Development of CD40-targeted bifunctional scFv-TRAIL fusion proteins that induce TRAILR1- and TRAILR2-specifc cell death and dendritic cells activation

Entwicklung CD40 gerichteter bifunktioneller scFv-TRAIL Fusionsproteine die TRAILR1- und TRAILR2-spezifischen Zelltod und dendritischen Zellaktivierung induzieren

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submitted by

Mohamed El-Sayed Ahmed Mohamed El-Mesery

PhD German-Egyptian Research Long Term Scholarship (GERLS) holder

from

Biochemistry Department, Faculty of Pharmacy
Mansoura University, Mansoura, Egypt

Würzburg 2014

Submitted on: ……………………………………………………………

Members of the promotion committee:

Chairperson: Prof. Dr. Jörg Schultz

Primary Supervisor: Prof. Dr. Harald Wajant

Supervisor (Second): Prof. Dr. Ralf Bargou

Supervisor (Third): Prof. Dr. Thomas Müller

Date of Public Defence: ……………………………………………..…………

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The current work was achieved in the period from 1. April 2011 till 30. April 2014 in the Division of Molecular Internal Medicine, University Hospital of Würzburg under the supervision of Prof. Dr. Harald Wajant.

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For my parents, my wife and my children

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1. Introduction

1.1. Receptors and ligands of the TNF family

1.1.1. TNF receptor superfamily (TNFRSF)

Receptors and ligands of the tumor necrosis factor (TNF) superfamily participate in a wide range of biological processing including cell differentiation, proliferation, apoptosis, survival and induction of inflammatory mediators such as cytokines and chemokines (Moran *et al.*, 2013). Therefore, it is no wonder that researchers have paid and still pay great attention on the investigation of receptors and ligands of TNF superfamily. Members of the TNFRSF typically consist of three major domains: an extracellular domain, which binds the corresponding TNF ligand, a transmembrane domain and an intracellular domain, which interacts with adapter proteins and various kinases (Aggarwal, 2003; Bodmer *et al.*, 2002; Locksley *et al.*, 2001). The assignment of a protein to the TNFRSF bases on the presence of one to six copies of a conserved cysteine rich domain (CRD) (Locksley *et al.*, 2001).

Except a few soluble or GPI-anchored decoy receptors (DcRs), all members of the TNFRSF are single spanning transmembrane receptors which typically activate proinflammatory and cytotoxic signaling pathways after stimulation by their corresponding TNF ligand (Locksley *et al.*, 2001). The transmembrane members of the TNFRSF can be further classified into two groups: TNFR associated factor (TRAF)-interacting or nondeath receptors and death receptors (DRs) (Figure 1) (Bodmer *et al.*, 2002). The nondeath receptors of the TNFRSF, such as CD40 and TNFR2, interact directly with members of the TRAF adapter protein family and stimulate signaling pathways resulting in the activation of nuclear factor κB (NFκB) and mitogen-activated protein kinases (MAPK) (Chakrabarti *et al.*, 2007; D'Aversa *et al.*, 2008; McLeish *et al.*, 1998; Tanimura *et al.*, 2005). On the other hand, DRs are characterized by intracellular domain containing a conserved protein-protein interaction domain, the death domain (DD). By help of the DD, some DRs, such as CD95 (Fas), TRAILR1 (DR4) and TRAILR2 (DR5), trigger apoptotic and/or necrotic cell death via DD-containing adapter proteins and caspase-8 (Locksley *et al.*, 2001).

1.1.2. TNF ligand family

The name giving TNF itself is a proinflammatory molecule and that is why there are many research trials to develop antibodies or Fc fusion proteins that inhibit or interfere with TNF signaling pathways as successful tools for the treatment several immune and inflammatory diseases such as rheumatoid arthritis (RA) and Crohn’s disease (Denmark and Mayer, 2013; Paula and Alves, 2014). Many other ligands of the TNF family have similarly implicated in the

stimulation of T and B lymphocytes and antigen-presenting cells such as dendritic cells (DCs) but now it has been recognized that TNF ligands also regulate non-lymphoid cells (Croft *et al.*, 2012). Based on the broad functions and distribution of TNF ligands, researchers have paid more attention to discover and analyze all members of TNF superfamily as additional or alternative therapeutic targets for patients suffering from inflammatory or autoimmune diseases (Figure 1) (Bodmer *et al.*, 2002).

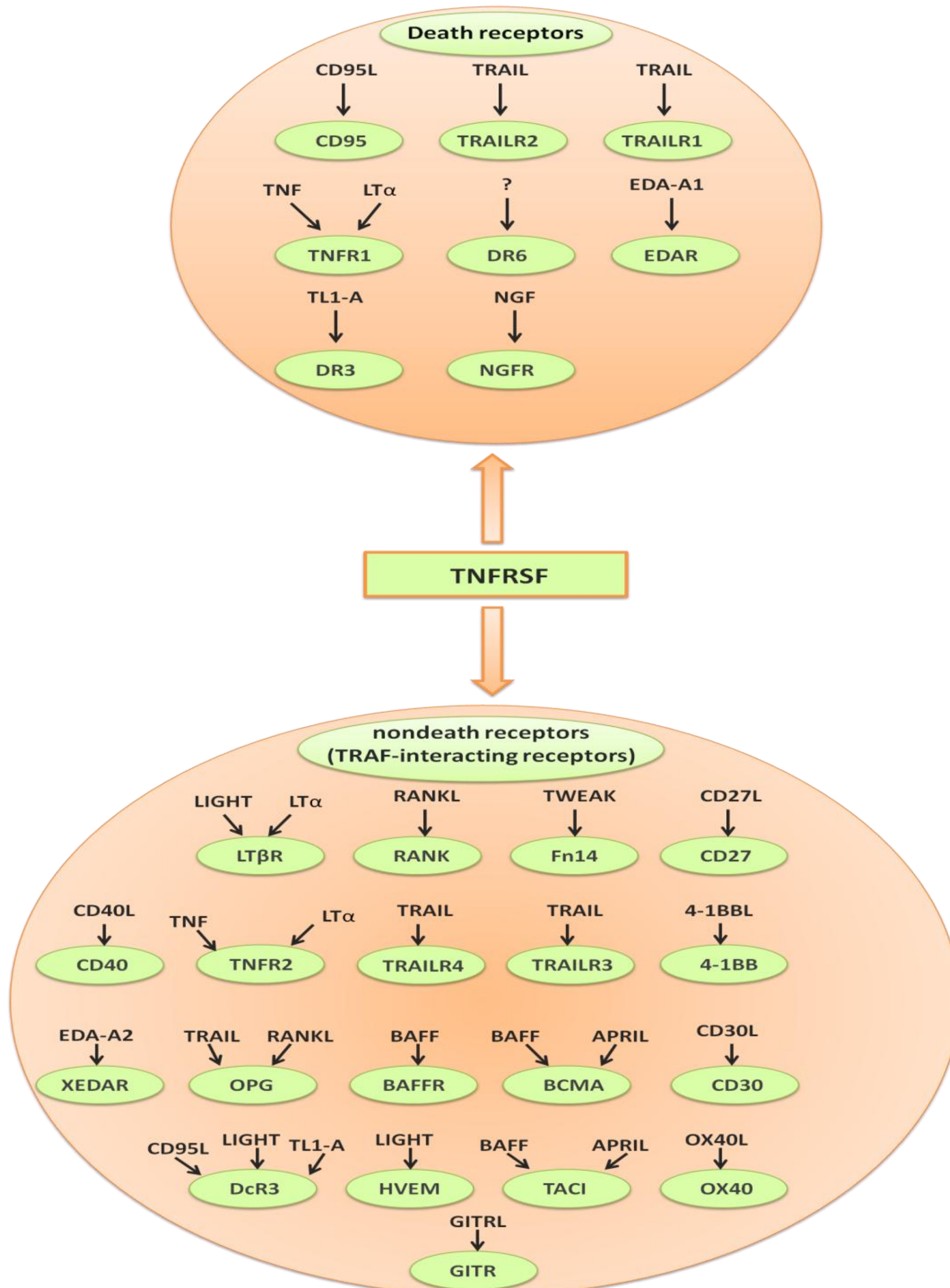


Figure 1: Receptors and ligands of the TNF superfamily.

For example, the TNF ligand OX40L, which stimulates the TNFR OX40, has acquired research interest due to its broad distribution and regulation of variable cell types such as T cells, B cells, natural killer (NK) cells and DCs. Indeed, OX40L and OX40 are considered as proinflammatory molecules that were well proved to participate in the etiology of many inflammatory diseases such as asthma, colitis, diabetes and atherosclerosis (Croft, 2010). Furthermore, the OX40L/OX40-system is required for survival and proliferation of T memory due to its ability to induce antiapoptotic proteins (Gramaglia *et al.*, 2000; Hori, 2006; Rogers *et al.*, 2001). Another member of TNF superfamily is CD30L which binds to CD30. The expression of CD30 is not only restricted to malignant tumors such as Hodgkin lymphoma but also to T-cells and other many cell types (Kennedy *et al.*, 2006; Schirrmann *et al.*, 2013). Regarding the therapeutic benefits after discovery of CD30L/CD30-pathway, it was revealed that blocking of this pathway attenuates the progress of inflammatory diseases such as diabetes and asthma (Oflazoglu *et al.*, 2009).

Likewise, CD70 is also another member of the TNF superfamily that is expressed on B-cells, activated T-cells and mature dendritic cells (mDCs). CD70 exerts its biological function via binding to a member of TNFRSF known as CD27 which is expressed on various types of T-cells, some types of B-cells, NK and NKT cells (Denoeud and Moser, 2011; Nolte *et al.*, 2009). Actually, it is well proved that CD27 activation plays a vital role in triggering survival signals and differentiation of T-cells via activation of both classical and alternative NFκB signaling (Gerondakis *et al.*, 2012; Ramakrishnan *et al.*, 2004). Moreover, CD70 is highly expressed in a variety of hematologic malignancies and also on solid tumors and thus represents a novel target for antitumor drugs (Diegmann *et al.*, 2005; Junker *et al.*, 2005; Ryan *et al.*, 2010; Wischhusen *et al.*, 2002). Indeed, immune-inhibitory effects were reported as a consequence of expression of CD70 on tumor cells and have been attributed to exhaustion of the T-cell pool and accumulation Tregs in the tumor (Claus *et al.*, 2012; van Gisbergen *et al.*, 2009). Therefore, CD27/CD70-pathway deserves more research interest in the field of cancer therapy as an interesting target for blocking the immune-inhibitory effects of CD70-expressing tumor cells (Vinay and Kwon, 2009).

Although the previously mentioned TNF ligand/TNFR-systems provide therapeutic benefits through their blockade, there are other members of the TNF superfamily that elicit antitumor activity or immune suppression through their stimulation. Agonistic antibodies of the TNFR 4-1BB, for example, which is naturally stimulated by 4-1BBL, exhibit antitumor activity in some murine tumor models (Kim *et al.*, 2001; Melero *et al.*, 1997; Shi and Siemann, 2006). The antitumor activity of agonistic 4-1BB-specific antibodies has been attributed to an increase of cytotoxic T-lymphocyte and NK cell activity (Tansey and Szymkowski, 2009; Vinay and Kwon, 2011). Unfortunately, there are reports that agonistic 4-1BB antibodies are associated

with adverse effects (Croft, 2009; Salek-Ardakani and Croft, 2010; Tansey and Szymkowski, 2009; Vinay and Kwon, 2011). CD40 is another prominent member of the TNFRSF that is targeted in clinical studies with agonistic antibodies (see details under section 1.5).

Worth mentioning, some ligands of TNF superfamily and agonistic antibodies of some TNRSF members are currently in clinical trials for treatment of cancer patients due to their ability to trigger apoptosis and antitumor activity upon binding to their corresponding DRs. For example, the TNF ligand CD95L (FasL) stimulates the DR CD95 (Fas). The CD95L/CD95-system represents an effector mechanism of cytotoxic T-lymphocytes against viral infection and transformed cells. Moreover, its expression on NK cells increases in response to CD16 engagement and other cytokines such as IL2 and IL12 (Eischen *et al.*, 1996). In addition, stress inducing agents, such as chemotherapy, radiation or viral infection, can trigger CD95L release in various cell types (Pinkoski and Green, 1999). In addition to CD95L release under stress condition, this molecule plays also a vital role under physiological conditions to control different biological processes such as skin homeostasis, erythroid differentiation and angiogenesis in the eye (De Maria *et al.*, 1999; Hill *et al.*, 1999; Janssen *et al.*, 2003; Kaplan *et al.*, 1999). Actually, CD95L is an interesting candidate for treatment of cancer patients due to its ability to induce apoptosis and consequent tumor cell death. The apoptotic activity of CD95L results from its binding to CD95 and triggering the recruitment of the adaptor molecule Fas-associated death-domain (FADD). The latter subsequently recruits and activates the initiator caspase which is known as caspase-8 and stimulates apoptosis (Kischkel *et al.*, 1995; Muzio *et al.*, 1996). Unfortunately, systemic activation of CD95 triggers deadly side effects in the liver which currently limit the use of CD95L and agonistic CD95 antibodies as safe antitumor drugs. It is thus a novel research challenge to widen the safety margin of CD95 targeting by the development of therapy concepts/drugs that allow tumor localized activation of CD95 (Guicciardi and Gores, 2009; Wajant *et al.*, 2005).

1.2. TRAIL/TRAILR-system and activation of apoptosis

1.2.1. Classification of TRAILRs

TRAIL is a member of the TNF superfamily which is important for immune surveillance and represents also a defensive function against tumor development as was proved by experiments in TRAIL-deficient mice (LeBlanc and Ashkenazi, 2003). Interestingly, molecules targeting the TRAIL DRs are considered as safe antitumor drugs as TRAIL DRs induce apoptosis preferentially in many cancer cells but have little or no cytotoxicity against normal cells. Actually, TRAIL induces apoptosis upon binding to its DD-containing receptors,

TRAILR1 (DR4) and/or TRAILR2 (DR5) (LeBlanc and Ashkenazi, 2003). Three other TRAILRs lack a functional DD and are thus unable to induce apoptosis. Two of these TRAILRs are Decoy receptor 1 (DcR1) and osteoprotegerin (OPG), without a cytoplasmic domain and their overexpression is in some tumor cells responsible for TRAIL resistance against apoptosis. The fifth TRAILR is also named as a DcR, DcR2 (TRAILR4) as it interferes with TRAIL-induced apoptosis similar to the other DcRs (Lane *et al.*, 2013; Pan *et al.*, 1997; Sheridan *et al.*, 1997). However, TRAILR4 has a cytoplasmic domain with a truncated DD and it is thus possible that this receptor triggers death-independent signaling pathways (Degli-Esposti *et al.*, 1997).

1.2.2. Mechanisms of TRAIL DR-induced apoptosis

Induction of apoptosis by TRAIL starts with binding of the molecule to TRAILR1 and/or TRAILR2 and triggering of receptor trimerization. Similar as in the CD95L/CD95-system, activation of the TRAIL DRs results in the recruitment of the DD-containing adaptor molecule FADD via a DD–DD interaction. Receptor-bound FADD in turn via a second protein-protein interaction domain, named death effector domain (von Pawel *et al.*), recruits procaspase-8/-10 and triggers their activation by oligomerization-induced proximity (Kischkel *et al.*, 2000). Apoptosis signaling is further transmitted by the ability of active caspase-8/-10 to convert procaspase-3 into active caspase-3 (Figure 2). Moreover, caspase-8 can further stimulate apoptosis via other pathway known as the intrinsic pathway which starts with the cleavage and the activation of the pro-apoptotic Bcl-2 protein, Bid (Green, 2000). Cleaved Bid activates Bax and Bak and triggers their oligomerization and subsequent pore formation in the outer mitochondrial membrane that leads to the release of the pro-apoptotic factors, cytochrome c and SMAC/DIABLO into the cytosol (Du *et al.*, 2000; Verhagen *et al.*, 2000). The released cytochrome c then binds to apoptosis-inducing factor-1 (Apaf1) and procaspase-9 resulting in the assembly of the apoptosome which triggers the release of caspase-9 which subsequently activates caspase-3. Of similar importance is that SMAC can antagonize the anti-apoptotic activity of xIAP which blocks caspase-3 activation downstream of caspase-8. Thus, DR-induced caspase-8 mediated BID cleavage and activation of the intrinsic pathway enhance apoptosis induction by the extrinsic pathway. Moreover, executioner caspases such as caspase-3 can cleave caspase-8 providing a positive feedback in the apoptotic caspase cascade (Kroemer and Reed, 2000). Indeed, caspase-8 mediated activation of caspase-3 and other executioner caspases is sufficient to trigger apoptosis in some cell types, while in other cell types the intrinsic pathway is necessary for the fulfilment of DR-induced apoptosis. Thus, conclusively, tumor cells are classified into type I tumor cells, which are independent on the intrinsic mitochondrial pathway, and type II tumor

cells which are dependent on the intrinsic mitochondrial pathway to trigger apoptosis (Figure 2) (Maas *et al.*, 2010).

Interestingly, a tumor suppressor protein known as p53 plays a vital role in the intrinsic but also in the extrinsic pathway pathway. p53 is a transcription factor and is activated in response to a variety of cellular stress conditions such as DNA damage or oxidative stress following chemotherapy or radiation therapy. The role of p53 is either to inhibit cell cycle through regulation of p53-responsive genes such as p21 and p27 or to trigger apoptosis through activation of the intrinsic pathway by some proapoptotic members of Bcl-2 family such as Bax, PUMA and Noxa but also by stimulation of the extrinsic pathway by upregulation of TRAILR2, CD95 and CD95L (Chandrasekaran and Richburg, 2005; Vogelstein and Kinzler, 2004; Yu and Zhang, 2005). Therefore, resistance to chemotherapy or irradiation may be attributed to mutations in p53 which are often detected in tumor cells.

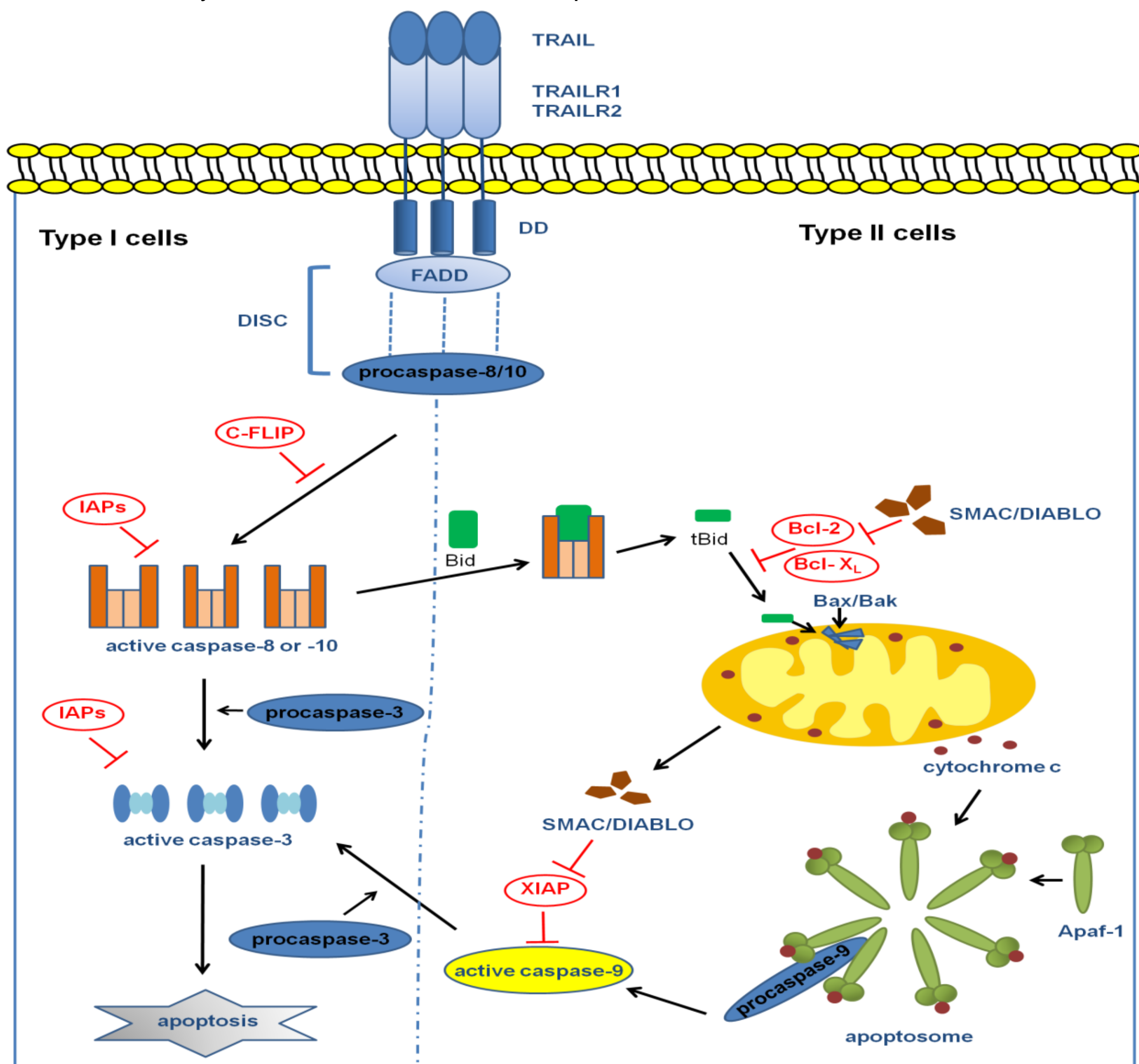


Figure 2: Mechanism of apoptosis induction via TRAILR1 and/or TRAILR2 stimulation. Details are discussed in the text.

1.2.3. Clinical trials for TRAIL DR targeting therapies

TRAIL attracts more research interest than other ligands of the TNF superfamily as a strong apoptosis inducing agent and a safe antitumor drug due to its ability to induce cell death mainly in tumor cells with little or no detected cytotoxicity in non-transformed cells and tissues (Newsom-Davis *et al.*, 2009). Although TRAIL-knockout mice display no developmental defects and grow in a normal manner, they are more susceptible to tumor initiation and display accelerated growth of malignancies and reduced apoptosis (Akazawa *et al.*, 2013; Cretney *et al.*, 2002; Finnberg *et al.*, 2005; Grosse-Wilde *et al.*, 2008; Zerafa *et al.*, 2005). As far as the role of TRAIL/TRAIL DRs in the induction of apoptosis and inhibition of tumor growth was confirmed, clinical research has paid great attention to investigate recombinant TRAIL or other TRAIL DRs targeting agents such as agonistic antibodies. Dulanermin is an example of recombinant TRAIL that acts as an agonist for both TRAILR1 and TRAILR2. Actually, dulanermin is now in phase II clinical trials and is evaluated in combination with other agents regarding its efficacy as an antitumor drug (Wainberg *et al.*, 2013). Unfortunately, recombinant TRAILR1/TRAILR2 agonistic ligands are associated *in vivo* with rapid clearance from the circulation and a short half life due to their small size (Herbst *et al.*, 2010; Kelley *et al.*, 2001).

In addition to recombinant TRAIL, TRAIL DRs can be stimulated with agonistic TRAILR1/TRAILR2 antibodies. It is worth saying that many TRAILR2 agonistic antibodies are in phase II trials such as conatumumab, drozitumab and lexatumumab (Holland, 2013). In addition, mapatumumab is an example for TRAILR1 agonist which is also in phase II trials (Holland, 2013; von Pawel *et al.*, 2013). Interestingly, TRAILR1/TRAILR2-agonistic antibodies provide more advantages than recombinant ligands. One of these advantages is that they have higher affinity to TRAIL DRs and bind with limited affinity to DcRs or OPG (Kruyt, 2008). Moreover, the half life of TRAIL DRs agonistic antibodies are longer than recombinant TRAIL and thus can be applied at lower doses (Duiker *et al.*, 2006). In addition, TRAILR1/TRAILR2-agonistic antibodies were reported to activate antibody-dependent cell-mediated cytotoxicity (ADCC) against tumor cells expressing TRAIL DRs (Maddipatla *et al.*, 2007). Unfortunately, phase I/II studies on TRAILR1/TRAILR2-agonistic antibodies have not proved their success as promising antitumor drugs (Holland, 2013). One reason for the later is that the *in vivo* activity of these agonistic antibodies is directly related to their binding to Fcɣ receptors (FcɣRs) and the subsequent cross linking of the antibodies (Wilson *et al.*, 2011). Therefore, the limited or the low expression of FcɣRs in the *in vivo* tumor environment interferes with the antibody cross linking and the induction of the antitumor activity.

1.2.4. Mechanisms of resistance against TRAIL-induced apoptosis

Although TRAIL is a potent inducer of apoptosis and death of tumor cells via TRAIL DRs stimulation, the presence of TRAIL DRs does not always reflect TRAIL sensitivity. Some tumors, such as chronic lymphocytic leukemia (CLL), meningioma and astrocytoma, are TRAIL resistant despite considerable expression of TRAIL DRs on their surfaces (Dyer *et al.*, 2007). Against the background of the considerable interest on TRAIL DR targeting for tumor therapy, knowledge of TRAIL resistance mechanism is an interesting and important research challenge. The mechanisms of TRAIL resistance are variable and cell type dependent. For example, TRAIL resistance can be attributed to the overexpression of DcRs, DcR1 and/or DcR2, which protect cancer cells from TRAIL binding to TRAIL DRs and thus prevent subsequent induction of apoptosis (LeBlanc and Ashkenazi, 2003; Morizot *et al.*, 2011; Pan *et al.*, 1997; Sheridan *et al.*, 1997). Unfortunately, DcRs are not always responsible for TRAIL resistance because scientific research failed to find a significant correlation between TRAIL resistance in most tumor cells and the expression of DcRs (Zhang *et al.*, 1999).

The most powerful inhibitor of TRAIL-induced apoptosis is presumably cellular FLICE-inhibitory protein (c-FLIP) which blocks caspase-8 binding on FADD and/or forms heteromers with procaspase-8 with limited activity and thus inhibits apoptotic DISC activity and interrupts the apoptosis cascade already at the receptor level (Figure 2) (Irmler *et al.*, 1997; Safa and Pollok, 2011). Many research trials have been directed to sensitize TRAIL-induced apoptosis through downregulation of c-FLIP level (Bijangi-Vishehsaraei *et al.*, 2010; Seo *et al.*, 2013). In addition to c-FLIP, other inhibitors of TRAIL-induced apoptosis were detected such as inhibitor of apoptosis proteins (IAP) which is a family of caspase inhibitory proteins including X-linked IAP (XIAP), c-IAP1, c-IAP2 and survivin (Figure 2) (Schimmer *et al.*, 2004). Needless to say that many research trials revealed a great success to sensitize tumor cells toward TRAIL-induced apoptosis by antagonizing IAPs (Allensworth *et al.*, 2013; Finlay *et al.*, 2013; Park *et al.*, 2013). In the same scenario, Bcl-2 is considered as antiapoptotic protein conferring TRAIL resistance in some tumor cells and its suppression sensitizes tumor cells to TRAIL-induced apoptosis (Li *et al.*, 2011; Zhang *et al.*, 2012). Moreover, tumor cells can trigger resistance to the intrinsic pathway of apoptosis via mutations in tumor suppressor proteins such as p53 that interferes with the release of some Bcl-2 family members as mentioned before (Vogelstein and Kinzler, 2004; Yu and Zhang, 2005).

1.3. Exogenous activation of TNFRs

The major initial character of all members of TNF ligands is that they are expressed as transmembrane proteins which are transformed naturally to soluble trimeric ligands by proteolytic processing or alternative splitting. These soluble TNF ligands still contain the TNF homolgy domain and therefore have the ability to bind to their corresponding members of the TNFRSF (Wajant *et al.*, 2013). Antibodies and recombinantly produced soluble TNF ligand variants are developed and used as research tools for exogenous activation of the TNFRSF. Indeed, both types of reagents are under consideration for TRAIL DR-targeted therapies in clinical studies. A major consideration for TNFR-specific antibodies in general and TRAIL DR-specific antibodies in particular is that their binding to FcɣRs can be extremely important for their agonistic activity. This means that the availability of FcɣRs expressing cells in the microenvironment of the tumor as well as the isotype of the antibody are of overwhelming importance for *in vivo* activity (Dhein *et al.*, 1992; Li and Ravetch, 2011; Vonderheide and Glennie, 2013; Wilson *et al.*, 2011). In addition, agonistic antibodies can activate *in vivo* immune cells and stimulate immune functions such as ADCC. With respect to recombinantly produced soluble TNF ligands, members of the TNFRSF respond differently to soluble ligand molecules in contrast to membrane-bound TNF ligands that always mediate strong receptor activation. Despite the strong activation of some members of the TNFRSF by soluble ligands, other members are unable to mediate signaling even after binding to soluble ligands (Wajant *et al.*, 2013). This particular also attains to TRAILR2 (Wajant *et al.*, 2001).

1.4. Response of TRAIL DRs to soluble recombinant TRAIL and strategies to improve its activity

Concerning the response of the TRAIL DRs to soluble recombinant TRAIL, there is evidence that TRAILR1 equally responds to the membrane bound form of TRAIL and the soluble ligand, whereas TRAILR2 signals only in response to membrane bound form of TRAIL (Kelley *et al.*, 2005; Wajant *et al.*, 2001). Despite research trials, it is difficult to explain the reasons for the inability of TRAILR2 to trigger apoptosis after binding to soluble TRAIL. However, it was proved that soluble TNF ligands become active after oligomerization in supramolecular clusters (Berg *et al.*, 2007; Wajant *et al.*, 2001). According to this finding, the first strategy to enhance the activity of soluble TRAIL was secondarily oligomerization. Fortunately, there is a proof of the success of this strategy in cell lines expressing only TRAILR2 such as Jurkat cells that revealed significant induction of cell death with soluble TRAIL ligands oligomerized with anti-Flag antibodies (Berg *et al.*, 2007). As far as oligomerization of soluble ligands revealed enhanced activity, research trials have continued

to improve the activity of soluble TRAIL ligands with the help of genetic engineering to design hexameric and nonameric death ligands that showed superior activity as compared to trimeric ligands (Bremer *et al.*, 2009; Greaney *et al.*, 2006; Holler *et al.*, 2003; Lamanna *et al.*, 2013; Wyzgol *et al.*, 2009). Hexameric death ligands can be produced through the design of fusion proteins of soluble TRAIL with a N-terminally Fc-immunoglobin-1 domain that exhibit a significant increase in activity and do not need further oligomerization with anti-Flag antibodies (Wajant *et al.*, 2013). Another strategy to obtain oligomerized ligand is the design of single chain polypeptide where three subunits of the TRAIL molecules are connected by polypeptide linker sequences (Krippner-Heidenreich *et al.*, 2008). Interestingly, fusion proteins of soluble TRAIL with a single chain antibody fragment (scFv) recognizing a cell surface antigen revealed enhanced TRAIL activity after anchoring to the cell surface antigen and mimiced the action of transmembrane TRAIL (Wajant *et al.*, 2013). These scFv-TRAIL fusion proteins trigger not only significant receptor activation and apoptosis induction but they also provide target antigen-restricted apoptosis induction on cells that express this specific antigen on their surface or on cells in the direct neighborhood of such cells (Bremer *et al.*, 2004b). Moreover, scFv-TRAIL fusion proteins represent a novel strategy to obtain bifunctional molecules which on one side are able to stimulate apoptosis through stimulation of TRAIL DRs by the TRAIL domain and on the other side are able to stimulate/block other specific cellular function by the scFv-domain binding to a specific cell surface antigen (de Bruyn *et al.*, 2010). As implied by previous findings, scFv-TRAIL represents a novel strategy to improve the activity of soluble TRAIL and the following table represents some scFv-TRAIL fusion proteins (Table 1).

Table 1: scFv fusion proteins of soluble TRAIL.

TRAIL Fusion protein	Targeted cell surface antigen	Reference
scFv:CD70-TRAIL	CD70	(Trebing *et al.*, 2014)
scFvM58-sTRAIL	MRP3	(Wang *et al.*, 2013a)
scFv-EHD2-scTRAIL	EGFR	(Seifert *et al.*, 2013)
Ad-KDRscFv:sTRAIL	VEGF	(Yang *et al.*, 2012)
scFv-scTRAIL	Extracellular domain of ErbB2	(Schneider *et al.*, 2010)
Anti-MCSP:TRAIL	MCSP	(de Bruyn *et al.*, 2010)
scFvCD33:sTRAIL	CD33	(ten Cate *et al.*, 2009)
scFvCD19:sTRAIL	CD19	(Stieglmaier *et al.*, 2008)
scFvCD7:sTRAIL	CD7	(Bremer *et al.*, 2005a)
scFv425:sTRAIL	EGFR (blocking antibody)	(Bremer *et al.*, 2005b)
scFvC54:sTRAIL	EGP2	(Bremer *et al.*, 2004a)
MBOS4-TRAIL	FAP	(Wajant *et al.*, 2001)

1.5. CD40 and its role in cancer immunotherapy

As mentioned before, members of TNFRSF are broadly expressed in the cells of immune system such as 4-1BB, CD27, OX40 and CD40. CD40 is composed of a protein of 277 amino acids. These amino acids include a large extracellular domain of 193 amino acids, transmembrane region of 22 amino acids and a short cytoplasmic C-terminus composed of 62 amino acid (Loskog and Eliopoulos, 2009). Concerning CD40L/CD40-system, CD40 mediates signaling mainly dependent on recruitment of adaptor proteins of the TRAF family upon binding to its corresponding ligand, CD40L (Bishop *et al.*, 2007). Despite the absence of intrinsic kinase activity in cytoplasmic tail of CD40, TRAFs are able to conduct CD40 to the intracellular signaling components and activate protein kinases resulting in the recruitments of many signaling pathways, such as JNK, ERK, MAPK and NFκB, that are responsible for the reported CD40 activities (Eliopoulos, 2008; Loskog and Eliopoulos, 2009).

CD40 is constitutively expressed on antigen presenting cells (APCs) such as B cells and DCs and a range of tumor cells. Therefore, CD40 represents an interesting therapeutic target due to its activity in immune cells and as a tumor target antigen. Concerning its role in immune regulation, stimulation of CD40 on APCs leads to a wide range of cellular responses such as maturation of DCs and subsequent secretion of cytokines, induction of antigen presentation via stimulation of CD40 on B cells and stimulation of antigen specific T cells (Vonderheide and Glennie, 2013). With regard to CD40 expression on tumor cells, almost all mature B-cell tumors display high CD40 expression such as Hodgkin lymphoma, NHL and CLL (Banchereau *et al.*, 1994; O'Grady *et al.*, 1994; Wang *et al.*, 1997). Moreover, CD40 expression is not limited only to B-cell malignancies but it has recently also been detected on some solid tumors such as melanoma, breast, neck, prostate and ovary tumors (Ottaiano *et al.*, 2002; Pellat-Deceunynck *et al.*, 1994).

The important role of immune system stimulation as an anticancer therapy has been recently proved in many cancer types (Gao *et al.*, 2013). Therefore, CD40 represents an attractive target for immunotherapy in cancer treatment because of its wide expression on different malignancies and cells of the immune system. Indeed, stimulation of CD40 acts as a bridge between the immune response and the antitumor activity due to the release of effector immune cells after stimulation of CD40 on DCs, such as $CD8^+$ cytotoxic T lymphocytes, NK cells and M1 macrophages, which in turn play a vital role in antitumor immunity (Loskog and Eliopoulos, 2009). In the view of CD40 role in tumor cells, agonistic CD40 antibodies reveal inhibition of tumor growth and potent antitumor efficacy alone or in combination with chemotherapy (Khong *et al.*, 2013; Vardouli *et al.*, 2009). Moreover, they can also inhibit

postoperative cancer recurrence and metastasis in some murine tumor models (Khong et al, 2013).

Actually, there are two hypothesises regarding the mechanism of antitumor activity of CD40 antibodies. The first one is related directly to disruption of tumor proliferation after inhibition of CD40L/CD40-pathway and the ability of the CD40 antibody to trigger antibody-dependent phagocytosis of tumor cells. The second one is related indirectly to CD40 stimulation in immune system and the release of effector immune cells which in turn mediate antitumor immunity (Moran *et al.*, 2013). It is worth saying that some CD40 agonistic antibodies are now in phase 1 studies as safe effective antitumor drugs which can be used alone or in combination with other agents such as chemotherapeutic drugs to synergize their antitumor activity (Beatty *et al.*, 2013; Hussein *et al.*, 2010). The following table lists some agonistic CD40 antibodies which were well proved in the clinical research for their antitumor efficacy (Table 2).

Table 2: Some agonistic CD40 antibodies used in the clinical research as antitumor drugs.

Agonistic CD40 antibodies	Types of the treated tumors	References
Chi Lob 7/4	Advanced solid tumors and Lymphoma (ongoing research)	(Vonderheide and Glennie, 2013)
CP-870,893	Advanced pancreatic ductal adenocarcinoma (solid tumor)	(Beatty *et al.*, 2013)
FGK45	AB1-HA mesothelioma tumor in mice	(Khong *et al.*, 2013)
ADX40	Murine B-cell lymphoma model in mice	(Carlring *et al.*, 2012)
Dacetuzumab (SGN-40)	NHL	(Lewis *et al.*, 2011)
G28-5	Lymphoma xenografted mice	(Francisco *et al.*, 1997)

1.6. The role of DCs in immune system and the effect of tumors on their function

1.6.1 The role of DCs in immune system

DCs are considered as a small subgroup of immune cells that are originated from the bone marrow and then found in nearly every tissue in the human body as a prime line of defence especially on body surfaces such as skin, on mucosal surfaces such as gastrointestinal tract and in immunological organs such as spleen and lymph nodes (Steinman and Banchereau, 2007). DCs are considered the best professional APCs that have the ability to initiate, coordinate and regulate the adaptive immune responses. Indeed, DCs act as a messenger in

the immune system that recognizes foreign antigens, as an innate immune response, then transfers the information to T and B cells leading to differentiation of naive T cells into diverse T helper lymphocytes representing an adaptive immune reaction. Therefore, DCs are considered as link between innate and adaptive immune system (Banchereau *et al.*, 2000; Levings *et al.*, 2005; Pulendran *et al.*, 1999).

Initially, DCs are present in an immature status at homeostatic condition that is characterized by lower MHC class II and costimulatory molecules expression. Then, when DCs recognize and ingest foreign antigens, they are transformed from the immature state to the mature state in presence of proinflammatory cytokines and this mature state is distinguished with upregulation and downreulgation of different markers. MHC class II molecules, CD80, CD86, CD40, OX40L and the CCR7 are examples of molecules undergoing upregulation on mDCs while CCR6 is downregulated on mDCs. Hence, mDCs gain the ability to migrate to lymph nodes and activate naive T lymphocytes and thus trigger an antigen-specific response (Benencia *et al.*, 2012).

1.6.2. Effect of tumors on DCs function

Concerning DCs role in tumor recognition, DCs are able to recognize tumor antigens and trigger adaptive immune response in an antigen-specific way to eradicate tumors. Therefore, DCs are considered as good candidates for cancer immunotherapy (Palucka and Banchereau, 2012). Unfortunately, tumor cells can bypass the response of DCs and disrupt their function through different inhibitory pathways which may start either early during DCs formation or appear at a later stage. The earlier inhibitory effect of tumor cells is attributed to their interference with the differentiation of monocytes into DCs by forcing the differentiation towards macrophages with the help of costimulatory molecules such as IL6 and macrophage colony stimulating factor (Chomarat *et al.*, 2000). The later interference of tumor cells with DCs function could be attributed to the tumor secretion of inflammatory mediators, such as IL10 which interferes with DC maturation, and other factors, such as lactoferrin and CD47 that bind to protein-α on the surface of phagocytes and then trigger inhibitory signals interfering with phagocytosis (Chao *et al.*, 2010; Palucka and Banchereau, 2012; Yanofsky *et al.*, 2013). Thus, recent research trials have been directed toward bypassing these inhibitory effects of tumor cells and restoration of DCs activity to trigger potent antitumor immunity (Kuhn and Ronchese, 2013; Li *et al.*, 2013; Wang *et al.*, 2013b).

1.7. Aim of the work

Some tumors are preferentially killed via only one of the two TRAIL DRs. Furthermore, there is evidence that combination therapies of TRAIL DRs targeting with sensitizing drugs may have side effects on normal cells. Against this background, one aim of this work was to evaluate TRAIL mutants that exhibit preferential binding to either TRAILR1 or TRAILR2 for their usefulness in the construction of scFv-TRAIL fusion proteins to have the option to circumvent side effects related to the activation of the TRAIL DR not relevant for antitumor activity in a certain tumor type. A second aim was to test with a scFv derived of a CD40-specific antibody whether it is possible to construct bifunctional scFv-TRAIL fusion proteins enabling TRAIL DR and DCs stimulation.

2. Materials

2.1. Chemicals, reagents and cell culture mediums for the cell culture

Substance	Company
1kb DNA-ladder	Fermentas, St. Leon-Rot, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma, Deisenhofen, Germany
Acetic acid	J. T. Baker, Leibzig, Germany
Acrylamide (30 %)	Carl Roth, Karlsruhe, Germany
Agar	Carl Roth, Karlsruhe, Germany
Agarose	Carl Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	AppliChem, Darmstadt, Germany
Ampicillin	Carl Roth, Karlsruhe, Germany
Anti-CD14-coated beads	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-Flag M2 agarose beads	Sigma, Deisenhofen, Germany
Bovine serum albumin (BSA)	Sigma, Deisenhofen, Germany
Crystal violet (CV) powder	Carl Roth, Karlsruhe, Germany
Cycloheximide (CHX)	Sigma, Deisenhofen, Germany
Dimethyl sulfoxide (DMSO)	Carl Roth, Karlsruhe, Germany
DMEM medium	PAA, Pasching, Austria
Ethanol	J. T. Baker, Leibzig, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe, Germany
Fetal bovine serum (FCS)	PAA, Pasching, Austria
Flag peptide	Sigma, Deisenhofen, Germany
Geneticin disulfate (G418-Sulfate)	Carl Roth, Karlsruhe, Germany
Granulocyte-macrophage colony-stimulating factor (GM-CSF)	Miltenyi Biotec, Bergisch Gladbach, Germany
IL1β	R&D Systems, Wiesbaden, Germany
IL4	Miltenyi Biotec, Bergisch Gladbach, Germany
IL6	Immuno tools, Friesoythe, Germany
Iodoacetamide	Sigma, Deisenhofen, Germany
Killer-TRAIL	Enzo Life Sciences, Lörrach, Germany
Lipopolysaccharide (LPS)	Sigma, Deisenhofen, Germany
Lymphocyte separation medium	PAA, Pasching, Austria
Methanol	J. T. Baker, Leibzig, Germany
Nonfat dried milk powder	Sigma, Deisenhofen, Germany
Paraformaldehyde	Carl Roth, Karlsruhe, Germany
Penicillin-Streptomycin (100 x)	PAA, Pasching, Austria
Peptone	Carl Roth, Karlsruhe, Germany
Phosphatase inhibitor II	Sigma, Deisenhofen, Germany
Phosphate buffered saline (PBS)	PAA, Pasching, Austria
Polymyxin B (PMB)	InvivoGen, Toulouse, France
Prestained protein marker (broad range)	New England Biolabs, Frankfurt, Germany

Prostaglandin E2 (PGE2)	Biomol, Hamburg, Germany
Protease inhibitor cocktail	Roche, Mannheim, Germany
Protein G agarose	Roche, Mannheim, Germany
RPMI 1640 Medium	PAA, Pasching, Austria
Silver gel marker (low molecular weight)	GE Healthcare, Garching, Dassel, Germany
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe Garching, Germany
Sucrose	Sigma, Deisenhofen, Germany
Tetramethylethylenediamine (TEMED)	Sigma, Deisenhofen, Germany
Tris	Carl Roth, Karlsruhe, Germany
Triton X-100	Sigma, Deisenhofen, Germany
Trypsin-EDTA solution (10X)	PAA, Pasching, Austria
Tween-20	Carl Roth, Karlsruhe, Germany
Yeast extract	Carl Roth, Karlsruhe, Germany
β-Mercaptoethanol	Sigma, Deisenhofen, Germany

2.2. Enzymes

Enzyme	Company
T4-Ligase	Fermentas, St. Leon-Rot, Germany

All enzymes used for cloning TRAIL variants and TRAIL fusion proteins were obtained from Fermentas, St. Leon-Rot, Germany.

2.3. Antibodies

Antibody	Source	Company
Anti-caspase-3	Rabbit polyclonal, #9662	Cell Signaling Technology, Beverly, MA, USA
Anti-caspase-8	Mouse IgG2b, clone C15	Enzo Life Sciences, Lörrach, Germany
Anti-caspase-9	Rabbit polyclonal, #9502	Cell Signaling Technology, Beverly, MA, USA
Anti-CD14-PE	Mouse IgG1, clone 134620	R&D Systems, Wiesbaden, Germany
Anti-CD40-PE	Mouse IgG1, clone HB14	Miltenyi Biotec, Bergisch Gladbach, Germany
Anti-CD83-PE	Mouse IgG1, clone HB15e	R&D Systems, Wiesbaden, Germany
Anti-CD86-PE	Mouse IgG1, clone 37301	R&D Systems, Wiesbaden, Germany
Anti-FADD	Rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg, Germany
Anti-Flag mAb M2	Mouse IgG1 monoclonal	Sigma, Deisenhofen, Germany
Anti-Flag mAb M2-FITC	Mouse IgG1	Sigma, Deisenhofen, Germany
Anti-FLIP (NF6)	Mouse IgG1 monoclonal	Enzo Life Sciences, Lörrach, Germany

Anti-IκBα	Mouse monoclonal, clone L35A5	Cell Signaling Technology, Beverly, MA, USA
Anti-JNK	Rabbit polyclonal, #9252	Cell Signaling Technology, Beverly, MA, USA
Anti-mouse IRDye 800	Goat polyclonal	LI-COR Bioscience, Bad Homburg, Germany
Anti-mouse-HRP	Rabbit polyclonal	Dako-Cytomation, Glostrup, Denmark
Anti-PARP	Mouse IgG1, clone 7D3-6	BD Biosciences, Heidelberg, Germany
Anti-pIκBα	Rabbit polyclonal, #2859	Cell Signaling Technology, Beverly, MA, USA
Anti-pJNK	Rabbit polyclonal, #9251	Cell Signaling Technology, Beverly, MA, USA
Anti-rabbit-HRP	Goat polyclonal	Dako-Cytomation, Glostrup, Denmark
Anti-rabbit-HRP	Goat polyclonal, #7074	Cell Signaling Technology, Beverly, MA, USA
Anti-TRAILR1	Rabbit polyclonal	Merck Chemicals, Schwalbach, Germany
Anti-TRAILR1-PE	Mouse IgG1, Clone 69036	R&D Systems, Wiesbaden, Germany
Anti-TRAILR2	Rabbit monoclonal, clone D4E9	Cell Signaling Technology, Beverly, MA, USA
Anti-TRAILR2-PE	Mouse IgG2B, clone 71908	R&D Systems, Wiesbaden, Germany
Anti-TRAILR3-PE	Mouse IgG1, clone 90906	R&D Systems, Wiesbaden, Germany
Anti-TRAILR4-PE	Mouse IgG1, clone 104918	R&D Systems, Wiesbaden, Germany
Anti-tubulin	Mouse monoclonal	Dunn Labortechnik, Asbach, Germany
Mouse IgG1-PE	Clone 11711	R&D Systems, Wiesbaden, Germany
Mouse IgG2B-PE	Clone 133303	R&D Systems, Wiesbaden, Germany

2.4. Kits

Kit	Company
Gaussia Luciferase Assay	New England Biolabs, Frankfurt, Germany
Human IL12 ELISA DuoSet	R&D Systems, Wiesbaden, Germany
OptEIA IL8-ELISA	BD Biosciences, Heidelberg, Germany
Pierce ECL Western Blotting Substrate	Fermentas, St. Leon-Rot, Germany
Pierce® Silver Stain	Fermentas, St. Leon-Rot, Germany

Pure Yield Plasmid Miniprep/Midiprep System	Promega, Mannheim, Germany

2.5. Instruments and disposable materials/equipments

Instrument or material/equipment	Company
96-well ELISA plates (high binding)	Greiner, Frickenhausen, Germany
Agfa Curix 60 processing maschine	Agfa, Düsseldorf, Germany
Black 96-well ELISA plates	Greiner, Frickenhausen, Germany
Casting chambers for SDS-PAGE	PeqLab, Erlangen, Germany
Cell culture bottles	Greiner, Frickenhausen, Germany
Cell culture petri dishes	Greiner, Frickenhausen, Germany
Cell culture plates	Greiner, Frickenhausen, Germany
Centrifuge Rotana 460R	Hettich, Tuttlingen, Germany
CO_2 incubator Heraeus Cell Safe	Heraeus, Hanau, Germany
Cryotubes	Greiner, Frickenhausen, Germany
Dialysing tubes, Viking, MWCO 15kDa	Carl Roth, Karlsruhe, Germany
Electrophoresis system "Mini-Protean Tetra Cell"	BioRad, München, Germany
Eppendorf tubes, 1,5 ml und 2 ml	Eppendorf, Hamburg, Germany
Equibio EasyjecT Plus electroporator	PeqLab, Erlangen, Germany
Flow cytometer FACScaliber	BD Biosciences, Heidelberg, Germany
Flow cytometry tubes	Falcon, Heidelberg, Germany
Heat block	PeqLab, Erlangen, Germany
LI-COR Odyssey® Infrared Imager	LI-COR Biosciences, Lincoln, USA
Lucy 2 luminometer/ELISA-reader	Anthos Labtec, Krefeld, Germany
MACS LS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS multistand	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Microcentrifuge 5417C	Eppendorf, Hamburg, Germany
Nitrocellulose membranes, 0,2 µM pore size	Whatman, Dassel, Germany
PCR-Thermocycle Primus	MWG Biotech, Ebersberg, Germany
Pipetus	Hirschmann Laborgeräte, Eberstadt, Germany
Polyallomer tubes	Seton, Los Gatos, CA, USA
Polypropylene tubes	Greiner, Frickenhausen, Germany
Power supply EPS 301	GE Healthcare, Garching, Germany
Sterile filters (0,2µm)	Sarstedt, Nümbrecht, Germany
Sterile plastic Pasteur pipettes	Hartenstein, Würzburg / Versbach, Germany
Ultracentrifuge OPTIMA-L70	Beckman Coulter, Krefeld, Germany
Well plates for cell culture	Greiner, Frickenhausen, Germany
Wet/tank blotting system	PeqLab, Erlangen, Germany

Whatman papers	Hartenstein, Würzburg / Versbach, Germany

2.6. Preparations and buffers

Preparation	**Prescription**
Assay diluent	1 x PBS 10 % (v/v) FCS
Blot buffer 10x	0,025 M Tris 0,192 M glycin 20 % (v/v) methanol pH 8,3
CV staining solution	20 % (v/v) methanol 0,5 % (w/v) CV powder
ELISA coating buffer	8,4 g/l $NaHCO_3$ 3,56 g/l Na_2CO_3 pH 9,5
Laemmli buffer (SDS-PAGE, 4 x)	8 % (w/v) SDS 10 % β-Mercaptoethanol 40 % gycerol 0,2 M Tris pH 8 0,04 % bromphenol blue
LB medium	10 g peptone 5 g yeast extract 10 g/l NaCl
Lysis buffer for immunoprecipitation (IP)	I M Tris-Hcl pH 7.4 2M NaCl 100 % glycerol 100 % triton volume adjusted to 1 L with distilled water
MACS buffer	1 x PBS 0,5 % (w/v) BSA 2 mM EDTA
MTT lysis buffer	250ml dimethyl formamide 75g SDS pH 4,7 (adjusted with acetic acid) volume adjusted to 500ml with distilled water
MTT solution	500 mg MTT powder 10 ml DMSO
PBS	0,02 M Na phosphate 0,7 % (w/v) NaCl pH 7,2
PBST	1 x PBS 0,05 % (v/v) tween-20
PBST in milk	1 x PBS

	0,05 % (v/v) tween-20 5 % (w/v) nonfat dried milk powder
Running buffer 10x (SDS-PAGE)	0,05 M Tris 0,38 M glycin 0,004 M SDS pH 8,3
Separating gel buffer (SDS-PAGE)	1,5 M Tris 0,015 M SDS pH 8,8
Stacking gel buffer (SDS-PAGE)	0,5 M Tris 0,015 M SDS pH 6,8
TAE buffer	2 M Tris 1 M acetic acid 0,1 M EDTA pH 8,3
TBS	0,02 M Tris 8 % (w/v) NaCl pH 7,6
TBST	1 x TBS 0,05 % (v/v) tween-20
TBST in milk	1 x TBS 0,05 % (v/v) Tween-20 5 % (w/v) nonfat dried milk powder

2.7. Cells

2.7.1. Eukaryotic cells

The human cancer cell lines used for this work were already available in the Division of Molecular Internal Medicine, University Hospital of Würzburg.

Cell line	**Source**	**Origin of cancer**
786-O	Institution's own stock	Human kidney carcinoma
BJAB	Institution's own stock	Human B-cell lymphoma
HEK293	Institution's own stock	Human embryonic kidney
HeLa	Institution's own stock	Human cervical carcinoma
HeLa-CD40	Stably transfected cell lines established the Division of Molecular Internal Medicine, University Hospital of Würzburg	Human cervical carcinoma
HT1080	Institution's own stock	Human fibrosarcoma
HT1080-CD40	Stably transfected cell lines established the Division of Molecular Internal Medicine, University Hospital of Würzburg	Human fibrosarcoma
HT29	Institution's own stock	Human colorectal adenocarcinoma

Jurkat	Institution's own stock	Human T-cell lymphoma
Mino	Institution's own stock	Mantle cell lymphoma
OVCAR3	Institution's own stock	Human ovarian carcinoma
Panc89	Institution's own stock	Human pancreatic carcinoma
Rec-1	Institution's own stock	Mantle cell lymphoma

2.7.2. Dendritic cells (DCs)

Blood of healthy donors were obtained from the Institute of Clinical Transfusion Medicine and Hemotherapy, University Hospital of Würzburg (Oberdürrbacher Str 6, 97080 Würzburg). Then, monocytes were freshly purified and isolated. Monocytes were differentiated into immature dendritic cells (iDCs) after one week of treatment with IL4 and GM-CSF (see section 3.14.1).

2.7.3. Prokaryotic cells

NEB 5-alpha Competent *E.Coli* was obtained from New England Biolabs Company, Frankfurt, Germany.

2.8. Plasmids

The expression plasmids for each protein used in this work were cloned by my own or with the help of other members in the Division of Molecular Internal Medicine, University Hospital of Würzburg. Then, HEK293 cells were transfected with the corresponding plasmid by my own to produce the required proteins (see section 3.2 and 3.3). TRAILmutR1 and TRAILmutR2 genes were ordered as synthetic genes from Life Technologies Company, Darmstadt, Germany.

3. Methods

3.1. Cell culture

All cell lines used in this work were cultivated under standard conditions (5 % CO_2, 37 °C) in RPMI 1640 medium with 10 % FCS (heat inactivated at 56 °C for 30 min) except OVCAR3 cells which were cultivated in DMEM medium with 10 % FCS. Adherent cells were harvested after incubating the cells with trypsin-EDTA solution for 10-30 min and then the cells were centrifuged at 1200 rpm for 4 min. The cells were counted under the microscope using hemocytometer before seeding on cell culture plates for further experiments. The rest of the cells were diluted to the ratio of 1:5 till 1:10 and further cultivated in fresh medium with 10 % FCS and were regularly freezed at -80 °C in 1 ml freezing medium (10 % DMSO in FCS) using cryotubes.

3.2. Cloning and production of the expression plasmids

The encoding plasmids for the trimeric TRAIL variants, Fc-fusion proteins of TRAIL variants, various scFv:G28-TRAIL fusion proteins, scFv:G28-2xFlag-GpL and scFv:G28-Fc-GpL were designed by cloning the corresponding DNA fragments encoding TRAIL aa 95-281 (accession number U37518), chicken tenascin-C (TNC) aa 110-139, human IgG1 aa 222-447 *Gaussia princeps* luciferase (GpL), a Flag epitope and scFv G28-5 derived from the human CD40 specific mAb G28-5 (accession number AJ853736) into pCR3 plasmid behind a leader of Ig. Then, the corresponding plasmids were transformed in competent *E.coli* and the obtained clones were controlled in the next day for the success of the cloning using the corresponding digestion enzymes to check the size of cutting fragments using horizontal electrophoresis unit. Afterwards, the positive plasmids were produced in *E.coli* in the presence of LB medium and isolated after purification steps. Finally, the sequence of each plasmid was proved and the plasmids were ready for transfection in HEK293 cells for protein production.

3.3. Protein production

The expression plasmids were further transfected in HEK293 with electroporation technique. First, HEK293 cells were harvested after incubation with trypsin-EDTA solution for 10 min at 37 °C in incubator and then the cells were centrifuged at 1200 rpm for 4 min. 50×10^6 cells/ml of HEK293 cells were further electroporated with the corresponding expression plasmids (40 µg) in 1 ml of the culture medium containing 10 % FCS and 1 % penicillin-streptomycin using 4-mm cuvette and an Easyject Plus electroporator (PeqLab)

(250 V, 1800 µF, maximum resistance). Afterwards, the transfected cells were transferred in large Petri dishes in the presence of RPMI 1640-Medium, 2 % FCS and 1 % penicillin-streptomycin and left in incubator at 37 °C. After 7 days, the supernatant were collected and centrifuged at 4600 rpm for 10 min to discard the dead cells. To establish stably transfected HEK293 cells, the cells were transferred directly after the transfection in large cell culture bottles in the presence of RPMI 1640-Medium, 10 % FCS and 1 % penicillin-streptomycin and left in incubator overnight at 37 °C. Then, the positive transfected cells were selected by adding G418-Sulfate (0.5 mg/ml) for four weeks. The status of protein production was controlled regularly by measuring the protein concentration using Western Blot technique after boiling protein samples for 5 min at 95 °C (see section 3.9). The nitrocellulose membranes were then incubated overnight with anti-Flag mAb M2 and incubated in the next day for one hour with the second antibody anti-mouse IRDye 800 after washing the first antibody. Finally, the nitrocellulose membranes were scanned using LI-COR Odyssey® Infrared Imager to determine the concentration of each protein depending on the intensity of the detected bands of the corresponding proteins and a standard protein of known concentration.

3.4. Protein purification

The protein supernatants produced by HEK293 cells were further purified using affinity chromatography on agarose beads of anti-Flag M2. The beads were settled in a column and then washed with autoclaved TBS. Then, the supernatant was applied on the column with adjusted flow rate of at least one drop/30 sec. The column was left at 4 °C until all the flow-through was collected. Afterwards, the beads were washed with TBS and the bound protein molecules were eluted from the beads in 0,5 ml fractions using TBS containing 100 µg/ml of Flag peptide and the flow rate was at least one drop/min. The eluted proteins were dialyzed against PBS overnight at 4 °C and then sterile filtered in the next day and stored at -20 °C for further analysis. The percent of protein recovery after purification was controlled by measuring the protein concentration in all of the followings: the supernatant before purification, the flow-through, the elution-fractions, the TBS washing flow-through after purification and beads using Western Blot technique (see section 3.3 and 3.9).

3.5. FACS analysis

3.5.1. Detection of cell surface markers

The cells were counted and 10^5 cells/marker were transferred to U shape 96-well plates. The plates were centrifuged for 4 min at 1200 rpm and the cells were washed 2 times with

PBS and then incubated for 30 min at 4 °C with the antibody that detects the required cell surface marker and its corresponding isotype control according the instruction of the manufacturer. Afterwards, the cells were washed 3 times with PBS and transferred in 200 µl PBS into flow cytometry tubes and analyzed with the FACSCalibur.

3.5.2. scFv:G28-TRAIL binding to CD40-transfectant cells

HeLa-CD40, HT1080-CD40 and their corresponding parental cells were counted and washed as mentioned in the previous section and then incubated with scFv:G28-TRAIL (500 ng/ml) or left untreated for 30 min at 4 °C. Then, the cells were washed 3 times with PBS and incubated again 30 min at 4 °C with anti-Flag mAb M2-FITC. Finally, the cells were washed 3 times with PBS and transferred in 200 µl PBS into flow cytometry tubes and then analyzed with the FACSCalibur.

3.6. Equilibrium binding studies

3.6.1. Binding studies in HEK293 cells transiently transfected with the various TRAILRs

HEK293 cells were transiently transfected with the expression plasmids of the corresponding TRAILR using electroporation technique as mentioned before in protein production section (see section 3.3). HEK293 cells were also transfected in parallel with plasmid of the empty vector to determine nonspecific binding. The transefected cells were seeded on large Petri dishes and left in incubator at 37 °C overnight. In the next day, the cells were harvested from plates after incubation with trypsin-EDTA solution and counted as 5-10 x 10^5 cells/group and prepared in 200 µl medium (RPMI 1640, 10 % FCS) in 1,5 ml Eppendorf tubes. Then, the cells were stimulated for 1 h with the increasing concentrations of the GpL fusion proteins of the different TRAIL variants at 37 °C. Afterwards, cells were centrifuged for 5 min at 4000 rpm and washed 5 times with 1 ml ice cold PBS. After the final washing step, cells were harvested from the Eppendorf tubes in 50 µl medium (RPMI 1640, 0,5 % FCS) and transferred to black 96-well plates. The cell bound GpL-TRAIL intensity was assayed using the *Gaussia* Luciferase Assay Kit and a Lucy 2 Luminometer according to the protocol of the manufacturer. To calculate specific binding values, nonspecific binding values were subtracted from total binding values of the corresponding TRAIL variant. GraphPad Prism 5.0 program (GraphPad Software, Inc.) was used to calculate K_D-values by non-linear regression equation.

3.6.2. Binding studies in Jurkat and HT1080 cells

Jurkat cells were counted, prepared and stimulated as mentioned before in the transiently transfected HEK293 cells. Nonspecific binding was determined by pretreating Jurkat cells for 1 h at 37 °C in the incubator with 10 µg/ml of TRAILR2-specific anti-rabbit anti-serum.

In case of HT1080 cells, cells were counted 2-3 x 10^5 cells/well and seeded in 24-well plates in 1 ml medium (RPMI 1640, 10 % FCS) and left in incubator overnight at 37 °C. In the next day, the old medium was absorbed and fresh medium with and without 10 µg/ml of TRAILR2-specific anti-rabbit anti-serum were added on the cells in plates to determine nonspecific binding. The plates were left in incubator for 1 h at 37 °C and then the different groups were treated for 1 h with the increasing concentrations of the GpL fusion proteins of the corresponding TRAIL variant at 37 °C. Afterwards, the 24-well plates were washed 10 times with ice cold PBS and the rest of PBS was removed perfectly from the plates. The plates were left on ice and then the cells were scratched in 55 µl medium (RPMI 1640, 0,5 % FCS) and 50 µl were transferred to black 96-well plates to measure cell bound GpL-TRAIL intensity as mentioned before in case of HEK293-transfected cells. In case of both Jurkat and HT1080 cells, nonspecific binding values of the groups pretreated with 10 µg/ml of TRAILR2-specific anti-rabbit anti-serum were subtracted from total binding values of the corresponding TRAIL variant to calculate specific binding values.

3.7. In vitro binding studies

Black 96-well ELISA plates were coated with 0,5 ug/ml of protein G overnight at 4 °C in refrigerator. In the next day, plates were loaded with ~ 1 µg/ml of TRAILR1(ed)-Fc or TRAILR2(ed)-Fc or remained untreated to determine nonspecific binding. The unbound molecules were removed and then GpL fusion proteins of the different TRAIL variants were added on the plates for 1 h at 37 °C. The unbound molecules were removed and then the luciferase intensity of each TRAIL variant were assayed using the *Gaussia* Luciferase Assay Kit and a Lucy 2 Luminometer according to the protocol of the manufacturer.

3.8. Immunoprecipitation (IP) analysis

TRAILR-complex was analyzed using IP method. Panc89 and HT29 cells were seeded on large Petri dishes. The plates were left in incubator until they were completely full with the cells. Afterwards, the cells were treated with the corresponding Fc-TRAIL fusion proteins (1 µg/ml, 2 h) in 8 ml medium. Then, the cells were harvested on ice in 50 ml falcon tubes and the volume was completed to 50 ml with ice cold PBS. The falcon tubes were centrifuged for 3 min at 2300 rpm and the supernatant was discarded and then the pellets were centrifuged

again with 50 ml ice cold PBS. The pellets were further suspended in 1,5 ml IP lysis buffer with protease inhibitor in 2 ml Eppendorf tubes and left for 20 min on ice. Then, the Eppendorf tubes were centrifuged for 5 min at 5000 rpm (4 °C) and the supernatants were transferred to new 2 ml Eppendorf tubes. The supernatants were centrifuged again for 45 min at 14000 rpm (4 °C). 200 µl of the supernatants from each group was stored at -20 °C for further analysis of lysates by Western Blot and the rest were used for the IP experiment. 10 ng/ml of each Fc-TRAIL variant was added to the corresponding negative control group. Afterwards, the supernatant of each group was mixed with 40 µl agarose beads in 2 ml Eppendorf tubes and left overnight on a roller at 4 °C. In the next day, the Eppendorf tubes were centrifuged for 30 sec at 5000 rpm (4 °C). The supernatants were then discarded using 1 ml insulin syringes. Then, 2 ml IP lysis buffer without protease inhibitor was added on the beads and the Eppendorf tubes were centrifuged again for 30 sec at 5000 rpm (4 °C). The previous step was repeated for other 3 times and then the supernatants were removed completely using 1 ml insulin syringes. In the next step, 60 µl 4× Laemmli buffer and 60 µl IP lysis buffer were added on the beads in Eppendorf tubes and the mixture was further heated at 80 °C for 15 min. Afterwards, the Eppendorf tubes were left on ice for 10 sec and then centrifuged for 5-10 sec. Finally, the supernatants from each group were carefully transferred away from the beads in other new Eppendorf tubes using 1 ml insulin syringe and stored at -20 °C for further analysis of immunoprecipitates by Western Blot.

3.9. Western Blot

3.9.1. SDS-PAGE

The proteins were separated using SDS-PAGE. First, the separating gel was prepared from 0,374 M Tris (pH 8,8) , 0,0035 M SDS and 12 % acrylamide and then polymerized using 0,1 % APS and 0,1 % TEMED. Isopropanol was added on the surface of the separating gel immediately after pouring of the gel and before its polymerization to obtain a straight surface. After polymerization, isopropanol was removed and the stacking gel was added which was composed of 6 % acrylamide in 0,123 M Tris (pH 6,8) , 0,00375 M SDS, 0,1 % APS and 0,1 % TEMED. The sample chambers were inserted in the stacking gel before polymerization. After polymerization, these sample chambers were removed and the samples were added using micropipette to run electrophoretic separation at 120 V and 400 mA for 95 min in case of small gels and 105 min in case of large gels.

3.9.2. Blotting on nitrocellulose membranes

The proteins separated by SDS-PAGE were blotted on nitrocellulose membranes using Wet/tank blotting system. Nitrocellulose membranes and Whatman papers were cut perfectly to the same size of the gels and wetted with blot buffer directly before use. The blotting process was started by pressing the nitrocellulose membranes directly on the gels in the blotting chamber as a sandwich in the following order:
anode - 2 wet Whatman papers - nitrocellulose membrane- gel - 2 wet Whatman papers - kathode.
The blotting process was finished after 90-150 min at room temperature, 90 V and 400 mA.

3.9.3. Membrane detection

After blotting, nonspecific binding of the nitrocellulose membranes was blocked by washing the membrane for 1 h with PBST-milk on the shaker. After that, the membrane was washed 3 times with PBST for 30 min and then incubated overnight at 4 °C on the shaker with the required 1st antibody in PBST or TBST according to the manufacturer. In the next day, the membranes were washed again three times with PBST or TBST for 30 min and then the 2nd antibody in PBST or TBST-milk was added on the membranes for 1h at room temperature on the shaker. Finally, the membranes were washed 3 times with PBST or TBST for 30 min and then detected either with ECL-system or using LI-COR Odyssey® Infrared Imager.

3.10. Cell viability assays

In case of adherent cell lines (HT29, HT1080, HeLa, HeLa-CD40, HT1080-CD40, 786-O and OVCAR3), 20×10^3 cells/well were seeded in 96-well plates with 100 µl medium containing 10 % FCS (RPMI 1640 medium for all cell lines or DMEM medium for OVCAR3 cell line) and left overnight in incubator at 37 °C. In the next day, the plates were full with cells and ready to start the experiments. All adherent cells were sensitized with CHX (2.5 µg/ml) for 30 min before stimulating with TRAIL constructs. The suspension cell lines (Jurkat, Mino, BJAB and Rec-1) were counted (60×10^3 cells/well) and seeded in 96-well plates and then stimulated in the same day in the absence of CHX. In the coculture assay, Jurkat cells (60×10^3 cells/well) were seeded together with Rec-1 (6×10^3 cells/well) in 96-well plates. In case of oligomerization with anti-Flag mAb M2, the corresponding TRAIL variant was incubated for 30 min with 1 µg/ml of anti-Flag mAb M2 in a separate 96-well plate and then added on the cells. To determine CD40-binding dependent enhancement of apoptosis, scFv:G28-Fc-GpL (2 µg/ml) and scFv:G28-2xFlag-GpL (2 µg/ml) were used as competitors and added to the cells 30 min before the corresponding scFv:G28-TRAIL fusion protein.

Untreated group represented 100 % living cells whereas the group treated with a cytotoxic cocktail (100 ng/ml of Fc-CD95L, 5 µg/ml of CHX and 0,5 % sodium azide) represented 0 % living cells.

In the next day, cell viability was determined using CV staining in case of adherent cells or MTT staining in case of suspension cells. In case of CV staining, the supernatants were removed from the plates and 80 µl/well of the CV staining solution was added on the plates and left for 15 min at room temperature. Afterwards, the excess CV staining was removed by immersing plates in distilled water. The plates were left to dry at room temperature before adding 180 µl methanol/well on the plates and then the plates were left on the shaker for 15 min. Finally, the plates were measured at 595 nm using Lucy 2 Luminometer. In case of suspension cells, 35 µl/well of MTT solution diluted with PBS (1:2,5) was added on the cells and the plated were incubated at 37 °C in incubator for 2 h. Then, 90 µl/well of MTT lysis buffer was added on the plates. The plates were covered with aluminum foils and left overnight at room temperature on the shaker and measured in the next day using Lucy 2 Luminometer at 570 nm.

3.11. Total cell lysates

Total cell lysates were prepared by harvesting the cells in ice-cold PBS and centrifuging them at 2300 rpm for 3 min at 4 °C. Then, the pellets were lysed in 4× Laemmli buffer with freshly added phosphatase inhibitor mixture II and protease inhibitor. Afterwards, cell lysates were sonficated for 20 sec and then boiled for 5 min at 95 °C. Finally, total cell lysates were centrifuged for 10 min at 14000 rpm and stored at -20 °C for further analysis by Western Blot.

3.12. Silver staining

The purified scFv:G28-TRAIL was separated by SDS-PAGE and then the gel was stained using Pierce® Silver Stain Kit according to the instructions of the manufacturer.

3.13. IL8 ELISA

HeLa and HeLa-CD40 cells were seeded (2×10^4 cells/well) in 96-well plates in 100 µl medium (RPMI 1640, 10 % FCS) and left overnight in incubator at 37 °C. The old medium was removed in the next day and cells were stimulated in triplicates in the absence of CHX with the indicated concentrations of scFv:G28-TRAIL and Killer-TRAIL prepared in fresh medium. The plates were left in incubator at 37 °C and after 8 h the supernatants were collected and freezed at -20 °C for further IL8 production analysis using the BD OptEIA Human IL8 ELISA Set according to the instructions of the manufacturer.

3.14. Isolation, cultivation and stimulation of monocyte-derived DCs

3.14.1. Preparations of DCs

Blood of healthy donors obtained from the Institute of Clinical Transfusion Medicine and Hemotherapy, University Hospital of Würzburg (Oberdürrbacher Str 6, 97080 Würzburg) were used to isolate PBMCs. Blood was transferred to 50 ml falcon tubes and then diluted slowly with sterile PBS (1:1) and transferred carefully on the surface of 15 ml lymphocyte separation medium in other 50 ml falcon tubes. The tubes were then centrifuged at 1800 rpm without brake for 15 min at 22 °C. Then, the buffy coat layer between plasma and lymphocyte separation medium was transferred carefully to another 50 ml falcon tube using sterile plastic Pasteur pipettes and mixed homogenously with 15 ml of sterile PBS-EDTA (PBS with 2 mM EDTA). The volume was then completed to 50 ml with PBS-EDTA and the tubes were centrifuged for 10 min at 1300 rpm. Afterwards, the supernatants were discarded and cells were suspended in 15 ml sterile PBS without EDTA and the volume was completed to 50 ml with PBS. The cells were centrifuged again for 10 min at 1300 rpm. The resuspension of the cells in PBS and centrifugation steps were repeated again. Then, the cells were suspended in 6 ml MACS buffer containing 200 µl anti-CD14-coated beads and left for 20 min at 4 °C in refrigerator. Afterwards, MACS buffer was added to the cells to complete the volume to 50 ml and then the cells were centrifuged for 10 min at 1300 rpm (8 °C). In the next step, the cells were suspended in 6 ml MACS buffer and applied on MACS LS column using MACS multistand and MACS separator. Finally, $CD14^{+}$-monocytes were harvested in 6 ml MACS buffer and centrifuged for 4 min at 1200 rpm. Monocytes were transferred to 10 cm Petri dish in the presence of 8 ml medium (RPMI 1640, 10 % FCS, 1 % penicillin-streptomycin). Purity of monocytes was controlled immediately after isolation by FACS analysis of CD14 expression. To differentiate monocytes into iDCs, monocytes were treated with IL4 (30 ng/ml) and GM-CSF (50 ng/ml) every 2 days for 1 week.

3.14.2. FACS analysis of monocytes, iDCs and mDCs

Monocytes were scanned on the same day of isolation by FACS analysis to detect the positive expression of CD14 as control for the purity of monocytes. iDCs were evaluated after one week of treatment with IL4 and GM-CSF by FACS analysis for the absence of CD14 and the expression of CD40, CD83 and CD86. mDCs were generated by treating iDCs for 24 h with TNF (1 µg/ml) or with Gold standard (mixture of 20 ng/ml of TNF which is a purified protein produced in HEK293 cells, 10 ng/ml of IL1β, 20 ng/ml of IL6 and 1 µg/ml of PGE2) and then scanned by FACS analysis for the same markers as in case of iDCs. To test the ability of scFv:G28-TRAIL fusion proteins to maturate iDCs, iDCs (1×10^{6} cells/well) were

seeded on 12-well plates and treated for 48 h with the indicated concentrations of the corresponding TRAIL fusion protein, Killer-TRAIL, scFv:G-28-TNC-GpL, Gold standard (mixture of 20 ng/ml of TNF which is a purified protein produced in HEK293 cells, 10 ng/ml of IL1β, 20 ng/ml of IL6 and 1 μg/ml of PGE2) and then cells were harvested and scanned by FACS analysis for the cell surface expression of both CD83 and CD86. Handling of cells and the procedures of FACS staining were the same as mentioned before (see section 3.5.1).

3.14.3. Cell viability assays

iDCs or mDCs prepared by treating iDCs for 24 h with TNF (1 μg/ml) or with Gold standard (mixture of 20 ng/ml of TNF which is a purified protein produced in HEK293 cells, 10 ng/ml of IL1β, 20 ng/ml of IL6 and 1 μg/ml of PGE2) were seeded (4×10^4 cells/well) in 96-well plates in 100ul medium (RPMI 1640, 10 % FCS, 1 % penicillin-streptomycin) in the presence of IL4 (30 ng/ml) and GM-CSF (50 ng/ml) and treated with the indicated concentrations of scFv:G-28-TRAIL fusion proteins and Killer-TRAIL for 24 h. In the next day, cell viability was determined using MTT assay (see section 3.10).

3.14.4. IL12 ELISA

iDCs were counted (4×10^4 cells/well) and then seeded in 96-well plates in 100 μl medium (RPMI 1640, 10 % FCS, 1 % penicillin-streptomycin) in the presence of IL4 (30 ng/ml) and GM-CSF (50 ng/ml). Then, the cells were treated in triplicates with the various scFv:G28-TRAIL fusion proteins, Killer-TRAIL, gold standard (mixture of 20 ng/ml of TNF which is a purified protein produced in HEK293 cells, 10 ng/ml of IL1β, 20 ng/ml of IL6 and 1 μg/ml of PGE2), control supernatant of mock-transfected HEK293 cells and scFv:G28-TNC-GpL. The plates were left overnight in incubator and then in the next day the plates were centrifuged for 2 min at 1200 rpm and the supernatants were collected and analyzed for IL12 production using R&D Systems Human IL12 ELISA DuoSet kit according to the instructions of the manufacturer.

LPS contamination in scFv:G28-TRAIL fusion proteins was controlled using two methods. The first one was the heat inactivation of 200 ng/ml of the different TRAIL fusion proteins parallel with LPS (100 ng/ml) for 30 min at 70 °C. After heat inactivation, both TRAIL fusion proteins and LPS were tested in regard to IL12 production in iDCs. The second experiment was to test the ability of PMB (50 μg/ml) to interfere with the IL12 production in iDCs induced by scFv:G28-TRAIL (200 ng/ml) or LPS (20 ng/ml).

3.15. Statistical analysis

Figures shown in this current work were designed by Microsoft Office Excel 2007, GraphPad Prism 5.0 program (GraphPad Software, Inc.) and CorelDRAW Graphics Suite X4 software. K_D-values for the different TRAIL constructs were also calculated using GraphPad Prism 5.0 program by non-linear regression equation.

4. Results

4.1. Characterization of TRAILR1- and TRAILR2-specific TRAIL mutants

TRAIL is known to induce apoptosis by binding to two DRs, TRAILR1 and TRAILR2 (LeBlanc and Ashkenazi, 2003). Therefore, these two receptors have received more research interest than the other TRAILRs and there are now considerable research efforts to characterize TRAIL mutants that exhibit preferential binding to TRAILR1 or TRAILR2 (MacFarlane *et al.*, 2005b; Reis *et al.*, 2010). Indeed, there are various reasons arguing for the development of TRAIL DR-specific mutants. It may be a way to attenuate the potential adverse effects mediated upon the activation of the TRAIL DR type other than the TRAIL DR required for induction of apoptosis in the targeted tumor cells. This concept bases on the previous finding that some tumors show an apoptotic response preferentially through stimulation of only one of the two TRAIL DRs. For example, CLL and pancreatic tumors show an apoptotic response mainly through stimulation of TRAILR1 although TRAILR2 is also expressed on these cells (Lemke *et al.*, 2010; MacFarlane *et al.*, 2005a; Stadel *et al.*, 2010). Moreover, there is evidence that TRAIL DR-specific mutants exhibit a higher specific activity than the wild type molecule due to reduced formation of TRAILR1-TRAILR2 heterocomplexes which seem to be less active than homotrimeric TRAIL DR complexes (Reis *et al.*, 2010).

As was the aim of our work to develop TRAIL variants with reduced side effects and a high specific activity on tumor cells, we wanted to exploit such mutants also in our project. Therefore, we characterized two recently published TRAIL DR-specific mutants that elicited promising specificities and activity features. Initially, we introduced the mutations of these two TRAIL mutants in our basic TNC-TRAIL construct, one conferring specificity for TRAILR1 (TRAILmutR1) G131R/R149I/S159R/N199R/K201H/S215D and the other for TRAILR2 (TRAILmutR2) Y189Q/R191K/Q193R/H264R/I266L/D267Q (MacFarlane *et al.*, 2005b; Reis *et al.*, 2010).

4.1.1. Binding studies with TRAIL mutants and TRAIL wild type

Initially, we analysed the binding of TNC-TRAILmutR1 and TNC-TRAILmutR2 to the different TRAILRs in cellular equilibrium binding studies. For this purpose, we tagged the TNC-TRAIL variants N-termnally with the GpL which enables quite sensitive detection and quantification of the tagged protein (Lang *et al.*, 2012; Tannous *et al.*, 2005). Then, we performed equilibrium binding studies with HEK293 cells transiently transfected with TRAILR1, TRAILR2, TRAILR3 and TRAILR4. Mock-transfected HEK293 cells were used to

determine nonspecific binding. The surface expression of the TRAILRs in both TRAILRs- and mock-transfected HEK293 cells were evaluated with FACS analysis before performing the binding studies not only to prove the success of TRAILR-transfection but also to analyze the expression of endogenous TRAILRs on the mock-transfected HEK293 cells. Actually, our results revealed that mock-transfected HEK293 cells expressed no endogenous TRAILR1 and TRAILR3 and had only moderate expression of endogenous TRAILR2 and TRAILR4 (Figure 3).

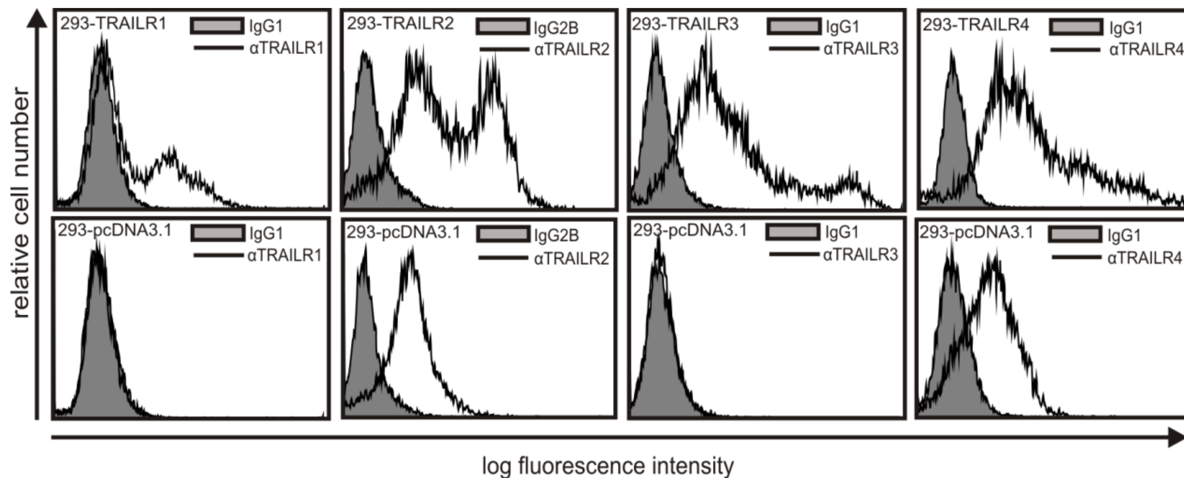


Figure 3: FACS analysis of cell surface expression of the various TRAILRs on TRAILR- and mock-transfected HEK293 cells.

HEK293 cells transfected with TRAILR1, TRAILR2, TRAILR3 and TRAILR4 expression constructs were analyzed along with empty plasmid transfected cells by FACS to determine cell surface expression of the various TRAILRs.

According to the results of these binding studies, GpL-TNC-TRAIL has K_D-values for TRAILR1 and TRAILR2 of 3450 and 880 pM respectively (Figure 4). The measured K_D-value of the interaction of GpL-TNC-TRAILmutR1 with TRAILR1 was 2590 pM (Figure 4). Concerning the binding of GpL-TNC-TRAILmutR1 to TRAILR2, there was also a significant binding to TRAILR2-transfected HEK293 cells but the maximal specific binding was significantly lower than that of GpL-TNC-TRAILmutR2 and GpL-TNC-TRAIL (Figure 4). Thus, the observed binding/K_D-value of GpL-TNC-TRAILmutR1 in the TRAILR2-transfectants most likely does not reflect binding to TRAILR2 but may be attributed to the binding to endogenous TRAILR4 and/or heterocomplexes of TRAILR2 and endogenous TRAILR4. This idea was supported by the further binding studies in the Jurkat and HT1080 cell lines expressing only or dominantly TRAILR2 (Figure 5a) (Sprick *et al.*, 2000). Here we found that GpL-TNC-TRAILmutR1, in contrast to GpL-TNC-TRAIL and GpL-TNC-TRAILmutR2, failed to show

specific TRAILR2 binding (Figure 5b). Regarding GpL-TNC-TRAILmutR2, the affinity of the binding to TRAILR2-transfected HEK293 cells was 720 pM while there was no detectable binding to the TRAILR1-transfectants (Figure 4). Furthermore, we also analyzed specific binding of the two TRAIL mutants to TRAILR3- and TRAILR4-transfected HEK293 cells and we found that GpL-TNC-TRAILmutR1 bound to both TRAILR3 and TRAILR4 while GpL-TNC-TRAILmutR2 showed only weak binding to TRAILR4, as compared to TRAIL and TRAILmutR1, and failed to bind TRAILR3 (Figure 4).

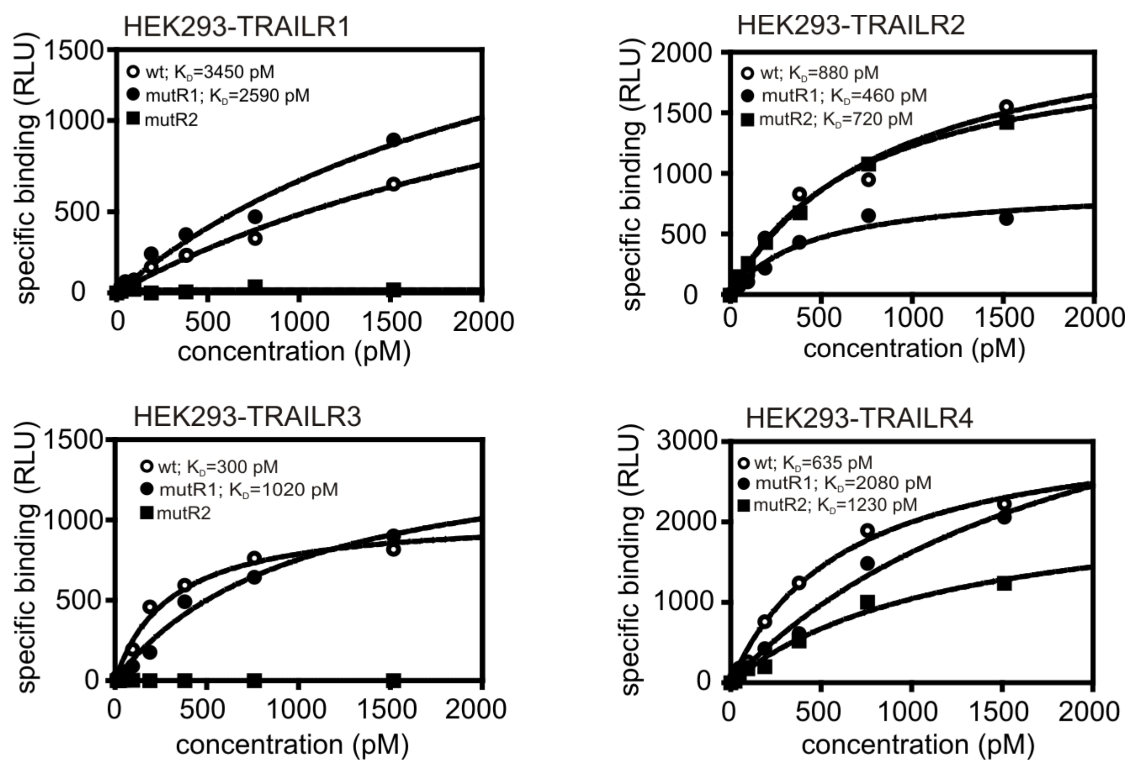
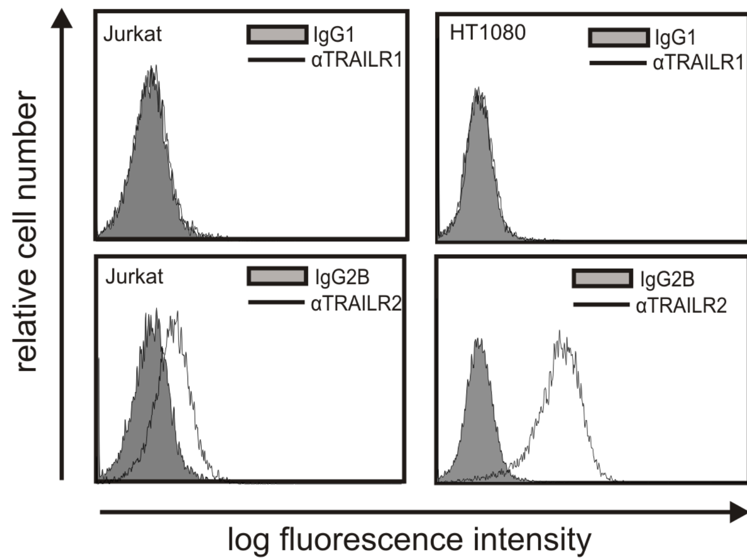


Figure 4: Binding studies in TRAILR-transfected HEK293 cells to evaluate the binding properties of TRAIL mutants with specificity for TRAILR1 and TRAILR2.

Expression constructs encoding the four types of cell bound TRAILRs were transiently transfected in HEK293 cells. The next day, equilibrium binding studies were performed at 37°C with GpL-TNC-TRAIL, GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2. Nonspecific binding was determined using mock-transfected HEK293 cells.

a)



b)

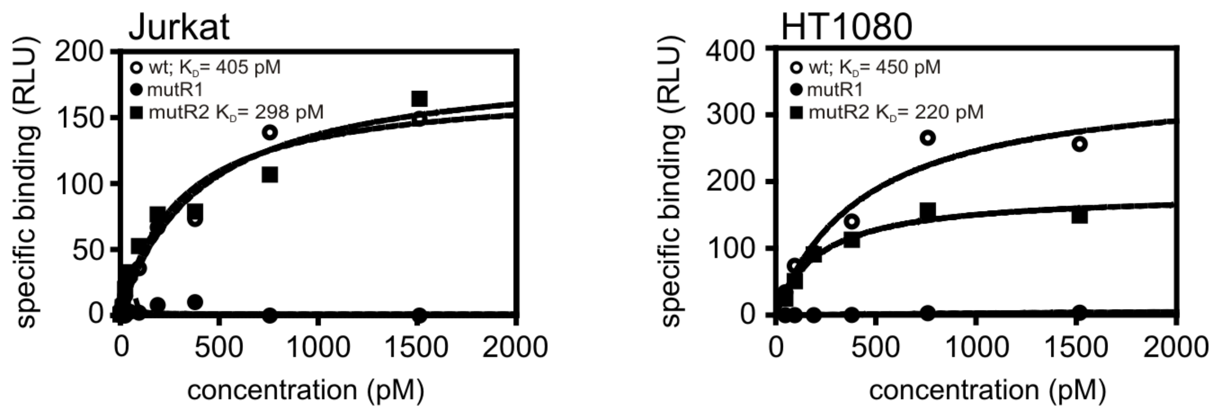


Figure 5: Binding studies in cell lines expressing endogenous TRAILR2 but no TRAILR1.

a) Jurkat and HT1080 cells were analyzed by FACS with respect to the cell surface expression of TRAILR1 and TRAILR2. b) Equilibrium binding studies were performed with the various GpL-TRAIL fusion proteins and Jurkat and HT1080 cells. Cells pretreated with 10 µg/ml of a TRAILR2-specific anti-rabbit anti-serum were used to determine non-specific binding.

We also performed cell-free binding studies with immobilized TRAILR1-Fc and TRAILR2-Fc and the results confirmed that GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2 efficiently discriminates between TRAILR1 and TRAILR2 (Figure 6).

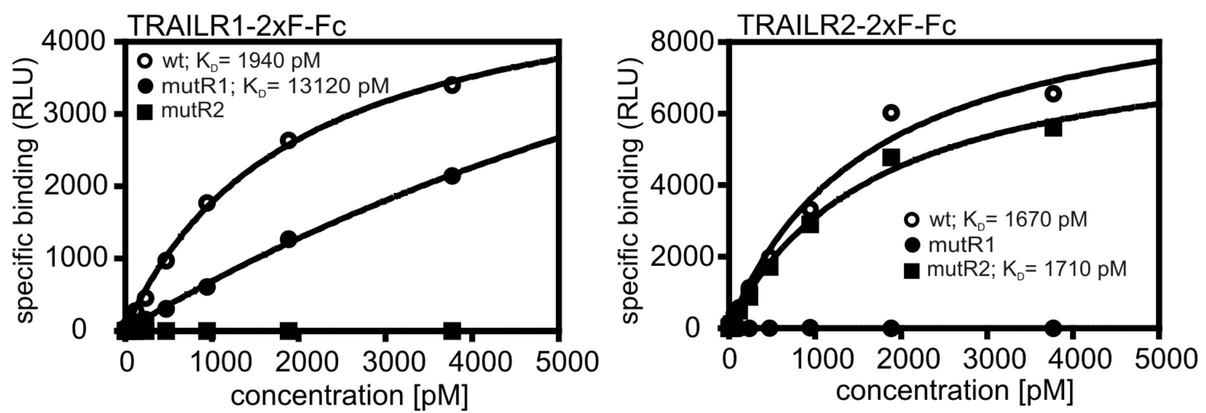


Figure 6: *In vitro* binding studies with immobilized TRAILR1-Fc and TRAILR2-Fc.

Protein G (0,5 µg/ml) was used to coat black 96-well ELISA plates. Then, these plates were loaded with TRAILR1(ed)-Fc or TRAILR2(ed)-Fc (~ 1 µg/ml) or remained untreated to determine later non-specific binding. Unbound molecules were removed and then GpL-TNC-TRAIL, GpL-TNC-TRAILmutR1 and GpL-TNC-TRAILmutR2 were added at the indicated concentrations of for 1 h at 37°C. Finally, the well-associated luciferase activity was quantified and specific binding was calculated as the difference of total binding (wells with immobilized TRAILR1/2-Fc) and nonspecific binding. (This experiment was done with the help of Johannes Trebing, PhD student in the research group of Prof. Dr. Wajant).

4.1.2. Immunoprecipitation (IP) analysis

To further confirm the selective binding of the TRAIL mutants to TRAILR1 and TRAILR2, we performed IP experiments. For this purpose, we generated Fc-fusion proteins of TRAIL, TRAILmutR1 and TRAILmutR2. The fusion of the TRAIL variants with the human IgG1 Fc domain by genetic engineering resulted in the formation of hexameric proteins which provided two advantages. First, they allowed easy IP of ligand bound receptor complexes. Second, the hexamerization provided a substitute for the known need of oligomerization of soluble trimeric TRAIL variants to achieve optimal activity (Berg *et al.*, 2007; Wajant *et al.*, 2001). Consistent with the results of the binding studies, there was no significant presence of TRAILR2 in Fc-TRAILmutR1 immunoprecipitates and no detectable level of TRAILR1 in Fc-TRAILmutR2 immunoprecipitates whereas both receptors were easily detectable in immunoprecipitates of the Fc-TRAIL stimulated Panc89 and HT29 cells (Figure 7).

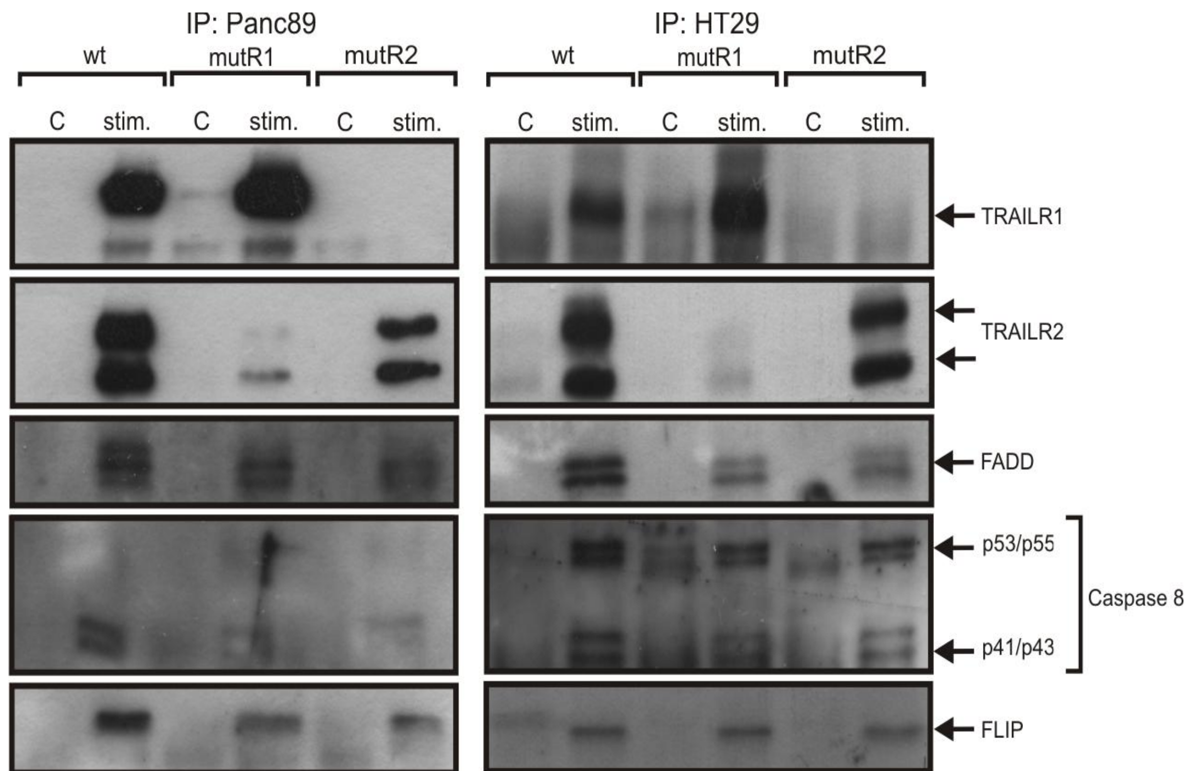


Figure 7: Immunoprecipitation (IP) with TRAILR1 and TRAILR2 discriminating TRAIL mutants.

Fc fusion proteins of the indicated TRAIL variants were incubated with Panc89 and HT29 cells for 2 h (37°C) and were then immunoprecipitated by help of protein G agarose. Western Blot was used to analyze immunoprecipitates with respect to the presence of the indicated proteins. Lysates of untreated cells supplemented with 10 ng of the corresponding Fc fusion proteins were also subjected to IP and served as negative control groups (c).

4.1.3. Cell viability assays

To evaluate the functional consequences of selective binding of TRAIL mutants to TRAILR1 or TRAILR2, we analyzed Jurkat, HT1080 and HT29 cells with respect to cell death induction (Figure 8a,b). In these experiments, the TNC-TRAIL variants were used with and without anti-Flag mAb M2 oligomerization. Our results revealed that oligomerized TRAILmutR1 induced significant cell death only in the case of HT29 cells which, in contrast to Jurkat and HT1080 cells, express significant amounts of TRAILR1 (Figure 8a). On the other hand, oligomerized TRAILmutR2 induced significant cell death in the all cell lines which all express endogenous TRAILR2 (Figure 8a). In all cases, the non-oligomerized TRAIL variants were far less active. Although it was shown previously that anti-Flag oligomerization of the Flag-

tagged TNC-TRAIL enhanced the cell death induction via TRAILR2, we marshalled in this current research the first-time investigation of the enhanced cell death induction via both TRAILR1 and TRAILR2 after anti-Flag oligomerization of the specific TRAIL mutants (Figure 8a) (Berg *et al.*, 2007). In addition, we also analyzed the cell viability of the different cell lines using the hexameric Fc fusion proteins of the TRAIL variants described above in the absence of oligomerization with anti-Flag mAb M2 (Figure 8b). In contrast to soluble trimeric TRAIL, Jurkat cells showed significant cell death with Fc-TRAIL and Fc-TRAILmutR2 in the absence of oligomerization with anti-Flag mAb M2 (Figure 8b). Thus, these results confirmed the above mentioned concept that hexameric ligands of some members of TNF superfamily exhibit superior activity and they can act as a substitute for the known need of oligomerization of soluble trimeric ligands (Holler *et al.*, 2003; Wyzgol *et al.*, 2009). Consistent with the cell viability results of TNC-TRAIL variants, it was not unexpected that Fc-TRAILmutR1 showed no cell death in Jurkat cells which express only TRAILR2 (Sprick *et al.*, 2000). Likewise, it showed also no cytotoxic effect in case of HT1080 cells (Figure 8b). On the other hand, all of the three Fc-TRAIL variants exhibited significant cell death at different degrees in HT29 cell lines which express both TRAILR1 and TRAILR2 (Figure 8b).

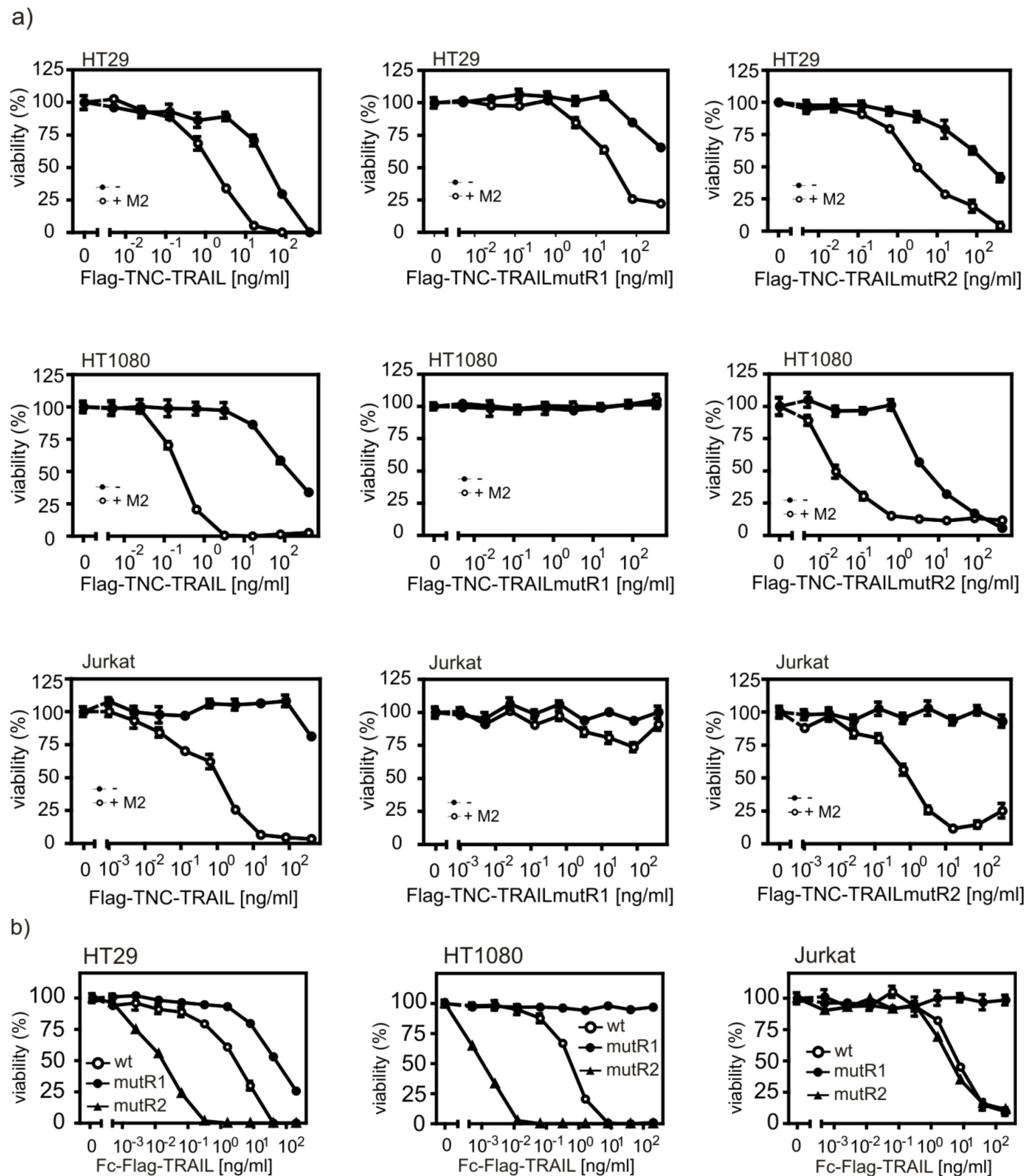


Figure 8: Cell viability assays discriminating between TNC-TRAIL and Fc-TRAIL constructs of TRAIL mutants in cell lines expressing endogenous TRAILR1 and/or TRAILR2.

a) HT29, HT1080 and Jurkat cells were challenged in triplicates with the indicated concentrations of Flag-TNC-TRAIL, Flag-TNC-TRAILmutR1 Flag-TNC-TRAILmutR2 in the presence and absence of 1 µg/ml of the anti-Flag mAb M2. b) Cells were stimulated in triplicates with the indicated concentrations of Fc-Flag-TRAIL, Fc-Flag-TRAILmutR1 and Fc-Flag-TRAILmutR2. Next day, cellular viability was determined using the MTT assay or CV staining. HT29 and HT1080 cells were challenged in the presence of 2.5 µg/ml of CHX which sensitizes these cell lines for apoptosis induction.

In addition to cellular viability, we also evaluated processing of caspases and caspase substrates in TRAIL-treated cells by Western Blot. Consistent with the results of the viability assays, Western blotting confirmed the inability of TRAILmutR1 to induce apoptosis in the absence of TRAILR1 expression. There were no processing of caspases and the caspase-3 substrate PARP in the TRAILR1-negative HT1080 cells while these signs of apoptosis were easily detectable in the HT29 cells (Figure 9). In contrast, oligomerized TRAILmutR2 induced caspase processing in both cell lines in accordance with their positive TRAILR2 expression Thus, the results of binding studies, viability assays and Western Blot analysis of caspase processing conclusively support the strong TRAILR1/TRAILR2 binding preference of the various TRAILmutR1 and TRAILmutR2 proteins.

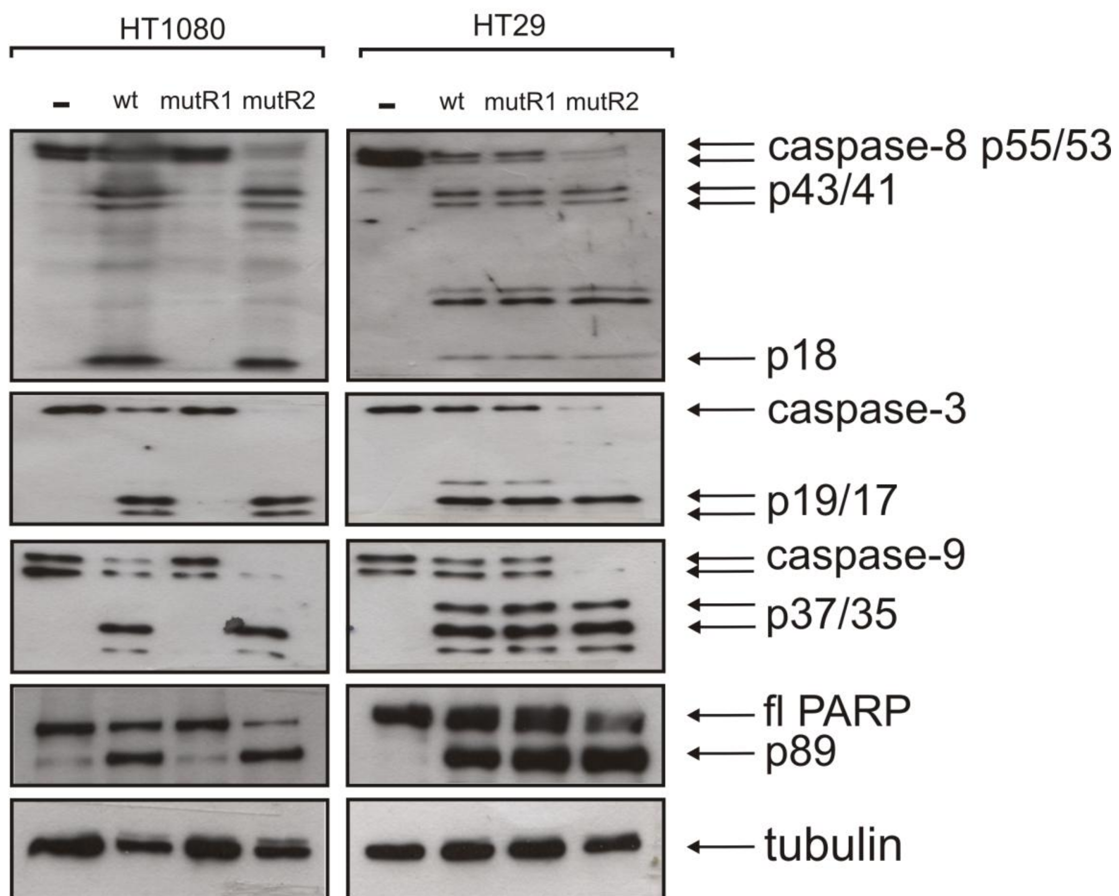


Figure 9: Western Blot analysis cells challenged with the TRAILR1 and TRAILR2 discriminating TRAIL mutants.

HT1080 and HT29 cells were treated for 4-6 h with 50 ng/ml of the various Flag-TNC TRAIL variants oligomerized with 1 µg/ml of the anti-Flag mAb M2 and then total cell lysates were analyzed by Western Blot to detect the presence of indicated proteins. The cells were sensitized with CHX 2,5 µg/ml.

4.2. Targeting and activation of CD40 with scFv-TRAIL fusion proteins

4.2.1. Design and production of scFv-TRAIL fusion protein targeting CD40

As discussed in the introduction, cell surface anchoring of fusion proteins of TRAIL with scFv antibody against cell surface expressed antigens is a well proved option to enhance the activity of soluble TRAIL and to convert it to a pseudo-membrane TRAIL molecule (table 1). Therefore, we constructed a fusion protein of soluble TRAIL with a CD40-specific scFv-domain to achieve targeting to CD40. The fusion protein is termed in the following as scFv:G28-TRAIL and consists of a Ig signal peptide followed by a scFv derived from the human CD40-specific mAb G28-5 and aa 95-281 of human TRAIL encompassing its C-terminal TNF homology domain. We further included an internal Flag epitope and the short 3 kDa trimerization domain of TNC between the scFv and TRAIL domains (Figure 10). The Flag epitope serves to facilitate detection and purification of the protein while the TNC domain stabilizes the trimeric assembly of the fusion protein which is acquired by the TRAIL domain (Wyzgol *et al.*, 2009).

Figure 10: Domain architecture of the scFv:G28-TRAIL fusion protein.

Scheme of scFv:G28-TRAIL. scFv:G28, scFv derived from the human CD40-specific mAb G28-5; Flag epitope; TNC, trimerization domain, aa 110-139 of chicken TNC; TRAIL, aa 95-281 of human TRAIL.

The scFv:G28-TRAIL fusion protein was produced using HEK293 cells stably transfected with the corresponding expression plasmid and then the supernatants were collected and subjected to affinity chromatography purification on anti-Flag agarose. This resulted in adequately pure preparations of the scFv-TRAIL fusion protein as was evident from SDS-PAGE analysis with subsequent silver staining of the gel (Figure 11).

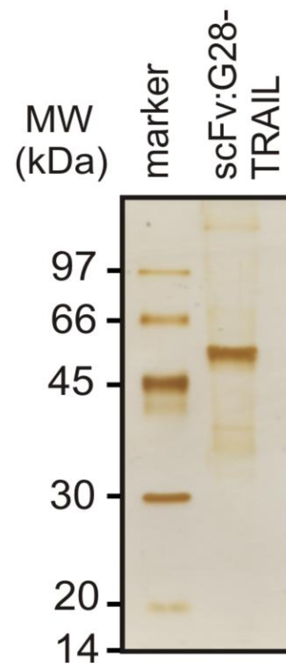


Figure 11: Evaluation of the purity of the scFv:G28-TRAIL fusion protein.

Anti-Flag affinity chromatography purified scFv:G28-TRAIL (100 ng) was subjected to SDS-PAGE and visualized by silver staining.

4.2.2. Evaluation of inherent functionality of scFv and TRAIL domain of scFv:G28-TRAIL fusion protein

First, we controlled that the principle functionality of TRAIL domain is preserved in the scFv:G-28-TRAIL fusion protein using viability analysis in CD40-negative Jurkat cells. Although TNC-TRAIL showed no significant toxicity in Jurkat cells without oligomerization, scFv:G28-TRAIL fusion protein showed a significant cytotoxic effect on Jurkat cells between 20 and 200 ng/ml (Figure 12). Moreover, both molecules elicited a comparable strong cytotoxic effect on Jurkat cells upon oligomerization with the anti-Flag mAb M2 recognizing the internal Flag tag of the two molecules (Figure 12). As mentioned before, it is well proved from previous studies that soluble TRAIL trimers can only induce significant cell death in Jurkat cells upon oligomerization (Kelley *et al.*, 2005; Wajant *et al.*, 2001). Therefore, it seems that scFv:G28-TRAIL preparations contained a minor fraction of oligomerized molecules that was responsible for the observed apoptosis induction at higher concentrations but otherwise had similar capability to induce apoptotic activity as compared to TNC-TRAIL (Figure 12).

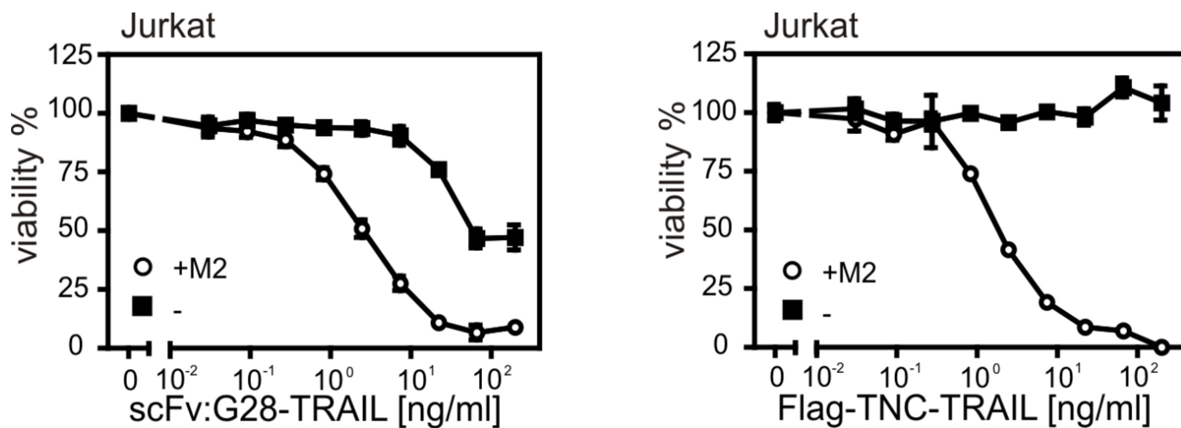


Figure 12: Evaluation of the inherent functionality of TRAIL domain of the scFv:G28-TRAIL fusion protein.

The indicated concentrations of scFv:G28-TRAIL or its conventional counterpart TNC-TRAIL were added to Jurkat cells (60 x 10^3 per well; 96-well plate, triplicates). Cellular viability was determined the next day by using the MTT assay.

Furthermore, we analyzed the functionality of the scFv:G28 domain by evaluation of the binding of the scFv:G28-TRAIL fusion protein to HeLa and HT1080 cells transfected with CD40 as compared it to their corresponding parental cells. FACS analysis revealed that the scFv:G28-TRAIL fusion protein efficiently binds to the cell lines transfected with CD40 as shown by the strong signal with anti-Flag mAb M2-FITC and exhibits only weak binding, due to TRAILR binding via the TRAIL domain, to the parental cell lines expressing no CD40 (Figure 13). Therefore, this experiment provided the proof for the functionality of the scFv:G28 domain of the fusion protein and its ability to target CD40-positive cells.

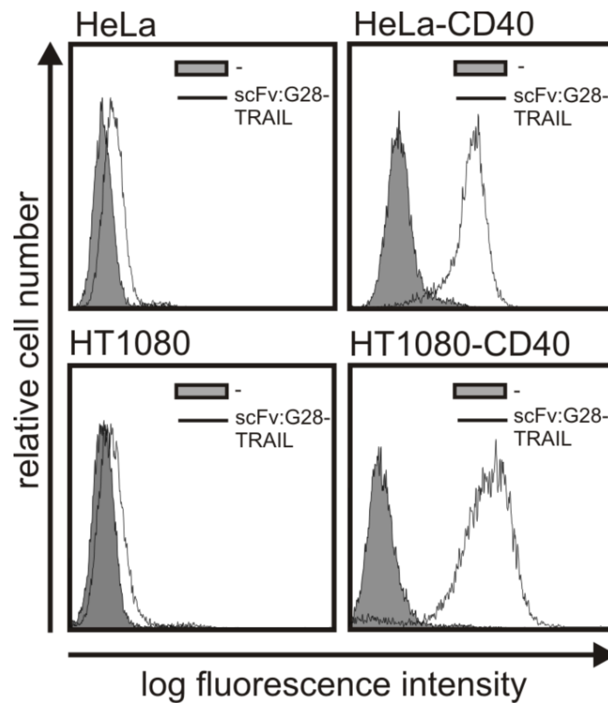


Figure 13: Analysis of CD40 binding of the scFv:G28-TRAIL fusion protein by FACS analysis.

scFv:G28-TRAIL (500 ng/ml) was incubated with HeLa and HT1080 cells parallel with the corresponding CD40 transfectants. After three washes, FACS was used to detect bound molecules using anti-Flag mAb M2-FITC.

4.2.3. Analysis of CD40-dependent enhancement of apoptosis induction by CD40-targeted TRAIL fusion proteins

As shown by us and others, scFv fusion proteins of soluble TRAIL exhibit enhanced antitumor activity (Table 1). Therefore, we expected that scFv:G28-TRAIL fusion proteins exhibit enhanced apoptotic activity on tumor cells in a CD40-dependent manner. To prove this idea, we performed viability assays using HT1080-CD40 and HeLa-CD40 transfectants along with their corresponding CD40-negative counterparts. The different cell lines were challenged with increasing concentrations of scFv:G28-TRAIL in the presence of the apoptosis sensitizer CHX (2,5 µg/ml) and the following day the viability of the cells were determined by CV staining. There was only partial killing in the case of the parental HeLa and HT1080 cells even at high concentrations of 200 ng/ml, whereas the HT1080-CD40 and HeLa-CD40 transfectants were already efficiently killed at concentrations of below 10 ng/ml. There was a shift of approximately two orders of magnitude in the ED_{50}-values of cell death induction towards lower concentrations (Figure 14).

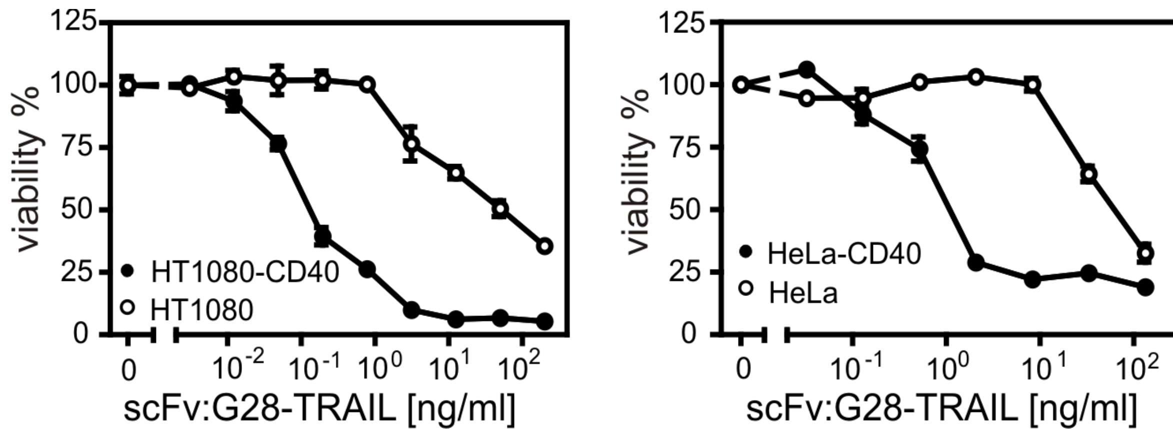


Figure 14: CD40-dependent enhancement of cell death-induction by scFv:G28-TRAIL.

HT1080 and HeLa cells along with corresponding CD40 transfectants were seeded in 96-well plates. Next day, cells were stimulated in triplicates with the indicated concentration of scFv:G28-TRAIL. One day later, CV staining was used to determine cellular viability. Cells were sensitized with CHX (2.5 µg/ml) to enhance apoptosis.

Furthermore, we confirmed the CD40-dependent enhancement of apoptosis induction by scFv:G28-TRAIL in different cell lines expressing endogenous CD40 by challenging the cells with scFv:G28-TRAIL in the presence and absence of a Fc fusion protein of scFv:G28 that competes with this fusion protein for CD40 binding (Figure 15a,b). In all CD40-expressing cells, cell death induction was inhibited in the presence of scFv:G28-Fc indicating that scFv:G28-TRAIL is able to trigger a potent cell death response in a CD40-anchoring depenent manner. Indeed, there was no CD40-dependent enhancement of apoptosis in the CD40-negative Jurkat cell line (Figure15a,b). Thus, conclusively, our results confirmed that the scFv:G28-TRAIL fusion protein has the expected ability to trigger enhanced cell death upon binding to cell surface exposed CD40.

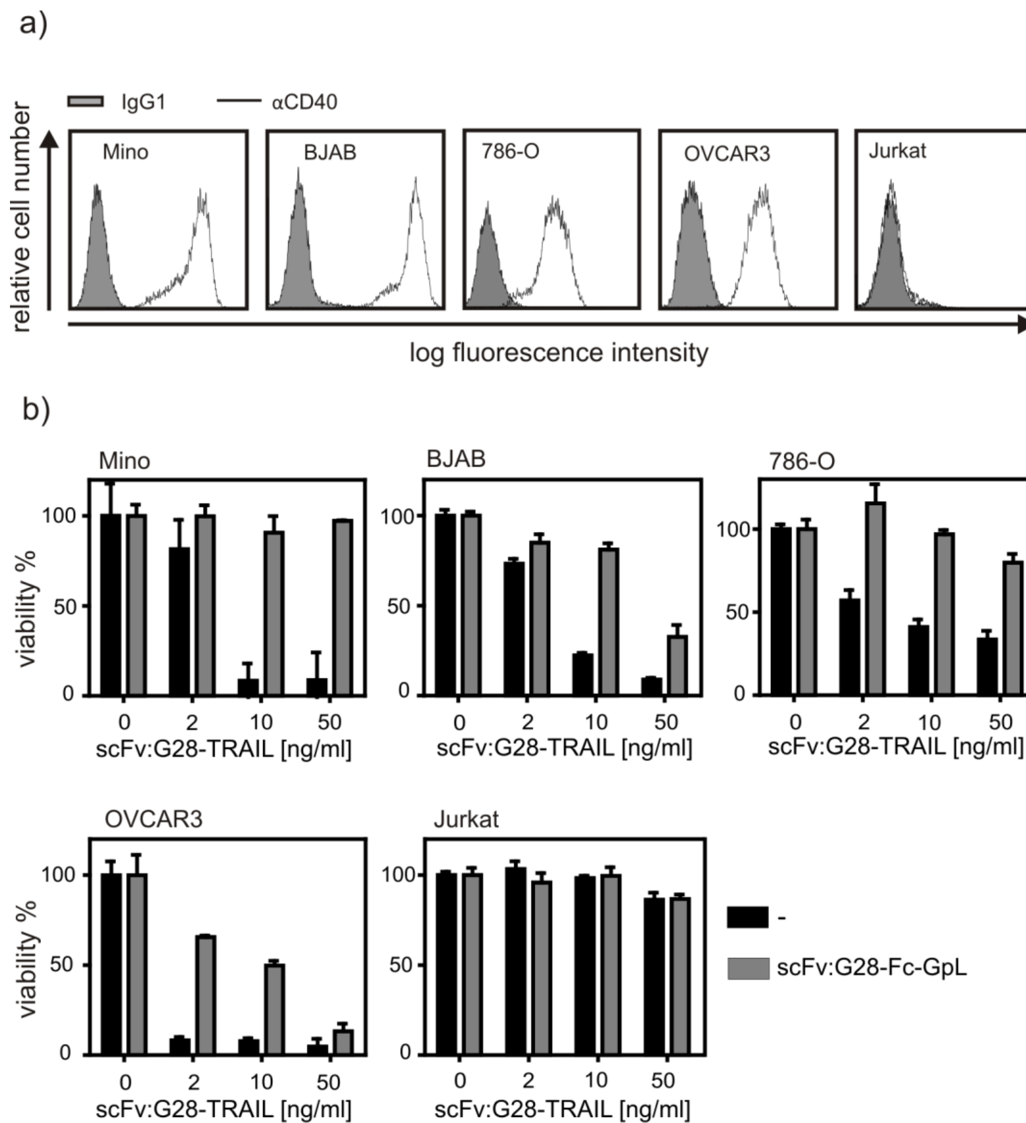


Figure 15: CD40-dependent enhancement of cell death-induction by scFv:G28-TRAIL in cell lines expressing endogenous CD40.

a) CD40 expression was analyzed by FACS in the indicated cell lines. b) Cells were seeded in 96-well plates and challenged in triplicates with the indicated concentrations of scFv:G28-TRAIL in the presence and absence of scFv:G28-Fc-GpL (2 µg/ml) as a competitor. Cellular viability was determined the next day by CV staining or using the MTT assay. OVCAR3 and 786-O cells were sensitized with CHX (2.5 µg/ml) to enhance apoptosis.

Consistent with the results of the viability assays, there was also a stronger activation of caspases in CD40 expressing cells as compared to the corresponding parental cells or the group pretreated with the scFv:G28-Fc fusion protein (Figure 16). These results confirmed the idea that the detected enhancement of cell death-induction with scFv:G28-TRAIL in CD40 expressing cells is due to enhanced activation of caspases.

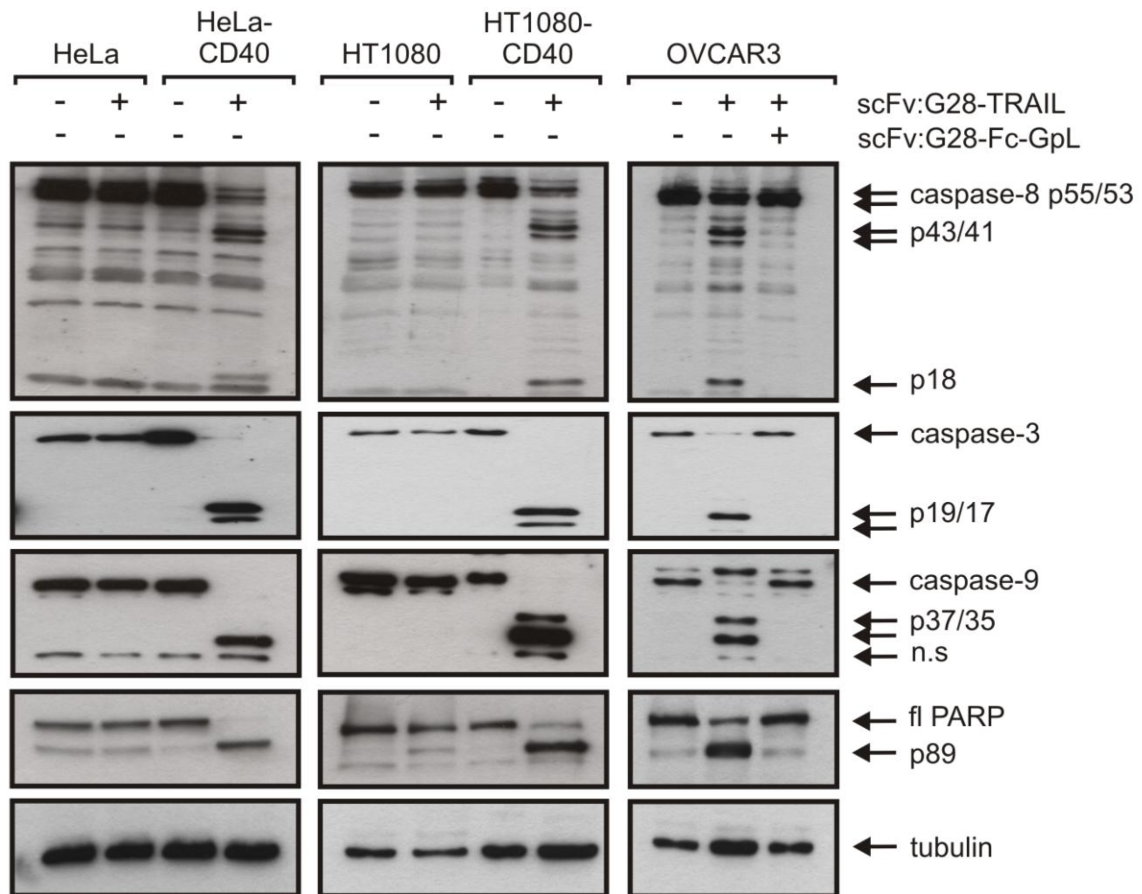


Figure 16: Analysis of caspase activation by scFv:G28-TRAIL in CD40 expressing cells.

The indicated cell lines were treated with scFv:G28-TRAIL (10 ng/ml) in the presence or absence of scFv:G28-Fc-GpL (2 µg/ml) as a competitor for 5 h. Cells were sensitized with CHX (2.5 µg/ml) to enhance apoptosis. Then, total cell lysates were analyzed by Western Blot for the presence of the indicated proteins.

4.2.4. CD40-bound scFv:G28-TRAIL induces cell death in CD40-negative bystander cells

It has been reported that a scFv fusion protein of soluble TRAIL exhibits a potent apoptotic effect on antigen negative bystander cells in the presence of target antigen positive cells (Bremer *et al.*, 2005b). Therefore, we wondered if CD40-bound scFv:G28-TRAIL could also exhibit an apoptotic effect on neighboring CD40-negative cells which express TRAIL DRs and are TRAIL sensitive. To prove this idea, we used CD40-expressing Rec-1 cells which are TRAIL-resistant and CD40-negative Jurkat cells which are only killed by oligomerized or cell surface immobilized TRAIL trimers (Figure 17a,b) (Wajant *et al.*, 2001).

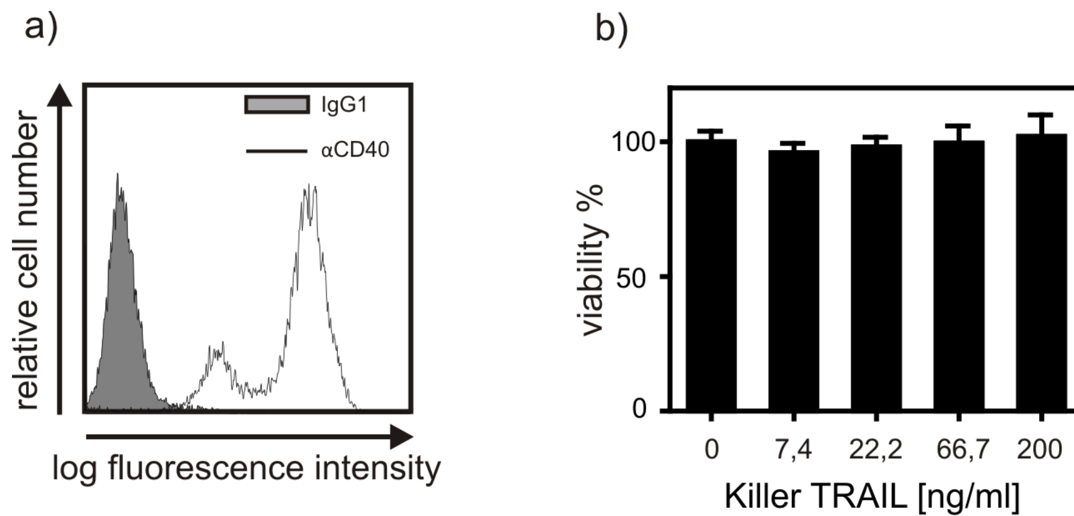


Figure 17: Analysis of CD40 expression and TRAIL resistance of Rec-1 cells.

a) CD40 cell surface expression on Rec-1 cells was analyzed by FACS. b) Rec-1 cells were seeded in 96-well plates and treated with the indicated concentrations of Killer-TRAIL. Cellular viability was analyzed in the next day using the MTT assay.

As shown in (Figure 12), scFv:G28-TRAIL induces cell death in Jurkat cells only between 20 and 200 ng/ml without oligomerization with anti-Flag mAb M2 and there is no significant cell death at lower concentrations. In addition, individual cultures of Jurkat and Rec-1 cells treated with 5 ng/ml of scFv:G28-TRAIL revealed no caspase activation (Figure 18a). However, scFv:G28-TRAIL induced significant cleavage of caspases in cocultures of the two cell lines (Figure 18a). Moreover, cell death induction and caspase activation in cocultures by scFv:G28-TRAIL were diminished in the presence of scFv:G28-2xFlag-GpL as a competitor confirming that this paracrine effect was fully dependent on the binding of scFv:G28-TRAIL fusion protein to CD40 on Rec-1 cells (Figure 18a,b).

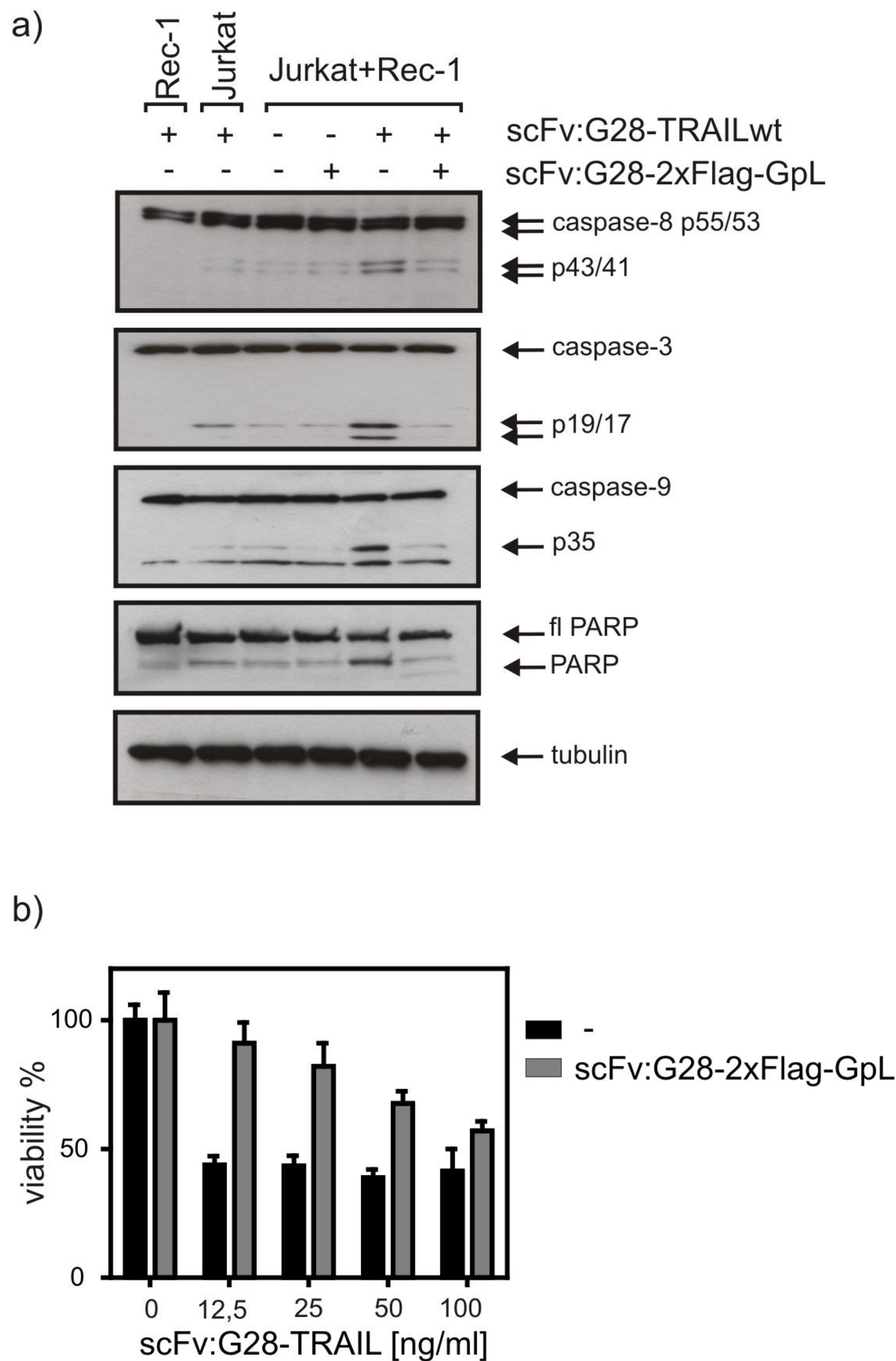


Figure 18: Binding of scFv:G28-TRAIL to TRAIL resistant CD40-positive cells confers the ability to induce apoptosis in TRAIL sensitive CD40-negative bystander cells.

a) Individual cultures or cocultures of Rec-1 and Jurkat cells were stimulated for 6 h with scFv:G28-TRAIL (5 ng/ml) in the presence and absence of scFv:G28-2xFlag-GpL (2 µg/ml) as a competitor for CD40 binding of scFv:G28-TRAIL. Then, cells were harvested for Western Blot analysis of caspase processing. b) Cocultures of Rec-1 and Jurkat were challenged with the indicated concentrations of scFv:G28-TRAIL in the presence and absence of scFv:G28-2xFlag-GpL (2 µg/ml) as a competitor. The next day, cellular viability was analyzed using the MTT assay.

4.2.5. Analysis of CD40-dependent activity of TRAILR1- and TRAILR2-specific scFv:G28-TRAIL fusion proteins

As far as CD40-dependent activity of the scFv:G28-TRAIL fusion protein was confirmed, we extended our work to scFv:CD40-targeted TRAILmutR1 and TRAILmutR2 fusion proteins. The idea behind this work was to combine the TRAIL DRs-specific binding of these TRAIL mutants with the CD40-restricted activity of scFv:G28-TRAIL. Therefore, we analyzed the degree of CD40-anchoring dependent enhancement of apoptotic activity of both scFv:G28-TRAILmutR1 and scFv:G28-TRAILmutR2 in HT1080- and HeLa-CD40 transfectants. Similar to scFv:G28-TRAIL, both scFv:G28-TRAILmutR1 and scFv:G28-TRAILmutR2, revealed significant enhanced induction of cell death upon CD40 binding (Figure 19).

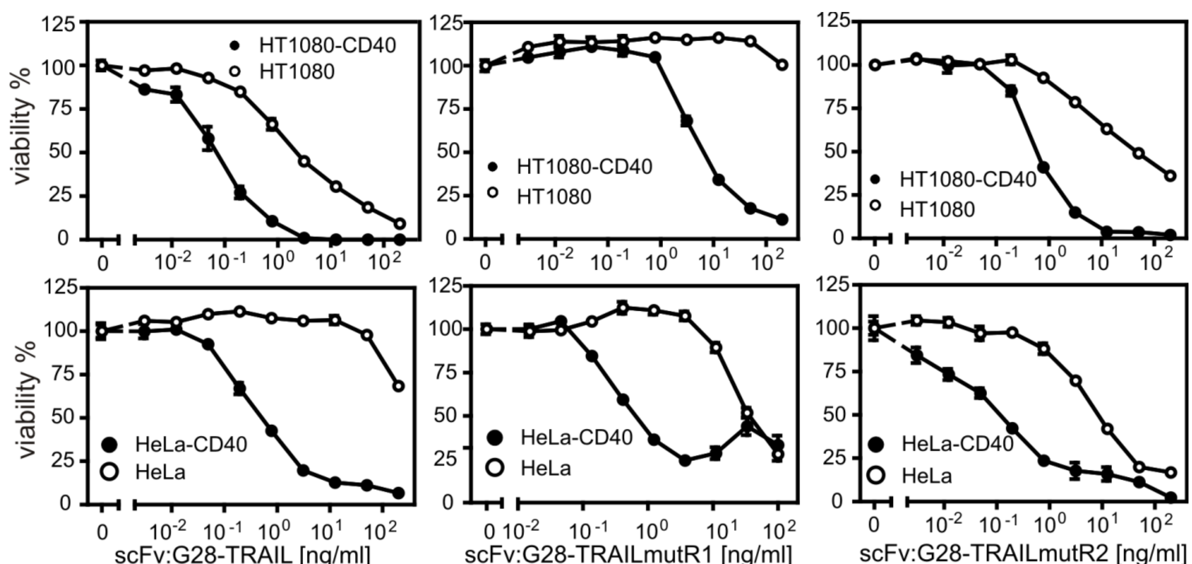


Figure 19: Analysis of apoptosis induction by scFv:G28 of TRAILR1- and TRAILR2-specific TRAIL mutants.

HT1080-CD40 and HeLa-CD40 transfectants along with the corresponding parental cell lines were seeded in 96-well plates. Next day, cells were challenged in triplicates with the indicated concentrations of scFv:G28-TRAIL, scFv:G28-TRAILmutR1 and scFv:G28-TRAILmutR2 in the presence of CHX (2.5 µg/ml). One day later, cellular viability was determined by CV staining.

Moreover, we also studied cell death-induction by scFv:CD40-targeted TRAILmutR1 and TRAILmutR2 fusion proteins on cell lines expressing endogenous CD40 (Figure 20). Consistent with the results obtained with the HeLa- and HT1080-CD40 transfectants, treatment of Mino and OVCAR3 with scFv:CD40-TRAILmutR1 and scFv:CD40-TRAILmutR2

in the absence and presence of scFv:G28-Fc-GpL also revealed CD40-restricted caspase activation and cell death induction (Figure 20, 21).

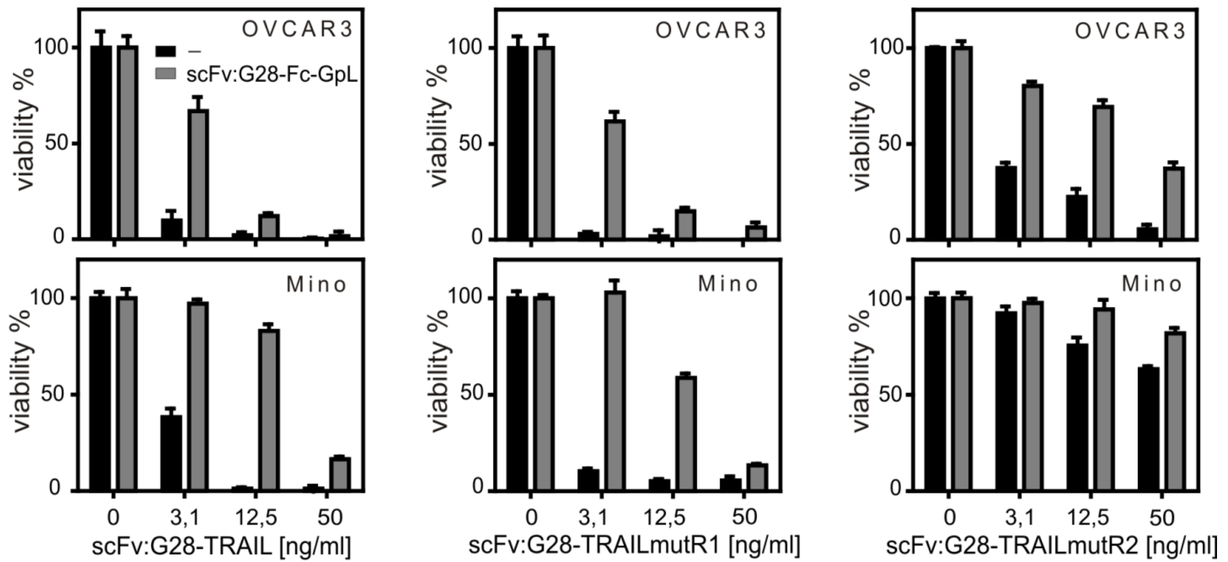


Figure 20: CD40-dependent induction of cell death by scFv:G28-TRAILmutR1 and scFv:G28-TRAILmutR1 in cell lines expressing endogenous CD40.

OVCAR3 and Mino cells were seeded in 96-well plates and challenged with the indicated concentrations of the various scFv:G28-TRAIL fusion proteins with and without pretreatment with scFv:G28-Fc-GpL (2 µg/ml) for 30 min. Cellular viability was determined the next day by CV staining in the case of OVCAR3 cells or by the MTT assay in the case of Mino cells. OVCAR3 cells were sensitized with CHX (2.5 µg/ml) to enhance apoptosis.

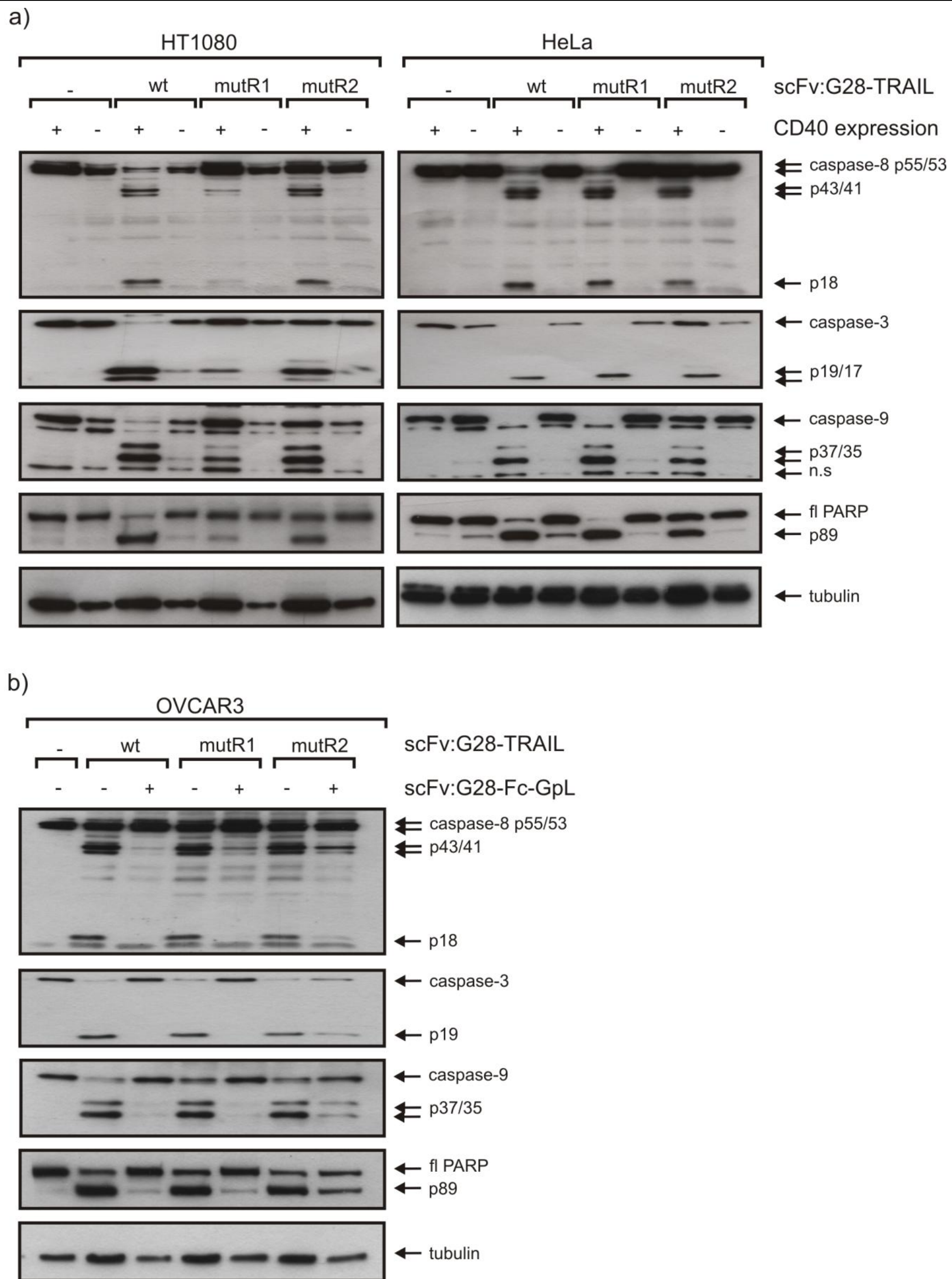


Figure 21: Analysis of CD40-dependent activation of caspases by scFv:G28 fusion proteins of TRAILR1- and TRAILR2-specific TRAIL mutants.

a) HeLa-CD40 and HT1080-CD40 transfectants and the corresponding control cells were stimulated for 5 h with 10 ng/ml of the various scFv:G28-TRAIL variants in the presence of CHX (2.5 µg/ml). b) OVCAR3 cells were similarly stimulated in the presence and absence of scFv:G28-Fc. Cells were harvested and total cell lysates were analyzed by Western Blot for the presence of the indicated proteins.

4.2.6. Analysis of effects of scFv:G28-TRAIL on CD40 signaling

4.2.6.1. scFv:G28-TRAIL triggers CD40 signaling in HeLa-CD40 cells

Some antibodies targeting members of the TNFRSF reveal agonistic activity after oligomerization or FcɣR binding (Dhein *et al.*, 1992; Li and Ravetch, 2011; Vonderheide and Glennie, 2013). Indeed, the parental antibody G28-5 used to construct the scFv domain of the scFv:G28 constructs acts as a potent CD40 agonist that rapidly crosslinks cell surface CD40 (Francisco *et al.*, 1996; Gaspari *et al.*, 1996). Therefore, we asked whether scFv:G28-TRAIL could act as an agonist of CD40. To answer this, we initially analyzed CD40 signaling in HeLa-CD40 transfectants. HeLa-CD40 and in parallel the parental HeLa cells were stimulated with scFv:G28-TRAIL and analyzed with respect to IL8 production as an indicator for CD40 activation. This experiment revealed that scFv:G28-TRAIL induces significant IL8 production only in HeLa-CD40 cells but not in HeLa cells (Figure 22a). It was shown before that the TRAIL DRs can also trigger robust IL8 production in HeLa, however, only upon sensitization for TRAILR1/2 signaling by CHX and blockade of apoptosis (Wajant *et al.*, 2000). Therefore, we avoided the proinflammatory IL8-inducing effect of the TRAIL domain of the scFv:G28-TRAIL fusion protein by screening IL8 production in the absence of CHX. To confirm the absence of TRAIL-induced IL8 production, a commercially available active form of TRAIL, Killer-TRAIL was screened in parallel with the scFv:G28-TRAIL fusion protein. Killer-TRAIL exerted only a weak IL8-inducing effect in HeLa and HeLa-CD40 cells in the absence of CHX (Figure 22a). On the other hand, both Killer-TRAIL and the scFv:G28-TRAIL fusion protein trigger apoptosis in HeLa-CD40 cells with more or less similar efficiency under CHX-sensitized conditions (Figure 22b). Therefore, the detected scFv:G28-TRAIL induced increase of IL8 in the absence of CHX can be attributed to the scFv:G28 domain of the scFv:G28-TRAIL fusion protein and is largely independent from TRAIL DR signaling.

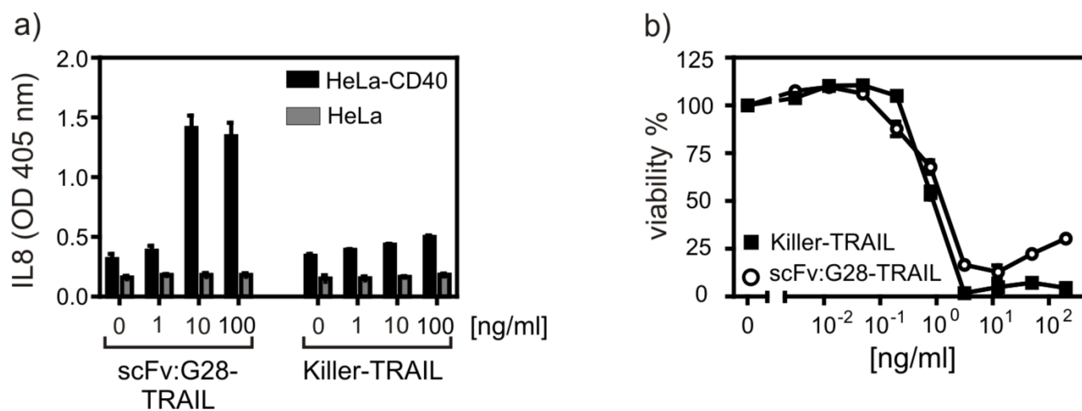


Figure 22: Stimulation of CD40 signaling by scFv:G28-TRAIL.

a) HeLa-CD40 transfectants along with the parental HeLa cells were seeded in 96-well plates and then stimulated in triplicates with the indicated concentrations of scFv:G28-TRAIL and Killer-TRAIL. After 8 h the supernatants were collected and analyzed by ELISA for the presence of IL8. b) HeLa-CD40 transfectants were seeded in 96-well plates and challenged in triplicates with the indicated concentrations of scFv:G28-TRAIL and Killer-TRAIL in the presence of CHX (2.5 µg/ml). CV staining was used to determine the cell viability the next day.

Furthermore, we analyzed the ability of the scFv:G28-TRAIL fusion protein to activate the classical NFκB pathway by detection of the degradation of IkBα. HeLa-CD40 cells were challenged with both scFv:G28-TRAIL and Killer-TRAIL in the absence of CHX and there was significant IκBα degradation after 7 min treatment with scFv:G28-TRAIL while Killer-TRAIL again showed no effect. Moreover, there was significant accumulation of p-JNK, indicative for activation of the proinflammatory JNK pathway, after 7 min in the case of stimulation with scFv:G28-TRAIL but not in response to Killer-TRAIL (Figure 23). This experiments thus provided additional evidence that the scFv:G28-TRAIL fusion protein stimulates CD40 signaling by help of its scFv:G28 domain independent from its TRAIL domain.

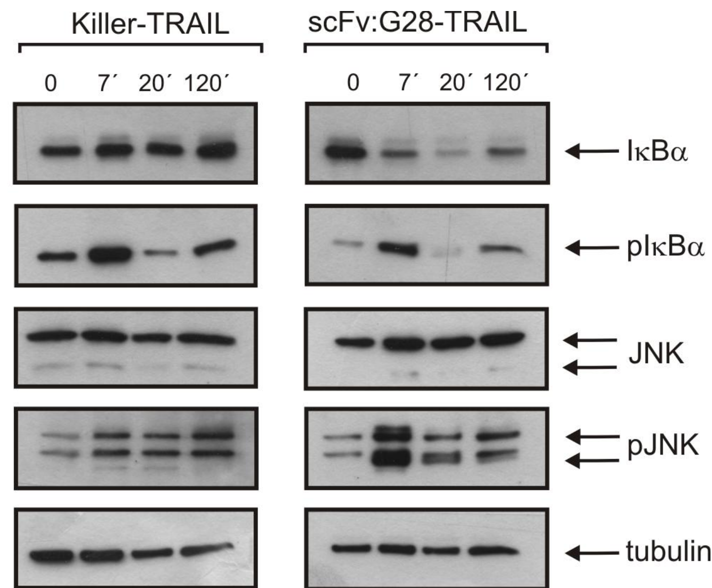


Figure 23: scFv:G28-TRAIL but not Killer-TRAIL robustly activates classical NFκB and JNK signaling in HeLa-CD40 cells.

HeLa-CD40 cells were challenged with 200 ng/ml of scFv:G28-TRAIL and Killer-TRAIL for 7, 20 and 120 min. The total cell lysates were then analyzed to detect the indicated proteins.

4.2.6.2. Stimulation of DCs maturation

One of the most important consequences of CD40 activation *in vivo* is the induction of DCs maturation which is required to enhance antitumor immunity (Vonderheide and Glennie, 2013). Therefore, we analyzed the ability of scFv:G28-TRAIL to trigger maturation of monocyte-derived DCs. Actually, iDCs are characterized by high expression of CD40, low expression of CD83 and CD86 and the absence of CD14 expression while mDCs are characterized by upregulation of the cell surface molecules CD83 and CD86 (Figure 24a) (Figdor *et al.*, 2004; Goxe *et al.*, 1998; Zhu *et al.*, 2005). Although DCs exhibit strong expression of CD40, there was no or only very low cell death-induction in iDCs and mDCs treated with scFv:G28-TRAIL fusion proteins (Figure 24b).

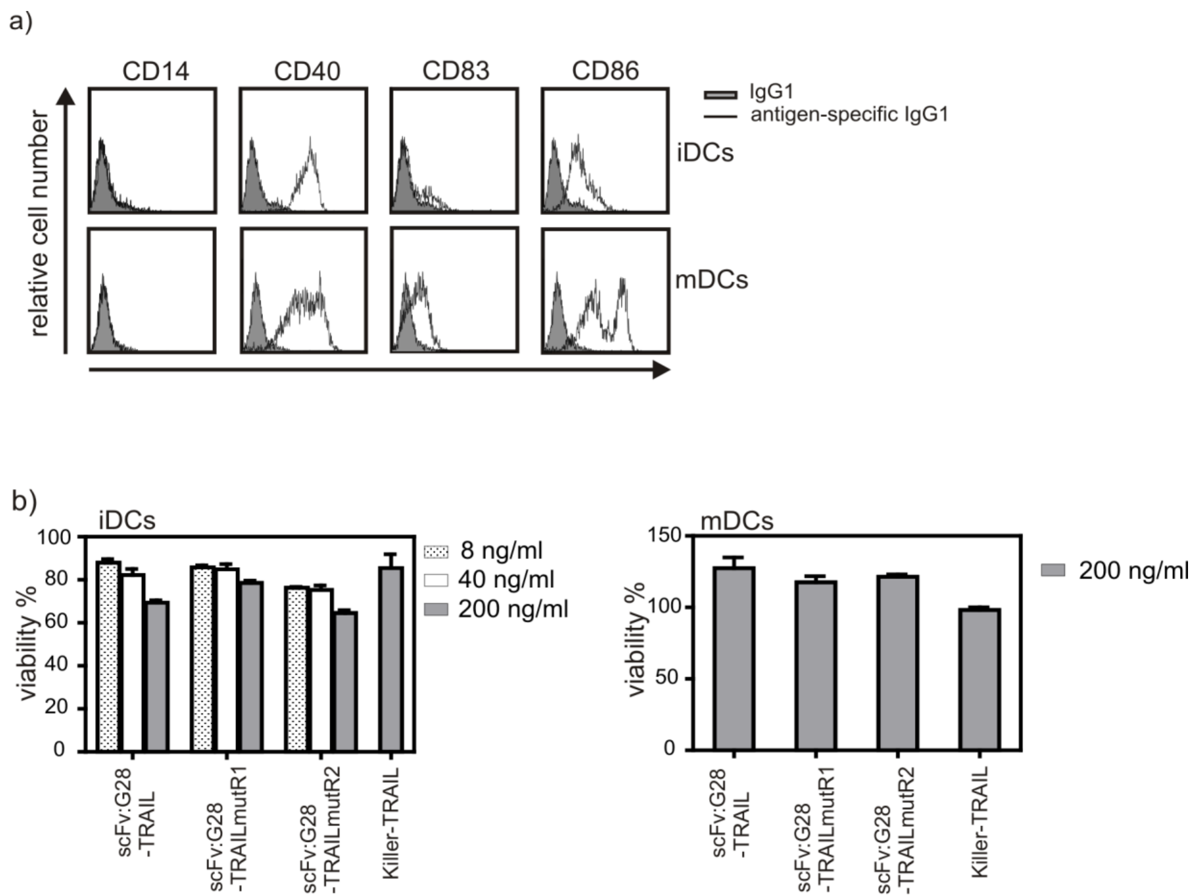


Figure 24: FACS screening of iDCs and mDCs and evaluation of cellular viability of scFv:G28-TRAIL-treated iDCs and mDCs.

a) FACS analysis of the cell surface expression of the indicated proteins on iDCs and mDCs that have been matured with the "gold standard" mixture for DCs maturation (20 ng/ml of TNF, 10 ng/ml of IL1β, 20 ng/ml of IL-6 and 1 µg/ml of PGE2). b) Both iDCs and mDCs were seeded in 96-well plates and stimulated with the indicated concentrations of scFv:G28-TRAIL variants, Killer-TRAIL or a cytotoxic cocktail (100 ng/ml of Fc-CD95L, 5 µg/ml of CHX and 0.5 % sodium azide). Cellular viability was analyzed in the next day using the MTT assay and normalized according to cells treated with the cytotoxic cocktail.

Next, we analyzed the ability of scFv:G28-TRAIL to stimulate DCs maturation and compared its effect to a mixture of TNF, IL6, IL1β and PGE2, the gold standard for *in vitro* DCs maturation. We found that all scFv:G28-TRAIL variants as well as a TNC trimerization domain-containing variant of the sole scFv:G28 domain (scFv:G28-TNC-GpL) were able to stimulate maturation of DCs. Indeed, the scFv:G28 domain-containing reagents were as effective as the gold standard in the upregulation of CD83 and CD86 and the induction of IL12 release. On the other hand, Killer-TRAIL was unable to mature DCs which proved that DCs maturation was dependent on the induction of CD40 signaling rather than TRAILR signaling (Figure 25a,b).

It is well known that LPS is a potent stimulant for DCs maturation. That is why it was worth to control that the detected DCs maturation induced by the scFv:G28-TRAIL fusion proteins were really related to the protein components rather than to any residual LPS contamination (Buelens *et al.*, 1997; Dowling *et al.*, 2008; Lapteva *et al.*, 2007). As implied by previous findings, LPS is heat resistance and PMB suppresses LPS effects (Morrison and Ryan, 1979; Rietschel *et al.*, 1993; Tynan *et al.*, 2012). According to these facts, we designed our strategy to control the absence of LPS contamination in our protein preparations. We found that the IL12 release triggered by LPS was not affected by heat inactivation while IL12-induction by the various scFv:G28-TRAIL samples was completely abrogated after heat inactivation (Figure 25b,c). Vice versa, the IL12 release induced by scFv:G28-TRAIL was not significantly affected by PMB treatment while the LPS-induced IL12 release was strongly inhibited after PMB treatment (Figure 25c). The observed reciprocal effect of heat-inactivation and PMB treatment on LPS- and scFv:G28-TRAIL-induced IL12 production in iDCs confirmed that the latter was mainly mediated by scFv:G28-TRAIL and not by unexpected LPS contaminations. Thus, conclusively, our results reveal that scFv:G28-TRAIL fusion proteins act as bifunctional molecules that not only induce cell death in tumor cells via their TRAIL domain upon CD40 binding but also stimulate DCs maturation via their scFv:G28 domain.

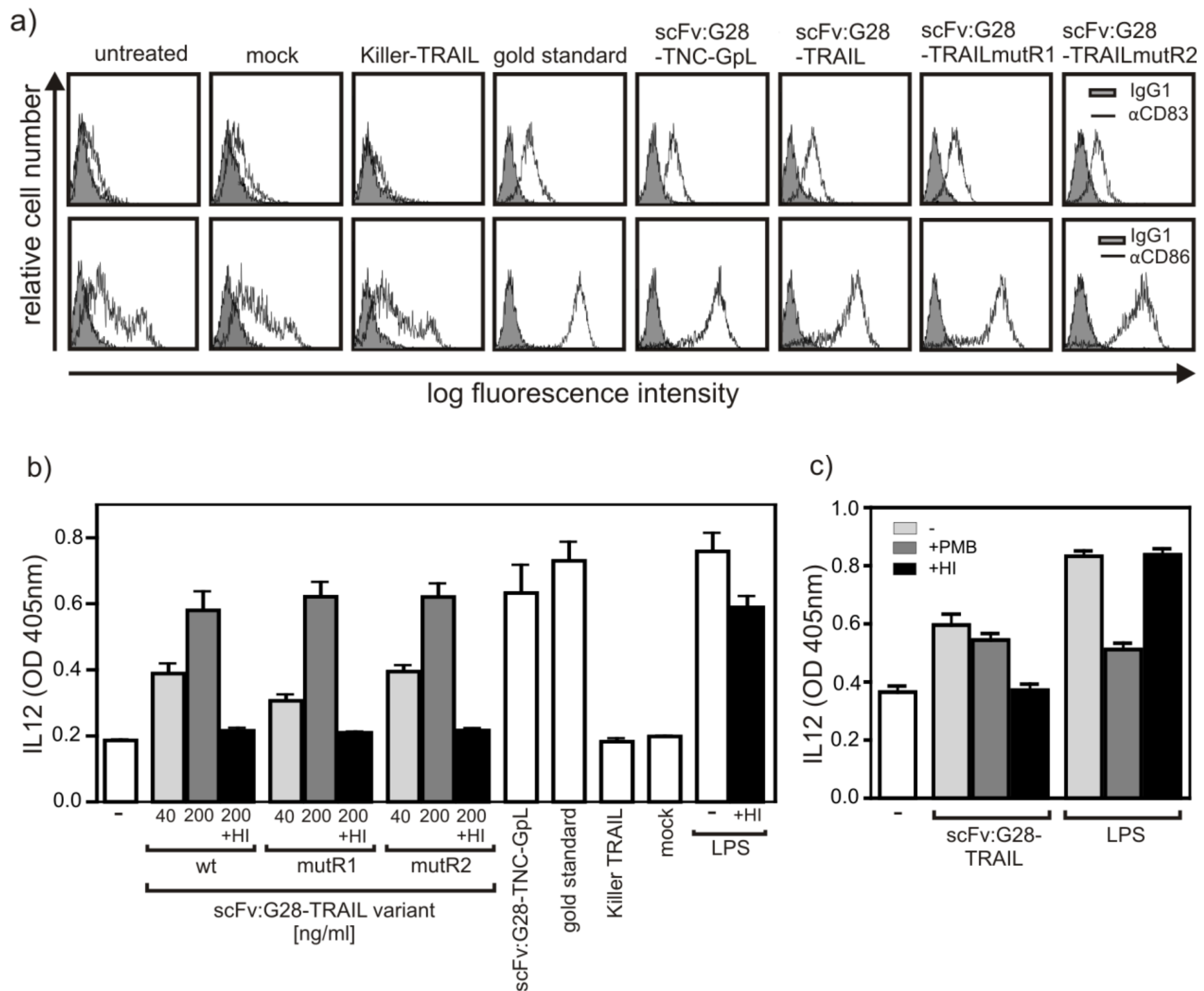


Figure 25: Induction of DCs maturation by scFV:G28-TRAIL fusion proteins.

a) iDCs were treated for 48 h with the various scFv:G28-TRAIL variants (200 ng/ml), scFv:G28-TNC-GpL (200 ng/ml), a tenascin-C trimerization domain-containing fusion protein of scFv:G28 and GpL or with 200 ng/ml of Killer-TRAIL. Mock- transfected HEK293 cells supernatant was included as a negative control for scFv:G28-TNC-GpL, scFv:G28-TRAILmutR1 and scFv:G28-TRAILmutR2. FACS analysis of the cell surface expression of CD83 and CD86 was used to evaluate DCs maturation. The different groups were compared to an untreated group and DCs matured with the “gold standard” mixture as a positive control. b) iDCs were seeded in 96-well plate and challenged in triplicates with the indicated concentrations of the various scFv:G28-TRAIL variants as well as with 200 ng/ml of Killer-TRAIL, the gold standard mixture, 100 ng/ml of LPS, scFv:G28-TNC-GpL and a control supernatant of mock-transfected HEK293 cells. As indicated, heat-stable endotoxin contaminations were controlled by heat-inactivated (HI) samples at 70°C for 30 min. Supernatants were collected from DCs after 24 h and analyzed for the production of IL12 by ELISA. c) iDCs were seeded in 96-well plate and treated in triplicates with scFv:G28-TRAIL (200 ng/ml) or 20 ng/ml of LPS. Where indicated, samples were heat-inactivated (HI) at 70°C for 30 min or treated with PMB (50 µg/ml). DCs supernatants were again assayed by ELISA after 24 h for IL12 production.

5. Discussion

5.1. Characterization of TRAILR1- and TRAILR2-specific TRAIL mutants

Despite the intensive worldwide research, cancer remains a devastating, often poorly treatable disease. The major challenge in treating cancer patients is to find antitumor therapies that exhibit selective antitumor activity only on tumor cells without significant cytotoxic effects on normal cells. TRAIL, which is a member of TNF ligand family, is well proved as a potent inducer of apoptosis in many tumor and transformed cells without major toxic effects on normal cells (Bernardi *et al.*, 2012; den Hollander *et al.*, 2013; Newsom-Davis *et al.*, 2009; van Dijk *et al.*, 2013). Therefore, it is no surprise that several groups and companies have developed and evaluated TRAIL/TRAIL DR-based cancer therapeutics. In fact, recombinant TRAIL and TRAIL DR-targeting antibodies are in phase I and II trials. As implied by these trials, TRAIL DR-targeting therapies are considered as a safe antitumor therapy either alone or in combination with other agents (Dimberg *et al.*, 2013; Hellwig and Rehm, 2012). TRAIL DR-targeting therapies have a good safety profile but show also only moderate antitumor activities (Dimberg *et al.*, 2013; Hellwig and Rehm, 2012).

TRAIL or TRAIL DRs-targeting therapies induce tumor cell death via binding to two TRAIL DRs; TRAILR1 and TRAILR2 (Newsom-Davis *et al.*, 2009). Therefore, the scientific research has unfailingly continued to differentiate between TRAILR1 and TRAILR2 signaling pathways by the development of TRAIL mutants that exhibit preferential binding to TRAILR1 or TRAILR2 (MacFarlane *et al.*, 2005b; Reis *et al.*, 2010). In this work, recently published TRAIL DR-specific mutants were investigated, one conferring specificity for TRAILR1 (TRAILmutR1) G131R/R149I/S159R/N199R/K201H/S215D and the other for TRAILR2 (TRAILmutR2) Y189Q/R191K/Q193R/H264R/I266L/D267Q (MacFarlane *et al.*, 2005b; Reis *et al.*, 2010). According to our results, TRAILmutR1 bound strongly to TRAILR1 and showed no binding to endogenous TRAILR2 or weak binding to TRAILR2 transiently expressed on HEK293 cells, as compared to TRAIL and TRAILmutR2 (Figure 4, 5). The weak detected binding of TRAILmutR1 to TRAILR2 transiently expressed in HEK293 cells may be related to the binding of TRAILmutR1 to TRAILR4 and/or heterocomplexes of TRAILR2 and TRAILR4 expressed endogenously on HEK293 cells (Figure 3). Concerning TRAILmutR2, it showed only significant binding to TRAILR2 either expressed transiently on HEK293 cells or endogenously with no detectable binding affinity to TRAILR1 transiently expressed on HEK293 (Figure 4, 5). The results of these binding studies were confirmed by *in vitro* binding studies with immobilized TRAILR1-Fc and TRAILR2-Fc and by IP analysis of cell lines expressing both endogenous TRAILR1 and TRAILR2 which revealed that TRAILmutR1 bound strongly only to TRAILR1 and on the other hand TRAILmutR2 displayed strong

binding only to TRALR2 with no detectable binding to TRAILR1 (Figure 6, 7). As implied by previous findings, TRAIL induces apoptosis in tumor cells by binding to TRAILR1 and/or TRAILR2 which leads to a research question whether the detected apoptosis in a given tumor is related to TRAILR1 or TRAILR2 (MacFarlane *et al.*, 2005a; Newsom-Davis *et al.*, 2009). As far as the preferential binding of TRAILmutR1 and TRAILmutR2 to TRAILR1/TRAILR2 was proved in this current work, these novel TRAIL mutants provide a novel option for future research that aims to differentiate between the biological responses of TRAILR1 or TRAILR2. Moreover, this preferential binding to TRAILR1 or TRAILR2 provides a possibility to bypass the potential side effects triggered from the activation of the TRAIL DR type not required for the induction of apoptosis in the targeted tumor cells (Lemke *et al.*, 2010; MacFarlane *et al.*, 2005a; Stadel *et al.*, 2010). Although so far unwanted side effects are not a major problem in TRAIL based therapies, this may change with the introduction of TRAIL DR-targeting reagents with higher activity and/or when novel potent sensitizers for TRAIL-induced cell death are used in combination therapies.

The selective binding of TRAILmutR1 and TRAILmutR2 translated into selective induction of cell death as revealed by the viability assays in cell lines expressing endogenous TRAIL DRs with the various trimeric Flag-TNC-TRAIL variants in the presence and absence of oligomerization with anti-Flag mAb M2 (Figure 8a). Indeed, these viability assays provided two important results/conclusions. The first one is that TRAILmutR1 was only able to induce cell death in cells expressing TRAILR1 such as HT29 cells with no significant cell death in cell lines expressing only or mainly TRAILR2 such as Jurkat and HT1080 cells, whereas TRAILmutR2 showed significant cell death-induction in all cell lines expressing TRAILR2. The second conclusion is that oligomerization with anti-Flag M2 mAb enhanced the ability of soluble trimeric TRAILR1- and TRAILR2-specific TRAIL to trigger cell death. Although the role of oligomerization with anti-Flag M2 mAb was proved before to enhance TRAIL/TRAILR2 cell death induction, the current work proved for the first time that TRAILR1-mediated cell death is also enhanced in response to oligomerized TRAILmutR1 (Berg *et al.*, 2007; Berg *et al.*, 2009).

Depending on previous findings that fusion of the human IgG1 Fc domain with soluble TNF ligands results in forced hexamerization and enhanced activity that replaces the need of secondary oligomerization with for example anti-Flag M2 mAb, we designed also Fc-TRAIL variants of the two TRAIL mutants (Holler *et al.*, 2003; Wyzgol *et al.*, 2009). The mutated hexameric TRAIL fusion proteins exhibited TRAILR1/2-selective cell death in the same manner as in case of the corresponding oligomerized trimeric TRAIL variants (Figure 8b). The oligomerization-mimiking effect of the hexameric structure of the Fc-TRAIL fusion proteins was particular obvious in Jurkat cells which responded with cell death in the

absence of oligomerization with anti-Flag M2 mAb in the case of the hexameric fusion proteins of TRAIL and TRAILmutR2 but not when their soluble trimeric counterparts were used (Figure 8a,b).

5.2. Targeting and activation of CD40 with scFv-TRAIL fusion proteins

Although recombinant soluble TRAIL and TRAIL DRs-targeting therapies are well proved as safe antitumor drugs through clinical phase I and II trials, they have a limitation which is their moderate antitumor activity (Dimberg *et al.*, 2013; Hellwig and Rehm, 2012). This moderate antitumor activity may be attributed to two reasons. TRAIL resistance in tumor cells is the first reason which may overcome by combining TRAIL DR-targeting therapies with appropriate drugs that re-sensitize the tumor cells and work against TRAIL resistance (Dimberg *et al.*, 2013; Hellwig and Rehm, 2012). The second reason is related to the inappropriate capability of TRAIL DR-targeting therapies to trigger the full apoptotic signaling activities of TRAILR1/TRAILR2 (Wajant *et al.*, 2013).

Despite the fact that the molecular explanation for the different activities of TNFRSF complexes with soluble and membrane-bound or oligomerized ligand trimers is still unclear, there are strong lines of evidence that supramolecular clusters of the initially formed trimeric ligand-receptor complexes play an important role. For example, TRAILR2-specific antibodies exhibit enhanced stimulation of TRAILR2-singaling provided that they are oligomerized or cross linked *in vivo* after binding to FcɣRs expressing cells (Adams *et al.*, 2008; Natoni *et al.*, 2007; Wilson *et al.*, 2011). Therefore, TRAILR2-agonistic antibodies display weak *in vivo* activity in tumors with a microenvironment with limited FcɣRs expression. Furthermore, the ability of soluble TNF ligands to stimulate the corresponding member of TNFRSF might be enhanced by secondary ligand oligomerization or artificial cell surface immobilization of the ligand (Wajant *et al.*, 2013; Wang *et al.*, 2013a). The latter can be achieved by generation of fusion proteins of soluble TRAIL with an antibody domain recognizing a cell surface-exposed antigen. The latter approach provides many advantages in the field of antitumor therapy. The first advantage is that anchoring to a cell surface antigen overcomes the poor receptor-stimulating activity of soluble TRAIL (Wajant *et al.*, 2001; Wang *et al.*, 2013a). The second one is that this type of TRAIL fusion proteins restricts TRAIL activity mainly to the tumor area and thus reduces the danger of triggering unwanted side effects on normal cells (Bremer *et al.*, 2004a; Trebing *et al.*, 2014). In addition, TRAIL fusion proteins represent a way to obtain bifunctional molecules which not only exhibit antitumor activity by stimulating DRs expressed on the tumor cells but also trigger or block signals emanating from the targeted antigen via the binding of the antibody domain to its cell surface antigen (Bremer *et al.*, 2005b).

The improvement of soluble TRAIL activity by anchoring the latter to cell surface antigens was proved by us and others (Trebing *et al.*, 2014; Wajant *et al.*, 2001; Wang *et al.*, 2013a). Therefore, we were motivated in this work to design fusion proteins of TRAIL and the TRAILR1- and TRAILR2-specific mutants with a scFv against CD40, scFv:G28-5 (Clark *et al.*, 1988). The idea behind using the agonistic CD40 antibody-derived scFv for the construction of TRAIL fusion proteins is that CD40 is highly expressed on the huge majority of B-cell malignancies and on many solid tumors (Banchereau *et al.*, 1994; Ottaiano *et al.*, 2002; Pellat-Deceunynck *et al.*, 1994; Wang *et al.*, 1997). Moreover, CD40 is also expressed on many immune cells especially DCs and represents a powerful target for cancer immunotherapy (Moran *et al.*, 2013).

Initially, the scFv:G28-TRAIL fusion proteins were investigated with respect to the functionality of both the TRAIL domain and the scFv:G28 domain. Regarding the TRAIL domain activity, we performed viability assays of the TRAIL fusion proteins along with the corresponding soluble trimeric TRAIL in Jurkat cells which are known to express only TRAILR2 (Sprick *et al.*, 2000). Our results indicated that the TRAIL domain of the fusion proteins behaved in a similar manner as the corresponding soluble trimeric TRAIL variants. Thus, both were able to induce cell death particular potent upon oligomerization with anti-Flag M2 mAb (Figure 12). To analyze the functionality of the scFv domain, we exploited the fact that total cellular binding of the scFv:G28-TRAIL fusion protein will increase in the case of high CD40 expression. Indeed, our results revealed enhanced binding of the scFv:G28-TRAIL fusion proteins to HeLa-CD40 and HT1080-CD40 cells as compared to their parental CD40-negative cells (Figure 13). The major aim of designing the scFv:G28-TRAIL fusion proteins was to enhance the antitumor activity of TRAIL in a CD40-dependent manner. This aim was proved through viability assays which elicited an enhanced apoptotic activity upon binding to CD40 (Figure 14, 15b). The explanation of the later is that the artificial immobilization of the scFv-TRAIL fusion proteins to CD40 constitutes a state for these molecules in which they mimic the membrane bound form of TRAIL and thus trigger robust caspase activation and strong cell death (Figure 14, 15b, 16).

It has been previously shown that scFv fusion proteins of soluble TRAIL exhibit a potent apoptotic effect in antigen negative bystander cells after binding to the antigen positive cells (Bremer *et al.*, 2004b). Therefore, it was worth in this current research to test the ability of scFv:G28-TRAIL fusion protein to induce such a paracrine effect in CD40-negative cells. Indeed, after anchoring of the TRAIL fusion protein on CD40-positive cells, the TRAIL domain induced cell death in CD40-negative cells without the need of exogenous oligomerization (Figure 18, 26).

According to our results, scFv:G28-TRAIL fusion proteins exhibited a strong CD40-dependent enhancement of apoptosis and cell death. To clarify whether both or only one of the two TRAIL DRs exhibit improved responsiveness to CD40-bound scFc:G28-TRAIL, we introduced the previously mentioned TRAILmutR1 and TRAILmutR2 in the TRAIL part that confer preference to TRAILR1 or TRAILR2. Here, we marshaled the first time investigation of TRAILR1- and TRAILR2-enhanced responsiveness to CD40-bound fusion proteins of TRAIL DRs specific mutants, TRAILmutR1 and TRAILmutR2 (Figure 19, 20, 21).

CD40 represents a tumor associated marker which is expressed on many tumor cells and also on many immune cells and mediates different biological functions after its activation. That is why we paid a great attention to analyze CD40 signaling induced by our scFv:G28-TRAIL fusion proteins. scFv:G28-TRAIL was able to induce CD40 signaling in CD40 expressing tumor cells as indicated by the induction of IL8 production and significant IκBα degradation in HeLa-CD40 cells (Figure 22a, 23). Moreover, we analyzed stimulation of CD40 signaling by scFv:G28-TRAIL fusion proteins in monocytes-derived DCs. Despite the strong CD40 expression on both iDCs and mDCs, our scFv:G28-TRAIL fusion proteins induced weak or no significant cell death in iDCs and mDCs (Figure 24). Interestingly, the various scFv:G28-TRAIL fusion proteins triggered maturation of iDCs as indicated by the upregulation of maturation markers such as CD83 and CD86 and the induction of IL12 production (Figure 25). As it is well known that LPS is a potent trigger of DCs maturation, we controlled that the strong activation of DCs maturation by the CD40-targeted TRAIL fusion proteins was indeed related to the protein component and not to LPS contaminations (Figure 25b,c) (Dowling *et al.*, 2008; Lapteva *et al.*, 2007).

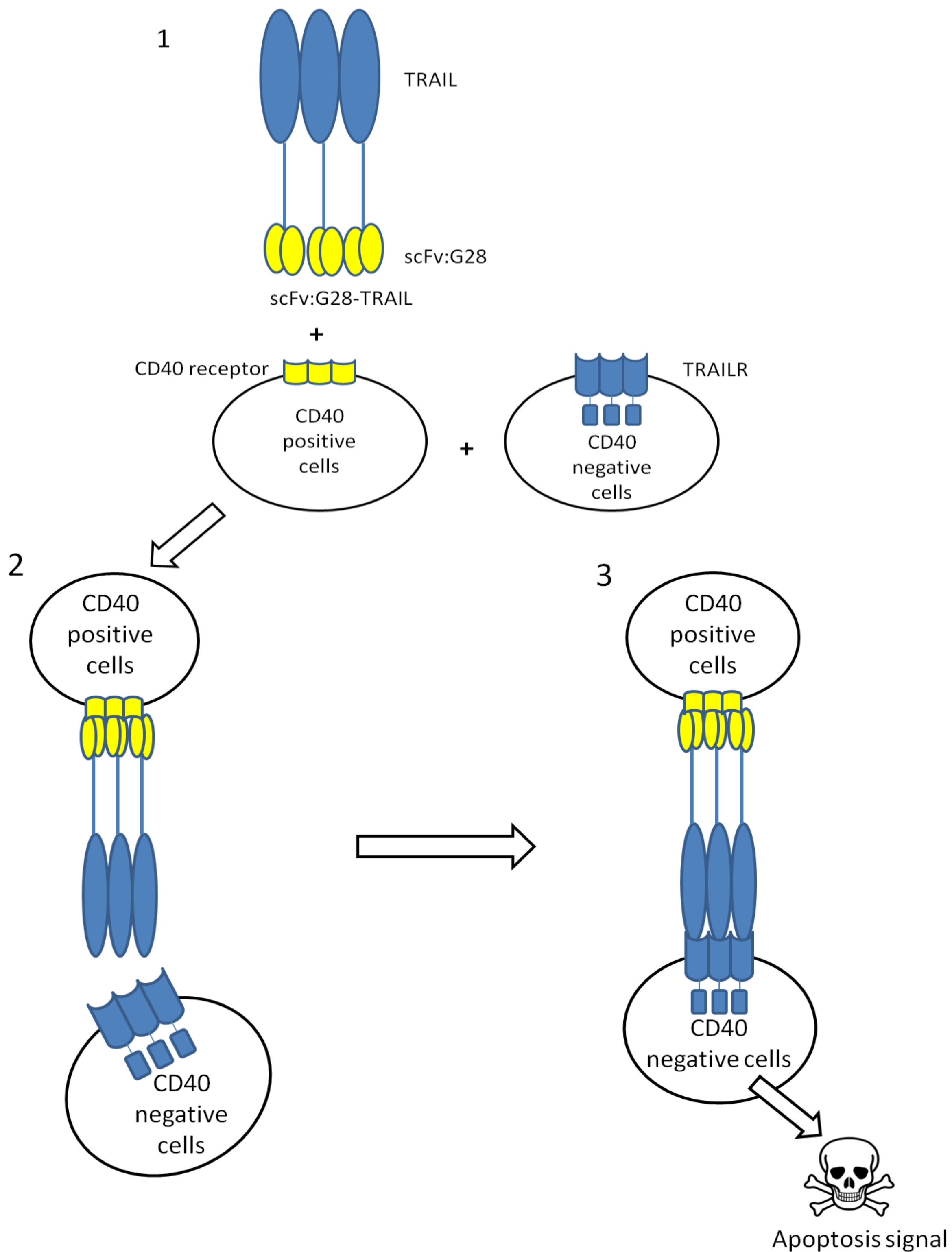


Figure 26: The paracrine effect of CD40-bound scFv:G28-TRAIL on TRAIL sensitive CD40-negative bystander cells.

In the presence of both CD40-positive cells and TRAIL sensitive CD40-negative cells, the scFv:G28 domain of the scFv:G28-TRAIL fusion protein will bind to CD40 on CD40-positive cells. Then, the free TRAIL domain of this artificially immobilized scFv:G28-TRAIL will mimic the activity of the membrane bound form of TRAIL that binds with full capacity to TRAIL DRs expressed on the surface of the neighboring CD40-negative cells and induces apoptosis.

As revealed by our results, scFv:G28 fusion proteins with soluble TRAIL variants have two advantages. The first one is the potential to limit the off-target effects of highly active TRAIL variants due to the CD40-binding dependent enhancement of the otherwise low activity of soluble TRAIL. This property attracts great attention in the field of TRAIL-based therapy because combination of TRAIL with other drugs confers the risk of the potential sensitization of normal cells for TRAIL-induced apoptosis (Dimberg *et al.*, 2013; Hellwig and Rehm, 2012). Actually, CD40-binding activity of scFv:G28-TRAIL fusion proteins exploits the advantage that CD40 is distributed on many tumor cells as well as on immune cells such as DCs (Moran *et al.*, 2013). Moreover, we proved also the CD40-dependent enhancement of the activity with TRAIL mutants that preferentially stimulate TRAILR1 or TRAILR2 (Figure 19, 20, 21). Therefore, needless to say that the combination of using such specific TRAIL mutants with CD40 targeting principally provide more safe antitumor therapies by attenuating the potential TRAIL-related side effects that are mediated by one TRAIL DR other than the required for apoptosis. The second advantage of these fusion proteins is the ability to trigger CD40 signaling (Figure 22a, 23, 25). Agonistic CD40 antibodies are currently investigated in clinical trials for cancer therapy due to their stimulatory effects on APCs and cytotoxic myeloid cells (Vonderheide and Glennie, 2013). Thus, conclusively, scFv:G28-TRAIL fusion proteins represent novel bifunctional molecules which are not only able to stimulate TRAIL DRs and mediate apoptosis in a CD40-dependent manner via the TRAIL domain but also stimulate CD40 signaling and activate DCs via the scFv:G28 domain (Figure 27).

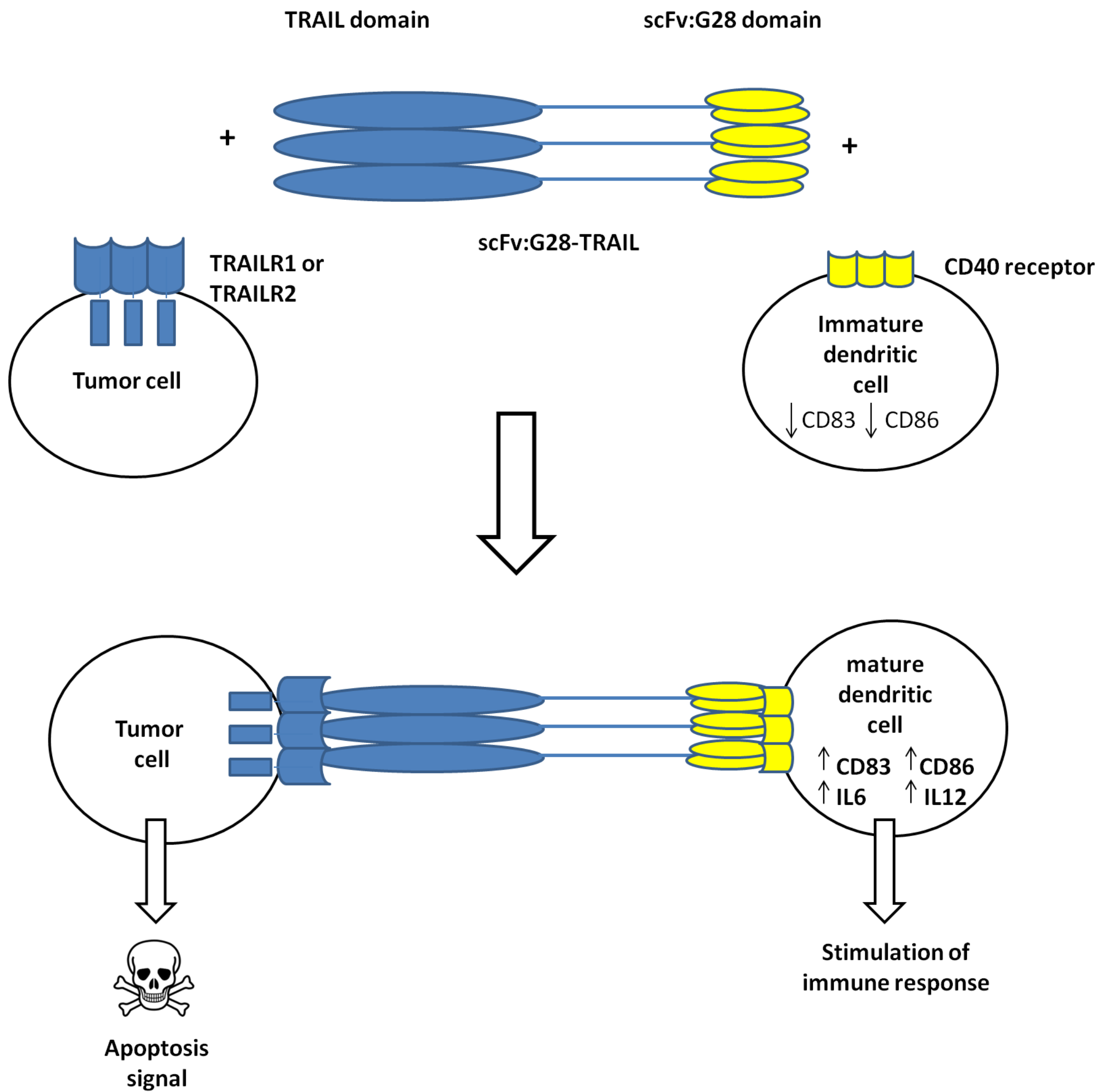


Figure 27: scFv:G28-TRAIL fusion protein represents bifunctional molecules.

TRAIL domain of scFv:G28-TRAIL fusion protein binds to the TRAIL DRs expressed on tumor cells leading to the activation of the apoptotic signal. Not only that but also scFv:G28 domain binds to CD40 on CD40 expressing cells such as iDCs leading to their maturation and stimulation of the immune response.

6. Summary

TRAIL is a member of TNF superfamily and mediates apoptosis by binding to two DRs, TRAILR1 and TRAILR2. Despite the fact that there are other TRAILRs, TRAILR1 and TRAILR2 receive the major research interest due to their ability to trigger apoptosis and their possible use as targets in tumor therapy. Due to the potential advantages of TRAILR1- or TRAILR2-specific targeting, we investigated recently published TRAIL DR-specific mutants, one conferring specificity for TRAILR1 (TRAILmutR1) and one for TRAILR2 (TRAILmutR2). It was well proved in this work that TRAILmutR1 shows specific binding to TRAILR1 and no specific binding to TRAILR2. TRAILmutR2 vice versa shows specific binding to TRAILR2 and no significant binding to TRAILR1. Moreover, these mutants were able to induce caspase activation and cell death in a TRAILR1/2-specific manner. Moreover, the enhancement of TRAILR2-induced apoptosis by secondary oligomerization of soluble wild-type TRAIL was confirmed for the TRAILR2-specifc TRAIL mutant and similar findings were made with the TRAILR1-specific TRAIL mutant.

The soluble form of TRAIL exhibits weak apoptotic activity as compared to transmembrane TRAIL. Therefore, there is the challenge in clinical research to improve the activity of soluble TRAIL. A second strategy besides the above mentioned oligomerization to improve soluble TRAIL activity is anchoring of the molecule to the cell surface, e.g. through the genetic fusion with a scFv domain recognizing a cell surface antigen. In this work, we generated fusion proteins of TRAIL, TRAILmutR1 and TRAILmutR2 with a scFv recognizing CD40 (scFv:G28). Initially, we analyzed the functionality of both the TRAIL domain and the scFv:G28 domain of the corresponding fusion proteins. TRAIL functionality was well proved through its ability to induce cell death in TRAIL sensitive cells such as Jurkat cells, provided that scFv:G28-TRAIL fusion proteins were oligomerized by anti-Flag mAb M2. Concerning the scFv:G28 domain, the fusion proteins showed enhanced binding affinity to cell lines expressing CD40 as compared to their parental CD40-negative cells. Consistent with previous studies investigating TRAIL fusion proteins with other cell surface antigen-targeting scFvs, the scFv:G28 fusion proteins with TRAIL, TRAILmutR1 and TRAILmutR2 showed enhanced induction of cell death in a CD40-dependent manner. Moreover, our results revealed that these fusion proteins have a significant paracrine apoptotic effect on CD40-negative bystander cells upon anchoring to D40-positive cells which are TRAIL resistant. Thus, the current work provides for the first time scFv fusion proteins of TRAIL and TRAILR1- and TRAILR2-specific TRAIL mutants with CD40-restricted activity. These fusion proteins provide the advantage of attenuating the off-target effects and the potential side effects of per se

highly active TRAIL variants on one hand due to the CD40-binding dependent enhancement of activity and on the other hand due to the differential use of TRAILR1 and TRAILR2.

CD40 represents a tumor associated marker which is expressed on many tumor cells but also on immune cells. Therefore, the last part of this work focused on the analysis of the ability of scFv:G28-TRAIL fusion proteins to induce CD40 signaling both in tumor cells and also in immune cells. It turned out that the scFv:G28-TRAIL fusion proteins are able to induce CD40 signaling in CD40-positive tumor cells but especially also in immune cells such as iDCs leading to their maturation and further activation of immune responses.

Taken together, this work provides novel bifunctional scFv-TRAIL fusion proteins which combine the induction of apoptosis via TRAIL DR with stimulation of CD40 signaling which possibly enhances antitumor immunity.

7. Zusammenfassung

TRAIL ist ein Mitglied der TNF-Superfamilie und vermittelt Apoptose durch die Aktivierung der Todesrezeptoren, TRAILR1 und TRAILR2. Obwohl es weitere TRAIL-Rezeptoren gibt, liegt das Hauptaugenmerk auf den beiden Apoptose induzierenden Rezeptoren TRAILR1 und TRAILR2 auf Grund ihrer möglichen Anwendung in der Tumortherapie. Wegen der möglichen Vorteile eines spezifischen TRAILR1- und TRAILR2-Targetings, haben wir kürzlich publizierte TRAIL-Todesrezeptor spezifische TRAIL Mutanten untersucht, von denen eine spezifisch für TRAILR1 (TRAILmutR1) und die andere spezifisch für TRAILR2 (TRAILmutR2) ist. Es konnte in dieser Arbeit sehr gut belegt werden, dass TRAILmutR1 spezifisch an TRAILR1 bindet und keine Bindung an TRAILR2 zeigte. Dem entsprechend zeigte die Variante TRAILmutR2 nur eine spezifische Bindung an TRAILR2 und keine signifikante Bindung an TRAILR1. Des Weiteren waren die Mutanten in der Lage, die Caspase-Aktivierung und den Zelltod TRAILR1/2-abhängig zu induzieren. Außerdem konnte eine Erhöhung der TRAILR2-induzierten Apoptose durch eine sekundäre Oligomerisierung der TRAILR2-spezifische TRAIL-Mutante erzielt werden. Ähnliche Ergebnisse zeigte die TRAILR1-spezifische TRAIL-Mutante.
Um die Aktivität des löslichen TRAIL Oligomerisierung unabhängig zu erhöhen, wurden in dieser Arbeit TRAIL-Fusionsproteine mit einem scFv (scFv:G28), der CD40 erkennt generiert. In Übereinstimmung mit früheren Studien, die mit TRAIL-Fusionsproteinen von anderen Zelloberflächenantigen-spezifischen scFvs wurden, zeigten die CD40-spezifischen scFv:G28 Fusionsproteine mit TRAIL, TRAILmutR1 und TRAILmutR2 eine verstärkte CD40-abhängige Induktion des Zelltods. Darüber hinaus zeigten unsere Ergebnisse, dass diese Fusionsproteine nach Bindung an CD40-positive Zellen einen parakrinen apoptotischen Effekt, auf umliegende CD40-negative Zellen haben. Diese Arbeit beschreibt somit zum ersten Mal scFv-TRAIL Fusionsproteine mit einer CD40-abhängigen TRAILR1- und TRAILR2-spezifischen Aktivität.
CD40 repräsentiert einen tumorassoziierten Marker, der in vielen Tumorzellen aber auch in Zellen des Immunsystems exprimiert wird. Aus diesem Grund fokussierte sich der zweite Teil dieser Arbeit auf die Analyse der Fähigkeit der scFv:G28-TRAIL Fusionsproteine, CD40-Signaling sowohl in Tumor- als auch in Immunzellen zu stimulieren. Es konnte festgestellt werden, dass die scFv:G28-TRAIL Fusionsproteine in der Lage sind, CD40-Signaling in CD40-positiven Tumorzellen, aber auch in Immunzellen, z.B. in iDCs, in denen die ScFv-TRAIL Fusionsproteine die Reifung und Aktivierung induzieren ohne Zelltod auszulösen.
Zusammengefasst beschreibt diese Arbeit neue bifunktionelle scFv-TRAIL Fusionsproteine, die die Induktion der Apoptose via TRAIL-Todesrezeptoren und die Stimulation des

kostimulatorischen CD40-Moleküls kombinieren, was zu einer synergistischen dualen Antitumor-Aktivität führen kann.

8. References

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9. Annex

9.1 DNA sequences

9.1.1. Flag-TNC-TRAIL

Signal peptide:	NT 1-84	aa 1-28
Flag:	NT 85-108	aa 29-36
TNC:	NT 115-204	aa 39-68
TRAIL:	NT 241-801	aa 81-267

```
  1 atg aac ttc ggg ttc agc ttg att ttc ctg gtc ctg gtg ctg aag ggc gtg cag
  1  M   N   F   G   F   S   L   I   F   L   V   L   V   L   K   G   V   Q
 55 tgc gag gtg aag ctg gtg cca cgc gga tcc gat tac aaa gac gat gac gat aaa
 19  C   E   V   K   L   V   P   R   G   S   D   Y   K   D   D   D   D   K
109 gat atc gcc tgt ggc tgt gcg gct gcc cca gac atc aag gac ctg ctg agc aga
 37  D   I   A   C   G   C   A   A   A   P   D   I   K   D   L   L   S   R
163 ctg gag gag ctg gag ggg ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt
 55  L   E   E   L   E   G   L   V   S   S   L   R   E   Q   G   T   G   G
217 ggg tct ggc ggc cgc ggt gaa ttc acc tct gag gaa acc att tct aca gtt caa
 73  G   S   G   G   R   G   E   F   T   S   E   E   T   I   S   T   V   Q
271 gaa aag caa caa aat att tct ccc cta gtg aga gaa aga ggt cct cag aga gta
 91  E   K   Q   Q   N   I   S   P   L   V   R   E   R   G   P   Q   R   V
325 gca gct cac ata act ggg acc aga gga aga agc aac aca ttg tct tct cca aac
109  A   A   H   I   T   G   T   R   G   R   S   N   T   L   S   S   P   N
379 tcc aag aat gaa aag gct ctg ggc cgc aaa ata aac tcc tgg gaa tca tca agg
127  S   K   N   E   K   A   L   G   R   K   I   N   S   W   E   S   S   R
433 agt ggg cat tca ttc ctg agc aac ttg cac ttg agg aat ggt gaa ctg gtc atc
145  S   G   H   S   F   L   S   N   L   H   L   R   N   G   E   L   V   I
487 cat gaa aaa ggg ttt tac tac atc tat tcc caa aca tac ttt cga ttt cag gag
163  H   E   K   G   F   Y   Y   I   Y   S   Q   T   Y   F   R   F   Q   E
541 gaa ata aaa gaa aac aca aag aac gac aaa caa atg gtc caa tat att tac aaa
181  E   I   K   E   N   T   K   N   D   K   Q   M   V   Q   Y   I   Y   K
595 tac aca agt tat cct gac cct ata ttg ttg atg aaa agt gct aga aat agt tgt
199  Y   T   S   Y   P   D   P   I   L   L   M   K   S   A   R   N   S   C
649 tgg tct aaa gat gca gaa tat gga ctc tat tcc atc tat caa ggg gga ata ttt
217  W   S   K   D   A   E   Y   G   L   Y   S   I   Y   Q   G   G   I   F
703 gag ctt aag gaa aat gac aga att ttt gtt tct gta aca aat gag cac ttg ata
235  E   L   K   E   N   D   R   I   F   V   S   V   T   N   E   H   L   I
757 gac atg gac cat gaa gcc agt ttt ttc ggg gcc ttt tta gtt ggc taa
253  D   M   D   H   E   A   S   F   F   G   A   F   L   V   G   -
```

9.1.2. Flag-TNC-TRAILmutR1

Signal peptide:	NT 1-84	aa 1-28
Flag:	NT 85-108	aa 29-36
TNC:	NT 115-204	aa 39-68
TRAILmutR1:	NT 241-801	aa 81-267

Mutations are indicated in **green color**.

```
  1 atg aac ttc ggg ttc agc ttg att ttc ctg gtc ctg gtg ctg aag ggc gtg cag
  1  M   N   F   G   F   S   L   I   F   L   V   L   V   L   K   G   V   Q
 55 tgc gag gtg aag ctg gtg cca cgc gga tcc gat tac aaa gac gat gac gat aaa
 19  C   E   V   K   L   V   P   R   G   S   D   Y   K   D   D   D   D   K
```

109 gat atc gcc tgt ggc tgt gcg gct gcc cca gac atc aag gac ctg ctg agc aga
37 D I A C G C A A A P D I K D L L S R
163 ctg gag gag ctg gag ggg ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt
55 L E E L E G L V S S L R E Q G T G G
217 ggg tct ggc ggc cgc ggt gaa ttc aca tct gag gaa acc att agc acc gtc cag
73 G S G G R G E F T S E E T I S T V Q
271 gag aaa cag cag aac att tca ccc ctc gtc cgg gaa cgg gga cca cag aga gtg
91 E K Q Q N I S P L V R E R G P Q R V
325 gcc gct cat att act ggc aca cgg **agg** cga tcc aat aca ctg agt agc ccc aac
109 A A H I T G T R **R** R S N T L S S P N
379 tcc aaa aac gaa aag gca ctg ggc **atc** aaa atc aat tca tgg gag agt agt agg
127 S K N E K A L G **I** K I N S W E S S R
433 **cgg** gga cat tcc ttt ctg tcc aac ctc cat ctc cga aac ggc gaa ctg gtg att
145 **R** G H S F L S N L H L R N G E L V I
487 cac gag aag ggc ttt tac tac atc tac tcc cag acc tac ttc aga ttt cag gag
163 H E K G F Y Y I Y S Q T Y F R F Q E
541 gag atc aag gaa **cgg** acc **cac** aac gac aaa cag atg gtc cag tac atc tac aaa
181 E I K E **R** T **H** N D K Q M V Q Y I Y K
595 tac acc **gac** tac ccc gac cct atc ctg ctc atg aaa tcc gct aga aat tca tgc
199 Y T **D** Y P D P I L L M K S A R N S C
649 tgg agc aag gat gcc gaa tac gga ctg tac tca atc tac cag ggc ggc att ttt
217 W S K D A E Y G L Y S I Y Q G G I F
703 gaa ctg aaa gag aac gat cgg atc ttc gtg tct gtc aca aac gaa cac ctc atc
235 E L K E N D R I F V S V T N E H L I
757 gac atg gat cac gag gcc tca ttc ttt ggc gct ttt ctg gtg gga tga
253 D M D H E A S F F G A F L V G -

9.1.3. Flag-TNC-TRAILmutR2

Signal peptide:	NT 1-84	aa 1-28
Flag:	NT 85-108	aa 29-36
TNC:	NT 115-204	aa 39-68
TRAILmutR2:	NT 241-801	aa 81-267

Mutations are indicated in **green color**.

1 atg aac ttc ggg ttc agc ttg att ttc ctg gtc ctg gtg ctg aag ggc gtg cag
1 M N F G F S L I F L V L V L K G V Q
55 tgc gag gtg aag ctg gtg cca cgc gga tcc gat tac aaa gac gat gac gat aaa
19 C E V K L V P R G S D Y K D D D D K
109 gat atc gcc tgt ggc tgt gcg gct gcc cca gac atc aag gac ctg ctg agc aga
37 D I A C G C A A A P D I K D L L S R
163 ctg gag gag ctg gag ggg ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt
55 L E E L E G L V S S L R E Q G T G G
217 ggg tct ggc ggc cgc ggt gaa ttc acc agc gaa gag aca atc agc acc gtg cag
73 G S G G R G E F T S E E T I S T V Q
271 gaa aag cag cag aac atc agc ccc ctc gtg cgc gaa agg ggc cct cag aga gtg
91 E K Q Q N I S P L V R E R G P Q R V
325 gcc gcc cac atc act ggc acc aga ggc aga agc aac acc ctg agc agc ccc aac
109 A A H I T G T R G R S N T L S S P N
379 agc aag aac gag aag gcc ctg ggc cgg aag atc aac agc tgg gag tct agc aga
127 S K N E K A L G R K I N S W E S S R
433 agc ggc cac agc ttt ctg agc aac ctg cac ctg aga aac ggc gag ctc gtg atc
145 S G H S F L S N L H L R N G E L V I
487 cac gag aag ggc ttc tac tac atc tac agc cag acc **cag** ttc **aag** ttc **cgg** gaa
163 H E K G F Y Y I Y S Q T **Q** F **K** F **R** E

```
541 gag atc aaa gag aat acc aag aac gac aag cag atg gtg cag tac atc tat aag
181  E   I   K   E   N   T   K   N   D   K   Q   M   V   Q   Y   I   Y   K
595 tac acc agc tac ccc gac ccc atc ctg ctg atg aag tcc gcc cgg aac agc tgc
199  Y   T   S   Y   P   D   P   I   L   L   M   K   S   A   R   N   S   C
649 tgg tcc aag gat gcc gag tac ggc ctg tac agc atc tac cag ggc ggc atc ttc
217  W   S   K   D   A   E   Y   G   L   Y   S   I   Y   Q   G   G   I   F
703 gag ctg aaa gag aac gac cgg atc ttc gtg tcc gtg acc aac gag cgg ctg ctg
235  E   L   K   E   N   D   R   I   F   V   S   V   T   N   E   R   L   L
757 cag atg gac cac gag gcc agc ttt ttc ggc gcc ttc ctc gtg gga tag
253  Q   M   D   H   E   A   S   F   F   G   A   F   L   V   G   -
```

9.1.4. GpL-Flag-TNC-TRAIL

GpL:	NT 1-555	aa 1-185
Flag:	NT 571-594	aa 191-198
TNC:	NT 601-690	aa 201-230
TRAIL:	NT 727-1287	aa 243-429

```
  1 atg ggc gtc aaa gtc ctg ttt gcc ctc atc tgt att gct gtc gct gag gct aaa
  1  M   G   V   K   V   L   F   A   L   I   C   I   A   V   A   E   A   K
 55 cca acc gag aat aat gag gat ttc aac atc gtg gct gtg gca tcc aat ttt gct
 19  P   T   E   N   N   E   D   F   N   I   V   A   V   A   S   N   F   A
109 acc acc gac ctc gat gcc gat cgg gga aaa ctg cct ggc aaa aaa ctg ccc ctg
 37  T   T   D   L   D   A   D   R   G   K   L   P   G   K   K   L   P   L
163 gaa gtg ctg aaa gag atg gag gcc aac gct aga aaa gct ggc tgt act aga gga
 55  E   V   L   K   E   M   E   A   N   A   R   K   A   G   C   T   R   G
217 tgt ctc atc tgc ctg tcc cac atc aag tgt acc cca aaa atg aaa aaa ttc atc
 73  C   L   I   C   L   S   H   I   K   C   T   P   K   M   K   K   F   I
271 cct ggc cgg tgt cac aca tac gag ggc gac aag gaa tct gct cag ggc gga atc
 91  P   G   R   C   H   T   Y   E   G   D   K   E   S   A   Q   G   G   I
325 gga gag gct att gtg gat att cct gaa att cct gga ttc aag gac ctg gag cct
109  G   E   A   I   V   D   I   P   E   I   P   G   F   K   D   L   E   P
379 atg gaa cag ttt atc gcc cag gtg gac ctc tgt gtc gat tgt aca act ggc tgc
127  M   E   Q   F   I   A   Q   V   D   L   C   V   D   C   T   T   G   C
433 ctg aaa ggg ctg gcc aat gtc cag tgt agt gac ctg ctg aaa aaa tgg ctg ccc
145  L   K   G   L   A   N   V   Q   C   S   D   L   L   K   K   W   L   P
487 cag aga tgt gcc act ttc gcc tct aaa att cag ggc cag gtc gac aaa atc aaa
163  Q   R   C   A   T   F   A   S   K   I   Q   G   Q   V   D   K   I   K
541 ggc gct gga gga gac tct gga gct gga tcc gat tac aaa gac gat gac gat aaa
181  G   A   G   G   D   S   G   A   G   S   D   Y   K   D   D   D   D   K
595 gat atc gcc tgt ggc tgt gcg gct gcc cca gac atc aag gac ctg ctg agc aga
199  D   I   A   C   G   C   A   A   A   P   D   I   K   D   L   L   S   R
649 ctg gag gag ctg gag ggg ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt
217  L   E   E   L   E   G   L   V   S   S   L   R   E   Q   G   T   G   G
703 ggg tct ggc ggc cgc ggt gaa ttc acc tct gag gaa acc att tct aca gtt caa
235  G   S   G   G   R   G   E   F   T   S   E   E   T   I   S   T   V   Q
757 gaa aag caa caa aat att tct ccc cta gtg aga gaa aga ggt cct cag aga gta
253  E   K   Q   Q   N   I   S   P   L   V   R   E   R   G   P   Q   R   V
811 gca gct cac ata act ggg acc aga gga aga agc aac aca ttg tct tct cca aac
271  A   A   H   I   T   G   T   R   G   R   S   N   T   L   S   S   P   N
865 tcc aag aat gaa aag gct ctg ggc cgc aaa ata aac tcc tgg gaa tca tca agg
289  S   K   N   E   K   A   L   G   R   K   I   N   S   W   E   S   S   R
919 agt ggg cat tca ttc ctg agc aac ttg cac ttg agg aat ggt gaa ctg gtc atc
307  S   G   H   S   F   L   S   N   L   H   L   R   N   G   E   L   V   I
973 cat gaa aaa ggg ttt tac tac atc tat tcc caa aca tac ttt cga ttt cag gag
325  H   E   K   G   F   Y   Y   I   Y   S   Q   T   Y   F   R   F   Q   E
```

```
1027 gaa ata aaa gaa aac aca aag aac gac aaa caa atg gtc caa tat att tac aaa
 343  E   I   K   E   N   T   K   N   D   K   Q   M   V   Q   Y   I   Y   K
1081 tac aca agt tat cct gac cct ata ttg ttg atg aaa agt gct aga aat agt tgt
 361  Y   T   S   Y   P   D   P   I   L   L   M   K   S   A   R   N   S   C
1135 tgg tct aaa gat gca gaa tat gga ctc tat tcc atc tat caa ggg gga ata ttt
 379  W   S   K   D   A   E   Y   G   L   Y   S   I   Y   Q   G   G   I   F
1189 gag ctt aag gaa aat gac aga att ttt gtt tct gta aca aat gag cac ttg ata
 397  E   L   K   E   N   D   R   I   F   V   S   V   T   N   E   H   L   I
1243 gac atg gac cat gaa gcc agt ttt ttc ggg gcc ttt tta gtt ggc taa
 415  D   M   D   H   E   A   S   F   F   G   A   F   L   V   G   -
```

9.1.5. GpL-Flag-TNC-TRAILmutR1

GpL:	NT 1-555	aa 1-185
Flag:	NT 571-594	aa 191-198
TNC:	NT 601-690	aa 201-230
TRAILmutR1:	NT 727-1287	aa 243-429

Mutations are indicated in **green color**.

```
   1 atg ggc gtc aaa gtc ctg ttt gcc ctc atc tgt att gct gtc gct gag gct aaa
   1  M   G   V   K   V   L   F   A   L   I   C   I   A   V   A   E   A   K
  55 cca acc gag aat aat gag gat ttc aac atc gtg gct gtg gca tcc aat ttt gct
  19  P   T   E   N   N   E   D   F   N   I   V   A   V   A   S   N   F   A
 109 acc acc gac ctc gat gcc gat cgg gga aaa ctg cct ggc aaa aaa ctg ccc ctg
  37  T   T   D   L   D   A   D   R   G   K   L   P   G   K   K   L   P   L
 163 gaa gtg ctg aaa gag atg gag gcc aac gct aga aaa gct ggc tgt act aga gga
  55  E   V   L   K   E   M   E   A   N   A   R   K   A   G   C   T   R   G
 217 tgt ctc atc tgc ctg tcc cac atc aag tgt acc cca aaa atg aaa aaa ttc atc
  73  C   L   I   C   L   S   H   I   K   C   T   P   K   M   K   K   F   I
 271 cct ggc cgg tgt cac aca tac gag ggc gac aag gaa tct gct cag ggc gga atc
  91  P   G   R   C   H   T   Y   E   G   D   K   E   S   A   Q   G   G   I
 325 gga gag gct att gtg gat att cct gaa att cct gga ttc aag gac ctg gag cct
 109  G   E   A   I   V   D   I   P   E   I   P   G   F   K   D   L   E   P
 379 atg gaa cag ttt atc gcc cag gtg gac ctc tgt gtc gat tgt aca act ggc tgc
 127  M   E   Q   F   I   A   Q   V   D   L   C   V   D   C   T   T   G   C
 433 ctg aaa ggg ctg gcc aat gtc cag tgt agt gac ctg ctg aaa aaa tgg ctg ccc
 145  L   K   G   L   A   N   V   Q   C   S   D   L   L   K   K   W   L   P
 487 cag aga tgt gcc act ttc gcc tct aaa att cag ggc cag gtc gac aaa atc aaa
 163  Q   R   C   A   T   F   A   S   K   I   Q   G   Q   V   D   K   I   K
 541 ggc gct gga gga gac tct gga gct gga tcc gat tac aaa gac gat gac gat aaa
 181  G   A   G   G   D   S   G   A   G   S   D   Y   K   D   D   D   D   K
 595 gat atc gcc tgt ggc tgt gcg gct gcc cca gac atc aag gac ctg ctg agc aga
 199  D   I   A   C   G   C   A   A   A   P   D   I   K   D   L   L   S   R
 649 ctg gag gag ctg gag ggg ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt
 217  L   E   E   L   E   G   L   V   S   S   L   R   E   Q   G   T   G   G
 703 ggg tct ggc ggc cgc ggt gaa ttc aca tct gag gaa acc att agc acc gtc cag
 235  G   S   G   G   R   G   E   F   T   S   E   E   T   I   S   T   V   Q
 757 gag aaa cag cag aac att tca ccc ctc gtc cgg gaa cgg gga cca cag aga gtg
 253  E   K   Q   Q   N   I   S   P   L   V   R   E   R   G   P   Q   R   V
 811 gcc gct cat att act ggc aca cgg agg cga tcc aat aca ctg agt agc ccc aac
 271  A   A   H   I   T   G   T   R   R   R   S   N   T   L   S   S   P   N
 865 tcc aaa aac gaa aag gca ctg ggc atc aaa atc aat tca tgg gag agt agt agg
 289  S   K   N   E   K   A   L   G   I   K   I   N   S   W   E   S   S   R
 919 cgg gga cat tcc ttt ctg tcc aac ctc cat ctc cga aac ggc gaa ctg gtg att
 307  R   G   H   S   F   L   S   N   L   H   L   R   N   G   E   L   V   I
```

973 *cac gag aag ggc ttt tac tac atc tac tcc cag acc tac ttc aga ttt cag gag*
325 H E K G F Y Y I Y S Q T Y F R F Q E
1027 *gag atc aag gaa* ***cgg*** *acc* ***cac*** *aac gac aaa cag atg gtc cag tac atc tac aaa*
343 E I K E **R** T **H** N D K Q M V Q Y I Y K
1081 *tac acc* ***gac*** *tac ccc gac cct atc ctg ctc atg aaa tcc gct aga aat tca tgc*
361 Y T **D** Y P D P I L L M K S A R N S C
1135 *tgg agc aag gat gcc gaa tac gga ctg tac tca atc tac cag ggc ggc att ttt*
379 W S K D A E Y G L Y S I Y Q G G I F
1189 *gaa ctg aaa gag aac gat cgg atc ttc gtg tct gtc aca aac gaa cac ctc atc*
397 E L K E N D R I F V S V T N E H L I
1243 *gac atg gat cac gag gcc tca ttc ttt ggc gct ttt ctg gtg gga tga*
415 D M D H E A S F F G A F L V G -

9.1.6 GpL-Flag-TNC-TRAILmutR2

GpL:	NT 1-555	aa 1-185
Flag:	NT 571-594	aa 191-198
TNC:	NT 601-690	aa 201-230
TRAILmutR2:	NT 727-1287	aa 243-429

Mutations are indicated in **green color**.

1 *atg ggc gtc aaa gtc ctg ttt gcc ctc atc tgt att gct gtc gct gag gct aaa*
1 M G V K V L F A L I C I A V A E A K
55 *cca acc gag aat aat gag gat ttc aac atc gtg gct gtg gca tcc aat ttt gct*
19 P T E N N E D F N I V A V A S N F A
109 *acc acc gac ctc gat gcc gat cgg gga aaa ctg cct ggc aaa aaa ctg ccc ctg*
37 T T D L D A D R G K L P G K K L P L
163 *gaa gtg ctg aaa gag atg gag gcc aac gct aga aaa gct ggc tgt act aga gga*
55 E V L K E M E A N A R K A G C T R G
217 *tgt ctc atc tgc ctg tcc cac atc aag tgt acc cca aaa atg aaa aaa ttc atc*
73 C L I C L S H I K C T P K M K K F I
271 *cct ggc cgg tgt cac aca tac gag ggc gac aag gaa tct gct cag ggc gga atc*
91 P G R C H T Y E G D K E S A Q G G I
325 *gga gag gct att gtg gat att cct gaa att cct gga ttc aag gac ctg gag cct*
109 G E A I V D I P E I P G F K D L E P
379 *atg gaa cag ttt atc gcc cag gtg gac ctc tgt gtc gat tgt aca act ggc tgc*
127 M E Q F I A Q V D L C V D C T T G C
433 *ctg aaa ggg ctg gcc aat gtc cag tgt agt gac ctg ctg aaa aaa tgg ctg ccc*
145 L K G L A N V Q C S D L L K K W L P
487 *cag aga tgt gcc act ttc gcc tct aaa att cag ggc cag gtc gac aaa atc aaa*
163 Q R C A T F A S K I Q G Q V D K I K
541 *ggc gct gga gga gac tct gga gct gga tcc gat tac aaa gac gat gac gat aaa*
181 G A G G D S G A G S D Y K D D D D K
595 *gat atc gcc tgt ggc tgt gcg gct gcc cca gac atc aag gac ctg ctg agc aga*
199 D I A C G C A A A P D I K D L L S R
649 *ctg gag gag ctg gag ggg ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt*
217 L E E L E G L V S S L R E Q G T G G
703 *ggg tct ggc ggc cgc ggt gaa ttc acc agc gaa gag aca atc agc acc gtg cag*
235 G S G G R G E F T S E E T I S T V Q
757 *gaa aag cag cag aac atc agc ccc ctc gtg cgc gaa agg ggc cct cag aga gtg*
253 E K Q Q N I S P L V R E R G P Q R V
811 *gcc gcc cac atc act ggc acc aga ggc aga agc aac acc ctg agc agc ccc aac*
271 A A H I T G T R G R S N T L S S P N
865 *agc aag aac gag aag gcc ctg ggc cgg aag atc aac agc tgg gag tct agc aga*
289 S K N E K A L G R K I N S W E S S R

```
 919 agc ggc cac agc ttt ctg agc aac ctg cac ctg aga aac ggc gag ctc gtg atc
 307  S   G   H   S   F   L   S   N   L   H   L   R   N   G   E   L   V   I
 973 cac gag aag ggc ttc tac tac atc tac agc cag acc cag ttc aag ttc cgg gaa
 325  H   E   K   G   F   Y   Y   I   Y   S   Q   T   Q   F   K   F   R   E
1027 gag atc aaa gag aat acc aag aac gac aag cag atg gtg cag tac atc tat aag
 343  E   I   K   E   N   T   K   N   D   K   Q   M   V   Q   Y   I   Y   K
1081 tac acc agc tac ccc gac ccc atc ctg ctg atg aag tcc gcc cgg aac agc tgc
 361  Y   T   S   Y   P   D   P   I   L   L   M   K   S   A   R   N   S   C
1135 tgg tcc aag gat gcc gag tac ggc ctg tac agc atc tac cag ggc ggc atc ttc
 379  W   S   K   D   A   E   Y   G   L   Y   S   I   Y   Q   G   G   I   F
1189 gag ctg aaa gag aac gac cgg atc ttc gtg tcc gtg acc aac gag cgg ctg ctg
 397  E   L   K   E   N   D   R   I   F   V   S   V   T   N   E   R   L   L
1243 cag atg gac cac gag gcc agc ttt ttc ggc gcc ttc ctc gtg gga tag
 415  Q   M   D   H   E   A   S   F   F   G   A   F   L   V   G   -
```

9.1.7 Fc-Flag-TRAIL

Signal peptide:	NT 1-51	aa 1-17
Fc:	NT 51-729	aa 18-243
Flag:	NT 814-837	aa 272-279
TRAIL:	NT 844-1404	aa 282-468

```
   1 atg gct atc atc tac ctc atc ctc ctg ttc acc gct gtg cgg ggc ctc gac aaa
   1  M   A   I   I   Y   L   I   L   L   F   T   A   V   R   G   L   D   K
  55 act cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc
  19  T   H   T   C   P   P   C   P   A   P   E   L   L   G   G   P   S   V
 109 ttc ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag
  37  F   L   F   P   P   K   P   K   D   T   L   M   I   S   R   T   P   E
 163 gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac
  55  V   T   C   V   V   V   D   V   S   H   E   D   P   E   V   K   F   N
 217 tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag
  73  W   Y   V   D   G   V   E   V   H   N   A   K   T   K   P   R   E   E
 271 cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac
  91  Q   Y   N   S   T   Y   R   V   V   S   V   L   T   V   L   H   Q   D
 325 tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca gcc
 109  W   L   N   G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A
 379 ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag gtg
 127  P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V
 433 tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc
 145  Y   T   L   P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T
 487 tgc ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat
 163  C   L   V   K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N
 541 ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ttg gac tcc gac ggC
 181  G   Q   P   E   N   N   Y   K   T   T   P   P   V   L   D   S   D   G
 595 tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg
 199  S   F   F   L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G
 649 aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag
 217  N   V   F   S   C   S   V   M   H   E   A   L   H   N   H   Y   T   Q
 703 aag agc ctc tcc ctg tct ccg ggt aaa aga tct ccg cag ccg cag ccg aaa ccg
 235  K   S   L   S   L   S   P   G   K   R   S   P   Q   P   Q   P   K   P
 757 cag ccg aaa ccg gaa ccg gaa gga tct ctg gag gtg ctg ttc cag ggg ccc gga
 253  Q   P   K   P   E   P   E   G   S   L   E   V   L   F   Q   G   P   G
 811 tcc gat tac aaa gac gat gac gat aaa gaa ttc acc tct gag gaa acc att tct
 271  S   D   Y   K   D   D   D   D   K   E   F   T   S   E   E   T   I   S
```

```
 865 aca gtt caa gaa aag caa caa aat att tct ccc cta gtg aga gaa aga ggt cct
 289 T   V   Q   E   K   Q   Q   N   I   S   P   L   V   R   E   R   G   P
 919 cag aga gta gca gct cac ata act ggg acc aga gga aga agc aac aca ttg tct
 307 Q   R   V   A   A   H   I   T   G   T   R   G   R   S   N   T   L   S
 973 tct cca aac tcc aag aat gaa aag gct ctg ggc cgc aaa ata aac tcc tgg gaa
 325 S   P   N   S   K   N   E   K   A   L   G   R   K   I   N   S   W   E
1027 tca tca agg agt ggg cat tca ttc ctg agc aac ttg cac ttg agg aat ggt gaa
 343 S   S   R   S   G   H   S   F   L   S   N   L   H   L   R   N   G   E
1081 ctg gtc atc cat gaa aaa ggg ttt tac tac atc tat tcc caa aca tac ttt cga
 361 L   V   I   H   E   K   G   F   Y   Y   I   Y   S   Q   T   Y   F   R
1135 ttt cag gag gaa ata aaa gaa aac aca aag aac gac aaa caa atg gtc caa tat
 379 F   Q   E   E   I   K   E   N   T   K   N   D   K   Q   M   V   Q   Y
1189 att tac aaa tac aca agt tat cct gac cct ata ttg ttg atg aaa agt gct aga
 397 I   Y   K   Y   T   S   Y   P   D   P   I   L   L   M   K   S   A   R
1243 aat agt tgt tgg tct aaa gat gca gaa tat gga ctc tat tcc atc tat caa ggg
 415 N   S   C   W   S   K   D   A   E   Y   G   L   Y   S   I   Y   Q   G
1297 gga ata ttt gag ctt aag gaa aat gac aga att ttt gtt tct gta aca aat gag
 433 G   I   F   E   L   K   E   N   D   R   I   F   V   S   V   T   N   E
1351 cac ttg ata gac atg gac cat gaa gcc agt ttt ttc ggg gct ttt tta gtt ggc
 451 H   L   I   D   M   D   H   E   A   S   F   F   G   A   F   L   V   G
1405 taa
 469 -
```

9.1.8. Fc-Flag-TRAILmutR1

Signal peptide:	NT 1-51	aa 1-17
Fc:	NT 51-729	aa 18-243
Flag:	NT 814-837	aa 272-279
TRAILmutR1:	NT 844-1404	aa 282-468

Mutations are indicated in **green color**.

```
   1 atg gct atc atc tac ctc atc ctc ctg ttc acc gct gtg cgg ggc ctc gac aaa
   1 M   A   I   I   Y   L   I   L   L   F   T   A   V   R   G   L   D   K
  55 act cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc
  19 T   H   T   C   P   P   C   P   A   P   E   L   L   G   G   P   S   V
 109 ttc ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag
  37 F   L   F   P   P   K   P   K   D   T   L   M   I   S   R   T   P   E
 163 gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac
  55 V   T   C   V   V   V   D   V   S   H   E   D   P   E   V   K   F   N
 217 tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag
  73 W   Y   V   D   G   V   E   V   H   N   A   K   T   K   P   R   E   E
 271 cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac
  91 Q   Y   N   S   T   Y   R   V   V   S   V   L   T   V   L   H   Q   D
 325 tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca gcc
 109 W   L   N   G   K   E   Y   K   C   K   V   S   N   K   A   L   P   A
 379 ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag gtg
 127 P   I   E   K   T   I   S   K   A   K   G   Q   P   R   E   P   Q   V
 433 tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc
 145 Y   T   L   P   P   S   R   D   E   L   T   K   N   Q   V   S   L   T
 487 tgc ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat
 163 C   L   V   K   G   F   Y   P   S   D   I   A   V   E   W   E   S   N
 541 ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ttg gac tcc gac ggc
 181 G   Q   P   E   N   N   Y   K   T   T   P   P   V   L   D   S   D   G
 595 tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg
 199 S   F   F   L   Y   S   K   L   T   V   D   K   S   R   W   Q   Q   G
```

649 aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag
217 N V F S C S V M H E A L H N H Y T Q
703 aag agc ctc tcc ctg tct ccg ggt aaa aga tct ccg cag ccg cag ccg aaa ccg
235 K S L S L S P G K R S P Q P Q P K P
757 cag ccg aaa ccg gaa ccg gaa gga tct ctg gag gtg ctg ttc cag ggg ccc gga
253 Q P K P E P E G S L E V L F Q G P G
811 tcc gat tac aaa gac gat gac gat aaa gaa ttc aca tct gag gaa acc att agc
271 S D Y K D D D D K E F T S E E T I S
865 acc gtc cag gag aaa cag cag aac att tca ccc ctc gtc cgg gaa cgg gga cca
289 T V Q E K Q Q N I S P L V R E R G P
919 cag aga gtg gcc gct cat att act ggc aca cgg **agg** cga tcc aat aca ctg agt
307 Q R V A A H I T G T R **R** R S N T L S
973 agc ccc aac tcc aaa aac gaa aag gca ctg ggc **atc** aaa atc aat tca tgg gag
325 S P N S K N E K A L G **I** K I N S W E
1027 agt agt agg **cgg** gga cat tcc ttt ctg tcc aac ctc cat ctc cga aac ggc gaa
343 S S R **R** G H S F L S N L H L R N G E
1081 ctg gtg att cac gag aag ggc ttt tac tac atc tac tcc cag acc tac ttc aga
361 L V I H E K G F Y Y I Y S Q T Y F R
1135 ttt cag gag gag atc aag gaa **cgg** acc **cac** aac gac aaa cag atg gtc cag tac
379 F Q E E I K E **R** T **H** N D K Q M V Q Y
1189 atc tac aaa tac acc **gac** tac ccc gac cct atc ctg ctc atg aaa tcc gct aga
397 I Y K Y T **D** Y P D P I L L M K S A R
1243 aat tca tgc tgg agc aag gat gcc gaa tac gga ctg tac tca atc tac cag ggc
415 N S C W S K D A E Y G L Y S I Y Q G
1297 ggc att ttt gaa ctg aaa gag aac gat cgg atc ttc gtg tct gtc aca aac gaa
433 G I F E L K E N D R I F V S V T N E
1351 cac ctc atc gac atg gat cac gag gcc tca ttc ttt ggc gct ttt ctg gtg gga
451 H L I D M D H E A S F F G A F L V G
1405 TGA
469 -

9.1.9. Fc-Flag-TRAILmutR2

Signal peptide:	NT 1-51	aa 1-17
Fc:	NT 51-729	aa 18-243
Flag:	NT 814-837	aa 272-279
TRAILmutR2:	NT 844-1404	aa 282-468

Mutations are indicated in **green color**.

1 atg gct atc atc tac ctc atc ctc ctg ttc acc gct gtg cgg ggc ctc gac aaa
1 M A I I Y L I L L F T A V R G L D K
55 act cac aca tgc cca ccg tgc cca gca cct gaa ctc ctg ggg gga ccg tca gtc
19 T H T C P P C P A P E L L G G P S V
109 ttc ctc ttc ccc cca aaa ccc aag gac acc ctc atg atc tcc cgg acc cct gag
37 F L F P P K P K D T L M I S R T P E
163 gtc aca tgc gtg gtg gtg gac gtg agc cac gaa gac cct gag gtc aag ttc aac
55 V T C V V V D V S H E D P E V K F N
217 tgg tac gtg gac ggc gtg gag gtg cat aat gcc aag aca aag ccg cgg gag gag
73 W Y V D G V E V H N A K T K P R E E
271 cag tac aac agc acg tac cgt gtg gtc agc gtc ctc acc gtc ctg cac cag gac
91 Q Y N S T Y R V V S V L T V L H Q D
325 tgg ctg aat ggc aag gag tac aag tgc aag gtc tcc aac aaa gcc ctc cca gcc
109 W L N G K E Y K C K V S N K A L P A
379 ccc atc gag aaa acc atc tcc aaa gcc aaa ggg cag ccc cga gaa cca cag gtg
127 P I E K T I S K A K G Q P R E P Q V

433 tac acc ctg ccc cca tcc cgg gat gag ctg acc aag aac cag gtc agc ctg acc
145 Y T L P P S R D E L T K N Q V S L T
487 tgc ctg gtc aaa ggc ttc tat ccc agc gac atc gcc gtg gag tgg gag agc aat
163 C L V K G F Y P S D I A V E W E S N
541 ggg cag ccg gag aac aac tac aag acc acg cct ccc gtg ttg gac tcc gac ggc
181 G Q P E N N Y K T T P P V L D S D G
595 tcc ttc ttc ctc tac agc aag ctc acc gtg gac aag agc agg tgg cag cag ggg
199 S F F L Y S K L T V D K S R W Q Q G
649 aac gtc ttc tca tgc tcc gtg atg cat gag gct ctg cac aac cac tac acg cag
217 N V F S C S V M H E A L H N H Y T Q
703 aag agc ctc tcc ctg tct ccg ggt aaa aga tct ccg cag ccg cag ccg aaa ccg
235 K S L S L S P G K R S P Q P Q P K P
757 cag ccg aaa ccg gaa ccg gaa gga tct ctg gag gtg ctg ttc cag ggg ccc gga
253 Q P K P E P E G S L E V L F Q G P G
811 tcc gat tac aaa gac gat gac gat aaa gaa ttc acc agc gaa gag aca atc agc
271 S D Y K D D D D K E F T S E E T I S
865 acc gtg cag gaa aag cag cag aac atc agc ccc ctc gtg cgc gaa agg ggc cct
289 T V Q E K Q Q N I S P L V R E R G P
919 cag aga gtg gcc gcc cac atc act ggc acc aga ggc aga agc aac acc ctg agc
307 Q R V A A H I T G T R G R S N T L S
973 agc ccc aac agc aag aac gag aag gcc ctg ggc cgg aag atc aac agc tgg gag
325 S P N S K N E K A L G R K I N S W E
1027 tct agc aga agc ggc cac agc ttt ctg agc aac ctg cac ctg aga aac ggc gag
343 S S R S G H S F L S N L H L R N G E
1081 ctc gtg atc cac gag aag ggc ttc tac tac atc tac agc cag acc **cag** ttc **aag**
361 L V I H E K G F Y Y I Y S Q T **Q** F **K**
1135 ttc **cgg** gaa gag atc aaa gag aat acc aag aac gac aag cag atg gtg cag tac
379 F **R** E E I K E N T K N D K Q M V Q Y
1189 atc tat aag tac acc agc tac ccc gac ccc atc ctg ctg atg aag tcc gcc cgg
397 I Y K Y T S Y P D P I L L M K S A R
1243 aac agc tgc tgg tcc aag gat gcc gag tac ggc ctg tac agc atc tac cag ggc
415 N S C W S K D A E Y G L Y S I Y Q G
1297 ggc atc ttc gag ctg aaa gag aac gac cgg atc ttc gtg tcc gtg acc aac gag
433 G I F E L K E N D R I F V S V T N E
1351 **cgg** ctg **ctg** **cag** atg gac cac gag gcc agc ttt ttc ggc gcc ttc ctc gtg gga
451 **R** L **L** **Q** M D H E A S F F G A F L V G
1405 tag
469 -

9.1.10. scFv:G28-Flag-TNC-TRAIL

Signal peptide:	NT 1-84	aa 1-28
scFv:G28-5:	NT 85-816	aa 29-272
Flag:	NT 823-846	aa 275-282
TNC:	NT 853-942	aa 285-314
TRAIL:	NT 979-1539	aa 327-513

1 atg aac ttc ggg ttc agc ttg att ttc ctg gtc ctg gtg ctg aag ggc gtg cag
1 M N F G F S L I F L V L V L K G V Q
55 tgc gag gtg aag ctg gtg cca cgc gga tcc gac atc gtg atg act cag aac cca
19 C E V K L V P R G S D I V M T Q N P
109 ctg tct ctg cct gtg tct ctg ggg gat gag gct agc att tct tgc cgc tca tct
37 L S L P V S L G D E A S I S C R S S
163 cag tca ctg gag aac tcc aat ggc aac acc ttc ctg aat tgg ttt ttc cag aaa
55 Q S L E N S N G N T F L N W F F Q K

```
 217 ccc ggc cag tca cct cag ctg ctc atc tac cga gtg agc aat cgg ttt agc gga
  73  P   G   Q   S   P   Q   L   L   I   Y   R   V   S   N   R   F   S   G
 271 gtg ccc gat cga ttc tct ggc tcc gga tct ggg acc gac ttt acc ctg aaa atc
  91  V   P   D   R   F   S   G   S   G   S   G   T   D   F   T   L   K   I
 325 tca cga gtg gag gcc gag gat ctg gga gtg tac ttc tgt ctc cag gtc aca cat
 109  S   R   V   E   A   E   D   L   G   V   Y   F   C   L   Q   V   T   H
 379 gtg cct tac aca ttt ggc ggc gga aca act ctc gaa atc aaa gga ggc gga ggc
 127  V   P   Y   T   F   G   G   G   T   T   L   E   I   K   G   G   G   G
 433 tcc ggc gga ggc gga tct ggc gga ggc ggg agt gat atc cag ctc cag cag tct
 145  S   G   G   G   G   S   G   G   G   G   S   D   I   Q   L   Q   Q   S
 487 ggc cct gga ctc gtc aaa cca tct cag agc ctg tct ctc acc tgt tct gtc acc
 163  G   P   G   L   V   K   P   S   Q   S   L   S   L   T   C   S   V   T
 541 gga tac tcc atc acc acc aac tac aac tgg aat tgg att cgg cag ttt cct ggg
 181  G   Y   S   I   T   T   N   Y   N   W   N   W   I   R   Q   F   P   G
 595 aac aaa ctc gaa tgg atg gga tac atc cga tac gac ggc act agt gaa tac acc
 199  N   K   L   E   W   M   G   Y   I   R   Y   D   G   T   S   E   Y   T
 649 cca tct ctc aaa aat cgg gtg tcc att acc cgg gac act tct atg aac cag ttc
 217  P   S   L   K   N   R   V   S   I   T   R   D   T   S   M   N   Q   F
 703 ttt ctc cga ctc acc tct gtg aca cct gag gat acc gcc aca tac tac tgt gct
 235  F   L   R   L   T   S   V   T   P   E   D   T   A   T   Y   Y   C   A
 757 aga ctg gac tac tgg ggg cag gga aca ctg gtg acc gtg tca tct gct tcc acc
 253  R   L   D   Y   W   G   Q   G   T   L   V   T   V   S   S   A   S   T
 811 aaa gga gga tcc gat tac aaa gac gat gac gat aaa gat atc gcc tgt ggc tgt
 271  K   G   G   S   D   Y   K   D   D   D   D   K   D   I   A   C   G   C
 865 gcg gct gcc cca gac atc aag gac ctg ctg agc aga ctg gag gag ctg gag ggg
 289  A   A   A   P   D   I   K   D   L   L   S   R   L   E   E   L   E   G
 919 ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt ggg tct ggc ggc cgc ggt
 307  L   V   S   S   L   R   E   Q   G   T   G   G   G   S   G   G   R   G
 973 gaa ttc acc tct gag gaa acc att tct aca gtt caa gaa aag caa caa aat att
 325  E   F   T   S   E   E   T   I   S   T   V   Q   E   K   Q   Q   N   I
1027 tct ccc cta gtg aga gaa aga ggt cct cag aga gta gca gct cac ata act ggg
 343  S   P   L   V   R   E   R   G   P   Q   R   V   A   A   H   I   T   G
1081 acc aga gga aga agc aac aca ttg tct tct cca aac tcc aag aat gaa aag gct
 361  T   R   G   R   S   N   T   L   S   S   P   N   S   K   N   E   K   A
1135 ctg ggc cgc aaa ata aac tcc tgg gaa tca tca agg agt ggg cat tca ttc ctg
 379  L   G   R   K   I   N   S   W   E   S   S   R   S   G   H   S   F   L
1189 agc aac ttg cac ttg agg aat ggt gaa ctg gtc atc cat gaa aaa ggg ttt tac
 397  S   N   L   H   L   R   N   G   E   L   V   I   H   E   K   G   F   Y
1243 tac atc tat tcc caa aca tac ttt cga ttt cag gag gaa ata aaa gaa aac aca
 415  Y   I   Y   S   Q   T   Y   F   R   F   Q   E   E   I   K   E   N   T
1297 aag aac gac aaa caa atg gtc caa tat att tac aaa tac aca agt tat cct gac
 433  K   N   D   K   Q   M   V   Q   Y   I   Y   K   Y   T   S   Y   P   D
1351 cct ata ttg ttg atg aaa agt gct aga aat agt tgt tgg tct aaa gat gca gaa
 451  P   I   L   L   M   K   S   A   R   N   S   C   W   S   K   D   A   E
1405 tat gga ctc tat tcc atc tat caa ggg gga ata ttt gag ctt aag gaa aat gac
 469  Y   G   L   Y   S   I   Y   Q   G   G   I   F   E   L   K   E   N   D
1459 aga att ttt gtt tct gta aca aat gag cac ttg ata gac atg gac cat gaa gcc
 487  R   I   F   V   S   V   T   N   E   H   L   I   D   M   D   H   E   A
1513 agt ttt ttc ggg gcc ttt tta gtt ggc taa
 505  S   F   F   G   A   F   L   V   G   -
```

9.1.11. scFv:G28-Flag-TNC-TRAILmutR1

Signal peptide:	NT 1-84	aa 1-28
scFv: G28-5:	NT 85-816	aa 29-272
Flag:	NT 823-846	aa 275-282
TNC:	NT 853-942	aa 285-314

TRAILmutR1: NT 979-1539 aa 327-513

Mutations are indicated in **green color**.

1 atg aac ttc ggg ttc agc ttg att ttc ctg gtc ctg gtg ctg aag ggc gtg cag
1 M N F G F S L I F L V L V L K G V Q
55 tgc gag gtg aag ctg gtg cca cgc gga tcc gac atc gtg atg act cag aac cca
19 C E V K L V P R G S D I V M T Q N P
109 ctg tct ctg cct gtg tct ctg ggg gat gag gct agc att tct tgc cgc tca tct
37 L S L P V S L G D E A S I S C R S S
163 cag tca ctg gag aac tcc aat ggc aac acc ttc ctg aat tgg ttt ttc cag aaa
55 Q S L E N S N G N T F L N W F F Q K
217 ccc ggc cag tca cct cag ctg ctc atc tac cga gtg agc aat cgg ttt agc gga
73 P G Q S P Q L L I Y R V S N R F S G
271 gtg ccc gat cga ttc tct ggc tcc gga tct ggg acc gac ttt acc ctg aaa atc
91 V P D R F S G S G S G T D F T L K I
325 tca cga gtg gag gcc gag gat ctg gga gtg tac ttc tgt ctc cag gtc aca cat
109 S R V E A E D L G V Y F C L Q V T H
379 gtg cct tac aca ttt ggc ggc gga aca act ctc gaa atc aaa gga ggc gga ggc
127 V P Y T F G G G T T L E I K G G G G
433 tcc ggc gga ggc gga tct ggc gga ggc ggg agt gat atc cag ctc cag cag tct
145 S G G G G S G G G G S D I Q L Q Q S
487 ggc cct gga ctc gtc aaa cca tct cag agc ctg tct ctc acc tgt tct gtc acc
163 G P G L V K P S Q S L S L T C S V T
541 gga tac tcc atc acc acc aac tac aac tgg aat tgg att cgg cag ttt cct ggg
181 G Y S I T T N Y N W N W I R Q F P G
595 aac aaa ctc gaa tgg atg gga tac atc cga tac gac ggc act agt gaa tac acc
199 N K L E W M G Y I R Y D G T S E Y T
649 cca tct ctc aaa aat cgg gtg tcc att acc cgg gac act tct atg aac cag ttc
217 P S L K N R V S I T R D T S M N Q F
703 ttt ctc cga ctc acc tct gtg aca cct gag gat acc gcc aca tac tac tgt gct
235 F L R L T S V T P E D T A T Y Y C A
757 aga ctg gac tac tgg ggg cag gga aca ctg gtg acc gtg tca tct gct tcc acc
253 R L D Y W G Q G T L V T V S S A S T
811 aaa gga gga tcc gat tac aaa gac gat gac gat aaa gat atc gcc tgt ggc tgt
271 K G G S D Y K D D D D K D I A C G C
865 gcg gct gcc cca gac atc aag gac ctg ctg agc aga ctg gag gag ctg gag ggg
289 A A A P D I K D L L S R L E E L E G
919 ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt ggg tct ggc ggc cgc ggt
307 L V S S L R E Q G T G G G S G G R G
973 gaa ttc aca tct gag gaa acc att agc acc gtc cag gag aaa cag cag aac att
325 E F T S E E T I S T V Q E K Q Q N I
1027 tca ccc ctc gtc cgg gaa cgg gga cca cag aga gtg gcc gct cat att act ggc
343 S P L V R E R G P Q R V A A H I T G
1081 aca cgg **agg** cga tcc aat aca ctg agt agc ccc aac tcc aaa aac gaa aag gca
361 T R **R** R S N T L S S P N S K N E K A
1135 ctg ggc **atc** aaa atc aat tca tgg gag agt agt agg **cgg** gga cat tcc ttt ctg
379 L G **I** K I N S W E S S R **R** G H S F L
1189 tcc aac ctc cat ctc cga aac ggc gaa ctg gtg att cac gag aag ggc ttt tac
397 S N L H L R N G E L V I H E K G F Y
1243 tac atc tac tcc cag acc tac ttc aga ttt cag gag gag atc aag gaa **cgg** acc
415 Y I Y S Q T Y F R F Q E E I K E **R** T
1297 **cac** aac gac aaa cag atg gtc cag tac atc tac aaa tac acc **gac** tac ccc gac
433 **H** N D K Q M V Q Y I Y K Y T **D** Y P D
1351 cct atc ctg ctc atg aaa tcc gct aga aat tca tgc tgg agc aag gat gcc gaa
451 P I L L M K S A R N S C W S K D A E
1405 tac gga ctg tac tca atc tac cag ggc ggc att ttt gaa ctg aaa gag aac gat
469 Y G L Y S I Y Q G G I F E L K E N D
1459 cgg atc ttc gtg tct gtc aca aac gaa cac ctc atc gac atg gat cac gag gcc
487 R I F V S V T N E H L I D M D H E A

1513 *tca ttc ttt ggc gct ttt ctg gtg gga tga*
505 S F F G A F L V G -

9.1.12. scFv:G28-Flag-TNC-TRAILmutR2

Signal peptide:	NT 1-84	aa 1-28
scFv: G28-5:	NT 85-816	aa 29-272
Flag:	NT 823-846	aa 275-282
TNC:	NT 853-942	aa 285-314
TRAILmutR2:	NT 979-1539	aa 327-513

Mutations are indicated in **green color**.

1 *atg aac ttc ggg ttc agc ttg att ttc ctg gtc ctg gtg ctg aag ggc gtg cag*
1 M N F G F S L I F L V L V L K G V Q
55 *tgc gag gtg aag ctg gtg cca cgc gga tcc gac atc gtg atg act cag aac cca*
19 C E V K L V P R G S D I V M T Q N P
109 *ctg tct ctg cct gtg tct ctg ggg gat gag gct agc att tct tgc cgc tca tct*
37 L S L P V S L G D E A S I S C R S S
163 *cag tca ctg gag aac tcc aat ggc aac acc ttc ctg aat tgg ttt ttc cag aaa*
55 Q S L E N S N G N T F L N W F F Q K
217 *ccc ggc cag tca cct cag ctg ctc atc tac cga gtg agc aat cgg ttt agc gga*
73 P G Q S P Q L L I Y R V S N R F S G
271 *gtg ccc gat cga ttc tct ggc tcc gga tct ggg acc gac ttt acc ctg aaa atc*
91 V P D R F S G S G S G T D F T L K I
325 *tca cga gtg gag gcc gag gat ctg gga gtg tac ttc tgt ctc cag gtc aca cat*
109 S R V E A E D L G V Y F C L Q V T H
379 *gtg cct tac aca ttt ggc ggc gga aca act ctc gaa atc aaa gga ggc gga ggc*
127 V P Y T F G G G T T L E I K G G G G
433 *tcc ggc gga ggc gga tct ggc gga ggc ggg agt gat atc cag ctc cag cag tct*
145 S G G G G S G G G G S D I Q L Q Q S
487 *ggc cct gga ctc gtc aaa cca tct cag agc ctg tct ctc acc tgt tct gtc acc*
163 G P G L V K P S Q S L S L T C S V T
541 *gga tac tcc atc acc acc aac tac aac tgg aat tgg att cgg cag ttt cct ggg*
181 G Y S I T T N Y N W N W I R Q F P G
595 *aac aaa ctc gaa tgg atg gga tac atc cga tac gac ggc act agt gaa tac acc*
199 N K L E W M G Y I R Y D G T S E Y T
649 *cca tct ctc aaa aat cgg gtg tcc att acc cgg gac act tct atg aac cag ttc*
217 P S L K N R V S I T R D T S M N Q F
703 *ttt ctc cga ctc acc tct gtg aca cct gag gat acc gcc aca tac tac tgt gct*
235 F L R L T S V T P E D T A T Y Y C A
757 *aga ctg gac tac tgg ggg cag gga aca ctg gtg acc gtg tca tct gct tcc acc*
253 R L D Y W G Q G T L V T V S S A S T
811 *aaa gga gga tcc gat tac aaa gac gat gac gat aaa gat atc gcc tgt ggc tgt*
271 K G G S D Y K D D D D K D I A C G C
865 *gcg gct gcc cca gac atc aag gac ctg ctg agc aga ctg gag gag ctg gag ggg*
289 A A A P D I K D L L S R L E E L E G
919 *ctg gta tcc tcc ctc cgg gag cag ggt acc gga ggt ggg tct ggc ggc cgc ggt*
307 L V S S L R E Q G T G G G S G G R G
973 *gaa ttc acc agc gaa gag aca atc agc acc gtg cag gaa aag cag cag aac atc*
325 E F T S E E T I S T V Q E K Q Q N I
1027 *agc ccc ctc gtg cgc gaa agg ggc cct cag aga gtg gcc gcc cac atc act ggc*
343 S P L V R E R G P Q R V A A H I T G
1081 *acc aga ggc aga agc aac acc ctg agc agc ccc aac agc aag aac gag aag gcc*
361 T R G R S N T L S S P N S K N E K A
1135 *ctg ggc cgg aag atc aac agc tgg gag tct agc aga agc ggc cac agc ttt ctg*
379 L G R K I N S W E S S R S G H S F L

1189 agc aac ctg cac ctg aga aac ggc gag ctc gtg atc cac gag aag ggc ttc tac
397 S N L H L R N G E L V I H E K G F Y
1243 tac atc tac agc cag acc **cag** ttc **aag** ttc **cgg** gaa gag atc aaa gag aat acc
415 Y I Y S Q T **Q** F **K** F **R** E E I K E N T
1297 aag aac gac aag cag atg gtg cag tac atc tat aag tac acc agc tac ccc gac
433 K N D K Q M V Q Y I Y K Y T S Y P D
1351 ccc atc ctg ctg atg aag tcc gcc cgg aac agc tgc tgg tcc aag gat gcc gag
451 P I L L M K S A R N S C W S K D A E
1405 tac ggc ctg tac agc atc tac cag ggc ggc atc ttc gag ctg aaa gag aac gac
469 Y G L Y S I Y Q G G I F E L K E N D
1459 cgg atc ttc gtg tcc gtg acc aac gag **cgg** ctg **ctg** **cag** atg gac cac gag gcc
487 R I F V S V T N E **R** L **L** **Q** M D H E A
1513 agc ttt ttc ggc gcc ttc ctc gtg gga tag
505 S F F G A F L V G *

9.2. List of abbreviations

`	Minute(s)
°C	Degree Celsius
aa	Amino acid(s)
ADCC	Antibody-dependent cell-mediated cytotoxicity
Apaf1	Apoptosis-inducing factor-1
APC(s)	Antigen presenting cell(s)
APS	Ammonium persulfate
Bcl-2	B-cell lymphoma 2
BID	BH3 interacting domain death agonist
BSA	Bovine serum albumin
Caspase	Cysteinyl aspartate specific protease
CCR6/7	C-C chemokine receptor 6/7
CD30	Tumor necrosis factor receptor superfamily member 8
CD30L	Ligand for tumor necrosis factor receptor superfamily member 8
CD40	Tumor necrosis factor receptor superfamily member 5
CD40L	Ligand for tumor necrosis factor receptor superfamily member 5
CD95 (Fas)	Tumor necrosis factor receptor superfamily member 6
CD95L (FasL)	Ligand for tumor necrosis factor receptor superfamily member 6
c-FLIP	Cellular FLICE-inhibitory protein
CHX	Cycloheximide
cIAP(s)	Cellular inhibitor of apoptosis protein(s)
CLL	Chronic lymphocytic leukemia
CO_2	Carbon dioxide
CRD	Cysteine rich domain
CV	Crystal violet
DC(s)	Dendritic cell(s)
DcR(s)	Decoy receptor(s)
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signaling complex
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DR(s)	Death receptor(s)
E.coli	*Escherichia coli*
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGP2	Epithelial Glycoprotein 2
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death-domain
FAP	Fibroblast activation protein
FcɣR(s)	Fcɣ receptor(s)
FCS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
fl	Full length
g	Gram

G418-Sulfate	Geneticin disulfate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GpL	*Gaussia princeps* luciferase
h	Hour(s)
HI	Heat-inactivated
HRP	Horseradish peroxidase
IAP(s)	Inhibitor of apoptosis protein(s)
iDC(s)	Immature dendritic cell(s)
IgG	Immunglobulin G
IL	Interleukin
IP	Immunoprecipitation
IκBα	Inhibitor of NFκB alpha
JNK	c-Jun N-terminal kinase
kDa	Kilodalton
LPS	Lipopolysaccharide
m	Milli (10^{-3})
M	Molar (Mol/Liter)
M2	Anti-Flag mAb
m2	Quadratmeter
mA	Milliampere
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCSP	Melanoma-associated chondroitin sulfate proteoglycan
mDC(s)	Mature dendritic cell(s)
MHC	Major histocompatibility complex
min	Minute(s)
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
n.s	Nonspecific
NFκB	Nuclear factor κB
NHL	Non-Hodgkin lymphoma
NK	Natural killer
NKT	Natural killer T cells
nm	Nanometer
NOXA	BH3-only member of the Bcl-2 protein family
NT	Nucleotide(s)
OD	Optical density
OPG	Osteoprotegerin
OX40	Tumor necrosis factor receptor superfamily member 4
OX40L	Ligand for tumor necrosis factor receptor superfamily member 4
p	Pico (10^{-12})
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBMCs	Peripheral blood mononucleated cell(s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGE2	Prostaglandin E2
pIκBα	Phosphorylated form of IκBα
pJNK	Phosphorylated form of JNK

PMB	Polymyxin B
PUMA	p53 upregulated modulator of apoptosis
RA	Rheumatoid arthritis
RLU	Relative light unit
rpm	Revolutions per minute
scFv	Single chain antibody fragment
SDS	Sodium dodecyl sulfate
sec	Second
SMAC	Second mitochondria-derived activator of caspases
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TNC	Tenascin-C
TNF	Tumor necrosis factor
TNFR(s)	Tumor necrosis factor receptor(s)
TNFRSF	Tumor necrosis factor receptor superfamily
TRAF(s)	TNF receptor associated factor(s)
TRAIL	TNF-related apoptosis inducing ligand
TRAILmutR1	TRAIL mutant conferring specificity to TRAILR1
TRAILmutR2	TRAIL mutant conferring specificity to TRAILR2
TRAILR(s)	TNF-related apoptosis inducing ligand receptor(s)
Tregs	Regulatory T cells
V	Volt
VEGF	Vascular endothelial growth factor
wt	Wild type
xIAP	X-linked inhibitor of apoptosis protein
α	Anti
μ	Micro (10^{-6})

9.3. List of publications originating from this thesis

1- CD70-restricted specific activation of TRAILR1 or TRAILR2 using scFv-targeted TRAIL mutants. Trebing J, **El-Mesery M**, Schäfer V, Weisenberger D, Siegmund D, Silence K, Wajant H. Cell Death Dis. 2014 Jan 30;5:e1035. doi: 10.1038/cddis.2013.555.

2- CD40-directed scFv-TRAIL fusion proteins induce CD40-restricted tumor cell death and activate dendritic cells. **El-Mesery M**, Trebing J, Schäfer V, Weisenberger D, Siegmund D, Wajant H. Cell Death Dis. 2013 Nov 14;4:e916. doi: 10.1038/cddis.2013.402.

9.4. Curriculum vitae

9.4.1. Personal information:

Place of birth: Belcas, Dakahlia, Egypt

Date of birth: 01/09/1983

Nationality: Egyptian

Marital status: Married

9.4.2. Education and scientific degrees:

10/2010-09/2014	Full PhD DAAD Scholarship, Division of Molecular Internal Medicine, University Hospital of Würzburg, Germany
10/2006-8/2008	Master degree in Pharmaceutical Science (Biochemistry), Faculty of Pharmacy, Mansoura University, Mansoura, Egypt. Title of master thesis “Evaluation of antitumor activity of some natural compounds in experimental animals.”
09/2000-05/2005	Bachelor of Pharmacy, degree excellent with honor, top student throughout the 5 year-period of study, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

9.4.3. Work experience:

2011-2014	Award full PhD DAAD scholarship, Division of Molecular Internal Medicine, University Hospital of Würzburg, Germany
2008-2010	Assistant lecturer of Biochemistry, Biochemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.
2005-2008	Demonstrator of Biochemistry, Biochemistry Department, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt.

Signature