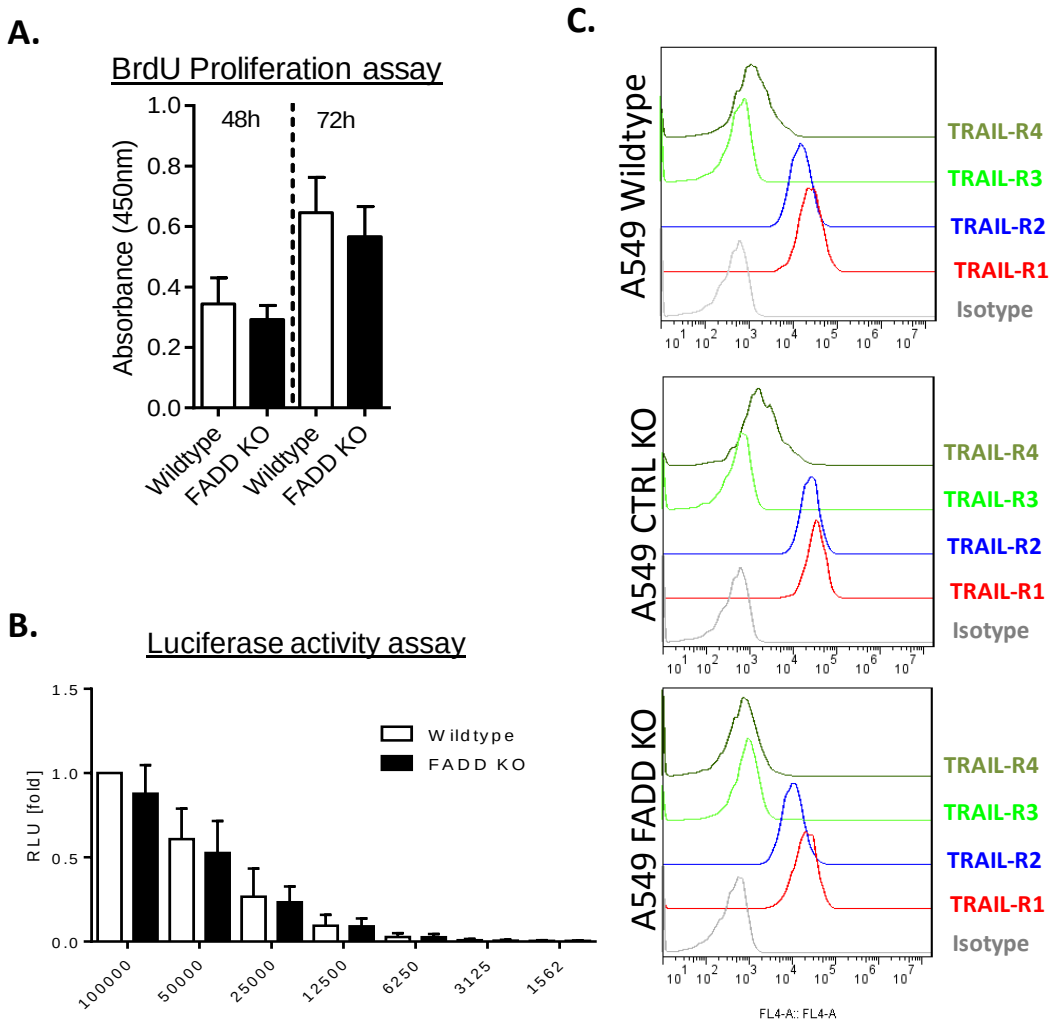


Figure 30. Validation of FADD KO cells for *in vivo* suitability

A. CTRL or FADD KO were subjected to proliferation assays by measuring BrdU incorporation after the indicated times. **B.** A549 were seeded at the indicated cell numbers and incubated with firefly luciferin the next day. Bioluminescence was quantified using a Mithras plate reader. **C.** Human TRAIL-R1-4 were stained on the indicated A549 cells and surface expression levels were determined by FACS.

3.3.5 FADD promotes tumour growth and correlates with tumour-supportive infiltrates

FADD is well-established as a central adaptor in the initiation of extrinsic apoptosis, induced by death ligands of the TNF superfamily. However, recent evidence from NSCLC and head and neck cancer patients indicates that high FADD expression levels correlate with poor survival prognosis, an unexpected finding given that FADD is mainly characterized as a pro-apoptotic adaptor molecule (Chen et al., 2005). Since FADD was observed to be crucial for cytokine secretion, it was hypothesized that its absence in cancer cells might modulate the tumour immune microenvironment and thereby possibly affect tumour growth. To investigate the role of cancer cell-expressed FADD in lung cancer growth, the previously generated FADD deficient A549 cells were utilized in an orthotopic xenograft mouse model of NSCLC. Strikingly, mice grafted with FADD KO cells had significantly lower tumour burden than FADD-proficient WT A549 cells as measured by photon flux (Figure 31A left panel) and pathological inspection of H&E sections (Figure 31A right panel).

Since the absence of FADD in tumour cells did not affect cell proliferation *in vitro* (Figure 31A), it was investigated whether the presence or absence of FADD might have affected the microenvironmental composition and thereby influenced tumour growth. In line with this, the levels of IL-8 and CXCL1 and CCL2, were significantly decreased in lungs containing FADD-deficient tumours (Figure 31B). Since these cytokines are described to recruit and polarise $CD11b^+$ cells, it was next assayed whether myeloid cell infiltrates were affected (Highfill et al., 2014; Roca et al., 2009). Interestingly, FADD-deficient tumours contained significantly fewer infiltrating $CD11b^+GR1^+$ cells with lower $CD206^+$ expression (Figure 31C, D). No difference in total $CD45^+$ cells was detected (Figure 31E). Expression of CD11b, GR1 and CD206 has been associated with alternatively activated myeloid cells, which can elicit tumour-supportive functions (Gabrilovich and Nagaraj, 2009; Allavena et al., 2008), thereby suggesting that absence of FADD in tumours leads to decreased accumulation of tumour-supportive immune cells. Therefore, FADD presence promotes growth of lung tumours, encourages the formation of a tumour-supportive chemo-/cytokine milieu and increases the recruitment of alternatively activated myeloid cells.

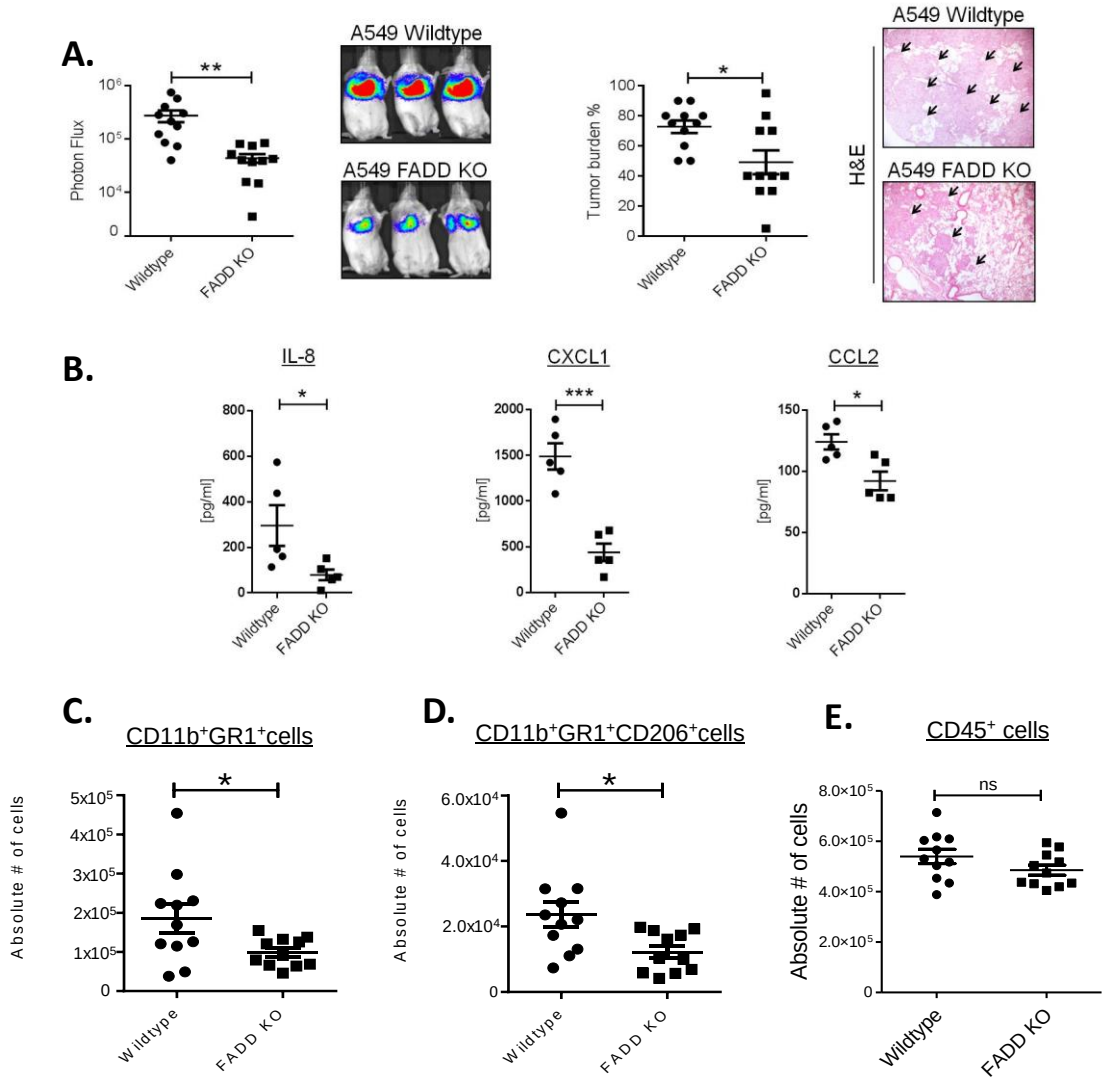


Figure 31. FADD promotes tumour growth and correlates with tumour-supportive immune infiltrates A. SCID/beige mice were injected with 2 x 10^6 luciferase expressing A549 Wildtype or FADD KO cells into the lateral tail vein. Tumour burden was assessed after 24 days, via bioluminescence imaging (left panel) and histological quantification of tumour burden (right panel). n = 11 per group. **B.** Indicated cytokines were quantified in lung homogenates by ELISA. **C.-D.-E.** Absolute number of $CD11b^+$ $Gr1^+$ (C) and $CD11b^+Gr1^+CD206^+$ cells (D) $CD45^+$ cells within tumour-bearing lungs.

3.3.6 Characterization of murine tumour cells for syngeneic *in vivo* models

Previous results indicated TRAIL- and FADD-mediated immune-modulatory effects *in vitro* and in a xenograft model. Due to the vast array of possible interactions within the tumour microenvironment, the output effect of any one immune subset on tumour burden highly context-dependent. Therefore, meaningful conclusions regarding an immune-modulatory role of TRAIL and FADD require the use of a fully immune competent model. In this effort, a suitable murine lung cancer cell line which exhibits high, reproducible intake rates in wildtype C57BL/6 mice was required. Three lung cancer cell lines, 802T4, 394T1 and 3LL were compared in their *in vivo* tumour take rate in C57BL/6 mice. After four weeks of growth, the mice were culled and assayed for tumour burden via visual inspection of the lung. Only the lungs of 3LL injected mice exhibited large nodules in all injected mice,

indicating a very high and reproducible tumour take rate (Figure 32A). Therefore, 3LL were selected for further characterization and tested for TRAIL-resistance and chemo-/cytokine secretion.

The cells were completely resistant to TRAIL, even at 1 ug/ml as seen in Figure 32C Left panel). The high resistance was however not due to lack of mouse TRAIL-R surface expression, as shown in Figure 32B. The responsiveness of 3LL cells to TRAIL is further supported by their ability to induce cytokines upon TRAIL stimulation *in vitro*, which is in line with results previously shown in this thesis (Figure 32C). Since 3LL had a high tumour take rate, were resistant to TRAIL and induced cytokines upon TRAIL stimulation, they represented a suitable cell line to study the role of TRAIL-induced cytokines in modulating the tumour immune microenvironment in an immune-competent setting.

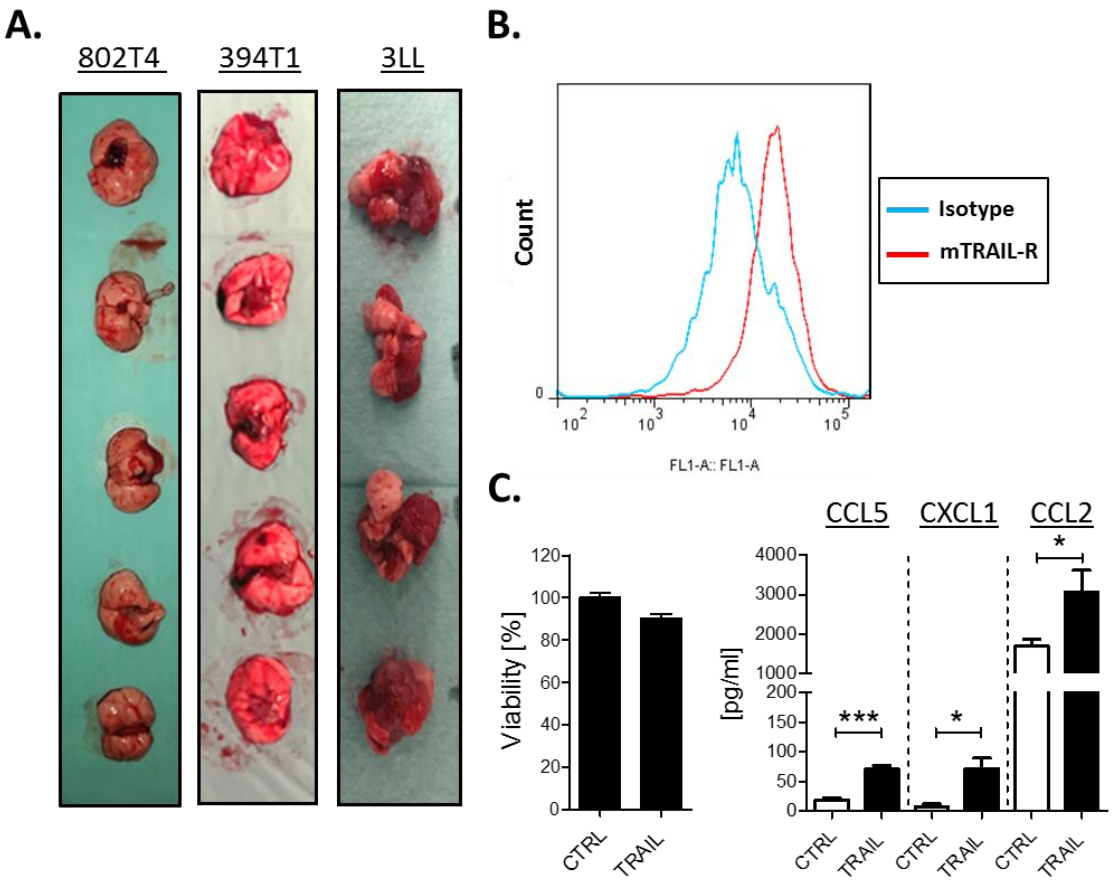


Figure 32. Establishment of a suitable syngeneic *in vivo* model

A. C57BL/6 lungs 4 weeks post i.v. injection of 5x10^4 of the indicated cells. **B.** Murine TRAIL-R was stained on 3LL and surface expression levels were determined by FACS. **C.** 3LL cells were stimulated with iz-mTRAIL [100 ng/ml]. After 24h, cell viability was determined by CellTiter-Glo and cytokine concentrations in the cell supernatants were measured via ELISA.

3.3.7 A tumour-supportive role for FADD in a syngeneic model of lung cancer FADD

To verify the immune-modulatory role of FADD in a syngeneic system, 3LL FADD KO cells were generated using CRISPR/Cas9 technology targeting exon 2 of murine FADD. After single cell cloning, two FADD KO clones were identified (Figure 33A). To verify a role of FADD in cytokine secretion, Wildtype and FADD KO 3LL cells were subsequently stimulated with TRAIL. Importantly, TRAIL-mediated cytokine production was decreased in both 3LL FADD KO clones, verifying the earlier findings in A549 FADD KO cells (Figure 33B).

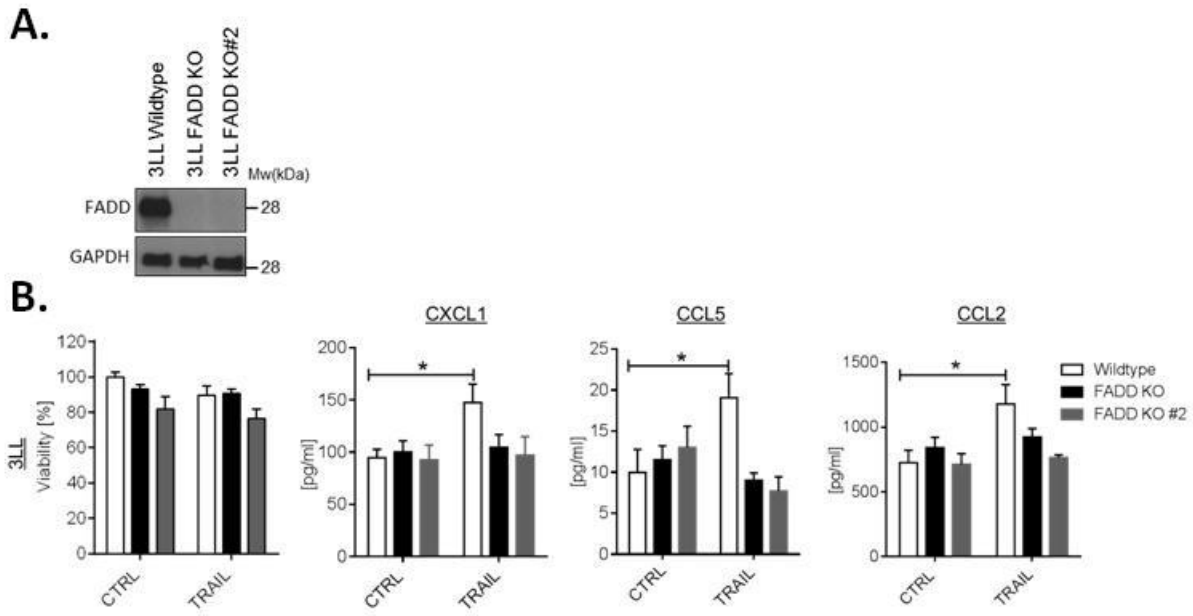


Figure 33. FADD is required for TRAIL-mediated cytokine production

A. Western Blot showing 3LL FADD KO cells. **B.** 3LL Wildtype and FADD KO were stimulated with iz-mTRAIL [1 µg/ml] for 24h followed by removal of supernatants and determination of the indicated cytokines by ELISA; cell viability was determined by CellTiter-Glo.

Next, 3LL wildtype and FADD KO cells were injected into C57BL/6 mice. Importantly, lung tumour burden of 3LL FADD KO was significantly decreased in comparison to 3LL wildtype cells, as quantified by lung weight and histological quantification (Figure 34A and B). Furthermore, the levels of lung $CD11b^+GR1^+$ cells were also significantly decreased in the lungs of mice injected with FADD-deficient cells (Figure 34C). The levels of $CD45^+$ cells were not significantly affected (Figure 34D). Therefore, tumour cell-expressed FADD promotes tumour burden and accumulation of $CD11b^+GR1^+$ cells, verifying the previously obtained results from the xenograft, and expanding their relevance to an immune competent mouse system.

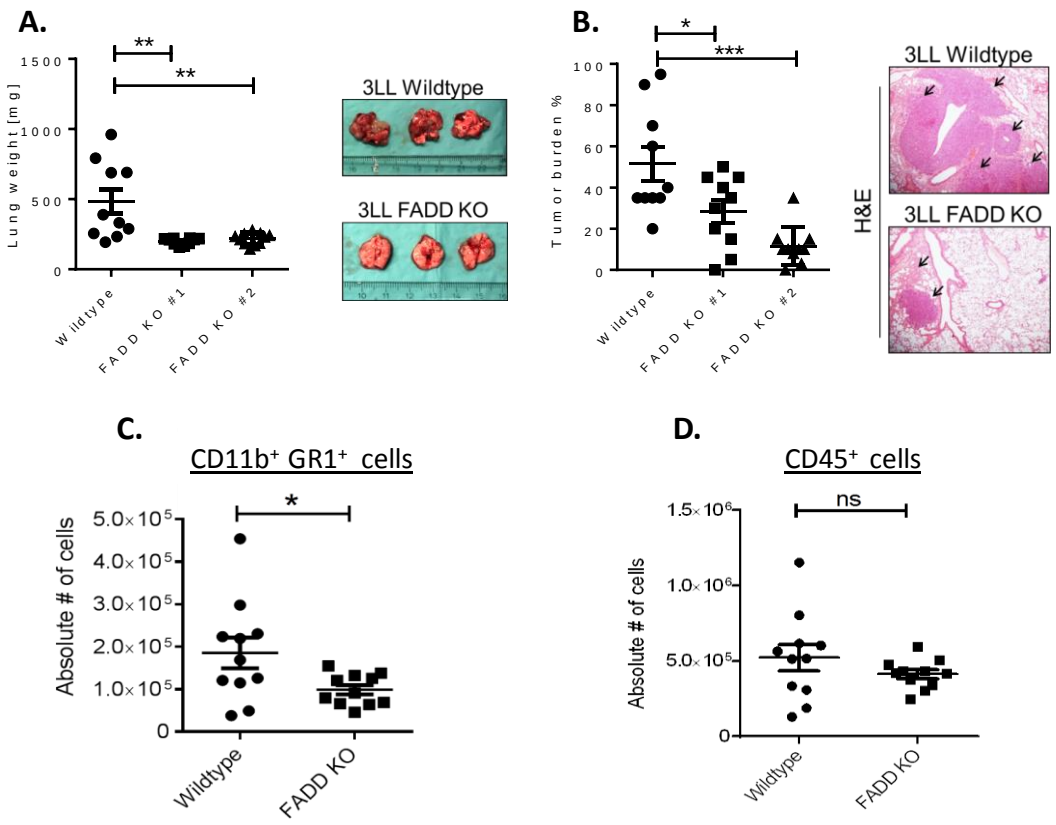


Figure 34. Tumour cell-expressed FADD promotes tumour growth and correlates with immune infiltrates in a syngeneic mouse model

A. C57BL/6 mice were injected with 5 x 10^5 3LL cells into the lateral tail vein. Lung weights were determined 28 days later. Representative lungs are shown. (**B**) Histological quantification of tumour in lungs from mice. Representative images of H&E-stained lung sections (5X magnification) are shown. **C.** Absolute number of $CD11b^+$ $Gr1^+$ and **D.** $CD45^+$ cells within tumour-bearing lungs.

In order to further exclude the possibility that other death ligands, which also utilize FADD in their signalling, were responsible for the observed modulation of the microenvironment, FADD KO 3LL were stimulated with CD95L and TNF. As seen in Figure 35A, CD95L stimulation did not elicit the induction of cytokines from 3LL Wildtype or FADD KO cells, whilst no significant difference in cytokine induction was observed between the two cell lines upon TNF stimulation. To confirm the activity of the employed CD95L, murine embryonic fibroblasts (MEF) were treated with different concentrations of the death ligand (Figure 35B). The MEFs were highly sensitive to CD95L-induced death, verifying the ligands activity. The result was further confirmed by demonstrating the presence or lack of CD95 surface expression in MEFs or 3LL cells, respectively (Figure 35C). Therefore, TRAIL is likely to be responsible for inducing the observed FADD-dependent modulation of the microenvironment in the syngeneic mouse model.

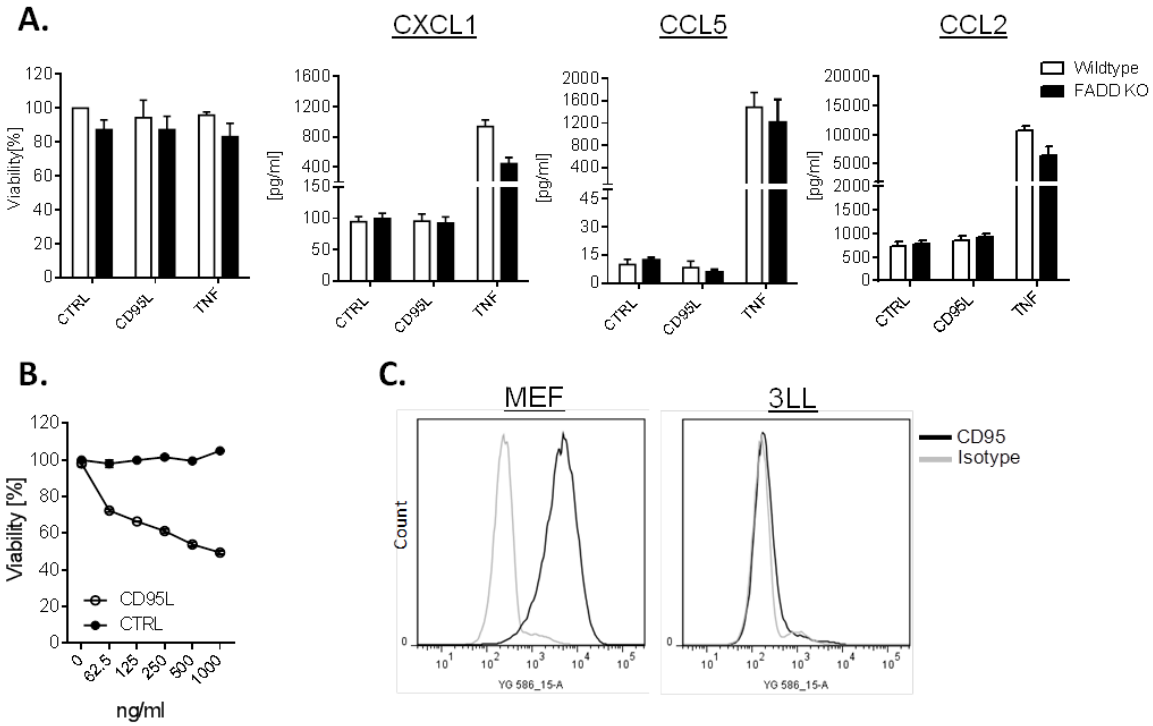


Figure 35. FADD does not differentially affect cytokine induction upon CD95L or TNF stimulation in 3LL cells

A.3LL cells were treated with TNF [50 ng/ml] or CD95L-FC [1 µg/ml] for 24h. Cell viability was determined by CellTiter-Glo and cytokines were quantified in supernatants via ELISA. **B.** MEF were stimulated with the indicated concentrations of CD95L-FC for 24h; cell viability was determined by CellTiter-Glo. **C.** Mouse CD95 was stained on the indicated cell lines and expression level was determined by FACS.

3.3.8 Generation of 3LL cells containing a mTRAIL-R stable knockdown and GFP construct

FADD-deficient human and mouse NSCLC cells were significantly impaired in their *in vivo* growth, which correlated with impaired recruitment of tumour-supportive, alternatively activated myeloid cells in a xenograft and syngeneic mouse model. Furthermore, FADD-proficiency was required by tumour cells to elicit the TRAIL-induced secretion of factors which polarise myeloid cells towards an alternatively activated state. These data strongly indicated that endogenous TRAIL might be involved in mediating the immune-modulatory effect observed *in vivo*. Therefore, it was next investigated whether TRAIL-R abrogation would have a similar effect.

To address the role of endogenous TRAIL in an immune competent background, 3LL cells were transfected with lenti-viral shRNA constructs targeting murine TRAIL-R (referred to as TRAIL-R). Construct sh23 had the most efficient knockdown as determined by western blot (Figure 36A). The knockdown efficiency was further confirmed by TRAIL-R surface staining which indicated a shift in the peak of shTRAIL-R, as compared to empty vector transfected cells (Figure 36B). 3LL cells were also stably transfected with a GFP-expression construct which was expressed in 99% of live 3LL cells, to offer a more

sensitive technique of assaying for tumour burden (Figure 36B). Importantly, pLKO.1 and sh23 transfected cells had equal levels of GFP expression, allowing for reliable relative tumour burden quantification *in vivo* (Figure 36B). In order to rule out cell-autonomous differences in proliferation, the proliferation rates of 3LL pLKO.1 and shTRAIL-R were compared *in vitro.* Both cells exhibited the same proliferative capacity as measured via BrdU incorporation after 48h and 72h in culture, rendering them suitable to assay for endogenous TRAIL/TRAIL-R-mediated, microenvironment modulating tumour-supportive effects *in vivo* (Figure 36C).

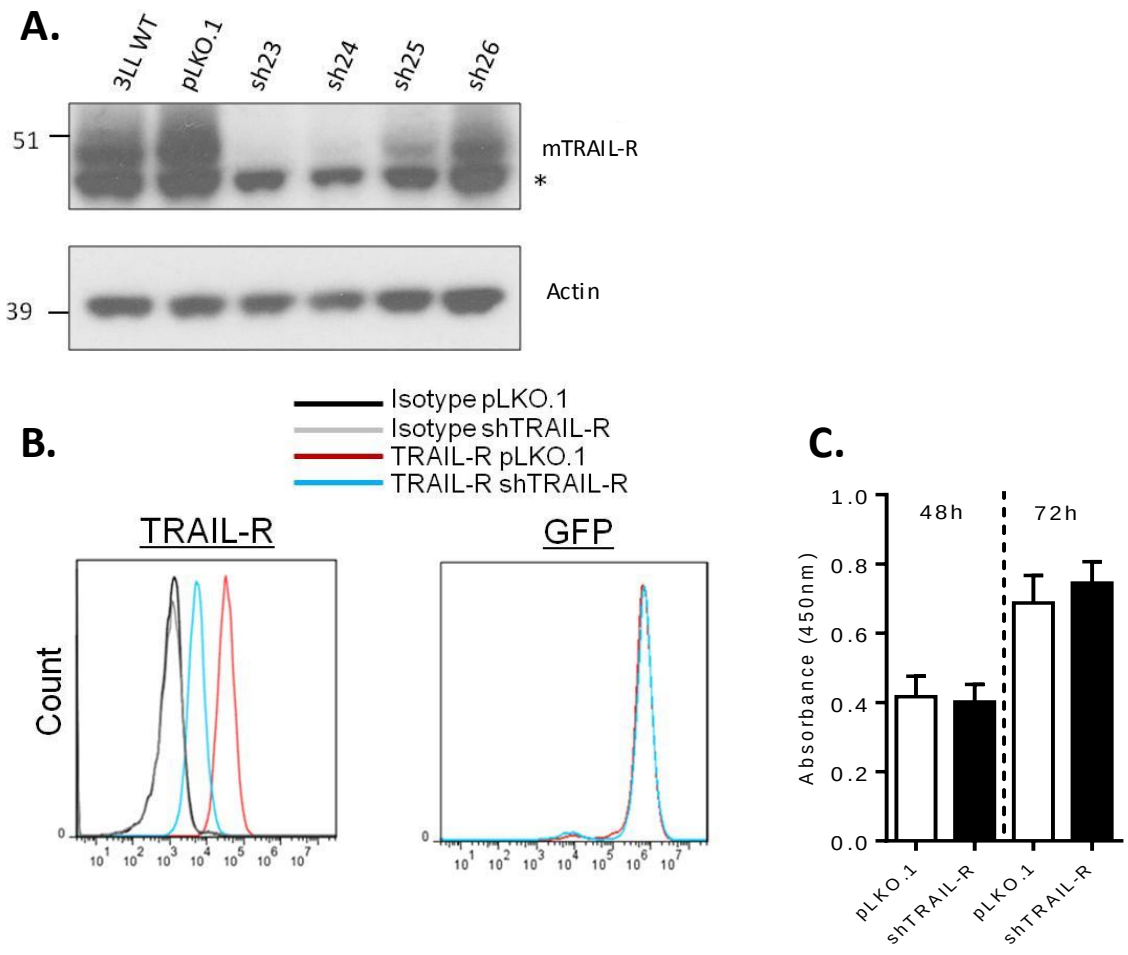


Figure 36. Generation of 3LL cells containing mTRAIL-R stable knockdown and GFP construct

A. 3LL cells were stably transfected with shRNA targeting murine TRAIL-R as described in the methods. Lysates of Wildtype, pLKO.1 or sh transfected cells were analysed for mouse TRAIL-R protein levels by western blot. **B.** Surface expression levels of mouse TRAIL-R and GFP levels of 3LL cells were determined by FACS. **C.** 3LL pLKO.1 or shTRAIL-R were subjected to proliferation assays by measuring BrdU incorporation after the indicated times.

3.3.9 Cancer cell-expressed TRAIL-R supports tumour growth and recruitment of tumour-supportive infiltrates in a host CCR2-dependent manner

In order to determine whether endogenous TRAIL is capable of eliciting the formation of a tumour-supportive microenvironment and promoting tumour progression in immune competent mice, C57BL/6 WT mice were injected with 3LL cells containing either a KD of mouse TRAIL-R (shTRAIL-R) or a vector control (pLKO.1), as well as GFP expression constructs. Measurement of GFP-positive cells in the lung and histological quantification of tumour burden indicated significantly decreased tumour burden in mice injected with shTRAIL-R 3LL, as compared to pLKO.1 3LL injected mice (Figure 37A). No evident difference in cell growth between pLKO.1 and shTRAIL-R cells was detected *in vitro* (Figure 36C). The decreased tumour burden in shTRAIL-R injected mice correlated with decreased CCL2 protein levels in shTRAIL-R cell-containing lungs (Figure 37C), indicating a decreased ability of shTRAIL-R 3LL cells to produce CCL2 *in vivo*. In accordance with the decreased ability to induce cytokines via endogenous TRAIL, $GR1^+CD11b^+$ cells were also decreased in the lung homogenates of shTRAIL-R injected mice (Figure 37D). The overall number of infiltrating $CD45^+$ cells was unchanged (data not shown). In accordance with data obtained in the xenograft and syngeneic models, lungs containing tumour cells proficient in TRAIL-mediated cytokine induction also showed higher levels of alternatively activated $CD206^+$ myeloid cells (Figure 37E). Therefore, tumour cell TRAIL-R expression is associated with increased tumour growth, CCL2 levels and alternatively activated myeloid cell infiltrates; verifying *in vivo* relevance of an immune-modulatory role of the endogenous TRAIL/TRAIL-R system in an immune competent setting.

CCL2 is a central chemoattractant for alternatively activated myeloid cells via its receptor CCR2 (Chun et al., 2015). Furthermore, CCL2 expression can polarise human peripheral blood $CD11b^+$ cells towards an anti-inflammatory, $CD206^+$ M2-like phenotype (Sierra-Filardi et al., 2014, Roca et al., 2009). Since increased CCL2 expression correlated with increased alternatively activated infiltrates in the lungs containing TRAIL-R-proficient 3LL cells, it was feasible that a CCR2 KO background would abrogate M2-like cell infiltration. As seen in Figure 37D and E, alternatively activated infiltrates were indeed equalized and remarkably, the equilibration also correlated with diminished differences in tumour burden between pLKO.1 and shTRAIL-R tumours in CCR2 KO mice (Figure 37A). Interestingly, the difference in CCL2 protein levels previously noted in Wildtype mice, was also apparent in CCR2 KO mice, demonstrating that it is not the mere extent of tumour burden which determines CCL2 levels, but instead the ability of tumour cells to produce CCL2, a capacity which is impaired in the absence of TRAIL-R (Figure 37C). These results provide *in vivo* evidence that expression of TRAIL-R on tumour cells is required for CCL2 production, which in turn facilitates pro-tumourigenic effects via host cell-expressed CCR2.

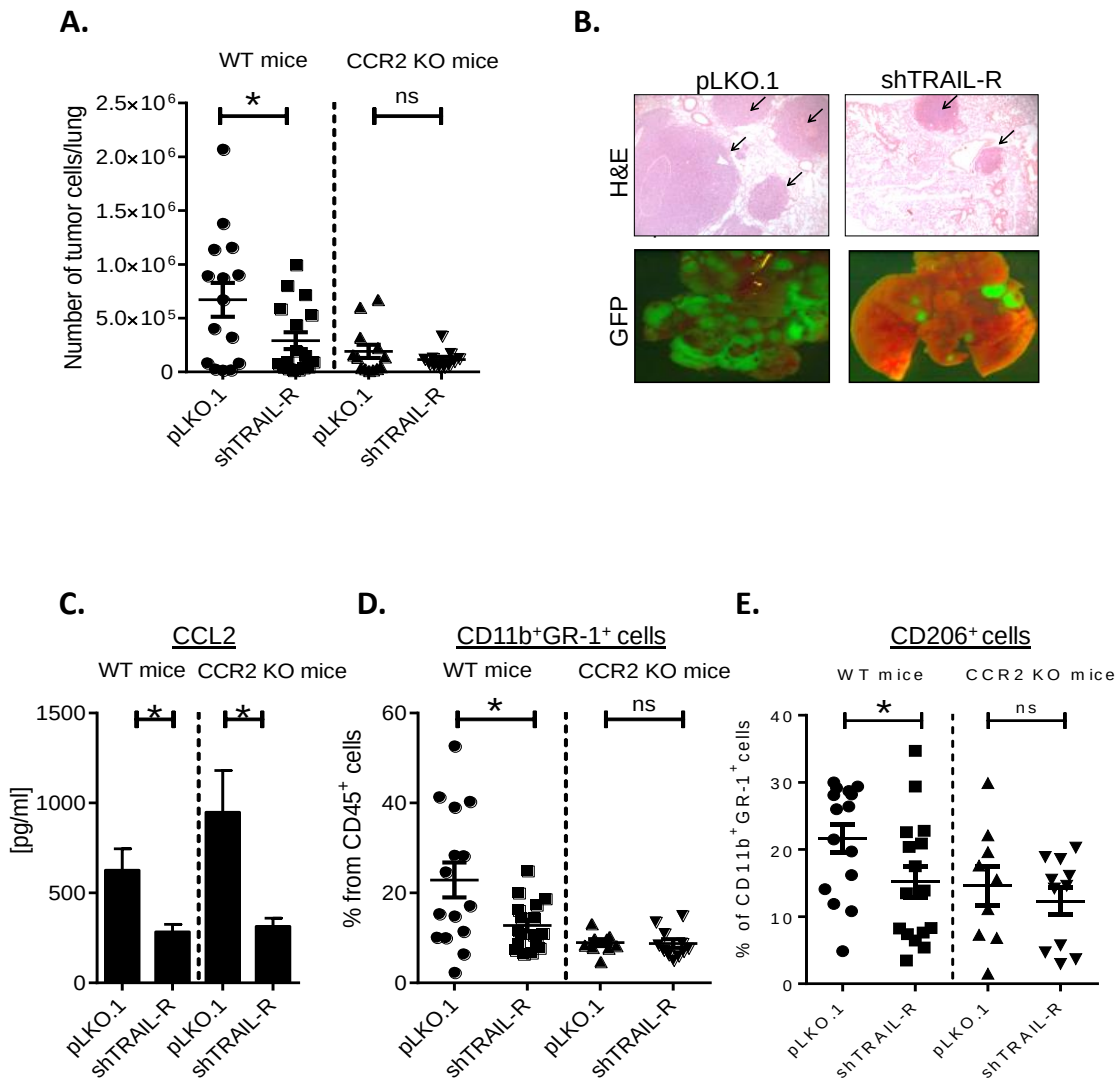


Figure 37. Cancer cell-expressed TRAIL-R supports tumour growth and recruitment of tumour-supportive infiltrates in a host CCR2-dependent manner. A. WT or CCR2 KO mice were injected with 5 x 10^5 3LL-GFP empty vector (pLKO.1) or shmTRAIL-R (shTRAIL-R) via the lateral tail vein and left to develop tumours for 28 days. Upon dissociation of lungs, the absolute number of tumour cells was determined by measuring GFP via FACS. WT mice pLKO.1 n=15, shmTR n=17; CCR2 KO mice pLKO=13, shmTR=11 B. Upper panel: H&E-staining of fixed lungs; Lower panel: GFP-positive 3LL-containing lungs as determined by bright field microscopy. C. CCL2 protein levels in lung homogenates from WT mice and CCR2 KO mice measured by ELISA. D. and E. Dissociated lungs were stained with fluorochrome-labeled antibodies for GR1 and CD11b (D) or GR1, CD11b and CD206 (E) and analysed by FACS.

3.3.10 Characterization of myeloid cell-derived tumour-supportive effects

Previous results in this thesis have demonstrated that endogenous TRAIL\TRAIL-R signalling promotes tumour growth and the accumulation of $CD11b^+GR1^+$ $CD206^+$ cells via CCR2. The abrogation of alternatively activated myeloid cell infiltrates correlated with diminished the tumour burden. As alternatively activated cells have previously been associated with tumour-supportive effects, it was feasible that this cell subset was responsible for the increased tumour growth in lungs of mice injected with TRAIL-R proficient cells. Previous literature shows that M2-like myeloid cells can elicit tumour-

supportive effects by immuno-suppression of antitumour $CD8^+$ effector T cells (Teff) as well as via directly promoting tumour growth (Lesokhin et al., 2012, Gabrilovich and Nagaraj, 2009, Mantovani and Sica, 2010, Toh et al., 2011). To elucidate potential roles of TRAIL/TRAIL-R-signalling in tumour cells and its associated infiltration of alternatively activated myeloid cells on T cell effector functions *in vivo*, the percentages Teff and Treg cells were as well as the Teff/Treg ratio was determined in lungs of tumour-bearing pLKO.1 and shTRAIL-R injected Wildtype mice. Interestingly, the lungs of mice containing pLKO.1 3LL cells exhibited increased Treg infiltrates and a significantly decreased Teff/Treg ratio, as compared to lungs containing 3LL cells with diminished TRAIL-R levels; thereby revealing the presence of a suppressive T cell compartment in the same condition previously associated with increased M2-like cells (Figure 38A, B and C and 37D, E). To specifically address, a direct role of $CD11b^+$ cells in tumour proliferation, $CD11b^+$ cells were isolated from 3LL tumour bearing lungs and cocultured with 3LL cells *ex vivo*; $CD11b^+$ and 3LL cells were also cultured alone as a control. Interestingly, the cocultured cells exhibited a 2.3 fold increase in proliferation relative to 3LL cells alone, as determined by BrdU incorporation (Figure 38D). Therefore, the M2-like cells found to accumulate in the microenvironment dependent on tumour cell-expressed TRAIL-R, correlate with a decreased Teff/Treg ratio *in vivo* and are likely to contribute to proliferation of 3LL cells *in vitro*.

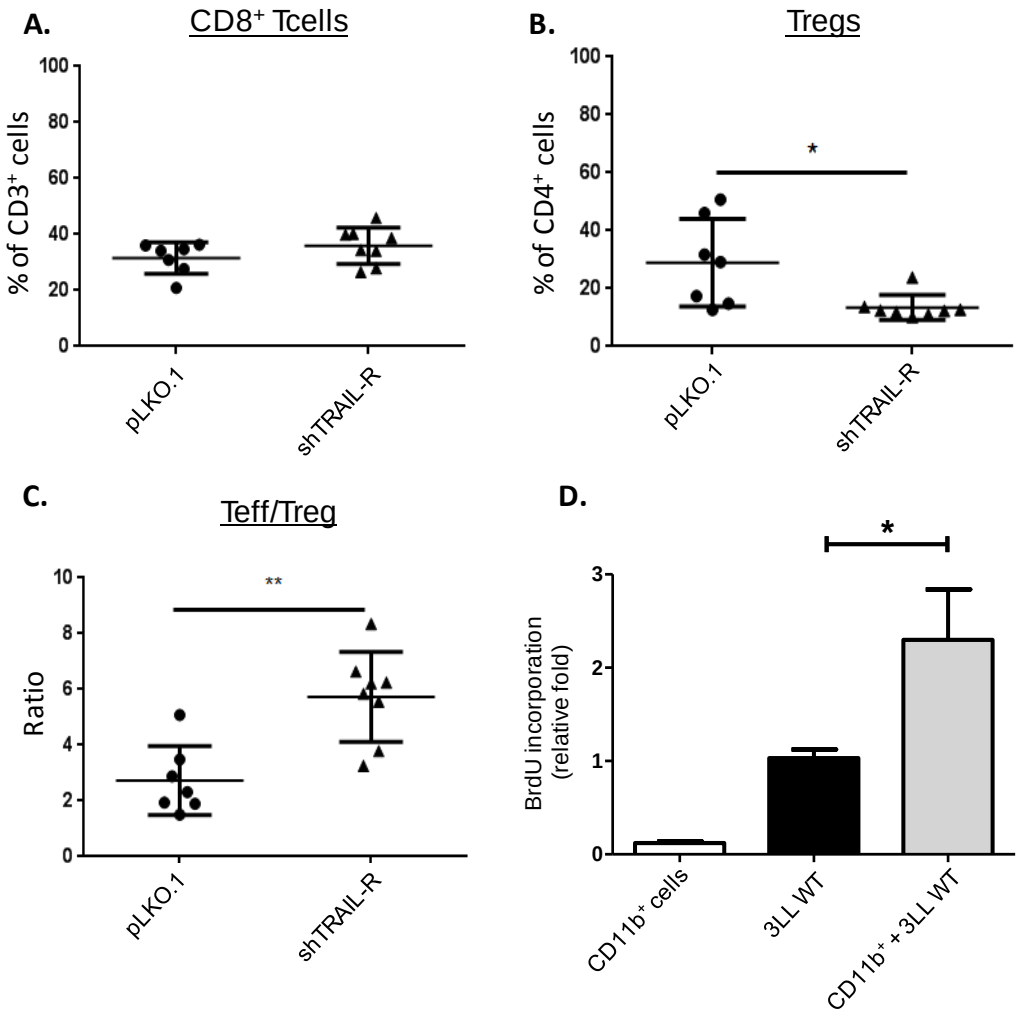


Figure 38. Characterization of M2-like effector functions

A-B.WT mice were injected with 5 x 10^5 3LL pLKO.1 or shTRAIL-R 3LL cells via the lateral tail vein and left to develop tumours for 28 days. Dissociated homogenates were stained with antibodies for CD3, CD8 (A) or CD3 CD4 Foxp3 (B) and analysed by FACS. C. Ratios of the absolute numbers of CD8 (Teff) and CD3 CD4 Foxp3 (Treg) positive cells are shown. **B.** Homogenates were generated as in (A) followed by staining for cell viability dye and CD11b. $CD11b^+$ cells were sorted and cultured with or without 3LL cells as indicated; 5 days later, proliferation was determined by BrdU incorporation. Fold change to 3LL WT alone is shown.

3.3.11 TRAIL and CCL2 correlate with a tumour-supportive immune profile in lung adenocarcinoma patients

Next it was evaluated whether TRAIL's pro-cancer immune-modulatory role would be reflected in gene expression data from patients. To this end, RNAseq data from a cohort of 489 lung adenocarcinoma patients, obtained from The Cancer Genome Atlas (TCGA), was subjected to bioinformatic co-expression analysis focusing on immune-cell markers and cytokines. Strikingly, TRAIL showed significant positive co-expression with 14 M2-type myeloid cell markers and cytokines associated with facilitating their expansion (Figure 39A, B). Importantly, these 14 factors included CCL2 as well as CD206, which were found to be upregulated in mice dependent on the endogenous TRAIL/TRAIL-R system. Expression of the myeloid marker CD33 was also correlated with TRAIL expression. In

line with this, expression of IL-6, which was not found to be regulated by TRAIL, also did not correlate with TRAIL expression in patients (Figure 39B).

Collectively, the data obtained in mice, together with the demonstrated correlations in humans suggested that a TRAIL/CCL2 axis is possibly involved in modulating the human tumour immune-environment. Therefore, composite TRAIL and CCL2 high versus low expression levels were analysed in respect to co-expression with factors involved in M2-type myeloid cell signalling. In line with a decisive role for CCL2, induced by endogenous TRAIL, in generating an alternatively activated immune environment, expression levels of 15 M2-type myeloid markers and cytokines, associated with facilitating their expansion significantly correlated with composite TRAIL/CCL2 levels (Figure 39C). Again, CD206 was amongst these 15 factors. Since TRAIL/TRAIL-R-induced CCL2 elicited its tumour-supportive effect via CCR2, it was also determined whether the immune cell markers and cytokines were co-regulated with composite TRAIL/CCR2 expression. Herein, 13 factors were identified to significantly correlate, providing a similar expression profile as before, with 10 of these matching with factors associated with TRAIL/CCL2 expression (Figure 39D). CD206 was again part of this group, indicative of its association with TRAIL/CCL2 as well as with TRAIL/CCR2. This implies a potential connection between TRAIL/CCL2/CCR2 in promoting the accumulation of M2-type myeloid cells within human tumours. Together, these data implicate endogenous TRAIL with an increase in tumour-supportive cytokines as well as M2-type markers, thereby extending the relevance of the findings in this thesis to lung adenocarcinoma patients.

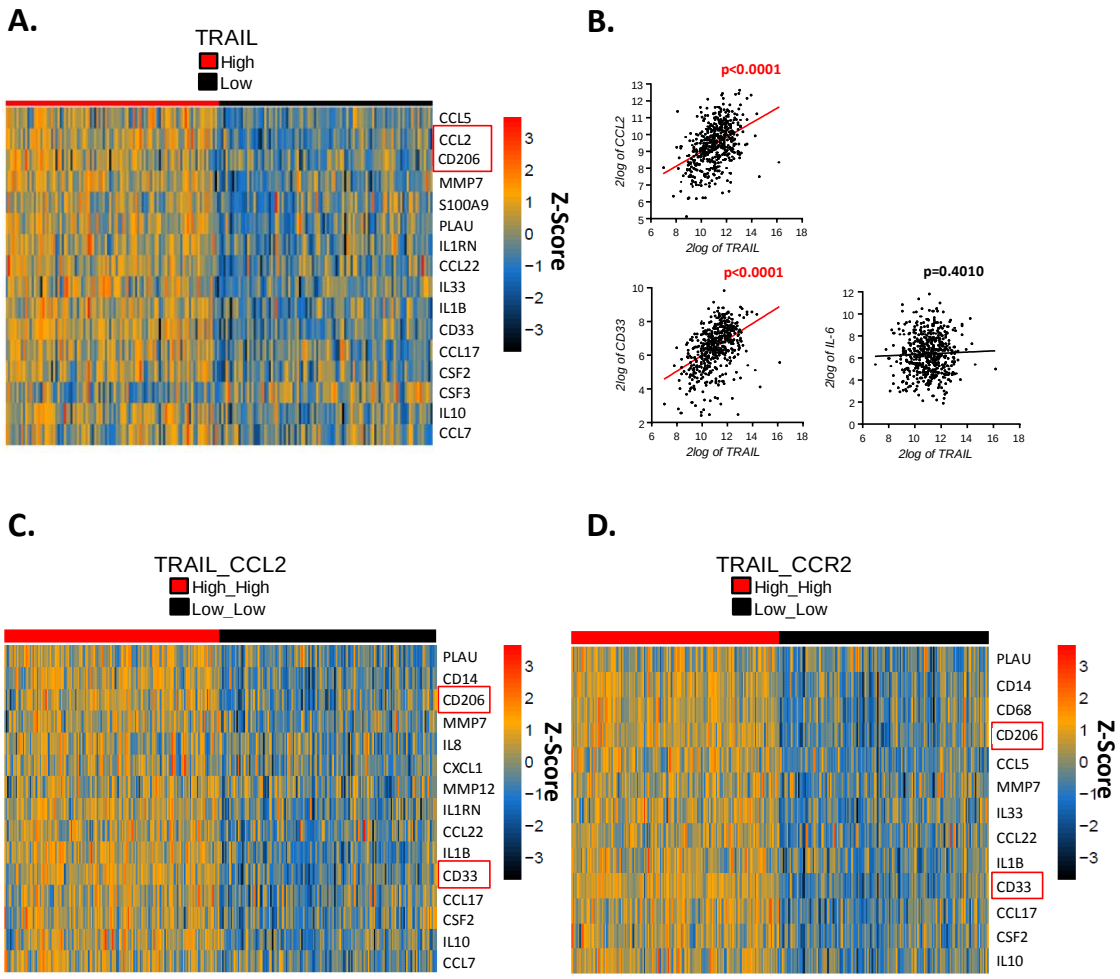


Figure 39. TRAIL and CCL2 correlate with a tumour-supportive immune profile in lung adenocarcinoma patients.

(A.-D.) RNAseq expression data from human lung adenocarcinoma biopsy samples (n=489) analysed for association of TRAIL (TNFSF10)/CCL2/CCR2 expression for a curated list of immune-related genes. **A.** Heatmap of genes significantly co-expressed (p=0.01) with TRAIL, showing log2 expression z-scores for 20% of samples with highest or lowest TRAIL levels. **B.** Correlation data for TRAIL versus CCL2, CD33 and IL-6 expression. **C.** As in (A), for 50% of samples with highest or lowest compound TRAIL and CCL2 expression. **D.** As in (C), for samples with highest or lowest compound TRAIL and CCR2 expression. In (B) the statistical significance of correlations was determined via Pearson's correlation coefficient. The linear regression curve is shown as a red or black line for significant or non-significant correlations respectively.

4 Discussion

Treatment with TRAIL is capable of specifically killing cancer cells without harming non-transformed cells (Walczak et al., 1999, Ashkenazi et al., 1999). However, the initial excitement regarding the potential of TRAIL monotherapy as an effective, clinical option for anti-cancer treatment was since dampened as roughly 50% of primary cancers turned out to be resistant to TRAIL (Ganten et al., 2006, de Miguel et al., 2016, Lemke et al., 2014b). On the contrary, some cancers even upregulate TRAIL-R expression, high levels of which are associated with poor prognosis (Spierings et al., 2003, Macher-Goeppinger et al., 2009, Ganten et al., 2009, Sanlioglu et al., 2007). *In vitro,* tumour cell line studies demonstrated that TRAIL treatment could induce proliferation, migration, invasion and neovascularisation, enhanced survival via ERK, and NF-κB activation and, importantly, cytokine secretion (Belyanskaya et al., 2008, Ishimura et al., 2006, Secchiero et al., 2004a, Secchiero et al., 2004b). *In vivo,* studies in mice have shown that TRAIL-treatment of apoptosis-resistant xenotransplanted cell lines induced liver metastasis of pancreatic cancer cell lines (Trauzold et al., 2006). Our laboratory has recently shown that endogenous TRAIL facilitates progression of *KRAS*-mutated cancers via cancer cell-autonomous Rac1 activation, independently of FADD (von Karstedt et al., 2015). However, although cytokines are major orchestrators of the immune microenvironment, the role of TRAIL-induced cytokines in tumour biology has not been investigated *in vivo*. This thesis identifies a distinct, additional tumour-supportive and immune-modulatory function of TRAIL-R signalling in cancer cells which requires FADD. Moreover, it provides the first unbiased analysis of the TRAIL-induced secretome, identifying cytokines and chemokines, and CCL2 in particular, as potential functional drivers therein. This dissertation further demonstrates that cytokine secretion is FADD-dependent. Finally, it is shown for the first time that endogenous TRAIL-elicited cytokines result in the polarisation of myeloid cells towards M2-like cells and the accumulation of such alternatively activated myeloid cells in the tumour microenvironment in a CCL2/CCR2-dependent manner, thereby contributing to tumour growth.

4.1 TRAIL induces a cytokine-rich secretome in surviving cancer cells

Secretome analyses have led to the identification of several disease biomarkers which are being developed for cancer and other diseases (Makridakis and Vlahou, 2010). TRAIL has previously been demonstrated to mediate the secretion of several cytokines as indicated in Table 1. However, signalling pathways involved in cytokine secretion such as NF-κB, JNK and MAPK can also mediate secretion of other factors which can influence the tumour biology e.g. matrix metalloproteases, cathepsins and coagulation factors and other

signalling molecules (Makridakis and Vlahou, 2010). As such, TRAIL has also been shown to induce the secretion of uPA, MMP-7 and MMP-9 shown to contribute to invasion *in vitro* (Trauzold et al., 2006). Due to their potential clinical relevance, it was important to define which particular factors, or subsets thereof are most crucial within TRAIL's secretome. The secretome analysis conducted in this thesis now determined that factors associated with cytokine and chemokine activity, in particular CCL2, IL-8, CXCL1 and CXCL5 are highly enriched (Figure 5). Although this analysis was the most unbiased analysis of the TRAIL-induced secretome to date, it was still based on the subset of secreted factors which were up regulated greater than 2 fold, which might result in overlooking some biologically important factors appearing below this cut-off. Although the TRAIL-induced secretome was highly enriched in cytokines and chemokines, it is noteworthy, and in line with previous reports, that proteases were also part of the secreted factors induced by TRAIL. The secretion of a disintegrin and matrix metalloproteases (ADAM) 9, 15 and 17 was upregulated 1.4 fold upon TRAIL stimulation. Interestingly, next to pro-invasive functions, proteases can elicit tumour supportive functions by engaging with cytokines and modulating their activity. This is the case as ADAMs can cleave and shed tumour supportive cytokines and chemokines which are initially produced as transmembrane isoforms, such as CXCL16 (ADAM10), CX3CL1 (ADAM16), CD95L (ADAM10), thereby promoting ligand activation (Reiss and Saftig, 2009). Furthermore cytokine receptors such as CSFR1 (ADAM17) can also be shed from the membrane and thereby function as a solubilised antagonist. As such, ADAMs can enable rapid activation or inhibition of extracellular signalling events mediated by cytokines which elicit tumour-supportive functions. In turn, it is not surprising that a large proportion of clinically approved therapies today use drugs targeting, or consisting of, secreted proteins or cell surface-associated membrane proteins. Interestingly, TRAIL itself is shed from the membrane by a currently unknown protease, whilst the biological significance of TRAIL shedding is also currently unknown. Therefore, due to the potential biological and clinical relevance, the existence of protease/cytokine crosstalk within TRAIL's secretome would be interesting to investigate.

The quantitative, label free, mass spectrometry analysis was further verified by ELISA, demonstrating robust inductions of CCL2, IL-8, CXCL1, CXCL5 in resistant lung, pancreatic and colorectal cancer cells (Figure 6). Cytokines and chemokines can also act synergistically regarding their elicited effects on immune cells (Proudfoot and Uguccioni, 2016). Interestingly, IL-8 has been shown to enhance the ability of low levels of CCL2 to enhance the migration of monocytes (Gouwy et al., 2008). Synergy between the two cytokines is further supported by increased extracellular signal-regulated kinase 1/2 phosphorylation and calcium mobilization in monocytes stimulated with a combination of IL-8 and CCL2; possibly indicating cooperative downstream signalling cascades of the two

cytokines during migration. Since potential cooperative regulatory mechanisms between CCL2, IL-8, CXCL1 and CXCL5 could influence their overall tumour effector functionality, it would be interesting to determine whether and if so how the cytokines of TRAIL's secretome might integrate in their overall tumour supportive effects.

This dissertation has demonstrated that TRAIL-mediated cytokine induction occurs in surviving lung, colorectal and pancreatic cancer cells and across species, thereby indicating that it is a cancer-wide phenomenon. Cytokines were released from cancer cells surviving TRAIL stimulation and the question remains as to why TRAIL, a death ligand, would elicit cytokine secretion in a wide array of cancer cells in the first place. The fact that the regulation of TRAIL-mediated cytokine induction is closely tied to factors associated with the apoptotic machinery suggests that cytokine induction may have originally been intended to facilitate the influx of immune cells to remove debris of the dead cells. This is in line with postulations made regarding the role of CD95L-mediated cytokine secretion (Cullen et al., 2013). However, in cancer, the opposite might occur as the mutated and TRAIL-resistant cell might rewire the pro-inflammatory signal towards a tumour supportive one. Thereby, the "find me" and "eat me" signals, may be modulated by the tumour microenvironmental milieu to facilitate cancer progression.

4.2 Molecular mechanism of TRAIL-mediated cytokine secretion: parallels to TNF

The ability of TRAIL to induce cytokines is undisputed; yet the mechanisms involved herein have remained elusive. Characterization of the non-apoptotic machinery could open potential avenues for the design of targeted treatments to pre-empt potentially undesired tumour-supportive roles of TRAIL-signalling. Therefore, this thesis investigates the overall mechanism for cytokine production as well as specific, required factors therein.

4.2.1 TRAIL-induced cytokine secretion is mechanistically distinct from the tumour supportive MPD/Rac1 axis

Expression of TRAIL-R1 and/or TRAIL-R2, and their adaptor FADD, is essential for the induction of TRAIL-mediated apoptosis. However, both receptors are also associated with promoting tumour growth, whilst independent studies demonstrate their ability to induce cytokines (Trauzold et al., 2006; Tang et al., 2009; von Karstedt et al., 2015). Our laboratory has recently shown that cancer-cell autonomous migration of *KRAS*-mutated cells was specifically mediated via the MPD of TRAIL-R2 (von Karstedt et al., 2015). In this study, the MPD was essential for promoting the FADD-independent activation of Rac1, which in turn facilitates cancer-cell autonomous migration and invasion. TRAIL-mediated cytokine production on the other hand, was negatively regulated by Rac1 activity. In line with our results showing an inhibitory role of Rac1 on cytokine production, Juncadella et

al. demonstrated that animals deficient in airway epithelial Rac1 exhibit increased concentrations of Th2 cytokines and T cell infiltration upon allergen challenge (Juncadella et al., 2013). Interestingly, TRAIL has been reported to be upregulated in the respiratory epithelium upon allergen exposure. Furthermore, TRAIL was crucial in facilitating CCL20 secretion and the recruitment of TH2 cells in a model of allergic airway disease (Weckmann et al., 2007). Therefore, it is likely that in airway epithelial cells, which give rise to the NSCLC cell line utilized in this thesis, Rac1 elicits an inhibitory function downstream of TRAIL-R engagement, which is in place to prevent autoinflammatory diseases. Interestingly, the inhibitory role of Rac1 in TRAIL signalling is different from its ability to promote NF-κB activation upon IL1β or IL-18 stimulation in Hela cells or B16F10 melanoma cells, respectively (Sulciner et al., 1996, Kim et al., 2008). It would be interesting to mechanistically decipher this negative feedback effect of Rac1 on TRAIL-mediated cytokine production and determine whether it is recapitulated in cells of tissues not native to the lung.

This thesis demonstrates that the secretion of cytokines by TRAIL does, unlike Rac1 activation, not depend on the MPD, but instead requires the DD and is dependent on FADD. Since TRAIL-R1 as well as TRAIL-R2 express a highly conserved DD, it is conceivable that both of these receptors are involved in TRAIL-mediated cytokine production. In line with this, stable KD of either TRAIL-R1 or TRAIL-R2 significantly reduced TRAIL's cytokine induction, as shown in Figure 26. The fact that cell autonomous migration was specifically mediated only by TRAIL-R2, not by TRAIL-R1, points out yet an additional mechanistic difference to cytokine secretion (von Karstedt et al., 2015). Therefore, TRAIL/TRAIL-R-mediated, FADD-dependent cytokine induction represents a distinct cancer-promoting function of this ligand/receptor system.

Interestingly, the CD95/CD95L axis, another death ligand/receptor system very similar to TRAIL/TRAIL-R has also been recently demonstrated to elicit cytokine secretion from dying cells (Cullen et al., 2013). The authors show that CD95L-induced cytokines can elicit the chemoattraction of THP-1 cells and PBMC-isolated neutrophils. CD95L can also facilitate tumour progression, although causative links between endogenous CD95L-mediated cytokine secretion and pro-tumour effects *in vivo* are yet to be made (Hoogwater et al., 2010). CD95L was ruled out as a possible contributor to tumour growth in the immune competent 3LL *in vivo* system employed in this thesis, as 3LL cells did not express CD95 as evidenced by surface staining (Figure 35). However, it cannot be excluded that in other cancers different death ligands may facilitate or cooperate with TRAIL to promote the accumulation of tumour-supportive immune cells.

The recent paper from our laboratory indicating cell-autonomous tumour-supportive roles of TRAIL and other studies on CD95 have shown that the presence of the activating *KRAS*

mutation can switch CD95 and TRAIL-R signalling from an apoptotic to a pro-invasive output (von Karstedt et al., 2015; Hoogwater et al., 2010). Interestingly, oncogenic context has also been demonstrated to regulate the ability of cancer cells to secrete cytokines. As such, expression of H-RasV12 was demonstrated to elicit the secretion of IL-8, which in turn promoted angiogenesis and cancer progression in a xenograft model (Sparmann and Bar-Sagi, 2004). In addition, studies by Borello et al. 2005 demonstrate that rearrangements of the receptor tyrosine kinase gene (RET) lead to the generation of the *RET/PTC1* oncogene which can mediate the upregulation of a proinflammatory program in papillary thyroid carcinoma (PTC) (Borrello et al., 2005). By exogenously expressing the RET/PTC1 oncogenes in thyrocytes, they demonstrated marked upregulations of CCL2, IL-8, G-CSF, matrix metalloproteinases and other tumour supportive secreted factors. These proinflammatory signatures were recapitulated in samples of patients with PTC. Although these studies implicate oncogenes with increasing the amenability of cancer cells to secreted cytokines, neither of them investigated which potential micoenvironmental triggers could be involved in mediating the secretion. Since TRAIL and also CD95L induce various cytokines, which independent studies associated with oncogenic regulation, it would be interesting to elucidate whether death ligand-mediated cytokine secretion underlies a specific oncogenic signature.

4.2.2 FADD and the non-enzymatic function of caspase-8 are crucial for complex formation and cytokine induction

The TRAIL-R adaptor protein FADD and caspase-8 are essential for induction of TRAIL-induced apoptosis. Paradoxically, both receptors and FADD have previously been independently associated with a secondary complex which is thought to induce gene-activatory signalling upon TRAIL stimulation. The complex is composed of RIP1, NEMO, TRAF2, caspase-8 and FADD but does not contain TRAIL-R1/R2 (Varfolomeev et al., 2005). This thesis has revealed that FADD is not only part of, but crucial for the formation of a caspase-8 containing secondary complex. Accordingly, both FADD and caspase-8 presence were required to elicit TRAIL-mediated cytokine secretion, which was blunted by FADD KO or caspase-8 KD (Figure 15 and 19). Therefore, FADD, the essential initial factor to interact with DD-containing TRAIL-R's in TRAIL-induced apoptosis, is also required for DD-dependent gene-activatory outputs (Sprick et al., 2000, Kischkel et al., 2000). Furthermore, very recent work from our group has also indicated a requirement of FADD for the recruitment of LUBAC, and its catalytic component HOIP, to the DISC and secondary complex (Lafont et al., 2017). Downstream of FADD, HOIP recruitment was shown to also block TRAIL-mediated necroptosis, as discussed below, rendering FADD also crucial for regulating necroptotic outputs of TRAIL signalling.

Whilst caspase-8 presence was required, its activity did not affect cytokine secretion independently of the crucial role of caspase-8 activity in mediating cell death. This is the case as caspase inhibition did not significantly affect TRAIL-mediated cytokine production in resistant cells, thereby implicating caspase-8 and FADD with crucial scaffolding roles in facilitating cytokine secretion. In line with this, Henry and Martin recently demonstrated the requirement of a 'FADDosome' for TRAIL-mediated cytokine production, which is composed of FADD, caspase-8 and RIP1. Within this complex, caspase-8 activity was dispensable, whilst caspase-8 did provide a platform for RIP1 recruitment, which was required for TRAIL-mediated cytokine production (Henry et al., 2017). Moreover, TRAIL-mediated cytokine induction could be rescued upon reconstitution of caspase-8 KO cells with an enzymatically inactive version of caspase-8. Further corroborating the results from this thesis, Henry and Martin demonstrate a requirement of TAK1 and NF-κB to mediate TRAIL-R's cytokine inducing arm. Functionally, the authors verify previous results from Varfolomeev et al., by showing that TRAIL-induced cytokines and chemokines can chemoattract myeloid cell subsets *in vitro*, but do not take this further to analyse the role of TRAIL-induced cytokines in a physiological setting.

Interestingly, several caspase-8 deletion and frameshift mutations have been found in hepatocellular carcinoma, colorectal, head and neck cancer and gastric carcinomas (Soung et al., 2005; Ghavami et al., 2009). These mutations frequently lead to the generation of mutant caspase-8 proteins defective in their ability to induce apoptosis but can still bind FADD (Shikama et al., 2003; Ando et al., 2013). Furthermore, the majority of caspase-8 mutants are also still capable of facilitating NF-κB activation (Ando et al., 2013). Therefore, it seems feasible that selective pressure directs cancer cell evolution toward loss of the cell death mediating protease activity of caspase-8, whilst maintaining its ability to facilitate gene activation and consequently cytokine secretion. Cancer cells thereby switch the anti-cancer death-signal towards a tumour-supportive pro-inflammatory outcome.

cFLIP, an inactive caspase-8 homolog, was also found in TRAIL's DISC and post-DISC complex in this thesis. It is also associated with several other complexes such as the apoptosis promoting RIP1/FADD/caspase-8 'Ripoptosome' and the necroptosis mediating RIP1/RIP3 necrosome wherein cFLIP likely functions to blunt caspase-8 activity (Sprick et al., 2000; Tenev et al., 2011; Oberst et al., 2011; Dillon et al., 2012). Since caspase-8 activity was however not required in TRAIL-mediated cytokine induction, cFLIP might serve a different function. Interestingly, recent studies by McFarlane and colleagues indicate that the role of cFLIP depends on which isoform, short or long, is mainly found in complex with procaspase-8. Predominant heterodimerization of procaspase-8 with $cFLIP_L$ was demonstrated to be capable of functioning as a DISC-activator by facilitating DED-

mediated recruitment of multiple procaspase-8 molecules, whilst $cFLIP_S$ blocks procaspase-8 activation (Hughes et al., 2016). Since caspase-8 presence was necessary for cytokine production, high levels of $cFLIP_L$ may actually enhance TRAIL-mediated cytokine induction, whilst $cFLIP_S$ may not have an effect. The potential differential effects of the cFLIP isoforms on TRAIL-mediated cytokine production would be appealing to address experimentally.

TRADD, another DED-containing protein, was also shown to promote cytokine production, as transient knockdown decreased TRAIL-mediated cytokine production (Figure 20). Surprisingly, TRADD was however not found to be associated with the DISC or secondary complex. The difficulty of localizing TRADD has also been indicated by Varfolomeev et al., possibly indicating a transient association of TRADD with the non-apoptotic TRAIL machinery. Although FADD and TRADD share high homology, the two adaptors are not redundant in meditating cytokine production, as cytokines were completely abrogated by FADD-deficiency. Therefore, TRADD possibly acts as an additional binding platform in TRAIL complex construction. Given that TRADD has been shown to interact with TRAF2 in the TNF-RSC, and TRAF2 was also demonstrated to be important in TRAIL mediated cytokine-production in this thesis, it is feasible that TRAF2 makes for at least one of TRADD's binding partners in TRAIL-signalling (Figure 21). It would be interesting to determine exactly where in the TRAIL complexes TRADD is found, and what its other binding partners might be.

4.2.3 HOIP: a previously unrecognized factor required for TRAIL-mediated cytokine production

Further downstream of DED-containing proteins, ubiquitylation plays an important role in TNF signalling (Walczak et al., 2012). As such, cIAP1/2 mediated ubiquitylation has recently been shown to promote the recruitment of LUBAC, another E3 ubiquitin ligase complex crucial for TNF-RSC stabilization. LUBAC is capable of diverting the TNF signalling output from inducing cell death towards enabling the full extent of NF-κB activation (Haas et al., 2009; Gerlach et al., 2011). Pharmacologic depletion of the cIAP1/2 by SMAC mimetics sensitises cells to TRAIL-induced death (Geserick et al., 2009, Fulda et al., 2002b). Given that the TRAIL/TRAIL-R and TNF/TNFR1 system employ a similar machinery in their gene-activatory signalling, it was feasible that LUBAC would also play a role in TRAIL signalling.

Results in this thesis have now identified HOIP, the catalytic core component of LUBAC, to promote TRAIL-mediated cytokine secretion, as HOIP KO cells exhibited reduced cytokine induction. This effect was specific to HOIP, as cytokine secretion was reinstated by reconstitution with Wildtype HOIP. Since the ablation of cIAP1/2 also blunted TRAIL-mediated cytokine production, and cIAPs recruit LUBAC into the TNF-RSC, it is tempting

to speculate that cIAP1/2 act upstream of HOIP to mediate recruitment of LUBAC into a TRAIL-induced signalling complex. As reconstitution with an enzymatically inactive HOIP mutant was not fully capable of rescuing cytokine secretion, but showed higher cytokine levels than the HOIP KO cells, it is possible that HOIP's role in TRAIL signalling involves both scaffolding- as well as activity-dependent functions. In line with results in this thesis, TNF-mediated gene activation also required HOIP activity (Haas et al., 2009) indicating possible similarities in the role of HOIP in TNF and TRAIL signalling (Figure 23). Interestingly, recent publications from our laboratory also describe potential scaffolding functions of HOIP, which was demonstrated to directly interact with the deubiquitinase CYLD; thereby antagonizing the linear chain forming ability of HOIP in TNF-signalling (Draber et al., 2015). Furthermore, very recent work from our group indicates that HOIP is found in both the TRAIL DISC and the post-DISC complex (Lafont et al., 2017). This study shows that HOIP can act as a scaffold, as specifically the presence of HOIP was required for preventing TRAIL-mediated necroptosis. In line with a role in promoting gene activation, HOIP was demonstrated to promote NF-κB activation, dependent on the presence of FADD, which was proposed to be mediated by the linear chain forming ability of HOIP. However, a presence dependent role of HOIP in gene activation, independently of a role in cell death, was not investigated. Nevertheless, previous work does demonstrate that HOIP can function to some extent as a binding platform in signalling complexes, independently of its linear chain forming ability. It would be interesting to investigate which factors directly interact with HOIP in TRAIL signalling to facilitate cytokine production, and how exactly LUBAC as a whole mediates gene-activatory outputs of TRAIL's non-apoptotic arm.

4.2.4 TRAIL-mediated cytokine induction requires TAK1 and NF-κB

Downstream of their receptor complexes both TNF- and TRAIL-signalling pathways were demonstrated to promote the activation of NF-κB signalling pathways (Wajant et al., 2003; Tang et al., 2009). However, the role of NF-κB in TRAIL-mediated cytokine induction has previously been shown in overexpression studies which could be prone to artefacts (Tang et al., 2009). Results in this thesis have demonstrated that TRAIL stimulation elicits the activation of NF-κB, dependent on the presence of FADD. Inhibition of NF-κB using the IKKα/β inhibitor TPCA-1, as well as silencing of p65 severely impaired TRAIL-mediated cytokine production (Figure 25). Since cytokine production was completely abrogated upon inactivation or silencing of NF-κB, it is likely that NF-κB is the core signalling pathway mediating the cytokine production in TRAIL-signalling. In line with NF-κB as a crucial pathway to mediate cytokine production, IL-8 and CCL2 production have previously been demonstrated to depend on NF-κB in TNF signalling, which was mediated by p65-p65

homodimers and p65-p50 heterodimers respectively (Andoh et al., 2000, Ritchie et al., 2004).

Further in support of a key role of NF-κB in TRAIL-mediated cytokine production, very recent work has demonstrated the recruitment of the NF-κB activating IKK complex into the TRAIL signalling complexes (Lafont et al., 2017). IKK complex activation is facilitated by phosphorylation via the TAK-TAB complex (Chen et al., 2006; Huang et al., 2004). This is supported by results in this thesis, which demonstrate a requirement of TAK1 activity and presence in TRAIL-mediated gene activation, as shown by decreased IκB phosphorylation and cytokine induction upon use of the TAK1 inhibitor 7-Oxozeanol (Figure 25). Furthermore, this effect was recapitulated by specific silencing of TAK1 expression (Figure 24). Although TAK1's role in gene-activatory signalling is supported, TAK1-presence has not previously been demonstrated within the TRAIL complexes. It is possible that its interaction here is rather transient and would therefore require detailed kinetics of receptor complex formation.

In summary, the molecular machinery required for mediating TRAIL-induced NF-κB activation and cytokine production is constructed around FADD and caspase-8 and is further composed of TRADD, TRAF2, cIAP1/2, LUBAC, the IKK complex and the TAK-TAB complex.

4.3 Novel functional insights on the role of TRAIL-induced cytokines in the modulation of the tumour microenvironment

Non-apoptotic TRAIL signalling has been shown to enhance proliferation, resistance to apoptosis and migration/invasion of cancer cells (Secchiero et al., 2004a, Secchiero et al., 2004b, Ishimura et al., 2006, Belyanskaya et al., 2008, von Karstedt et al., 2015). Although previous results in this thesis demonstrate the induction of various cytokines by TRAIL, the TRAIL-induced secretome did not induce any substantial proliferation or resistance-enhancing autocrine effects *in vitro*. This indicates that the tumour-supportive effects observed in other *in vitro* studies are likely mediated by TRAIL-induced cell-autonomous mechanisms and thus independent of autocrine cytokine signalling. However, the extent to which non-apoptotic signalling mediated cytokine production can elicit immune-modulatory effects, particularly regarding their potential *in vivo,* has not been investigated. Therefore, this section discusses novel, functional, *ex-* and *in vivo* aspects of the TRAIL-induced secretome uncovered in this thesis.

4.3.1 The TRAIL-induced secretome polarises monocytes to M2-like cells

Results from this thesis demonstrated a crucial requirement of FADD for TRAIL-induced secretion of a cytokine-rich secretome containing IL-8, CXCL1, CCL2 and CXCL5. The

investigation of potential paracrine roles revealed that TRAIL's secretome is capable of polarising monocytes towards alternatively-activated M2-like immune cells. This is the case as $CD14^+$ monocytes were polarised to HLA-$DR^{low/neg}$ $CD206^+$ cells upon incubation with conditioned media from TRAIL-treated, but not from untreated, NSCLC cells. Importantly, the polarisation was dependent on tumour cell expression of FADD, as conditioned media from TRAIL-treated, FADD-deficient cells was incapable of eliciting polarising effects (Figure 29). The observed polarisation state is characteristic of M2-like, alternatively activated myeloid cells which can be capable of eliciting tumour supportive functions (Gabrilovich and Nagaraj, 2009, Lesokhin et al., 2012; Mantovani and Sica, 2010; Toh et al., 2011). Although it remains unclear exactly which cytokine or possibly combination thereof is responsible for the polarisation towards an M2-like phenotype, previous studies indicate potential polarising functions of CCL2, which will be further discussed in subsequent chapters (Roca et al., 2009; Chun et al., 2015). In line with their potential tumour-supportive functions, the polarised cell subsets were able to promote EMT of A549 cells, as co-culture of polarised, M2-like cells with A549 cells decreased the cancer cell's E-cadherin levels. This effect was again dependent on the expression of FADD in tumour cells, as myeloid cells previously incubated with conditioned media from FADD KO cells were incapable of eliciting EMT in A549. As the polarised M2-like myeloid cells were not physically separated from the co-cultured A549 cells, the observed loss in E-cadherin could have been mediated by cell-cell contact dependent mechanisms, as well as via factors secreted by the polarised cells. Previous literature indicates that supernatants of M2-like cells were capable of decreasing E-cadherin levels of colorectal cancer cells via MMP9 secreted by the myeloid cells (Vinnakota et al., 2017). As mechanistic insights on myeloid function could enhance the understanding of myeloid cell functionality in light of TRAIL's tumour supportive immunomodulatory roles, it would be interesting to determine whether the polarised M2-like cells elicit EMT by secretion of matrix metalloproteases or via cell-cell contact based mechanisms. Overall these results demonstrate the previously uncharacterized ability of TRAIL's secretome to elicit the *ex vivo* polarisation of myeloid cells towards M2-like cells, dependent on tumour cell expressed FADD.

4.3.2 FADD promotes tumour growth and facilitates the formation of a tumour-supportive microenvironment

Previous findings indicate that the TRAIL-induced secretome of FADD-proficient cancer cells can drive the polarisation of myeloid cells towards M2-like cells *ex-vivo*. Further investigation of an *in vivo* role of FADD demonstrated that tumour cell-expressed FADD promotes tumour growth, as FADD KO NSCLC cells exhibited decreased lung tumour formation in a xenograft model in comparison to Wildtype cells (Figure 31). As an inherent proliferative advantage of FADD proficient cells was not present *in vitro*, it is likely that the

tumour immune-environment might contribute to tumour growth *in vivo* via mechanisms that require cancer cell expression of FADD. Although FADD is well established as a mediator of apoptosis, it has been implicated in facilitating tumour promotion in HCC and is associated with poor clinical outcome in head and neck and NSCLC cancer patients (Ehlken et al., 2014, Bowman et al., 2015, Rasamny et al., 2012). In HCC driven by NEMO-deficiency in liver parenchymal cells, FADD deletion was shown to rescue from aberrant apoptosis, hepatitis and carcinoma development independently of TRAIL-R. In contrast to HCC, lung cancer is commonly driven by NF-κB signaling resulting in cytokine induction, whilst in this model of HCC, abrogation of NF-κB signaling through NEMO-deletion results in inflammatory cell death which causes cancer progression (Meylan et al., 2009, Luedde et al., 2007, Maeda et al., 2005), implying divergent cancer etiologies for the two models. Yet, analysis of how these discrepancies may result in opposing roles of TRAIL-R will be interesting to investigate in the future.

Interestingly, results from this thesis demonstrate that tumour-cell expressed FADD increased the infiltration of M2-like myeloid cells as the lungs bearing FADD-proficient tumours exhibited increased absolute numbers of $CD11b^{+}GR1^{+}$ and $CD11b^{+}GR1^{+}CD206^{+}$ cells. These immune populations are equivalent to the HLA-$DR^{lo/neg}$ $CD206^{+}$ phenotype, observed *in vitro*, thereby indicating that TRAIL-induced polarisation is likely to occur *in vivo* in mice (Sevko and Umansky, 2013).

Further in line with this, the levels of IL-8, CXCL1 and CCL2, previously shown to be induced by TRAIL dependent on the presence of FADD, were also elevated in lungs containing FADD-proficient tumours, in comparison to lungs containing FADD KO tumours. As the observed myeloid subsets have previously been shown to elicit tumour supportive functions, by either directly engaging the cancer cell or by suppressing anti-tumour T cell activity, which is supported in this thesis, it is feasible that they might contribute to tumour growth (Gabrilovich and Nagaraj, 2009, Lesokhin et al., 2012).

In order to validate these findings in fully immune-proficient mice, a syngeneic model, utilizing C57BL/6 mice and murine 3LL cells was established. Importantly, the syngeneic model also recapitulated the previous findings from the xenograft, as two independent 3LL FADD KO clones exhibited decreased lung tumour burden in comparison to 3LL Wildtype cells (Figure 34). Furthermore, lungs populated with FADD-proficient 3LL cells also demonstrated significantly decreased absolute numbers of $CD11b^{+}GR1^{+}CD206^{+}$ to fully recapitulate the findings from the xenograft. The elevated numbers of these M2-like cells were independent of a possible overall increase in immune cell infiltration, as $CD45^{+}$ cells were not significantly altered between the two groups. The 3LL cells also exhibited FADD-dependent cytokine production upon TRAIL stimulation (Figure 32). Since FADD absence did not affect TNF-mediated cytokine secretion, and CD95L did not induce the secretion

of cytokines from 3LL cells, it is unlikely that these death ligands are responsible for the decreased tumour burden observed in the syngeneic FADD-deficient 3LL model. Furthermore, the previously demonstrated M2-polarising ability of the TRAIL-induced secretome further implicates TRAIL as the *in vivo* trigger which promotes M2-like cell accumulation *in vivo*. Therefore, tumour cell-expressed FADD mediates increased lung tumour burden, cytokine levels and the infiltration of tumour supportive, M2-like cells *in vivo;* a signalling cascade which in all likelihood is triggered by endogenous TRAIL/TRAIL-R signalling. In line with this, evidence from NSCLC patients indicates that high FADD mRNA expression correlates with poor prognosis of survival, although the composition of the tumour microenvironment was not addressed (Chen et al., 2005). In fact, FADD has recently been shown to promote lung cancer progression in a KRAS-driven, genetically engineered mouse model (Bowman et al., 2015). Interestingly, cancer cell-specific FADD deletion was also associated with decreased myeloid infiltrates. However, the myeloid cell polarisation state, or whether FADD had a causative role in their infiltration, was not investigated. It is therefore likely that TRAIL and its function as a promoter of a tumour-supportive immune-microenvironment may also play a role in this KRAS-induced genetic model.

4.3.3 The TRAIL-induced cancer secretome promotes a tumour-supportive immune microenvironment via CCR2 *in vivo*

Data in this thesis strongly indicated that a tumour cell TRAIL-R/FADD axis is involved in modulating the tumour immune environment. The specific role of the endogenous TRAIL/TRAIL-R system in tumour-supportive immune modulation was further verified in a syngeneic system, using 3LL cells containing a stable knockdown for TRAIL-R. In line with results from the FADD KO mouse models, lungs containing these shTRAIL-R 3LL cells exhibited significantly decreased tumour burden as compared to lungs containing empty vector-transfected (pLKO.1) cells (Figure 36). Since no significant differences between the two cell lines were observed *in vitro*, it was again likely that microenvironmental effects would account for the observed growth promoting role of TRAIL-R. Importantly, the levels of CCL2 and, accordingly, the levels of $CD11b^+$ $GR1^+$ and $CD11b^+$ $GR1^+$ $CD206^+$ cells, previously shown to be decreased in the xenograft and syngeneic FADD models, were also decreased in the lungs containing shTRAIL-R 3LL cells. The further investigation of potential *in vivo* mechanisms revealed that host CCR2 presence was required to mediate TRAIL's immune-modulatory role, as the difference in tumour burden between pLKO.1 and shTRAIL-R lung tumours was abrogated in CCR2 KO mice.

Whilst CCL2 is described as its principal endogenous ligand in humans and mice, CCR2 also binds CCL7, CCL8 and CCL11 (El Khoury et al., 2007, Naert and Rivest, 2013). Importantly however, CCL2, whose main receptor is CCR2, was the only CCR2 ligand

found to be induced by the TRAIL/TRAIL-R system (data not shown). This implicates CCL2 as the CCR2-ligand which is induced by endogenous TRAIL and mediates the CCR2-dependent modulation of the immune environment. However, it does not preclude, that other CCR2 ligands could serve a similar role in other systems.

The difference in levels of M2-like cell infiltrates, observed between pLKO.1 and shTRAIL-R 3LL containing lungs, was also neutralized in CCR2 KO mice, which is in line with their possible tumour supportive function *in vivo*. Importantly, it is feasible that the TRAIL/TRAIL-R system mediates the production of CCL2 *in vivo*, as CCL2 levels were still increased in pLKO.1 containing lungs as compared to shTRAIL-R containing lungs in CCR2 KO mice. Therefore, these data demonstrate that host cell-expression of CCR2 and tumour cell expression of TRAIL-R and CCL2 is required to enable pro-tumourigenic crosstalk between TRAIL/TRAIL-R and CCL2/CCR2; generating a tumour supportive myeloid compartment.

Although CCL2, was identified as the main immune-modulatory driver, this thesis also demonstrates CXCL1, IL-8 and CXCL5 to be induced by TRAIL dependent on FADD expression in tumour cells. CCL2/CCR2 interaction abrogated TRAIL/TRAIL-R signalling mediated accumulation of tumour supportive M2-like cells; yet this does not mean that CCL2 is the only tumour supportive driver cytokine. As such, various TRAIL-independent studies have demonstrated supportive functions of the other cytokines as previously indicated (Qian et al., 2011, Fader et al., 2010, Zhang et al., 2010, Singh and Lokeshwar, 2009, Begley et al., 2008, Dong et al., 2013). Interestingly, CXCL1, IL-8 and CXCL5 all bind to CXCR2, a receptor involved in PMN-MDSC trafficking, angiogenesis and metastasis (Nannuru et al., 2011, Matsuo et al., 2009, Steele et al., 2016). Since CCL2 can polarise as well as chemoattract M2-like cells, it is possible that abrogation of CCL2 alone has the strongest effect out of any one of these cytokines. However, the additional abrogation of CXCR2 may reveal further tumour supportive functions of TRAIL's secretome.

Whereas this thesis identifies that endogenous TRAIL-R-induced CCL2 and its activity on host-derived CCR2-expressing cells is crucial in tumour support, the source for TRAIL can be manyfold. In the case of the previously indicated A549 model, the tumour cells themselves could serve as the source as silencing of endogenous TRAIL reduces their cytokine secretion (data not shown). However, many stromal cell types, including various different immune cells such as monocytes, dendritic cells, T cells and natural killer cells and can express TRAIL and could therefore contribute to tumour promotion; possibly even via providing a positive feedback loop (Mariani and Krammer, 1998, Mirandola et al., 2004, Fanger et al., 1999, Griffith et al., 1999, Kayagaki et al., 1999). The identification of the

source providing the endogenous TRAIL to trigger cytokine production will be interesting to investigate in the future.

Interestingly, and in line with a role for TRAIL-induced cytokines in affecting the myeloid cell compartment, supernatants of TRAIL-treated HT1080 cells have previously been found to attract human macrophages *in vitro* (Varfolomeev et al., 2005). However, a biological function of TRAIL-induced cytokines in tumour biology has remained elusive. This thesis identifies that within the TRAIL-induced secretome, CCL2 fulfills a central function in the formation of a tumour-supportive myeloid compartment *in vivo*, via the engagement of CCR2 on host cells.

4.3.4 Relevance of the TRAIL/TRAIL-R-CCL2/CCR2 signalling axis in human lung cancer patients

The endogenous TRAIL/TRAIL-R axis mediates its tumour-supportive effects via host CCR2 in mice. The immune-modulatory link between TRAIL and CCL2 signalling was supported by data from humans, as the analysis of LUAD patient RNAseq data sets revealed positive correlations between TRAIL and CCL2 expression levels (Figure 39). Furthermore, TRAIL levels also positively correlated with markers of alternatively activated myeloid cell infiltrates, such as CD206, which were previously demonstrated to be increased in murine lungs. In line with this, CCL2 has been shown to facilitate tumour growth in various cancers including prostate (Li et al., 2009), breast (Soria et al., 2008), and lung cancer (Cai et al., 2009), and to be elevated in the lungs of NSCLC patients (Arenberg et al., 2000, Rivas-Fuentes et al., 2015). CCL2 has also been shown to mediate its tumour-supportive ability by acting as a potent chemoattractant for M2-like cells such as MDSCs (Fujita et al., 2011) and unpolarised monocytes, in addition to being able to contribute to polarisation of monocytes to MDSC by increasing their CD206 expression (Roca et al., 2009). As supernatants of TRAIL-treated cells were also able to polarise human $CD14^+$ cells towards HLA-$DR^{lo/neg}$ $CD206^+$ cells *in vitro*, it is conceivable that TRAIL-induced cytokines may not only recruit myeloid cells to, but also promote their polarisation within the human tumour microenvironment. Since a potential immune-modulatory role of the endogenous TRAIL/TRAIL-R-CCL2/CCR2 axis is supported in humans, immunotherapeutic approaches involving blockade of the TRAIL/TRAIL-R system on its own, or in combination with existing immune checkpoint blockers would be interesting to pursue; as discussed in the following section.

4.3.5 Relevance of TRAIL's immunomodulatory role for therapeutic approaches in cancer

To date, TRAIL therapy approaches have involved recombinant human TRAIL and antibodies directed against the DD containing receptors TRAIL-R1 and TRAIL-R2.

Unfortunately, these TRAIL-R agonists provided insufficient agonistic activity and have failed in clinical trials, as most primary cancer cells exhibit resistance to TRAIL-based monotherapy (Lemke et al., 2014b). Although more potent TRAIL agonists are currently in development, results from this thesis and the recent publication by our lab indicate that TRAIL antagonism might be an alternate strategy worth pursuing (von Karstedt et al., 2015). In accord, antagonism of TRAIL signalling using the chimeric fusion protein TRAIL-R2:Fc, which consists of the extracellular domain of TRAIL-R2 and the Fc portion of human IgG1, significantly decreased tumour burden in a NSCLC xenograft model (von Karstedt et al., 2015). Based on data from this thesis, antagonizing the endogenous TRAIL ligand, rather than blocking individual receptors, is likely the most effective approach as both DD-containing receptors contribute to the generation of TRAIL's secretome (Figure 26). As such, TRAIL antagonism could benefit patients by blocking both the cancer cell autonomous pro-invasive functions of TRAIL, as well as its tumour-supportive immunomodulatory secretome.

Immune modulation can be a powerful tool in cancer therapy; as such the current standard of care ICB options ipilimumab (anti-CTLA4) and nivolumab (anti-PD-1) have shown substantial clinical benefit for patients with renal cell carcinoma, urothelial carcinoma, ovarian cancer and demonstrated 50% regression and considerable survival benefit for patients with advanced melanoma (Sharma and Allison, 2015). Antagonizing these checkpoint inhibitory molecules liberates CD8 T-cells from inhibition and facilitates their anti-tumour killing ability (Tumeh et al., 2014). The results in this thesis demonstrate that the recruited M2-like cells are capable of directly promoting tumour growth, as BrDU incorporation was increased in *ex-vivo* co-culture of sorted $CD11b^+$ cells with 3LL cells (Figure 38). Next to their direct effects on tumour growth, M2-like cells frequently enhance tumour progression via suppression of anti-tumour T cells (Noy and Pollard, 2014). Therefore, inhibition of endogenous TRAIL could facilitate a similar T cell activatory function.

However, ICB still has its limitations which can be caused by distinct immune checkpoints elicited by the tumour and/or its immune microenvironment, to render ICB ineffective (Hodi et al., 2014, Ruffell et al., 2014, Smyth et al., 2016). Most recent studies indicate that these CTLA4, PD-1/PD-L1 independent suppressive barriers can be overcome by further adjuvant therapy which boosts the anti-tumour T cell activity (Zhu et al., 2014, Winograd et al., 2015, Dammeijer et al., 2017). One possible way to circumvent any additional inhibition to T cell efficacy is to target suppressive cells of the myeloid compartment and/or cytokines associated with their effector functions and accumulation in the tumour microenvironment. In line with this, antagonizing the chemokine receptor CCR1 via the small molecule CCX9588 synergistically reduced the tumour burden when combined with

anti-PD-L1 treatment in a preclinical breast cancer model (Jung et al., 2015). As CCX9588 treatment was associated with decreased MDSC infiltrates the authors attributed their observed synergy to the abrogation of MDSC accumulation. Furthermore, prompted by high levels of tumour secreted IL-8 and intra-tumour MDSCs expressing IL-8's receptor, CXCR-2, Highfill et al. hypothesized that abrogation of MDSC chemoattraction by blocking CXCR-2 may unleash any breaks they impose on anti-PD-1 treatment efficacy in a rhabdomyosarcoma model. They approached their hypothesis using mice deficient in CXCR-2 as well as CXCR-2 neutralizing antibodies; in both settings, the resulting abrogation of MDSC trafficking synergized with anti-PD-1 therapy to decrease tumour burden and enhance mouse survival respectively (Highfill et al., 2014). These results indicate that targeting essential myeloid cell trafficking hubs can have beneficial effects in combination with ICB. Therefore, antagonizing endogenous TRAIL, and its MDSC recruiting secretome, via TRAIL-R2:Fc treatment might similarly synergize with anti-PD-1 and would be an interesting strategy to pursue.

The feasible rationale to antagonize TRAIL-R and the failure of TRAIL monotherapy highlight the importance of suitable biomarkers to delineate which patients would benefit from either of the two opposing therapy options. High expression of the o-glycosidase GALNT14 has been proposed as a marker for TRAIL-sensitive cancer cells, but its expression failed to significantly correlate with a clinical response in a randomized phase II study of advanced NSCLC patients (Soria et al., 2011). Alternatively, high TRAIL-R1/2 expression levels could function as indicators of sensitivity. On the other hand, in light of TRAIL's tumour supportive effects high receptor expression alone may just as well indicate that the tumour benefits from endogenous TRAIL signalling and has been positively selected for increased receptor expression. Distinguishing whether to activate or block TRAIL signalling would thus require further markers, one of which could be *KRAS* mutation (von Karstedt et al., 2015). In line with KRAS as a feasible additional marker, high TRAIL-R2 expression correlated with poor prognosis in patients with *KRAS*-mutated pancreatic and colorectal carcinoma. Since mutated *KRAS* can enable endogenous TRAIL to facilitate cancer proliferation and invasion, TRAIL antagonism rather than treatment might be more feasible in patients bearing a *KRAS* mutation.

Next to KRAS, mutations in caspase-8 could also function as suitable biomarkers. Tumour-associated caspase-8 mutations have been described for a large subset of cancers including gastric carcinomas, hepatocellular carcinoma, HNSCC and colorectal cancer (Soung et al., 2005). These mutations frequently abrogate TRAIL-mediated apoptotic signalling but enhance TRAIL's non-apoptotic, gene activatory signalling. In line with this gain of function mutation, studies by Ando et al. demonstrated that overexpression of a caspase-8 point mutant, previously identified in a HNSCC cell line, significantly increased

NF-κB in comparison to overexpression of wildtype caspase-8 (Ando et al., 2013). Since this thesis demonstrates that TRAIL's tumour supportive secretome is driven by NF-κB, it is likely that these caspase-8 mutations also facilitate the influx of M2-like myeloid cells. Therefore, tumour associated caspase-8 mutations could function as a biomarker to indicate cancers which might respond to antagonism of TRAIL signalling. Which mutations of caspase-8 in particular, or of other components of the TRAIL pathway, are suitable in this regard should be a main pillar of future TRAIL research. As such, high throughput screenings of large cell line collections with established mutations could hold great promise towards the identification of suitable biomarkers to distinguish patients which might benefit from either TRAIL treatment or antagonism (Garnett et al., 2012).

Summary and Outlook

In this thesis, the molecular and functional characteristics of the TRAIL-induced secretome have been investigated. The first unbiased analysis to date of this secretome was performed in this thesis and revealed that the cytokines CCL2, CXCL1, CXCL5 and NAMPT are highly enriched herein. TRAIL-mediated cytokine secretion was identified to be a cancer-wide phenomenon present across species and cancer types as it was demonstrated in both murine and human lung cancers as well as in human colorectal, pancreatic and cervical cancer cells. This indicates high potential for the functional significance of TRAIL-mediated cytokine induction in tumour biology.

Endogenous TRAIL-induced cytokine production was demonstrated to occur via a mechanism distinct from the cancer cell-autonomous TRAIL-induced activation of Rac1, which is triggered via the MPD of TRAIL-R, independently of its death domain and the adaptor protein FADD (von Karstedt et al., 2015). Instead, cytokine production via endogenous TRAIL/TRAIL-R requires FADD and the scaffolding function of caspase-8 but not its enzymatic activity. FADD was required for formation of the TRAIL-induced signalling complexes and TRAIL-mediated cytokine production. The analysis of potential regulatory factors of cytokine production further identified a requirement of several factors also found in the TNF-RSC, most importantly HOIP, a previously unidentified component in TRAIL-induced non-apoptotic signalling.

Whilst answering several questions regarding the mechanism of TRAIL-induced cytokine production the results of this thesis pose several new questions: 1. Are other constituents of the TRAIL-induced cancer secretome, e.g. various different proteases, also relevant in tumour biology? 2. Is TRAIL-induced cytokine production driven by (a) specific oncogenic signature(s)? 3. What is the exact role of HOIP and other LUBAC components in regulating cytokine production and how do TRADD and cFLIP exert their roles? 4. What is the

underlying mechanism by which activated Rac1 elicits negative feedback on TRAIL-mediated cytokine production?

The mechanism and functional consequences of TRAIL-induced cytokine production are summarized in Figure 40. This thesis demonstrates that endogenous TRAIL induces the FADD-dependent secretion of chemo-/cytokines which facilitate the polarisation of myeloid cells towards M2-like MDSC and TAM, that the accumulation of these M2-like cells in the tumour microenvironment depends on FADD-dependent signalling triggered by endogenous TRAIL/TRAIL-R and that this accumulation contributes to tumour growth. Importantly, TRAIL/TRAIL-R mediate this accumulation via cancer-cell-derived CCL2 and host-expressed CCR2. Together, the results obtained in this thesis reveal a previously unknown tumour immune-modulatory role of TRAIL biology in a pathophysiological setting and identify the production of CCL2, induced via endogenous TRAIL and TRAIL-R in a FADD-dependent manner, as a crucial driver of the accumulation of M2-like immune cells in the tumour microenvironment via host cell-expressed CCR2.

Results from this thesis indicate that the TRAIL/TRAIL-R induced cytokine secretome, with CCL2 at its core, possibly establishes myeloid trafficking and polarisation hubs, due to its enrichment in CCR2 as well as CXCR2 ligands. It would, therefore, be highly interesting to determine whether blocking TRAIL/TRAIL-R may limit the accumulation of alternatively activated myeloid cells, possibly attenuating not only their tumour growth-promoting effects but also their immune-suppressive function. The newly discovered link between endogenous TRAIL/TRAIL-R signalling and a tumour-supportive immune-microenvironment suggests that inhibiting the interaction of TRAIL with its receptors, or the use of inhibitors for components identified to be required for TRAIL-mediated cytokine production, might serve as an effective therapeutic option to limit the presence of tumour-supportive myeloid cells within the tumour microenvironment and thereby decrease tumour growth.

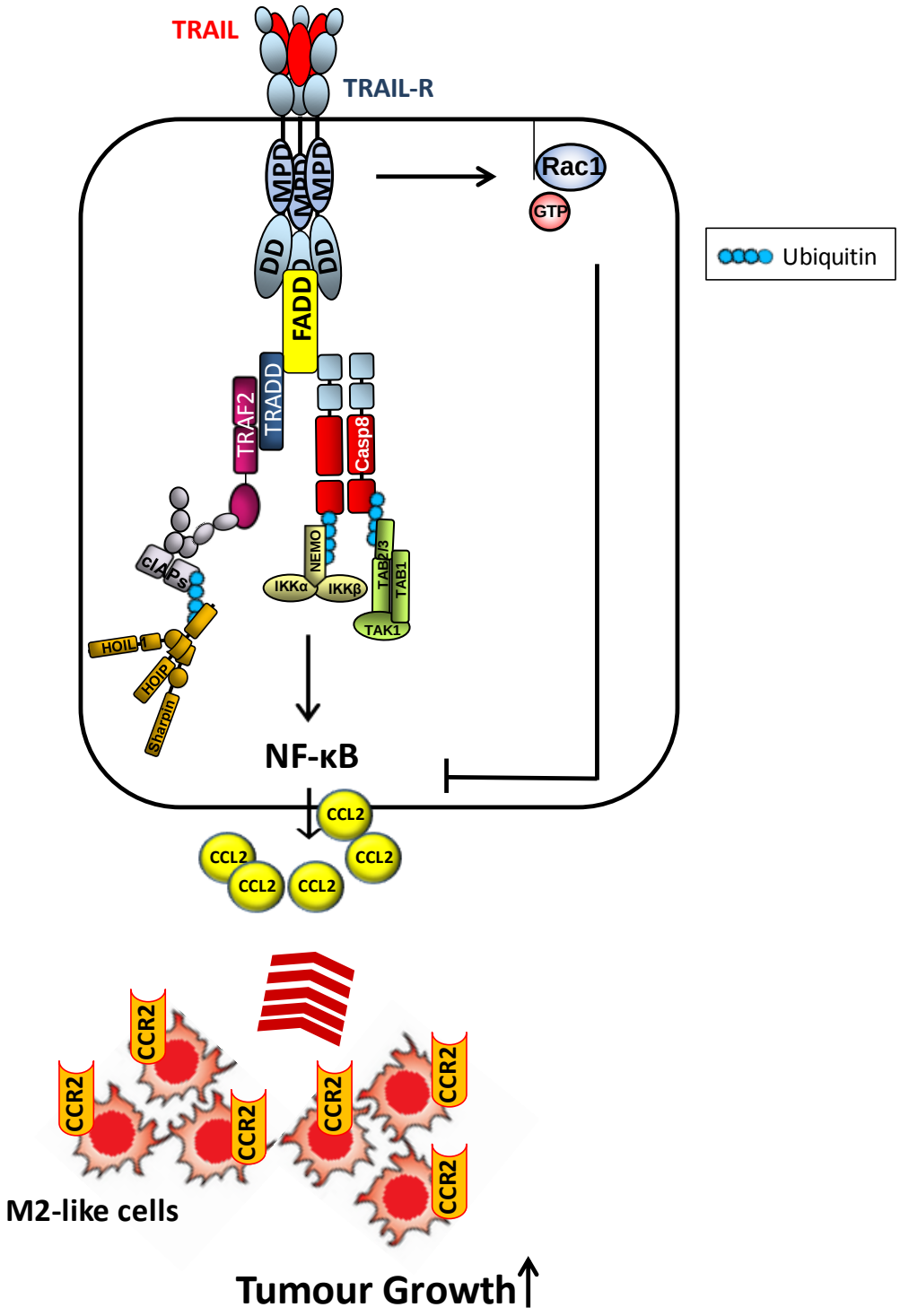


Figure 40. The TRAIL-induced cancer secretome promotes a tumour-supportive immune microenvironment via CCR2.

Endogenous TRAIL mediates TRAIL-R trimerization to promote the formation of a complex which can elicit the activation of NF-κB. Complex formation is instigated via the receptors death domains (DD) which bind the adaptor protein FADD. FADD binding next facilitates the recruitment of caspase-8 and other components including E3 ligases cIAP1/2 and LUBAC, that create nondegradative ubiquitin chains which in turn bind the effector molecules NEMO-IKKα-IKKβ and the TAK-TAB complex promoting non-apoptotic signalling. This facilitates the production of CCL2, which promotes the recruitment of tumour-supportive M2-like myeloid cells via CCR2, ultimately promoting tumour growth. The membrane proximal domain (MPD) of TRAIL-R2 can further elicit negative feedback on cytokine production in NSCLC cells via activation of the GTPase Rac1.

As with the mechanistic results, these new functional insights also pose several new questions with respect to the (patho)physiological role of the TRAIL/TRAIL-R system, not the least regarding its potential immune-modulatory role. Firstly, the source of the endogenous TRAIL driving this signalling remains to be determined. Another question is whether some of the other cytokines induced by the endogenous TRAIL/TRAIL-R system, e.g. IL-8, CXCL1, CCL2 and CXCL5, contribute to the tumour-supportive microenvironment, and if so, how? In the light of the current success of immune checkpoint blockade in cancer therapy, but perhaps more importantly also its limitations, a particularly intriguing question is whether inhibition of the suppressive environment generated by endogenous TRAIL/TRAIL-R-mediated signalling, may alter the outcome of immune checkpoint blockade, particularly in patients in which this novel paradigm in cancer therapy has so far shown limited efficacy.

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ABBREVIATIONS

µg	Microgram
µL	Microlitre
µM	micromolar
Apaf-1	Apoptosis-protein-associated-factor-1
APC	Antigen presenting cell
ATP	Adenosine Triphosphate
BAX	Bcl-2-associated protein X
Bcl-2	B cell lymphoma gene 2
Bcl-xL	B cell lymphoma gene x (long form)
Bid	BH-3 interacting Domain
BSA	Bovine serum albumin
CARD	Caspase activation and recruitment domain
CCL	CC-motif ligand
CCR	CC-motif receptor
cFLIP	cellular FLICE(caspase-8) inhibiting protein
CD95L	Apo1ligand/ FAS ligand
CRD	Cysteine rich domain
cIAP	cellular inhibitor of apoptosis protein
CXCL	CXC-motif ligand
CXCR	CXC-motif receptor
DC	Dendritic cells

DD	Death domain
DcR	Decoy receptor
DED	Death effector domain
DISC	Death-inducing signalling complex
DMBA	7,12-dimethyl-benz-anthracene
DMSO	Dimethylsulfoxid
DR	Death receptor
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial–mesenchymal transition
FADD	Fas-associated death domain protein
FCS	Fetal calf serum
FSC	Forward scatter
GPI	Glycosylphosphatidylinositol
HBS	HEPES-buffered Saline
HCC	Hepatocellular carcinoma
HEK	Human embryonic kidney
HRP	Horseradish peroxidase
IAP	Inhibitor of apoptosis protein
IFN	Interferon
IL	Interleukin
JNK	c-Jun N-terminal kinase
IZ-huTRAIL	isoleucine -Zipper-human -TRAIL
IZ-muTRAIL	isoleucine -Zipper-mouse -TRAIL
kB	kilobase

kd	knock-down
kDa	kilodalton
M	Molar (mol/L)
MAPK	Mitogen-activated protein kinase
Mcl-1	Myeloid cell leukemia-1
MCMV	Murine cytomegalovirus
MCP	Monocyte chemoattractant protein
MDSC	Mteloid derived suppressor cell
MMP	Matrix metalloproteinase
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MOMP	Mitochondrial outer membrane permeabilization
mRNA	messenger ribonucleic acid
mu	murine
MW	Molecular weight
NF-κB	Nuclear Factor -κB
ng	nanogram
NK cells	Natural killer cells
OPG	Osteoprotegerin
ORF	Open reading frame
OVA	Ovalbumin
P	phospho
PBS	Phosphate buffered saline

PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
R	Receptor
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe combined immunodeficiency
SDS	Sodiumdodecylsulfate
SDS-PAGE	Sodium-dodecylsulfate Polyacrylamide-Gelelectrophoresis
siRNA	Small interference RNA
SMAC	Super molecular activation cluster
SSC	Side scatter
STAT	Signal transducer and activator of transcription
TGFβ	Transforming growth factor β
TNF	Tumour necrosis factor
TRADD	TNFRSF1A Associated Via Death Domain
TRAIL	TNF-related apoptosis-inducing ligand
TPA	12-O-tetradecanoylphorbol-13-acetate
XIAP	X-chromosome linked IAP
VEGF	Vascular endothelial growth factor
wt	Wildtype