Generation and characterization of CD40-Flag-FasL, a novel Fas agonist devoid of systemic toxicity

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Index

1 Abbreviations

AA	Amino acid
ABTS	2, 2´-Azino-di-3-ethylenbenzthiaolinsulphonic acid
ADH	Alcohol dehydrogenase
AIF	Apoptosis-inducing factor
ALT	Alanine amino transferase
AMAZE	Antibody-Mediated Activation of Apoptotic Zytokine
AP	Alkaline phosphatase
APAF-1	Apoptotic protease-activating factor 1
APS	Ammonium-persulphate
AST	Aspartate amino transferase
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosin-triphosphate
BCA	Bicinchonic acid assay
BCIP	5-Brom-4-Chlor-3-Indolylphosphate-p-Toluidinsalt
Bcl-2	B-cell lymphoma (Protein) 2
BIR	Baculovirus IAP repeat
BH	Bcl-2 homology domain
bp	Base pair
BSA	Bovine serum albumin
°C	Degree centigrade
CAD	Caspase-activated DNAse
CARD	Caspase recruitment domain
Caspase	Cysteinyl aspartate-specific protease
cDNA	complementary DNA
CD40L	CD40 ligand
ced	cell death abnormal
cFLIP	cellular FLIP
CFP	Cian fluorescent protein
CHX	Cycloheximide
COMP	Cartilage oligomeric matrix protein

CO_2	Carbon dioxide
CRD	Cystein-rich domain
CTL	Cytotoxic T lymphocytes
dATP	desoxy-adenosine-triphosphate
DcR	Decoy receptor
DD	Death domain
DED	Death effector domain
DEPC	Diethyl-pyrocarbonate
DFF	DNA fragmentation factor
DIABLO	Direct IP binding protein with low pI
DISC	Death-inducing signaling complex
DMF	N, N-dimethyl-formamide
DMSO	Dimethyl sulphoxide
DNA	Deoxy-ribonucleic acid
DR	Death receptor
DTT	Dithiothreitol
EDAR	Ectodermal dysplasia receptor
EDTA	Ethylendiamine-tetra-acetic acid
egl	egg-laying defective
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular Signal-Regulated Kinase
F	Farad
FACS	Fluorescence Activated Cell Sorting
FasL	Fas-Ligand
FCS	Fetal calf serum
FADD	Fas-Associated Death Domain
FLICE	FADD-Like ICE
FLIP	FLICE-Inhibitory Protein
FLIP-L	FLIP-Long
FLIP-S	FLIP-Short
GFP	Green Fluorescent Protein

HEPES	2-[4-(2-Hydroxyethyl)-1-piperazino]-Ethansulphonic acid
HtrA2	High temperature requirement protein A2 (= Omi)
IAP	Inhibitor of Apoptosis Protein
ICE	Interleukin-1beta-Converting Enzyme
IFNγ	Interferon-γ
IKK	I-κB Kinase
I-κB	Inhibitor of NF-κB
IL-1	Interleukin-1
JNK	c-Jun N-terminal Kinase
kDa	Kilo-Dalton
LTα	Lymphotoxin alpha
mA	Milliampere
mAb	monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
min	Minute
MKK	MAPK Kinase
NAMAZE	Non-antibody mediated activation of apoptotic zytokine
NBT	p-Nitrotetrazoliumbluechloride
NF-κB	Nuclear Factor kappa B
NGF	Nerve Growth Factor
NGFR	NGF-Receptor
NK	Natural killer
NLS	Nuclear localization sequence
nm	Nanometer
NMR	Nuclear magnetic resonance
O/N	Over night
OPG	Osteoprotegerin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction

PE	Phycoerythrin
PEG	Polyethylene glycol
PLAD	Pre-ligand-binding assembly domain
PMSF	Phenylmethylsulphonylfluoride
PKB	Protein kinase B
RANK	Receptor activator of NF-κB
RANKL	Receptor activator of NF-κB ligand
RHD	Rel homology domain
RING	Really interesting new gene
RIP	Receptor-interacting protein
RNA	Ribonucleic Acid
RNAse A	Ribonuclease A
RPA	RNAse protection assay
RPM	Round per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
siRNA	small interfering RNA
SMAC	Second mitochondria-derived activator of caspase (=DIABLO)
TACE	TNFα Converting Enzyme
TCR	T-cell receptor
TEMED	N, N, N′, N′-Tetramethylendiamine
TGF-β	Transforming Growth Factor β
TNF	Tumour Necrosis Factor
TNFR	TNF Receptor
TRAF	TNF Receptor Associated Factor
TRAIL	TNF-Related Apoptosis-Inducing Ligand
TRAIL-R	TRAIL-Receptor
V	Volt
vFLIP	viral FLIP
W	Watt

WT	Wild type
XIAP	X-Chromosome-Linked Inhibitor of Apoptosis
YFP	Yellow fluorescent protein
zVAD-fmk	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketon

2 Summary

Recent studies have shown that soluble single chain antibody fusion proteins incorporating the extracellular domains of human TNF, FasL or TRAIL acquire functional properties similar to those of their corresponding membrane-bound forms, following interaction with surface antigens expressed by target cells (Wajant et al, 2001; Wuest et al, 2001; Samel et al, 2003). In the work presented here this principle is extended to include other selective protein-protein interactions. Soluble fusion proteins comprising, at the N-terminus, the extracellular domains of human CD40 (AA1-192), 4-1BB (AA1-186), TNFR1 (AA 1-211), TNFR2 (AA 1-257), RANK (AA 29-213) and, at the C-terminus, the extracellular domain of human FasL (AA 139-281) were generated. CD40 was also fused to the extracellular domain of human TRAIL (AA 95-281). In all cases the two domains were separated by the Flag sequence to facilitate the detection and purification of the fusion proteins, and all the DNA constructs had a leader sequence to target the resulting proteins to the secretory pathway. The proteins had MWs over the expected sizes, a tendency that was particularly striking for RANKed-Flag-FasL and CD40-Flag-FasL, probably due to post-translational modifications such as glycosylation. The proteins were subsequently analysed for their soluble FasL-like activity on HT1080 cells. Despite only moderate expression or barely detectable expression in the cases of TNFR1-Flag-FasL, 4-1BB-Flag-FasL and TNFR2-Flag-FasL, these proteins showed high specific activities. In sharp contrast to the above mentioned fusion proteins, supernatants of CD40-Flag-FasL and RANKed-Flag-FasL, which contained much more protein, showed very low and low specific activity, respectively. Since the activity of soluble FasL can be increased through multimerization (Schneider et al, 1998), the proteins were cross-linked via their internal Flag-tag. Surprisingly, it led to a decrease in activities for TNFR1-Flag-FasL, TNFR2-Flag-FasL and 4-1BB-Flag-FasL. The activity of RANKed-Flag-FasL could be increased by a factor of 50-100 but the cell death-inducing capacity of CD40-Flag-FasL was virtually unchanged by artificial cross-linking. The high specific activity observed for TNFR1-Flag-FasL, 4-1BB-Flag-FasL and TNFR2-Flag-FasL was thus likely the result of the presence of significant amounts of higher MW aggregates of homotrimers in the supernatants. RANKed-Flag-FasL was probably mostly non-

aggregated and was therefore activated upon cross-linking. The lack of any M2 cross-linking antibody-mediated effect in the case of CD40-Flag-FasL does not indicate a defect in the FasL part of the protein but might rather reflect, for example, steric hindrance of the M2 antibody. RANKed-Flag-FasL and CD40-Flag-FasL were thus subsequently further analysed with respect to their capacity for target-dependent activation. CD40-Flag-FasL was stably expressed in HEK293 cells and then affinity purified from supernatants of the stable cells. Western blotting analysis showed that CD40-Flag-FasL migrated with a MW of ~50 kDa under reducing conditions however, analysis of the native protein by gel filtration showed that CD40-Flag-FasL eluted with an apparent MW of 471 kDa, corresponding to a 9.4 mer but this is likely to be an overestimation as CD40-Flag-FasL behaved like a homotrimeric molecule. Binding experiments showed that CD40-Flag-FasL could be immobilized specifically by CD40L-expressing cells and, furthermore, that upon binding it induced clustering of Fas on neighboring cells. CD40-Flag-FasL induced apoptosis and NF-κB activation in a concentration-dependent manner in transfected HT1080 and KB cells expressing CD40L and not in parental cells, despite similar sensitivities of both parental and transfectant cells to cross-linked FasL. When RANKed-Flag-FasL was analysed in a setting allowing target antigen-mediated immobilization, the protein led to gene induction in a RANKL-dependent manner. The EC_{50} of CD40-Flag-FasL was shown to be ~ 4 logs lower in HT1080-CD40L compared to HT1080 cells. Cell death induced in CD40L-positive cells was accompanied by cleavage of pro-caspase-8 and activation of caspase-3 and could be completely inhibited by zVAD, Fas-Comp or an anti-human CD40L antibody. CD40-Flag-FasL induced apoptosis in a paracrine manner on by-stander cells and proved to be safe when assayed *in vivo* in mice for acute toxicity, whereas artificial cross-linking of CD40-Flag-FasL induced liver failure in a concentration-dependent fashion. The promising results obtained with CD40-Flag-FasL led to the cloning of a CD40-Flag-TRAIL construct that was shown to be capable of inducing both apoptosis and NF-κB in a CD40L-dependent manner. CD40-Flag-TRAIL-mediated apoptosis was shown to be TRAIL-specific, to result from the interaction between CD40/CD40L, and to occur via interaction with TRAIL-R2 in both KB-CD40L and HT1080-CD40L cells. These

promising results provide the basis for relatively simple development of safer i.e. less toxic FasL or TRAIL derivatives for the treatment of a variety of human pathologies.

3 Introduction

3.1 The Fas/FasL system

Fas (also called Apo-1, CD95 or TNFRSF6) was discovered in 1989 by two independent groups (Trauth et al, 1989; Yonehara et al, 1989). They reported the development of antibodies (IgG3 anti-APO-I and IgM anti-Fas, respectively) able to rapidly induce cell death in a variety of human cell lines. These antibodies were raised against a plasma membrane component of the SKW6.4 B human lymphoblastic cell line in one instance and against FS-7 fibroblasts in the other instance. Molecular cloning of the antigen recognized by the anti-Fas monoclonal antibody in 1991 (Itoh et al, 1991) allowed the classification of Fas as a member of the tumour necrosis factor receptor (TNFR) superfamily which contains to date 29 receptors (Aggarwal et al, 2003). The Fas antigen cloned by Nagata and his associates turned out to be identical to the APO-1 protein identified later by Krammer's group (Oehm et al, 1992). Fas is a 45 kDa type I-glycoprotein (N-terminal extracellular, C-terminal cytoplasmic) of 319 amino acids. Like most members of the TNFR superfamily it has a single transmembrane domain, an N-terminal cystein-rich extracellular domain and a C-terminal cytoplasmic domain (see Figure 1). It exists either as a membrane-bound or as a soluble protein. The extracellular region of TNFR family members is characterized by the presence of a cystein-rich domain (CDR) and members of this family carry at least one but typically several of these structural units. The extracellular part of human Fas comprises three CDRs. Additionally, a conserved domain in the first cystein rich repeat of Fas and of certain other TNFR family members has been identified, known as the pre-ligand-binding assembly domain (PLAD), that mediates specific ligand–independent homophilic association of receptors (Papoff et al, 1999; Chan et al, 2000; Siegel et al 2000). The PLAD is physically distinct from the part of the extracellular domain that forms the main contacts with the ligand, but is required for the assembly of Fas complexes that bind FasL and mediate signaling. TNF-related apoptosis inducing ligand receptor 1 (TRAIL-R1), CD40 and TNFR2 are other members of the TNFR superfamily showing such homotypic associations. The cytoplasmic tails of these receptors signal by interacting with two major groups of intracellular proteins: the TNF receptor-associated factors (TRAFs) and the

death domain (DD)-containing proteins. TNF-family receptors that have a DD sequence are called death receptors and Fas is the prototypic death receptor. The DD is a region of approximately 80 amino acid residues, first identified in the cytoplasmic domain of Fas and TNF-R1, that is responsible for transducing the death signal (Itoh et al, 1991 and 1993; Tartaglia et al, 1993). Besides Fas and TNF-R1 the group of death receptors comprises six additional members: death receptor (DR)3, DR4 or TRAIL-R1, DR5 or TRAIL-R2, DR6, p75-nerve growth factor receptor (NGFR) and ectodermal dysplasia receptor (EDAR). Death receptors can also mediate non-apoptotic signals and in fact EDAR and DR6 have so far not been shown to be strong apoptotic inducers but rather to activate NF-κB and MAP kinase members (reviewed in Wajant, 2003b). The tertiary structure of the DD of Fas, as revealed by NMR spectroscopy, consists of six anti-parallel amphipathic α-helices with an unusual topology that is found in other homophilic interaction domains (Huang et al, 1996). The latter include, besides the DD, the death effector domain (DED) found in Fas-associated death domain (FADD), for example, and the caspase recruitment domain (CARD) found in pro-caspase 9. The DDs and CARDs interact via electrostatic interactions (Fesik et al, 2000) whereas DEDs seem to bind through hydrophobic patches.

Human CD95 (Fas) is expressed on most normal cells including epithelial cells, fibroblasts and haematopoietic cells as well as on many transformed cells. The human Fas gene consists of nine exons and a variety of transcripts are derived from this gene by alternative splicing. Six variants of human Fas have been described so far that encode for proteins lacking the transmembrane domain but have retained the leader peptide and are thus secreted as soluble molecules. Most variants derive from a shift in the reading frame leading to premature stop codons. One of the variants contains all the three CRDs and should therefore retain full ligand binding capacity. Shorter variants, possibly lacking ligand binding capacity might still interfere with Fas signaling via PLAD-PLAD interaction with full length Fas. In agreement with this, splice variants can down regulate Fas-mediated apoptosis, suggesting a physiological role as modulators of Fas signaling (Ruberti et al, 1996).

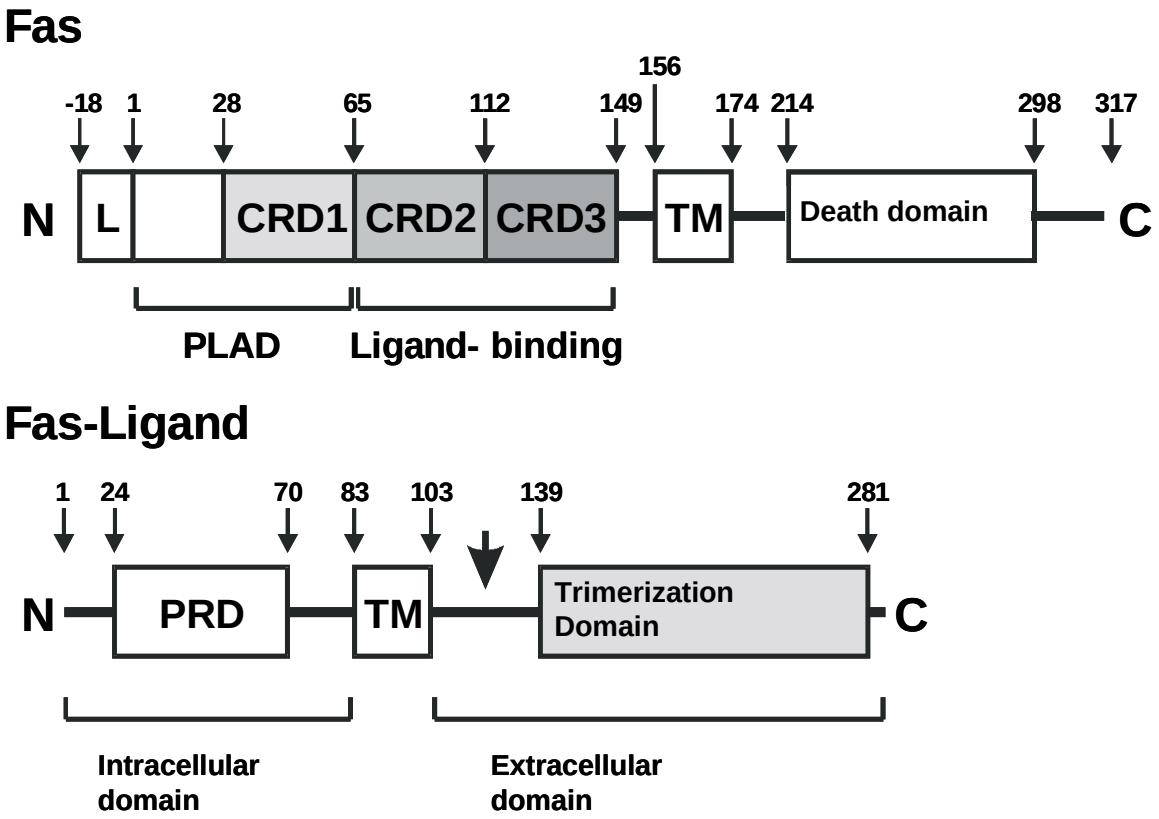

Figure 1. Structure of Fas and FasL (Wajant et al, 2003a). Numbering is based on the mature proteins. The cleavage site of FasL is indicated with a big arrow. CRD, cystein-rich domain; PLAD, pre-ligand-binding assembly domain; PRD, proline-rich domain; TM, transmembrane domain.

Human FasL (CD95L, APO-1L, TNFSF6, CD178) was cloned in 1993 by Suda and colleagues and is structurally similar to most members of the TNF family, that to date includes 19 different ligands (Suda et al, 1993; Aggarwal et al, 2003). It is a type II transmembrane protein that can be found as a 40 kDa membrane-bound or a 26 kDa soluble cytokine. FasL is characterized by an extracellular trimerization domain, a transmembrane domain and an intracellular proline-rich domain (see Figure 1).

The bioactive form of FasL is a homotrimer. It is believed that the membrane-integrated forms of the ligands of the TNF family form homotrimers, like their soluble counterparts, as revealed by crystallographic studies of soluble TNF, LTα, CD40L and others (Eck et al, 1988, 1989, 1992 and Jones et al, 1989). Similar to many other members of the TNF ligand family, the extracellular domain of FasL can be cleaved by metalloproteinases to release the soluble trimeric ligand (Suda et al, 1994; Tanaka et al, 1995). Both forms are capable of binding to Fas but only membrane-bound FasL can efficiently activate Fas. Soluble FasL has been reported to act as an antagonist preventing apoptosis induction by the membrane form of the ligand (Suda et al, 1997; Tanaka et al, 1998; Schneider et al,

1999). As mentioned earlier, the intracellular domain of FasL, contains a proline-rich domain which is thought to be responsible for sorting of FasL into secretory lysosomes as well as being involved in reverse signaling (Blott et al, 2001; Wenzel et al, 2001).

The cellular expression pattern of FasL, in contrast to that of Fas, seems to be limited to cells of the immune system such as activated T cells and natural killer (NK) cells although it is also expressed constitutively in the tissues of 'immune-privileged' sites Several groups have found that some tumour cells become resistant to Fas-induced apoptosis because they constitutively express FasL (Hahne et al, 1996). Such FasL expression on tumour cells counteracts the activity of cytotoxic T lymphocytes (CTL) and NK cells. This mechanism, termed the 'tumour counterattack', is thought to account for the ability of tumour cells to evade immune destruction.

3.2 TRAIL and its receptors

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL or Apo2L) was identified due to its sequence homology to FasL and consequently also belongs to the TNF ligand family (Willey et al, 1995; Pitti et al, 1996). TRAIL is expressed as a type II transmembrane protein of about 33-35 kDa, but the extracellular domain of the human protein, at least, can be proteolytically cleaved from the cell surface (Mariani et al, 1998; Liabakk et al, 2002). Like most members of the TNF ligand family, the extracellular domain of TRAIL forms a bell-shaped homotrimer that can bind three receptors at the interface between two of its subunits (Cha et al, 1999; Hymowitz et al, 1999, 2000; Mongkolsapaya et al, 1999). A zinc (Zn) ion bound by cysteins in the trimeric ligand is essential for optimal biological activity (Hymowitz et al, 1999; Bodmer et al, 2000). Because of this unique structural feature, the method of preparing recombinant soluble ligands may be important. Indeed some preparations of TRAIL lacking the Zn have reduced solubility and tend to aggregate, perhaps explaining the reported toxicity of certain TRAIL preparations to human hepatocytes (Jo et al, 2000; Lawrence et al, 2001). On the other hand, untagged trimeric TRAIL containing stoichiometric Zn did not induce significant cell death in human or cynomolgus monkey hepatocytes (Lawrence et al, 2001). The identification of two death receptors (TRAIL-R1/DR4; TRAIL-R2/DR5) that

can bind to TRAIL pointed to the mechanisms by which this ligand can induce apoptosis (Chaudhary et al, 1997; Mcfarlane et al, 1997; Pan et al, 1997; Screaton et al, 1997; Schneider et al, 1997; Sheridan et al, 1997; Walczack et al, 1997; Wu et al, 1997). In addition to these two receptors, three other receptors bind to TRAIL and appear to act as 'decoy' receptors. DcR1/TRAIL-R3 (LIT,TRID) and DcR2/TRAIL-R4 (TRUNDD) are closely homologous in their extracellular domains to DR4 and DR5 (Degli-Esposito et al, 1997; Masters et al, 1997; Mcfarlane et al, 1997; Pan et al, 1997, 1998; Schneider et al, 1997, 1998; Sheridan et al, 1997). DcR2 has a truncated, non functional DD, while DcR1 lacks a transmembrane domain and is membrane-anchored by a GPI linker. Both receptors are therefore incapable of transmitting an apoptotic signal, and seem to act as antagonists of the apoptosis-inducing TRAIL receptors by mechanisms not totally understood. A fifth receptor, the soluble TNFR family member osteoprotegerin (OPG), binds TRAIL in addition to RANKL (receptor activator of NF-κB ligand), a member of the TNF family involved in the regulation of osteoclastogenesis. Compared to the other TRAIL-Rs described above OPG has a lower affinity for TRAIL at physiological temperature (Emery et al, 1998; Truneh et al, 2000). Nevertheless, endogenously produce OPG can interfere in some cellular systems with TRAIL-induced apoptosis (Holen et al, 2002; Shipman and Croucher, 2003; reviewed by LeBlanc and Ashkenazi, 2003).

TRAIL is expressed on several normal human tissues including the lung, prostate, spleen and testis, as well as by activated human T cells and NK cells (Willey et al, 1995; Pitti et al, 1996; Jeremias et al, 1998). TRAIL-R1 and TRAIL-R2 are expressed on tumour cells as well as on normal human cells (Pan et al 1997; Screaton et al 1997; Schneider et al, 1997; Sheridan et al, 1997). Thus, unlike FasL or TNF, that are not constitutively expressed or have only limited expression, TRAIL and its death receptors are constitutively expressed in the organism under physiological conditions. Transcripts of TRAIL-R3 and TRAIL-R4 are found in several normal human tissues as well as on transformed cells. Various studies show that TRAIL potently induces apoptosis in a broad range of cancer cell lines but not in many normal cells (Ashkenazi et al 1999; Walczak et al, 1999). Although it has been shown that expression of TRAIL-R3/4 can protect against TRAIL-induced apoptosis in certain models, TRAIL sensitivity does not usually correlate with the expression of TRAIL decoy receptors, thus the level of

expression of TRAIL decoy receptors is not the major determining factor in the context of TRAIL sensitivity.

3.3 CD95 and TRAIL-R1/2 signaling pathways

3.3.1 The apoptotic pathway

3.3.1.1 Apoptosis: a historical overview

The fact that cell death occurs normally during vertebrate development was recognized over 150 years ago (Vogt, 1842), promptly forgotten and only re-discovered 100 years later (Glucksmann, 1951). Initially the analysis of cell death comprised mainly morphological considerations. In fact, between the late 1800's and 1960's, features of cell death such as cell shrinkage, chromatin condensation, break-up and engulfment of cells were recognized via light and electron microscopy (Bellairs, 1961 and reviewed by Clarke, 1996 and by Lockshin, 1997). In 1965 the term programmed cell death (PCD) was employed to describe this sort of cell death (Lockshin and William, 1965) followed in 1972 by the term 'apoptosis' (Kerr et al, 1972). Nevertheless, the study of physiological cell death in which an organism's cells activate intrinsic mechanisms for the purpose of killing themselves, remained relatively obscure until the late 1980's. A genetic understanding of cell death has primarily come from the study of the free-living nematode *Caenorhabditis elegans,* providing insights into the regulation of the cell death process and identifying a simple, but evolutionarily conserved model. During the ontogenesis of *C. elegans* 1090 cells are generated, 131 of which undergo PCD, the latter caused by a process that was shown to be specific for cell death and had no other role. These findings indicated that cell death in the worm is an active process, the only purpose of which is to remove unwanted cells (Horwitz et al, 1982).

The first physiological component of cell death to be identified that did not rely on morphology came with the recognition that the process is usually accompanied by rapid activation of endonucleases (Williams et al, 1974), but it took a further 17 years to identify the major endonuclease responsible (DNA fragmentation factor/caspase-activated DNAse: DFF/CAD) (Lu et al, 1997; Enari et al, 1998). The observation that

phosphatidyl serine is exposed on dying cells provided another convenient marker for apoptosis (Fadok et al, 1992). Increasing interest in the field came with the identification of the biochemical and genetic processes that implement it, beginning with the recognition of the first component of the cell death system, Bcl-2, the gene for which was cloned in 1986 (Tsujimoto et al, 1986; Cleary et al, 1986). It was cloned originally not because it was recognized as a gene concerned with cell death but because it is translocated in follicular lymphoma. It was initially assumed that Bcl-2 resembled other oncogenes involved in translocation such as abl and c-myc, and that it promoted cellular proliferation. However over-expression of Bcl-2 lead not to stimulation of cell division but to prevention of cell death when growth factor was removed (Vaux et al, 1988). These experiments not only identified Bcl-2 as a regulator of the apoptosis machinery, but showed that inhibition of cell death could ultimately lead to cancer in humans (reviewed by Vaux, 2002).

3.3.1.2 Molecular mechanisms of apoptosis induction

Fas and the TRAIL death receptors activate the apoptotic machinery by a common mechanism. These death receptors can be activated either by agonistic antibodies, cross-linked soluble ligands or by their membrane-bound ligands. In the case of Fas and TRAIL-R2 their soluble ligands induce only poor activation whereas the membrane-bound ligands are required for optimal signaling. TRAIL-R1 however can be activated both by soluble TRAIL and by membrane-bound TRAIL (Wajant et al, 2001). Recent studies demonstrated that a variety of factors, including anti-tumour reagents such as ET-18-0CH3 and ganciclovir (Gajate et al, 2001; Beltinger et al, 1999), UV light (Aragane et al, 1998), bile salts (Faubion et al, 1999), as well as cell shrinkage (Fumarola et al, 2001), induce FasL-independent aggregation of Fas. These findings suggest that Fas receptor can be activated either extracellularly, via FasL, or from within the cell independently of ligand interactions through ‘concentration’-driven aggregation (Aragane et al, 1998; Bennett et al, 1998; Sodemann et al, 2000), and emphasize the importance of receptor aggregation for initiation of Fas-associated signaling pathways.

After ligand binding, the pre-assembled signaling-inactive receptor complexes reorganize to signal competent receptor clusters. For Fas, development of strong intracellular signals has been correlated vith the formation of large receptor/ligand clusters (Algeciras-Schminch et al, 2002). It has been suggested that ceramide and lipid rafts are involved in clustering and internalization of Fas although this remains debatable and may be cell type-dependent (Cremesti et al, 2001; Grassme et al, 2001; Algeciras-Schminch et al, 2002; Hueber et al, 2002). The physiological implication of Fas internalization is still unknown and internalization of receptor ligand complexes seems not to be mandatory for the induction of Fas-mediated apoptosis (Schutze et al, 1999; Algeciras-Schminch et al, 2002). Since hexamers of FasL possess superior apoptotic capacity when compared to normal trimeric FasL, it has been speculated that a single ligated trimeric Fas aggregate recruits only one molecule of caspase-8 while at least two molecules of caspase-8 are necessary for the initial proteolytic activation (Holler et al, 2003). This could be one of the reasons for the need for large receptor clusters for efficient signaling.

Ligand-dependent clustering of receptors is a prerequisite for the assembly of the so-called death-inducing signaling complex (DISC) (Kischkel et al, 1995). As well as the ligands and receptors the DISC contains the DD-containing protein FADD, the initiator caspases pro-caspase-8 and pro-caspase-10 and cellular FLICE-inhibitory protein (cFLIP). FADD binds to ligated Fas, TRAIL-R1 or TRAIL-R2 via its C-terminal DD and, through its N-terminal DED, can recruit the pro-enzyme form of caspase-8. Pro-caspase-8 is composed of an N-terminal regulatory prodomain consisting of two DEDs and a C-terminal caspase homology domain. In the DISC several molecules of pro-caspase-8 are located in close proximity, leading to their activation by dimerization. Subsequently, the active caspase-8 dimer is stabilized by a two-step autoproteolytic process (Salvesen et al, 1999; Chen et al, 2002, Boatright et al, 2003; Donepudi et al, 2003). Caspases are cystein proteases that cleave after a loosely specific series of four amino acids and which absolutely require the presence of an aspartate residue at position P1 of their substrate (Earnshaw et al, 1999). These proteases are responsible for executing the apoptotic process. The apoptotic caspases perform different roles. The effector caspases-3, -6 and -7 are responsible for the cleavage of proteins characteristic of apoptosis and are responsible for most of the cleavage of the proteins that induce the

major morphological changes observed during programmed cell death. The initiator caspases, caspase-8, caspase-9 and caspase-10 transduce the first signals of apoptosis. As mentioned earlier, as well as two DEDs caspase-8 and caspase-10 contain a protease domain consisting of two subunits, p20 and p10. Caspase-8, the main initiator caspase, is expressed as two isoforms; caspase-8/a (p55) and caspase-8/b (p53) which are both recruited to the DISC. A first cleavage occurs between the p20 and the p10 subunits of the caspase homology domain of pro-caspase, resulting in a FADD-bound p43/p41 intermediate, which contains the DEDs and the p20 subunit. The p10 subunit remains non-covalently associated with the p43/41 intermediate. A second cleavage event between the two DEDs and the p20 subunit releases the mature enzymatically active caspase-8 from the DISC as a heterotetramer containing two p18 and two p10 subunits (Boatright et al, 2003; Donepudi et al, 2003). Several other proteins have been shown to be recruited to the DISC by direct interaction with DISC proteins. The roles of many of these proteins are not yet clear (reviewed by Peter & Krammer, 2003; Wajant, 2003b).

Two pathways of death receptors' apoptotic signaling have been described that depend on the quantity of activated caspase-8 produced at the DISC (Scaffidi et al, 1998). In type I cells a high production of caspase-8 at the DISC can directly process the effector caspase, caspase-3, leading to its activation and apoptosis of the cell. In type II cells however, only small amounts of caspase-8 are produced in the DISC. The DISC in those cells is formed quite poorly, little FADD is recruited and little caspase-8 is induced. Apoptosis in these cells depends on Bcl-2 family members. The latter consists of two functional classes of proteins including anti-apoptotic members such as Bcl-2, $Bclx_L$, Mcl1, Bfl1 and Brag1 as well as pro-apoptotic molecules such as Bax, Bad, Bid, Bik, Bim. Bcl-2 family members are characterized by up to four so-called Bcl-2 homology (BH) domains, with pro-apoptotic counterparts having typically only one BH3. Bcl-2 family members can interact with each other through a complex network of homo- and heterodimers. In type II cells the little caspase-8 induced cleaves the BH3-only domain-containing Bcl-2 family member Bid (Li et al, 1998; Luo et al, 1998), resulting in a pro-apoptotic fragment termed tBid (truncated Bid). This fragment induces the pro-apoptotic functions of mitochondria by causing aggregation of Bax or Bak (reviewed by Korsmeyer et al, 2000). Pores formed by oligomerized Bax/Bak then allow the exit of apoptogenic proteins,

including cytochrome c, the second mitochondria-derived activator of caspase/direct inhibitor of apoptosis protein (IAP)-binding protein (Smac/Diablo) and the serine protease HtrA2/Omi into the cytosol. Smac/Diablo and HtrA2/Omi interfere with the caspase-inibitory function of members of the IAP family, thereby promoting apoptosis. Following their release, the adaptor protein Apaf-1 and cytochrome c combine with seven molecules of dATP to form a large protein complex, the apoptosome, a sort of sub-mitochondrial DISC, where caspase-9 as initiator caspase is activated (Shi, 2002). Caspase-9 then activates caspase-3 resulting in apoptosis of the cell. Remarkably, active caspase-3 itself is able to process its upstream activators caspase-8 and caspase-9 establishing a self amplifying loop of caspase activation. Type I and type II cells can be distinguished experimentally by the fact that in type II cells, expression of the anti-apoptotic members of the Bcl-2 family such as Bcl-2 or $Bclx_L$ renders these cells resistant to death receptor-mediated apoptosis (Figure 2) (reviewed in Wajant, 2003b, 2004).

Fas kills cells not only by apoptosis induction but also, depending on the cellular context, by necrosis. Fas-induced necrosis requires the adaptor protein FADD and the Fas interacting kinase RIP, whereas caspase-8 seems to be dispensable (Holler et al, 2000; Matsumura et al, 2000).

3.3.1.3 Regulators of death receptor-induced apoptosis

Apoptosis induced by death receptors is principally regulated by the IAPs and the two cFLIP isoforms (Figure 2). As their name implies, the IAPs confer protection from death receptor-mediated apoptosis. The prototype IAP was described in baculoviral genomes in 1993, by means of a genetic screen to identify regulators of host-cell viability during virus infection (Crook et al, 1993). This discovery led to the identification of cellular orthologues in species as diverse as yeast, nematodes, flies and humans. Two motifs were identified in baculovirus IAPs: the baculovirus IAP repeat (BIR) and the really interesting new gene (RING) domain. RING-containing proteins can catalyze the degradation of both themselves and selected target proteins through ubiquitinylation. The BIR is a ~70-residue zinc binding domain, of which one to three copies are found in IAPs. The BIRs are essential for the anti-apoptotic properties of the IAPs, and in several cases this has

been attributed to the binding and inhibition of caspases. To date, eight human IAPs have been identified, the best characterized of which is the X-linked IAP (XIAP). XIAP is an extremely potent suppressor of apoptosis and these effects are mediated, at least in part, by its ability to suppress caspases directly (Devereaux et al, 1997). Indeed, XIAP, c-IAP1 and c-IAP-2 are thought to directly inhibit certain caspases, in particular caspase-3, caspase-7 and caspase-9 (Roy et al., 1997; Devereaux and Reed, 1999). Structure-function analysis of XIAP showed that the BIR3 domain of XIAP binds directly to the small sub-unit of caspase-9. The cleavage of caspase-9 is not required for activation, yet, paradoxically, cleavage seems to be required for the inactivation of caspase-9 by XIAP. The small subunit of caspase-9 is generated by means of proteolytic cleavage at the conserved aspartate residue at position 315, an event that exposes a segment, starting at residue 316, that is recognized by the BIR3 domain of XIAP (Sun et al, 2000; Srinivasula et al, 2001).

The crystallographic resolution of XIAP with caspase-3 and caspase-7 shows that the domain in XIAP that is essential for interaction lies in a small segment that is immediately amino-terminal to BIR2 (Chai et al, 2001; Huang et al, 2001; Riedl et al, 2001). This domain functions by reversible, high affinity binding to caspase-3 and caspase-7, and results in the stearic occlusion of normal substrates of these caspases. XIAP thus functions solely to mask the active site in the caspases.

Biochemical studies have lead to the identification of an intriguing IAP-interacting protein known as Smac/DIABLO, already referred to above. In non-apoptotic cells the protein is localized to the inter-membrane space of mitochondria, and it is released along with cytochrome c in response to apoptotic stimuli. Upon release from mitochondria Smac/Diablo binds XIAP, and probably several other IAPs, in a manner that displaces the caspases from XIAP (Chai et al, 2000; Srinivasula et al, 2001). Recent structural studies have lead to the identification of Omi/HtrA2, a serine protease that is released from mitochondria in addition to Smac/DIABLO and that promotes apoptosis induction in a similar way (Suzuki et al, 2001). However, the activities of XIAP and other IAPs are not restricted to caspase inhibition as there is compelling evidence for their role in cell-cycle regulation, protein degradation and caspase-independent signal-transduction cascades (NF-κB, JNK and TGF-β)(reviewed by Salvesen and Duckett, 2002).

FLIP proteins were initially identified by means of a bioinformatics screen for novel proteins with DED. Viral FLIP proteins (v-FLIP) were first detected in γ2-herpesviruses. Cellular FLIP was identified later (c-FLIP, also called CASH, Casper, CLARP, FLAME, I-FLICE, MRIT or ursupin) (Goltsev et al, 1997; Han et al, 1997; Hu et al, 1997; Inohara et al, 1997; Irmler et al, 1997; Shu et al, 1997; Srinivasula et al, 1997; Rasper et al, 1998). Several splice isoforms of c-FLIP have been described, two of which have been shown to be expressed at the protein level: the short FLIP ($FLIP_S$) isoform and the long FLIP ($FLIP_L$). The overall structure of $FLIP_L$ is similar to caspase-8 and caspase-10, but it lacks catalytic activity because several of the amino acids that are critically required for such activity have not been conserved (Cohen, 1997). However $FLIP_L$ contains an Asp (Asp 341) between the p20 and the p10-like domains of the caspase-homologous region, in a position that is well conserved in caspase-8 and caspase-10 and that can be cleaved in the context of death receptor signaling (Irmler et al., 1997; Scaffidi et al, 1999). Owing to their structural homology with the N-terminus of caspase-8 and 10 but highly variable C-terminal extensions, $FLIP_S$ and $FLIP_L$ can interfere with receptor-mediated activation of these caspases by different mechanisms. They bind to the DED-containing amino terminus of FADD, caspase-8 or caspase-10, but while $FLIP_S$ acts as a dominant negative inhibitor of these caspases by preventing processing and release of active caspase-8 or -10 from the receptor (Thome et al, 1997; Krueger et al, 2001), $FLIP_L$ can bind to both the DEDs and the caspase domain of caspase-8. Moreover, it has a somewhat different effect than $FLIP_S$. Indeed in the presence of $FLIP_L$, both caspase-8 and $FLIP_L$ are partially processed into a C-terminal p10 fragment and an N-terminal p43 fragment that stay bound to the receptor complex (Scaffidi et al, 1999; Krueger et al, 2001; Micheau et al, 2002). $FLIP_L$ provides considerably stronger or more complete protection against FasL- and TRAIL-induced cytotoxicity despite the fact that the partially processed caspase-8/$FLIP_L$ heterodimer in the DISC has substantial enzymatic activity (Chang et al, 2002; Micheau et al, 2002). It is conceivable that the partially processed caspase-8/$FLIP_L$ heterodimer has proteolytic activity with altered substrate specificity or membrane-restriction action, which could increase cell survival by cleavage-induced inactivation of cell death-promoting proteins. Alternatively, the presence of $FLIP_L$ may induce the recruitment of additional signaling molecules like TNF receptor-associated factor-1 and -

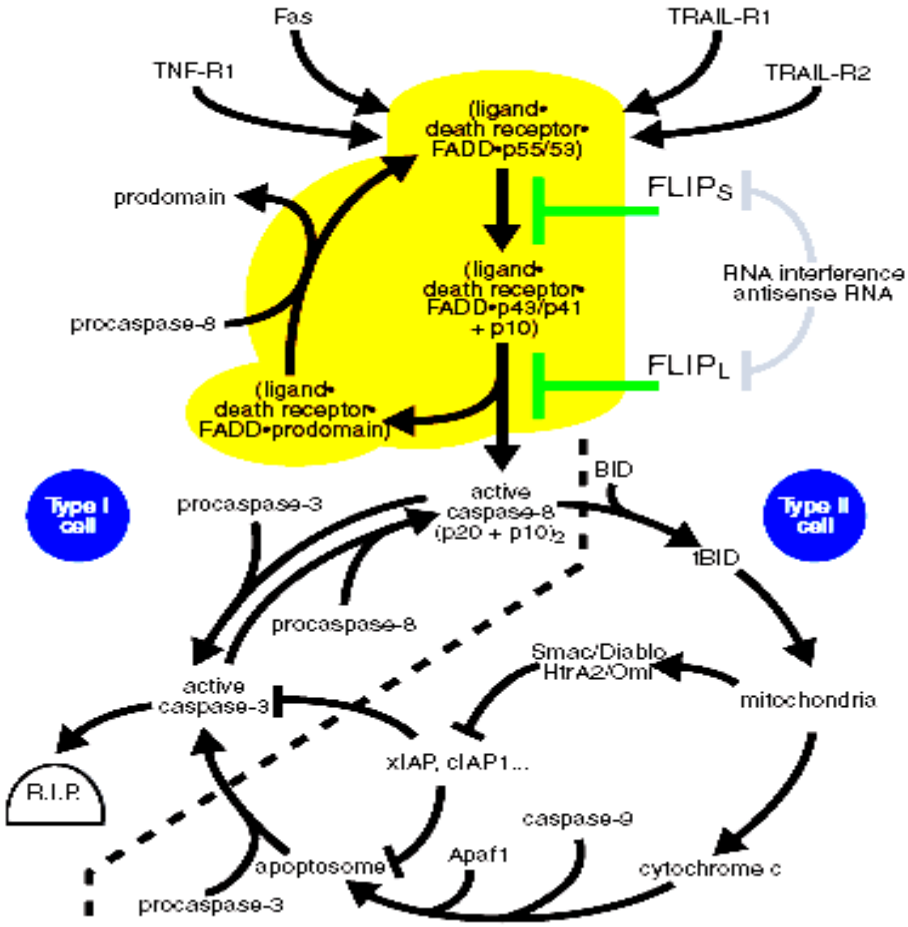

Figure 2. Apoptotic signaling pathways induced by death receptors. (Wajant, 2003c). In type I cells, death receptors (e.g., Fas) induce strong caspase-8 activation, which alone is sufficient to lead to robust processing of effector caspases such as caspase-3 and apoptosis induction (events shown left of the bold dashed line, golden brown region). In type II cells, only low amounts of caspase-8 are activated upon DISC formation. In addition, the action of effector caspases processed by caspase-8 is blocked by IAP proteins. Apoptosis induction in this type of cells is therefore dependent on a mitochondrial amplification loop, which triggers a second pathway leading to caspase-3 activation and furthermore interferes with the action of IAP proteins. IAP, inhibitor of apoptosis protein; BID, BH3- interacting domain death agonist; tBID, truncated BID (a caspase derived cleavage product of BID); TRAIL, tumour necrosis factor–related apoptosis-inducing ligand; SMAC, second mitochondria-derived activator of caspase; Diablo, direct IAP binding protein with low pI; HtrA2, human serine protease with significant similarity to *E. coli* HtrA (HtrA2 is also called Omi); Apaf1, apoptotic protease activating factor 1.

2 (TRAF1 and TRAF2) and Raf-1 within the Fas DISC. These proteins may link $FLIP_L$ to the activation of NF-κB and extracellular signal-regulated kinase (ERK)-dependent transcriptional pathway and thus lead to increased proliferation and/or survival of $FLIP_L$-

expressing cells. Since $FLIP_L$ is induced by NF-κB itself, such a mechanism could serve as a feedback amplification loop by further increasing $FLIP_L$ expression levels (Kreuz et al, 2001; Micheau et al, 2001).

3.3.2 Non-apoptotic signal transduction pathways

Signaling by the death receptors Fas and TRAIL-R1/2 has been extensively studied with respect to their apoptosis-inducing capabilities but just like TNF-R1, for which a broad range of non-apoptotic cellular responses have been described, it is becoming increasingly evident that gene induction is also a function of these receptors. Fas has been reported to enhance proliferation of TCR-stimulated T-cells, thymocytes (Alderson et al, 1993) and fibroblasts (Aggarwal et al, 1995; Freiberg et al, 1997; Jelaska & Korn, 1998) and soluble FasL has been shown to activate ERK1/2 in serum-starved fibroblasts (Ahn et al, 2001). The Fas/FasL system is also involved in inflammation, leading to recruitment of neutrophils (Seino et al, 1998; Ottonello et al, 1999; Hohlbaum et al, 2000; Waku et al, 2000; Behrens et al, 2001; Roth et al, 2001). Most importantly some reports have shown the ability of these receptors to induce signaling pathways like NF-κB and JNK (Mühlenbeck et al, 2000; Wajant et al, 2000; Kreuz et al, 2004).

3.3.2.1 Fas and TRAIL death receptors-mediated activation of the transcription factor NF-κB

NF-κB was discovered by Baltimore and co-workers in 1986 as a factor in the nucleus of B cells that binds to the enhancer of the kappa light chain of immunoglobulin (Sen and Baltimore, 1986). It has since been shown to be expressed ubiquitously in the cytoplasm of all cell types, from *Drosophila* to man. It translocates to the nucleus only when activated, where it regulates the expression of over 200 immune, growth, and inflammation genes. Currently, NF-κB is known to consist of a family of Rel-domain-containing proteins including the following: Rel (also known as cRel), Rel A (also known as p65 or NF-κB3), Rel B, NF-κB1(or p50), NF-κB2(or p52). All five of these proteins have a Rel homology domain (RHD) which serves as their dimerization, DNA-

binding and principal regulatory domain. The RHD contains a nuclear-localization sequence (NLS) at its C terminus that is rendered inactive in non-stimulated cells through binding to specific NF-κB inhibitors, known as the I-κB proteins. This *ankyrin* repeats-containing family of proteins consists of IκBα, IκBβ (IκBβ1 and IκBβ2), IκBγ, IκBε, bcl-3, NF-κB1 and NF-κB2. NF-κB1 and NF-κB2 are initially made as large precursor molecules, p105 and p100, respectively. Phosphorylation-dependent cleavage of p100 produces p52, whereas p105 is cleaved to p50. Interestingly, NF-κB1 and NF-κB2 are in essence an RHD fused through its c-terminus to an auto-inhibitory *ankyrin* repeat domain. So the two precursors, which can dimerize with the different Rel proteins, are trapped in the cytoplasm and can therefore function both as reservoirs for mature p50 and p52 subunits and as I-κBs. Activation of most forms of NF-κB, especially the most common form, the p50-RelA dimer, depends on phosphorylation-induced ubiquitination of the I-κB proteins. This sequential modification depends on two protein complexes: the I-κB kinase (IKK) complex and the E3 $^{\text{I-κB}}$ ubiquitin ligase complex. Once poly-ubiquitinated, the I-κBs undergo rapid degradation through the 26S proteasome and the liberated NF-κB dimers translocate to the nucleus, where they participate in transcriptional activation of specific target genes. The IKK complex is composed of three subunits, IKK-α, IKK-β and IKK-γ (or NEMO). IKK activity and classical NF-κB activation are absolutely dependent on the integrity of IKK-γ which serves as a regulatory subunit. However IKK-γ is not required for the activation of the alternative NF-κB signaling pathway which leads to nuclear translocation of p52-RelB dimers. Of the two catalytic subunits, the more important one for activation of the classical NF-κB signaling pathway is IKK-β. IKK-α kinase activity, however, is indispensable for activation of the alternative NF-κB signaling pathway as it is essential for inducible p100 processing (see Karin et al, 2004 and Wajant, 2004 for details).

Unlike death receptors such as TNFR-1, TRAMP, DR6 and EDA-R which preferentially transmit signals that induce specific gene expression, including NF-κB activation, Fas and the TRAIL death receptors primarily activate the apoptotic pathway. Nevertheless, gene induction is also a function of these receptors and some reports indicate their capacity to activate NF-κB (Schneider et al, 1997; Wajant et al, 2000). The mechanisms

of gene induction by these receptors have, however, not yet been clearly delineated. In most cell lines it does appear that NF-κB activation by these receptors depends on inhibition of protein synthesis. Moreover NF-κB activation is enhanced when apoptosis is inhibited by the use of caspase inhibitors. This is consistent with the notion that the NF-κB pathway negatively regulates the apoptotic programme by up-regulation of anti-apoptotic proteins, while ongoing apoptosis in turn interferes with activation of NF-κB by caspase-mediated cleavage of several of the components utilized by this signaling pathway. Fas and the TRAIL death receptors-mediated activation of the NF-κB pathway involves FADD, caspase-8 (although its enzymatic activity is not required), receptor interacting protein (RIP) and TNF receptor associated factor 2 (TRAF2), the latter conveying the signal initiated by the death receptors to the IKK complex. Although the role of $FLIP_L$ in NF-κB activation is more complex, $FLIP_L$ is indeed a short-lived inhibitor of NF-κB the expression of which is down regulated by protein synthesis inhibitors like CHX (Wajant et al, 2000; Kreuz et al, 2004).

3.4 FasL and TRAIL in therapy

Because of their strong apoptosis-inducing capacity, the therapeutic potential of Fas and the death receptors of TRAIL have been extensively investigated in the field of cancer. In fact, the ability of tumour cells to evade apoptosis is an essential feature of cancer development and progression as well as resistance to current therapeutic approaches, and mutations in the tumour suppressor gene p53 are frequently the cause of such resistance. Death ligands such as TRAIL and FasL have been shown to be able to trigger apoptosis independently of p53.

Apart from the well-established therapeutic approaches for the treatment of cancer, several promising alternatives targeting different components of the cell death pathway have been developed. Strategies including the use of anti-sense oligonucleotides or of small-molecule drugs to overcome the cytoprotective effects of Bcl-2 and $BcLx_L$ have been evaluated, for example. FLIP can be downregulated with antisense-based therapy, while the IAPs can be efficiently targeted with synthetic peptides that mimic Smac/DIABLO or HtrA2/Omi. Although not core components of the cell death

machinery certain signaling pathways like NF-κB, Akt/PKB, p53 have emerged as potential drug targets for modulating apoptosis (reviewed by Reed, 2003).

Here, we will focus more on the rationale for using FasL or TRAIL derivatives for cancer therapy.

In 1989, Trauth and colleagues demonstrated for the first time that triggering Fas with an agonistic monoclonal antibody had potential for cancer therapy (Trauth et al, 1989). However, when a similar approach was taken using an agonistic anti-mouse Fas Ab (Jo2), all animals died within 6 h of injection because of the toxic effects resulting from massive engagement of Fas receptors in normal tissues including the liver (Ogasawara et al, 1993). Thus the systemic administration of Fas agonists or recombinant FasL for therapeutic applications is at present impractical without additional measures to facilitate the targeting of these agents selectively to tumours. Locally applied FasL however can kill tumour cells very efficiently without systemic toxicity and may represent an approach for local tumour treatment (Rensing-Ehl et al, 1995).

TRAIL is a potent and more selective inducer of tumour cell apoptosis compared to FasL. Recombinant soluble trimeric TRAIL has been shown to induce apoptosis in cell lines from a broad spectrum of human cancers, whereas normal human non-tumour-derived cell types, with the exception of hepatocytes, neurons and astrocytes, are resistant to TRAIL (Jo et al, 2000; Nitsch et al, 2000). Aggregated TRAIL or membrane-bound TRAIL does, however, induce apoptosis in all TRAIL-sensitive cells. In mouse models *in vivo* treatment of immunodeficient mice bearing various human tumour types with recombinant soluble trimeric TRAIL has proven effective and safe (Ashkenazi et al, 1999; Walczack et al, 1999; Mitsiades et al, 2001; Pollack et al, 2001). At least three distinct forms of soluble recombinant TRAIL with potential for therapeutic use have been reported to date. A Flag-tagged form of recombinant soluble TRAIL generated by fusing the extracellular portion of TRAIL (AA 95-281) to an amino terminal Flag-tag is rendered cytotoxic for tumour cells when multimerized by cross-linking with an anti-Flag antibody (Bodmer et al, 2000). A second example is LZ-TRAIL, generated by fusing the extracellular portion of TRAIL (AA 95-281) to a modified yeast Gal-4 leucine zipper (LZ) that favors multimerization of TRAIL. This construct has been tested in a pre-

clinical animal model of cancer (Walczack et al, 1999). A third example comprises a fully recombinant version of TRAIL consisting only of the AA 114-281 of human TRAIL. The production of the latter was optimized by the addition of zinc and reducing agent to the cell culture media and extraction buffers, and by formulating the purified protein in such a way as to allow the formation of a TRAIL derivative that, unlike the other above-mentioned derivatives, forms homogeneous trimers. This construct is the least likely to cause toxicity in human patients and has shown promising results in preclinical and toxicological studies in small and large animals (Kelley et al, 2001).

Taken together all these data suggest that TRAIL and FasL and their derivates may prove useful for cancer therapy. However the putatively safe forms of TRAIL have only limited apoptosis-inducing capacity on those types of tumour that are predominantly sensitive to aggregated forms of TRAIL. Recent data suggest that recombinant TRAIL combined with cytotoxic drugs or radiation therapy can synergize to provide enhanced tumour destruction (reviewed by Wajant et al, 2002). Thus TRAIL and FasL either alone or in association with more conventional approaches such as chemotherapy and radiation therapy are increasingly being considered for cancer therapy.

3.5 Aim of the study

There still is a need to develop more sophisticated strategies that will allow better exploitation of the therapeutic potential of death receptor-induced apoptosis. One approach exploits the fact that non-aggregated soluble death receptor ligands have a limited capacity to activate death receptors compared to their membrane-bound forms and in some cases (FasL) they may even act as antagonists (Suda et al, 1993; Schneider et al, 1998; Tanaka et al, 1998). It has been shown that recombinant derivatives of the soluble ligands obtained by fusing the extracellular domains of TRAIL or FasL to single chain antibody variable regions converts these molecules into fully active ligands that mimic the activity of membrane-bound ligands upon target-dependent immobilization. Moreover these recombinant derivatives are devoid of systemic toxicity (Wajant et al, 2001; Samel et al, 2003). Here we used the same strategy to develop firstly FasL- and secondly TRAIL-based fusion proteins. The target structures here were not limited to

antibody-defined derivatives but expanded to include other selective protein-protein interactions. This approach provides the basis for relatively simple development of safer i.e. less toxic FasL or TRAIL derivatives for the treatment of a variety of human pathologies.

4 Material and Methods

4.1 Material

4.1.1 Chemicals, reagents and cell culture media

ABTS	Boehringer, Mannheim
ABTS buffer	Boehringer, Mannheim
Acrylamide solution 30 %	Roth, Karlsruhe
Anti-Flag-M2-agarose-beads	Sigma, Deisenhofen
APS	Sigma, Deisenhofen
BC Assay	Uptima, Interchim, France
BCIP	Roth, Karlsruhe
Bio-Rad-Solution	Bio-Rad, München
Caspase-3-substrate (Ac-DEVD-AMC)	Alexis, Grünberg
Chloroform	Roth, Karlsruhe
Cycloheximide	Sigma, Deisenhofen
DEPC	Sigma, Deisenhofen
DMSO	Roth, Karlsruhe
DTT	Roche Diagnostica, Penzberg
Ethidium bromide	Roche Diagnostic, Penzberg
Fas-Comp	Pascal Schneider University of Lausanne, Switzerland
Foetal calf serum (FCS)	PAA Lab. GmbH, Linz, Austria
Flag-peptide	Sigma, Deisenhofen
Flag-TRAIL	Pascal Schneider University of Lausanne, Switzerland
G418	Invitrogen, Karlsruhe
HBSS	Seromed, Berlin
Isoamylalcohol	Roth, Karlsruhe
Isopropanol	Roth, Karlsruhe
Lipofectamine	Invitrogen, Karlsruhe

Lipofectamine 2000	Invitrogen, Karlsruhe
Methanol	Roth, Karlsruhe
NBT	Roth, Karlsruhe
Nonidet-P40	Sigma, Deisenhofen
Protease-inhibitor-cocktail	Boehringer, Mannheim
RPMI 1640	Seromed, Berlin
Silver staining kit	Sigma, Deisenhofen
Superfect	Qiagen, Hilden
TEMED	Sigma, Deisenhofen
Trypsin/EDTA-solution	PAA LabGmbH,Linz
zVAD-fmk	Bachem, Heidelberg
1 kb and 100 bp	Fermentas, Stuttgart

Flag-FasL was produced by affinity chromatography from supernatants of stably transfected HEK293 cells (HEK293-Flag-FasL).

4.1.2 Solutions, buffers and agar plates

Agar plates	For 1 liter: 15 g agar-agar in LB medium + ampicillin 100 µg/ml
AP-buffer	1 M Tris/HCl pH 9.5, 1 M NaCl, 50 mM $MgCl_2$
Blotting buffer	25 mM Tris, 192 mM glycine, 20 % methanol, pH 8.3
Caspase-3-activity buffer	10 mM HEPES, 220 mM mannitol, 68mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, 1mM DTT, pH 7.4
Caspase-3-lysis buffer	200 mM NaCl, 20 mM Tris, 1% nonidet- P40 , pH 7.4
Coating-buffer	100 mM carbonate, pH 9.5 (for 1

	liter: 8.4 g $NaHCO_3$; 3.56 g Na_2CO_3)
DNA-buffer (6 x)	1 mM EDTA, 50 % glycerin, 0.0025 % bromophenolblue, 0.0025 % xylancyanol-bleu
Crystal violet solution	0.5 % crystal violet, 20 % methanol
LB media	For 1 liter: 10g peptone, 10 g yeast extract, 5 g NaCl
Lysis buffer for caspase-8	Tris/Hcl pH 7.4, 1 % v/vTriton X100 150 mM NaCl, 5 mM EDTA pH 7.4
0.1 M glycine HCl pH 3.5	0.1 M glycine and HCl acid up to pH 3.5.
Running buffer (SDS-PAGE)	0.05 M Tris, 0.38 M glycine, 0.004 M SDS, pH 8.3
PBS (10 x)	80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 1.4 M NaCl, pH 7.2
PBS-FCS	1 x PBS, 10 % FCS (v/v)
PBS-T	1 x PBS, 0.05 % (v/v) Tween-20
5 x loading buffer (SDS-PAGE)	0.05 M Tris-HCl, 5 mM EDTA, 100 mM DTT, 5 % SDS, 50 % glycerin, 0.5 % Bromophenolblue, pH 8,0
Stacking gel buffer (SDS-PAGE)	0.5 M Tris, 0.015 M SDS, pH 6.8
1 x TAE	40 mM Tris acetic, EDTA 1mM, pH 8.0
1 x TBS	127 mM NaCl, 20mM Tris pH 7.4
TBS-glycerol	TBS + 50% glycerol
TBST	TBS + 0.05% Tween20
TE-PBS	1 x Trypsin EDTA-solution in 1 x PBS
TBE (10 x)	0.9 M Tris, 0.9 M boric acid, 25 mM Na_2EDTA, pH 8.9
Running gel buffer (SDS-PAGE)	1.5 M Tris, 0.015 M SDS, pH 8.8

4.1.3 Freezing media

For eukaryotic cell lines: 10% DMSO in FCS, sterile filtered.

For bacteria: 70 % glycerol in H_20, autoclaved.

4.1.4 Antibodies and sera

Anti-Caspase-8 (human), mouse, monoclonal	Prof. Schulze-Osthoff, University of Düsseldorf
Anti-CD40L (human), mouse, monoclonal	Pharmingen, San Diego, USA
Anti-Fas (human), mouse, monoclonal (33-D7)	Marcus Peter, University of Chicago, USA
Anti-Flag (M2), mouse, monoclonal	Sigma, Deisenhofen
Anti-TRAILR1 (Fab+Fc)	IZI
Anti-TRAILR2 (Fab+Fc)	IZI
Anti-TRAIL (Apo 2L, etc), Mouse, monoclonals	A. Ashkenazi, Genentech Inc., USA.
Anti-mouse-IgG, alkaline phosphatase-conjugated, goat, $F(ab)_2$-Fragment	Sigma, Deisenhofen
Anti-mouse-IgG, R-phycoerythrin-conjugated, goat	Sigma, Deisenhofen
Mouse-IgG1 (MOPC 21)	Sigma, Deisenhofen

4.1.5 Enzymes

Restriction enzymes, T4 ligase were purchased from MBI Fermentas (Vilnius, Lithuania); the polymerase for the PCR reactions was from the kit *High Expand Polymerase* supplied by Roche.

4.1.6 Plasmids

pcDNA3	Invitrogen, Karlsruhe
pCR™3	Invitrogen, Karlsruhe PS435
Ig signal Flag-TRAIL-R2 in pCR3	Pascal Schneider (University of Lausanne)
pEYFPC-1	Clontech, Palo Alto, USA
PCR3RANK-Fc	Pascal Schneider (University of Lausanne)

The cloning experiments were performed with the chemical competent *E. coli* strains XL1-Blue and DH5α .

4.1.7 DNA and protein markers and reagents for DNA purification and isolation

MW standard for proteins was purchased from New England Biolabs and for DNA (1kb and 100bp) from MBI Fermentas.

Nucleobond kit (Maxi/Midi) and Nucleospin extract kit were from Macherey Nagel; Quiaquick gel extraction kit was from Qiagen. The deoxyoliginucleotides (dNTPs) used were from PeqLab.

4.1.8 Cell lines

HT1080	ATCC, Rockville, MD, USA
KB	Dr. Michael Kracht, Medizinische Hochschule Hannover
Cos 7	ATCC, Rockville, MD, USA
HEK293	ATCC, Rockville, MD, USA
CHO	ATCC, Rockville, MD, USA
HT1080-FLIP$_L$GFP and HT1080-Bcl-2GFP	Sebastian Kreuz (IZI)
HT1080-FAP	W. Rettig, Boehringer Ingelheim Pharma (Vienna, Austria)

4.2 Experimental Methods

4.2.1 Cloning

4.2.1.1 Templates

The cDNA of CD40 was isolated from the B cell lines RAJI and BJAB; that of 4-1BB from Jurkat cells stimulated with PMA and ionomycin. TNFR2 was obtained from the plasmid pEGFN-1-TNFR2 which contained the full length of TNFR2. TNFR1 was amplified from the template pPADTNF-R1. CD40L was cloned from cDNA isolated from BHK-CD40L, a baby hamster kidney cell line stably expressing CD40L.

4.2.1.2 Oligonucleotides

The oligonucleotides used to amplify CD40, 4-1BB, TNFR1, TNFR2, CD40L, FasL and TRAIL comprised forward primers:

CD40-F42-HindIII: 5'-CCC AAG CTT CTC GCC ATG GTT CGT CTG CCT CTG CAG-3'

4-1BB-F134-HindIII: 5'-CCC AAG CTT TTC ATC ATG GGA AAC AGC TGT TAC-3'

TNFR1-F198HindIII: 5'-CCC AAG CTT CTG TCT GGC ATG GGC CTC TCC ACC-3'

gfp-F581-HindIII-Pac1: 5'-CCC AAG CTT GTT AAT TAA CCG TCA GAT CCG CTA GCG CTA CCG-3'

CD40L-F34-Bgl2: 5'-GGA AGA TCT CAG AGC ATG ATC GAA ACA TAC AAC C-3'

EcoR1-RGD-RGD-FasL: 5'-CCG GAA TTC GGC CGG GGC GAC TCA CCC GGC CGG GGC GAC TCA CCC GAA AAA AAG GAG CTG AGG AAA GTG GCC-3'

TRAIL-F370-EcoRI: 5'-CCG GAA TTC TAC GCA TAT TAC ACC TCT GAG GAA ACC ATT TCT ACA G-3'

And reverse primers:

CD40-R623-BamHI-gga: 5'-CGC GGA TCC CAG CCG ATC CTG GGG ACC ACA GAC-3'

4-1BB-R695-Bgl2: 5'-GGA AGA TCT CGG AGA GTG TCC TGG CTC TCT GCG-3'

TNFR1-R838-BamHI: 5'-CGC GGA TCC GGT GCC TGA GTC CTC AGT GCC-3'

TNFR2-R863-BamHI: 5'-CGC GGA TCC GTC GCC AGT GCT CCC TTC AGC TGG-3'

CD40L-R832-Xho1: 5'-CCG CTC GAG CAC TGT TCA GAG TTT GAG TAA GCC-3'

FasL-R-Xho1: 5'-CCG CTC GAG GTG CTT CTC TTA GAG CTT ATA TAA GCC G-3'

TRAIL-Rstop-Xba1: 5'-TGC TCT AGA CCA GGT CAG TTA GCC AAC TAA AAA GGC-3'
All primers were synthesized by MWG Biotech, Ebersberg.

4.2.1.3 RNA isolation

The cell pellet from the relevant cell lines was resuspended in 1 ml peqGold RNAPure™ (PeqLab, Erlangen, Germany) and incubated for 5 min at RT. 200 µl of chloroform was then added to the reaction mixture and vortexed for 15 seconds. The test tubes were incubated for 10 min at RT then centrifuged (5 min, 13000 rpm, RT), allowing the separation of a white aqueous phase containing the RNA from the phenol phase and an inter-phase in between. The interphase and the phenol phase contained proteins and genomic DNA. RNA was precipitated from the aqueous phase by transferring to a fresh tube and addition of 0.5 ml of isopropanol, followed by vortex-mixing and incubation for 15 min at RT. The reaction mixture was then centrifuged at 13000 rpm for 15 min at 4°C. The supernatant was carefully removed and the RNA-containing pellet washed two times by addition of 0.5 ml 75 % ethanol followed by centrifugation at 13000 rpm for 5 min at 4°C and removal of the supernatant. After the RNA-pellet was air dried at RT for 20 min it was dissolved in 50 µl DEPC-H_2O. The RNA concentration was then determined with a photometer at a wavelength of 260 nm.

4.2.1.4 cDNA Preparation

The synthesis of cDNA was performed using the *First Strand cDNA Synthesis Kit* (MBI Fermentas) following the manufacturer's instructions.

4.2.1.5 PCR analysis

All fragments were amplified with a proof reading polymerase. We made use of the *Expand High Fidelity PCR System* kit (Roche Diagnostic, Penzberg, Germany) and the following programme: initial denaturation 94°C for 2 min; (94°C for 5s, 50-55°C for 30s,

72°C for 7 min) 10X then (94°C for 5s, 50-55°C for 30s, 72°C for 7 min) 20X and final elongation 72°C for 7 min.

4.2.1.6 Cloning strategies

For generation of the constructs PCR3CD40FasL, PCR341BBFasL, PCR3CD40TRAIL, PCR3TNFR2FasL we took advantage of a vector designated PS435-Ig signal-Flag-TRAIL-R2 in pCR3. This vector is a variant of the expression plasmid pCR3 (Invitrogen, Karlsruhe, Germany) in which the multiple cloning site of pCR3 is modified to include the Ig signal secretion peptide followed by the DNA sequence of Flag inserted respectively in the HindIII/BamHI and BamHI/EcoRI sites of pCR3. TRAIL-R2 was cloned as an EcoRI/XbaI fragment. The fusion proteins were generated in two steps:

In the first step, the extracellular domain of human FasL (AA139-281) was amplified as a PCR fragment with the primers (EcoR1-RGD-RGD-FasL-F/FasL-R-xho1).The PCR fragment was appropriately restricted and cloned into PS435-Ig signal-Flag-TRAIL-R2 in pCR3, exchanging it for the TRAIL-R2 component. We thus generated PS435-Ig signal-Flag-FasL in pCR3 or simply PCR3FasL. Similarly to PCR3FasL, PCR3TRAIL was obtained by amplifying the extracellular domain (AA 95-281) of human TRAIL with the primers (TRAIL-F370-3Y-EcoR1/TRAIL-Rstop-Xba1) and exchanging it for the TRAIL-R2 part.

The second step consisted of the cloning or sub-cloning of the targeting modules. For this purpose, human CD40 (AA 1-192) was obtained by RT-PCR using the pair of primers (CD40-F42-HindIII/ CD40-R623-BamH1-gga). After restriction with HindIII and BamHI the remaining PCR fragment was cloned into the corresponding sites of PCR3FasL giving PCR3CD40FasL. Similarly human 4-1BB (AA1-186) was amplified using the primers (4-1BB-F134-HindIII/41BB-R695-Bgl2). The HindIII/Bgl2 fragment was introduced into PCR3FasL giving PCR34-1BBFasL. Bgl2 is BamHI compatible.

The portion of the extracellular domain of TNFR1 (AA1-211) was obtained using the primers (TNF-R1-F198Hind III/TNFR1-R-838-BamHI) and introduced into PCR3FasL giving PCR3TNFR1FasL.

Subsequent to the cloning of PCR3CD40FasL, CD40 was excised from that plasmid with the restriction enzymes HindIII and BamHI and the fragment introduced into the corresponding sites of the intermediate vector PCR3TRAIL giving PCR3CD40TRAIL.
Using a similar procedure, the extracellular domain of TNFR2 (AA1-257) gained with the primer pair (gfp-F581-HindIII-Pac1/TNF-R2-R863-BamH1) was exchanged for the CD40 part of PCR3CD40FasL to give PCR3TNFR2FasL.
For the generation of CD40L-expressing KB or HT1080 cells, the full length human CD40L was amplified as a Bgl2/Xho1 fragment with the above-mentioned primer pair and cloned into the Bgl2/Sal1 sites of the expression vector pEYFPC-1 (Clontech Laboratories, Palo Alto, USA) generating the plasmid pEYFPC-1-CD40L.

4.2.2 Cell culture

All cell lines used in this work were cultured in RPMI 1640 supplemented with 5 % or 10 % heat-inactivated FCS. The cells were kept at 37°C in a thermostatically-controlled incubator in an atmosphere containing 5 %CO_2 and at 96 % humidity. To sub-culture adherent cell lines, the cells were washed with cell culture medium and incubated with TE-PBS until the cells detached from the bottom of the cell culture flask. Trypsin was inactivated with HBSS-2 % heat-inactivated FCS. The cells were pelleted by centrifugation at 1500 rpm for 5 min. The supernatant was removed and the cells resuspended in culture medium and seeded at the desired density. For long-term storage, the cell lines were harvested as described above, resuspended in 10 % DMSO in heat-inactivated FCS for cryo-preservation in 1.5 ml polypropylene cryo-tubes. To allow a slow and gradual freezing, the tubes were stored at -80°C in a container filled with isopropanol. After 24 hours the tubes were transferred to liquid nitrogen.

4.2.3 Transfection of eukaryotic cell lines

HEK293, COS7 and CHO cells were used for transient production of fusion proteins. Transfections were performed with either Lipofectamine™ Reagent, Lipofectamine™ 2000, Superfect™ transfecfion reagent or by electroporation.

For transfection with Lipofectamine: $2x10^5$ cells per well were cultured in 6-well-plates. The following day, for each well in a transfection, 2 µg of DNA + 7 µl of Lipofectamine were diluted in 800 µl of opti-MEM®, the mixture incubated at RT for 45 min to allow DNA-liposome complexes to form, and the complex solution overlayed onto the cells after removal of the culture medium. The cells were then incubated at 37°C for 5-6 h. Following incubation, 1 ml of medium with twice the normal concentration of serum was added. 18-24 h after initiation of transfection, the medium was replaced with either 2 ml opti-MEM® or 2 ml medium with 1 %FCS.

For transfection with Lipofectamine 2000: Cells were seeded in a 6-well-plate to achieve 90-95 % confluence at the time of transfection (O/N). For each transfection sample, DNA-Lipofectamine 2000 complexes were prepared as follows: 4 µg DNA was diluted in 250 µl opti-MEM® and in parallel 10 µl of Lipofectamine 2000 was mixed with 250 µl opti-MEM® and incubated at RT for 5 min. The diluted Lipofectamine was combined with the diluted DNA within 30 min and the mixture incubated for an extra 20 min at RT. The 500 µl DNA-Lipofectamine complex was added to each well and the cells incubated O/N, after which the transfection medium was removed and the cells supplemented with 2ml opti-MEM® or 2 ml medium with 1 % FCS with antibiotics if desired.

Electroporation: 2.5 x 10^7 cells were resuspended in 1 ml culture medium + 30 µg DNA and carefully mixed. 800 µl of the suspension was transferred into an electroporation cuvette (PeqLab, Erlangen, Germany) and the DNA electroporated into the cells at 250 V and 1800 F with (Easyject, PeqLab, Erlangen, Germany). The cells were subsequently seeded to the desired density in culture medium O/N. The medium was then replaced with opti-MEM® or medium with 1 % FCS.

4.2.4 Establishment of stable cell lines

For the establishment of stable cell lines expressing CD40L, KB cells were transfected by electroporation and HT1080 cells with Lipofectamine, in triplicates in 6-well-plates, with pEYFPC-1-CD40L, a plasmid with the neomycin resistance gene which allows selection with G418. 24 h post-transfection the cells were supplemented with medium containning 500 µg/ml G418. Stable transfectants were obtained after 3 weeks of selection. All the

clones were pooled, expanded and enriched for CD40L expression by 3 cycles of cell sorting with a FACStar plus (Becton Dickinson, San Jose, Ca., USA).
HEK293CD40-Flag-FasL cells expressing the fusion protein CD40-Flag-FasL were obtained by transfecting PCR3CD40-Flag-FasL into HEK293 with Lipofectamine 2000 in 6-well-plates. The following day, the cells were transferred to a 10 cm petri dish, and cultured for an additional day before starting the selection with 750 µg/ml G418 for 2-3 weeks. Single clones were pooled, expanded and analysed by Western blotting (WB) to detect protein production.

4.2.5 FACS analysis

FACS was used to detect cell surface expression of CD40L or Fas and to show binding of CD40-Flag-FasL and CD40-Flag-TRAIL to CD40L positive cells.
Approximately 10^6 cells were harvested and resuspended in 100 µl PBS-5 % FCS. Cells were then transferred to a 96-well-plate (V-shaped). For determination of CD40L cell surface expression, KB/KB-CD40L and HT1080/HT1080-CD40L were incubated with 5 µg/ml of anti-human CD40L monoclonal antibody, and for Fas expression cells were incubated with anti-Fas monoclonal antibody at a concentration of 5 µg/ml. To show binding of the fusion protein to its target the cells were treated with 1 µg/ml CD40-Flag-FasL or CD40-Flag-TRAIL for 1 h at 4°C followed by treatment with 5 µg/ml M2 anti-Flag antibody. Bound antibody or fusion protein was detected with R-phycoerythrin-labeled goat anti-mouse IgG antibody. Between each step the cells were washed with 200 µl of PBS-5 % FCS and analysis performed with an EPICS®XL-MCL (Beckman Coulter, Krefeld, Germany).

4.2.6 Production, concentration and affinity chromatography purification of CD40-Flag-FasL

4.2.6.1 Production and concentration of CD40-Flag-FasL

For the production of the fusion protein CD40-Flag-FasL, the pool of HEK293CD40-Flag-FasL cells producing 1-2 µg/ml was cultured to 50-60 % confluency in 20 ml of RPMI with 2.5 % FCS in 20 cell culture flasks. The stable cell lines were kept under

these conditions for 3 weeks and every 3-4 days the supernatants were collected and exchanged with fresh medium. All the supernatants were then pooled, giving a total of volume 4 liters. Prior to purification, the supernatant was concentrated 5 times at 4°C (to 800 ml) by dialysis on PEG 6000 in dyalisis bags (cut off 14 kDa, Roth, Karlsruhe, Germany). The concentrated supernatant was centrifuged at 4 000 rpm in a cell culture centrifuge and then stored at 4 °C until needed.

Preparation of dyalisis bags: before being used the dyalisis bags were boiled for 10 min in a 1 L beaker containing 10 g/l Na_2C0_3 The bags were then washed several times with water and autoclaved.

4.2.6.2 Affinity chromatograpy

CD40-Flag-FasL was recovered by affinity chromatography via the internal Flag-tag on an anti-Flag M2-agarose column followed by elution with Flag® peptide.

Briefly, the suspension of anti-Flag M2 affinity resin stored in 50 % glycerol buffer was transferred to a clean chromatography column. The 50 % glycerol buffer was allowed to drain off and the column washed 3 times with TBS and further washed with 3 sequential column volumes of 0.1 M glycine HCl pH 3.5 taking care not to leave the column in glycine HCl for more than 20 min. The resin was then equilibrated with 5 column volumes of TBS and the pH of the column adjusted if needed to 7.4.

The supernatant was loaded onto the column and allow to flow very slowly by gravity flow. The column was then washed with 10-20 column volumes of TBS and elution carried out by competition with 5 column volumes of a solution containing 100 µg/ml of Flag peptide. The fractions were dyalised O/N against PBS to remove excess Flag peptide and sterile filtered.

4.2.6.3 Determination of protein concentration

The commercially-available BCA assay, a colorimetric assay which involves the reduction of Cu2+ to Cu+ by peptidic bonds of proteins, was used to determine protein concentration. Bicinchonic acid (BC) chelates Cu+ ions with very high specificity to form a water-soluble purple coloured complex. This reaction is measured by the optical

absorbance of the final Cu+ complex at 562 nm. The absorbance is directly proportional to the protein concentration, with a broad linear range between 5-20 µg/ml up to 1-2 mg/ml. The protein concentration can be calculated directly from a reference curve obtained with a standard protein, commonly bovine serum albumin (BSA). The evaluation of protein concentration is performed in triplicates, in a 96-well-plate, according to the manufacturer's instructions. The plate contains also a serial dilution of BSA standard.

4.2.6.4 Silver staining

Silver staining was performed with an appropriate kit (Sigma, Munich, Germany) according to the manufacturers' instructions to assess the purity of the protein.

4.2.6.5 Gel filtration chromatography

325 µg in 650 µl of affinity purified protein were analysed by FPLC gel filtration chromatography using a Superdex 200 HR 10/30 column (Amersham Biosciences, Freiburg, Germany). The elution was performed in PBS with a flow rate of 0.5 ml/min, collecting fractions of 0.5 ml and recording the absorbance at 280 nm. For the molecular weight estimation, the column was calibrated with the following standard proteins: tyroglobulin (669 kDa), apoferritin (443 kDa), ADH (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). The fractions were then sterile filtered and stored at 4°C.

4.2.7 Bioactivities of fusion proteins

4.2.7.1 Cytotoxicity assays

HT1080/HT1080-CD40L, KB/KB-CD40L, co-cultures of SV80 cells with HT1080 cells or HT1080-CD40L, co-cultures of RANKL transfectants B16-C151 and mock transfected cells B16-C91 with HT1080 cells were grown in flat-bottomed 96-well plates at a density of 2 x 10^4 cells per well in 100 µl of culture medium for at least 5 h. The cells were then treated with cycloheximide at a final concentration of 2.5 µg/ml. Flag-TRAIL+M2 complexes, Flag-FasL+M2 complexes or fusion proteins were titrated onto the cells at the indicated concentrations in triplicates.

After 12-13 h, the medium was removed and the cells treated with a crystal violet solution (100 µl/well). After 20 min the excess colour was washed away with water and the plate air dried. Bound crystal violet was then dissolved in methanol (100 µl/well) and the plates placed on a shaker for 1-3 hours. The absorption at 550 nm was then measured with an ELISA reader (R5000, Dynatech, Guernsey, Great Britain).
Flag-FasL and Flag-TRAIL complexes were obtained by preincubation of Flag-FasL or Flag-TRAIL with the anti-Flag antibody M2 at 37°C for at least 20 min.

4.2.7.2 Neutralization assays

To show CD40-, FasL-, TRAIL-, TRAILR1/2- and caspase-dependent cell death, the cells or the fusion proteins were pre-incubated for 1 h either with 1 µg/ml anti-human CD40L), 4 µg/ml Fas-Comp, 20 µM zVAD, 1 µg/ml anti-TRAIL (Apo2L, E-11, 2G2) or 25 µg/ml anti-TRAILR1/2. Cell viability was determined by crystal violet staining after 12-13 h as described above. All experiments were performed in triplicate.

4.2.8 IL-8 ELISA

4.2.8.1 Stimulation of cells

KB/KB-CD40L, HT1080/HT1080-CD40L cells or a co-culture of HT1080 cells with RANKL transfectants B16-C151 and mock tranfected B16-C91 cells were seeded at 2 x 10^4 cells/well in 96-well plates. The following day the supernatant was removed to get rid of constitutively produced IL-8. After treatment of HT1080/HT1080-CD40L cells with 20 µM zVAD or of KB/KB-CD40L cells with 2.5 µg/ml cycloheximide and 20 µM zVAD, cells were stimulated as stated in the results section. 6 h post-stimulation, the plates were centrifuged and the supernatant carefully removed and stored at -80°C until needed.

4.2.8.2 Detection

The detection of IL-8 in the cell culture supernatants was performed using the OptEIA^{TM} IL-8 ELISA kit (Pharmingen, San Diego, USA). Briefly, 96-well ELISA plates were coated with 50 µl/well of an IL-8 capture antibody (diluted 1:250 in 0.1 M carbonate buffer. The plates were incubated overnight at 4°C. Non-specific sites were blocked with 100 µl /well of PBS-10 % FCS for 1 h followed by incubation with the supernatants for 2 hours. Finally the plates were incubated for 1 h with 50 µl/well of biotinylated IL-8 antibody and streptavidin HRP (1:250) in PBS-10 % FCS. Between each step, the plates were washed several times with 100 µl/well of PBS-0.05 % Tween 20. The assay was performed in triplicates. The detection was done by incubation with 100 µl/well of a solution of 1 mg/ml ABTS in ABTS buffer (Boehringer, Mannheim, Germany) until development of a persisting green color. Absorption at 405 nm was then determined with an ELISA plate reader (R5000, Dynatech, Guernsey, Great Britain). The plate contained also a human IL-8 standard to determine the concentration of IL-8 in each test sample. The amount of IL-8 contained in culture medium, as negative control, was also evaluated and substracted from the mean of each triplicate. All negative pg/ml values were arbitrarily set to 3 pg/ml, which is the lower level of sensitivity of the ELISA kit.

4.2.9 Western blotting

Used here for the detection of caspase-8 and for the analysis of supernatants for detection of protein production.

4.2.9.1 Preparation of lysates

HT1080/HT1080-CD40L or KB/KB-CD40L cells (1 x 10^6) were seeded in 6 cm Petri dishes. The following day, the cells were treated with cycloheximide at a final concentration of 2.5 µg/ml for 3 hours and finally treated with 40 ng/ml CD40-Flag-FasL for 0, 2 or 4 h. At each of these time points, cells were harvested, centrifuged and lysates prepared in a buffer containing 50 mM Tris pH 7.4, 1 % TritonX100, 150 mM NaCl, and 5 mM EDTA pH 7.4, supplemented with protease inhibitors (Roche, Mannheim,

Germany). Following centrifugation at 13000 rpm in a bench centrifuge, the protein concentration was determined by the Bradford assay.

4.2.9.2 SDS-PAGE

The resolution of the proteins according to their molecular weights was performed on a 13.5 % acrylamide gel. For 20 ml of 13.5 % running gel, 5 ml of running buffer was mixed with 9 ml 30 % acrylamide solution, 6 ml H_20, 340 µl APS-solution (10 % in H_2O) and 28 µl TEMED to start the polymerization reaction. The mixture was poured into the space created by two glass plates clipped together and separated with a 1.5 mm spacer. Isopropanol was used to overlay the gel to avoid evaporation of solution and maintain the correct concentration. After polymerization, the isopropanol was washed away with water and the gel overlayed with stacking gel. To prepare 14 ml of 13.5 % stacking gel, 5 ml stacking gel buffer was mixed with 2.4 ml 30 % acrylamide solution, 6.6 ml H_2O, 144 µl APS-solution und 12 µl TEMED. Before polymerization of the stacking gel, slots for sample loading were created in it by inserting a plastic comb. Electrophoresis was performed in a vertical electrophoresis chamber (PHASE, Lübeck, Germany) running at 30-45 mA, with 30 µg of protein in 5 x loading buffer that were boiled for 5 min at 95°C prior to loading.

4.2.9.3 Electroblotting

Proteins were electroblotted onto nitrocellulose membranes (BioTrace®NT, Pall Corporation, Pensacola, USA) as follows: 6 pieces of Whatman filter paper and 1 piece of nitrocellulose membrane were prepared and soaked in blotting buffer. After electrophoresis, the stacking gel was removed from the running gel and the latter briefly soaked in blotting buffer. Three layers of Whatman filter paper were placed in the blotting chamber and overlayed with the gel followed by the nitrocellulose membrane and finally 3 additional layers of Whatman filter paper. Air bubbles were carefully eliminated. Current was applied to the chamber such that the proteins migrate from the negative to the positive pole with an applied intensity of 1.5 mA per cm^2 (70 mA) per gel for 45 min.

4.2.9.4 Immunodetection

After electroblotting, non-specific sites on the nitrocellulose membrane were blocked by incubation for 30-45 min in 3 % low fat milk (w/v) in PBS-T. This was followed sequentially by incubation with primary antibody (1 µg/ml in PBS-T) for 2 hours at RT or at 4°C O/N, and then by incubation for 45 min at RT with an alkaline phosphatase (AP)-conjugated secondary antibody (1:10000 in PBS-T). Reactions were developed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrate. All steps were performed with agitation provided by an end-to-end shaker and between each step the membrane was washed three times for 5 min in PBS-T.

4.2.10 Measurement of caspase-3 activity

4.2.10.1 Preparation of cell lysates

HT1080/HT1080-CD40L, KB/KB-CD40L cells with CD40-Flag-FasL or CD40-Flag-TRAIL: 1 x 10^6 cells were seeded in 6 cm petri dishes. The following day, the cells were treated with 2.5 µg/ml cycloheximide for 3 hours and stimulated with 40 ng/ml CD40-Flag-FasL or 125 ng/ml CD40-Flag-TRAIL, the cells harvested with a cell scraper at different time points (0, 2, 4 h) and pelleted by centrifugation. The cell pellets were resuspended in 200 µl caspase-3 lysis buffer supplemented with a protease inhibitor cocktail and incubated for 30 min on ice. Supernatants were recovered by centrifugation at 4°C for 10 min at 13 000 rpm, the protein concentration estimated with the Bradford assay and active caspase-3 detected as described below.

4.2.10.2 Preparation of liver homogenates

To determine the activation of caspase-3 in the liver, liver homogenates were prepared as follows: a small portion of liver was homogenized with the plunger of a syringe in 500 µl caspase-3 lysis buffer supplemented with a protease inhibitor cocktail. The homogenate was centrifuged at maximum speed in a bench centrifuge and the supernatant aspirated. The protein concentration was then determined using the Bradford method.

4.2.10.3 Activity measurement

The evaluation of caspase-3 activity was performed in triplicates. In a 96-well plate 30 µg of protein were added to caspase-3 activity buffer so that the total volume was 250 µl.
1 µl /well of caspase 3-substrate (Ac-DEVD-AMC, 5 mM in DMSO) were added and the cleavage of the substrate was measured every 15 min for 1 h using a fluorescence spectrometer (FLUOstar, BMG Labtech, Offenburg, Germany), at an absorption wavelength of 390 nm and an emission wavelength of 460nm.

4.2.11 Animal experiments

Animal care and all the animal experiments were performed in accordance with Federal guidelines and were approved by University and State authorities. The mouse strains C57BL/6xCBA/J and Balb/c were purchased from Janviers breeders (LeGenest St Ile, France).
To investigate the systemic toxicity of CD40-Flag-FasL groups of 3 animals were treated as described in table 1a&b of the results section, by i.v. injections using the tail vein. Animals were sacrificed at the indicated time points and blood was collected by cardiac puncture to determine AST and ALT activities. Macroscopic liver damage was documented photographically and livers were frozen on dry ice and later at -80°C for the determination of caspase-3 activity.

4.2.12 AST and ALT measurements

Blood samples were taken by cardiac puncture with syringes containing 50 µl of heparin. After centrifugation at 4°C at 13000 rpm for 5 min, the plasma was taken and stored at -80°C until needed. As an indicator of liver damage, the plasma activity of the enzymes alanine amino transferase (ALT) and aspartate amino transferase (AST) was assessed by measuring the enzyme activities according to Bergmeyer (Bergmeyer et al, 1984) using an automated procedure. Mice were killed by Enfluran inhalation.

4.2.13 Confocal microscopical analyses

To analyse the functionality of HT1080-CD40L cells, HeLa-CD40 cells transfected with TRAF2-CFP or CFP only were co-cultured with HT1080-CD40L. For receptor clustering analysis, HT1080-CD40L or pEYFPC-1-transfected HT1080 cells were co-cultured with HeLa cells transfected with Fas-CFP overnight and stimulated with CD40-Flag-FasL (1 μg/ml). In both cases images of cells' section were taken with a TCS SL confocal laser scanning microscope from Leica. CFP and YFP were excited at 458 nm and 514 nm respectively and the data was analysed with the Leica confocal software and Adobe photoshop v8.0.

5 Results

5.1 Cloning of fusion proteins

The genes for the chimaeric fusion proteins used in this study were created by fusing DNA sequences encoding the two functional components: (1) the gene for the targeting modules and (2) the extracellular domain either of human FasL (AA 139-281) or of human TRAIL (AA 95-281). The two domains were separated by a sequence corresponding to the Flag-tag to facilitate detection and purification of the proteins. The targeting modules analysed were the extracellular domains of human CD40 (AA 1-192); 4-1BB (AA1-186); TNFR1 (AA1-211); TNFR2 (AA 1-257) and RANK (AA 29-213).

CD40 has been chosen for the possibility of targeting the autocrine/paracrine CD40/CD40L survival loop described in several malignancies or alternatively to target activated T cells. 4-1BB along with LIGHT, CD70, OX40, CD30 and their respective ligands 4-1BBL, HVEM, CD27, OX40L, CD30L, are costimulatory molecules that belong to the TNFR-TNF-family. Targeting these molecules could either enhance or curtail an ongoing immune response leading to several potential therapeutic benefits. TNFR1 and TNFR2 were considered in the context of control of recalcitrant inflammatory conditions. The interaction of RANKL with its two receptors (RANK and OPG) is critical for bone and calcium homeostasis. The targeting of RANKL could inhibit or ameliorate, for example, cancer-related osteolytic bone lesions and hypercalcaemia and may even play a role in preventing bone metastasis. In murine models of human multiple myeloma and prostate carcinoma, soluble RANK or OPG have been shown to prevent bone destruction and metastasis (Younes and Kadin, 2003).

To generate the plasmids PCR3CD40FasL, PCR34-1BBFasL, PCR3TNFR1FasL, PCR3TNFR2FasL and PCR3CD40TRAIL we took advantage of a vector designated PS435 Ig signal Flag-TRAIL-R2 in PCR3. This vector is a variant of the expression plasmid pCR3 (Invitrogen) in which the multiple cloning site of pCR3 is modified to include the Ig signal secretion peptide followed by the DNA sequence of the Flag-tag inserted respectively in the HindIII/BamHI and BamHI/EcoRI sites of pCR3. TRAIL-R2 was cloned as a EcoRI/XbaI fragment. The fusion proteins were generated in two steps:

in the first step, the extracellular domain of human FasL (AA139-281) was exchanged for the TRAIL-R2 component generating thus PCR3FasL (Figure 3).

Similarly to PCR3FasL, PCR3TRAIL was obtained by amplifying (AA 95-281) of the extracellular domain of human TRAIL and substituting it for the TRAIL-R2 fragment (Figure 3).

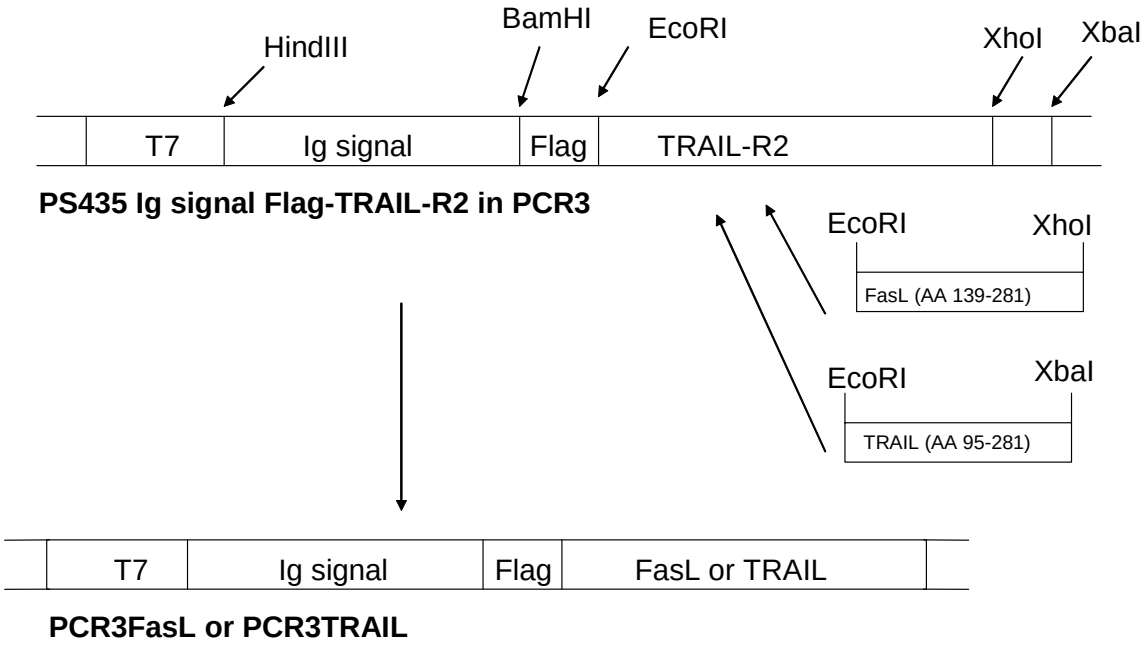

Figure 3. Schematic representation of ps435Ig signal Flag-TRAIL-R2, PCR3FasL and PCR3TRAIL

The second step consisted of the cloning or sub-cloning of the targeting modules. For this purpose the extracellular domains of human CD40, 4-1BB and TNFR1 including their leader sequences obtained by RT-PCR and were inserted in front of the Flag-tag giving the vectors PCR3CD40FasL, PCR34-1BBFasL, PCR3TNFR1FasL.

Subsequent to the cloning of PCR3CD40FasL, CD40 was excised from that plasmid with the restriction enzymes HindIII and BamHI and the fragment introduced into the corresponding sites of the intermediate vector PCR3TRAIL giving PCR3CD40TRAIL (Figure 4).

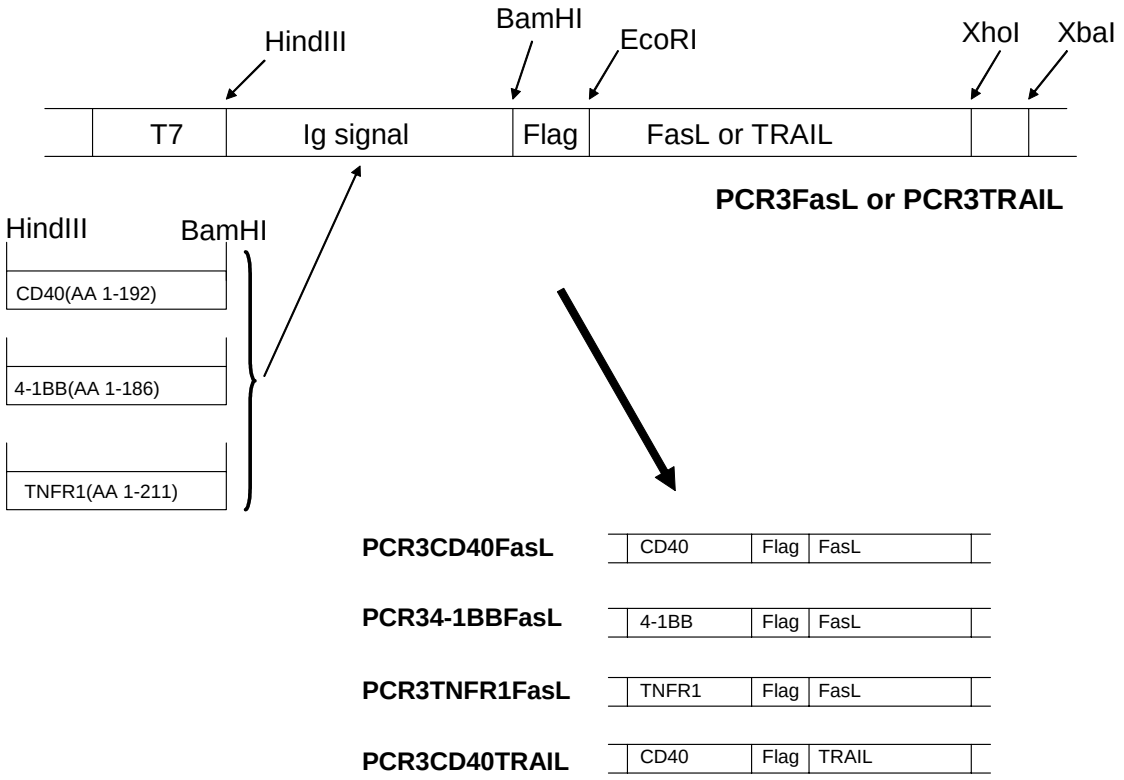

Figure 4. Schematic representation of PCR3CD40FasL, PCR34-1BBFasL, PCR3TNFR1FasL and PCR3CD40TRAIL

Using a similar procedure, the extracellular domain of TNFR2 with its own leader was exchanged for the CD40 fragment of PCR3CD40FasL to give PCR3TNFR2FasL (Figure 5).

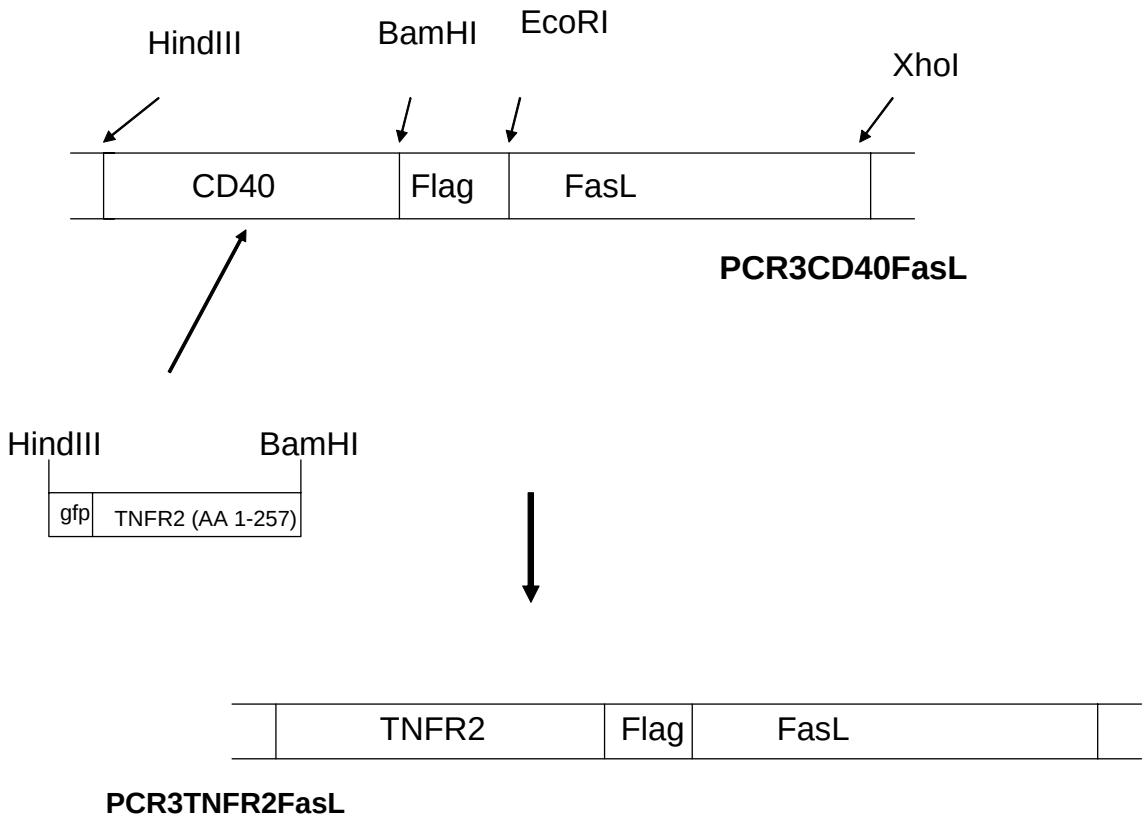

Figure 5. Schematic representation of PCR3TNFR2FASL

PCR3RANKedFasL was cloned from the plasmid hRANKed-Fc-pCR3, containing the HA (haemagglutinin) leader and the extracellular domain of hRANK fused to human Fc. The extracellular domain of human FasL was amplified and exchanged for the Fc portion of hRANKed-Fc-pCR3 giving PCR3RANKedFasL (Figure 6).

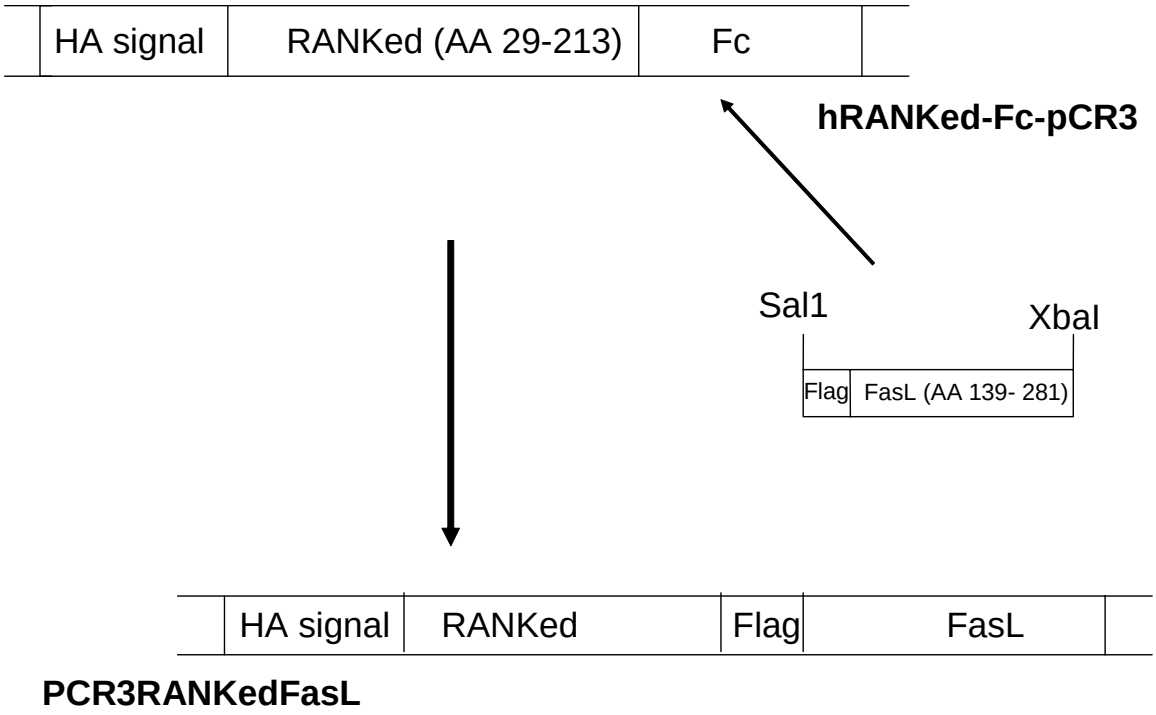

Figure 6. Schematic representation of PCR3RankedFasL

5.2 Expression of fusion proteins

The expression vectors described in section 5.1 were transiently transfected into either HEK293 cells or Cos-7 cells by lipofection. Supernatants from transfected cells containing the recombinant proteins were analysed by SDS-PAGE under reducing conditions followed by Western blotting to monitor expression of the proteins and to evaluate their molecular weights (MW).

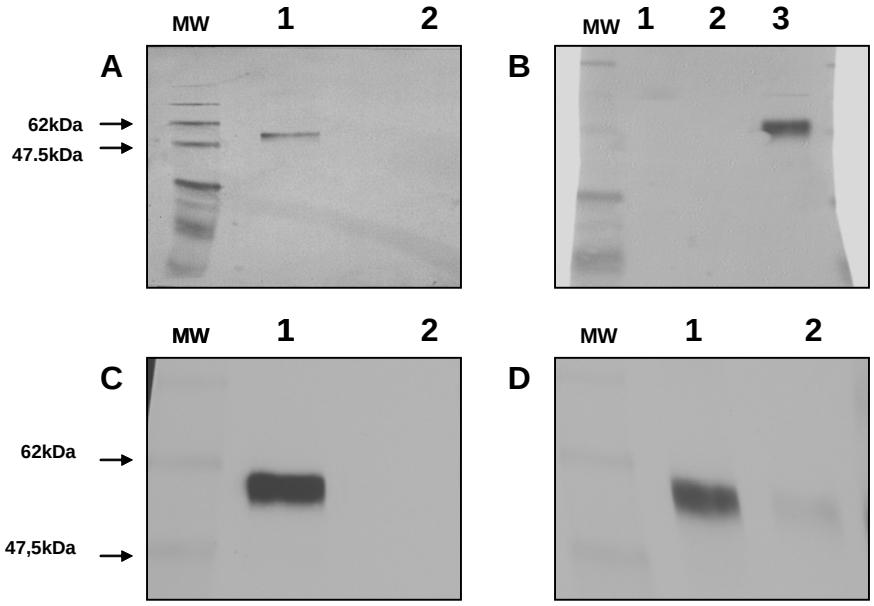

Figure 7. Transient expression of the different fusion proteins. Cos-7 or HEK293 cells were transiently transfected with **A**: PCR3TNFR1FasL **B**: PCR34-1BBFasL; **C**: PCR3CD40FasL; **D**: PCR3RANKedFasL. In each case 30 µl of cell supernatant were resolved on a 13.5 % SDS-PAGE gel under reducing conditions. Fusion proteins could be detected via the Flag-tag using an anti-Flag primary antibody (M2 at 1 µg/ml) and a goat anti-mouse IgG AP-conjugated second antibody. In all cases lane 1 contains supernatants (fusion proteins) and lane 2 a negative control (supernatant from mock transfected cells). In the case of PCR34-1BBFasL (panel B) we included 30 µl of supernatant of CD40-Flag-FasL in lane 3 as a positive control as the fusion protein was not expressed at detectable levels. MW: molecular weight markers.

All the fusion proteins except 4-1BB-Flag-FasL and TNFR2-Flag-FasL (data not shown) were detectable by Western blotting; CD40-Flag-FasL and RANKed-Flag-FasL were particularly well expressed (Figure 7). The theoretical MW based on the nucleotide sequence for 4-1BB-Flag-FasL, TNFR1-Flag-FasL, TNFR2-Flag-FasL, CD40-Flag-FasL, RANKed-Flag-FasL were: ~38kDa, ~41kDa, ~46kDa, ~39 kDa, ~38 kDa respectively. The proteins migrated in agreement with the expected sizes except for TNFR1-Flag-FasL, RANKed-Flag-FasL and even more strikingly CD40-Flag-FasL. CD40-Flag-FasL migrated in a similar way as sc40-Flag-FasL, a protein used as a

positive control that has a MW of around 50 kDa. The migration profiles of TNFR1-Flag-FasL, RANKed-Flag-FasL and CD40-Flag-FasL indicating high observed MWs probably resulted from post-translational modifications such as glycosylation, for example, that is a characteristic feature of members of the TNF/TNFR family.

5.3 Target-independent activities of fusion proteins

This work aimed to generate NAMAZE (non-antibody mediated activation of apoptotic zytokine) proteins for therapeutic purposes. To achieve this goal, several targeting structures were fused to FasL. In some cases, however, spontaneous aggregation of the molecules leads to target-independent FasL-mediated activities, thus requiring selection of suitable candidates for further investigation. Initial expression of proteins was therefore followed by further characterization in order to identify constructs exhibiting secondary aggregation as previously described for this type of reagent (Mueller, 2002; Samel et al, 2003). The soluble fusion proteins selected are indicative of the findings of this preliminary screening procedure.

To address the issue of spontaneous aggregation, we used the human fibrosarcoma cell line HT1080 that is highly sensitive to FasL (see Figure 15). Moreover these cells do not express any of the target structures required to immobilize the fusion proteins on the cell surface therefore precluding the intended protein-target interaction that would lead to induction of cell death via a membrane FasL-like apoptotic process (data not shown). HT1080 cells were treated with CHX to sensitize them to apoptosis and then incubated with sequential dilutions of supernatants containing either TNFR1-Flag-FasL, TNFR2-Flag-FasL, 4-1BB-Flag-FasL, RANKed-Flag-FasL or CD40-Flag-FasL. Despite only moderate expression or barely detectable expression in the cases of TNFR1-Flag-FasL, 4-1BB-Flag-FasL and TNFR2-flag-FasL (Figure 7 and data not shown for TNFR2-Flag-FasL) these proteins had a high specific activity (Figure 8 A&C closed symbols). In sharp contrast to the above mentioned fusion proteins, supernatants of CD40-Flag-FasL and RANKed-Flag-FasL, which contained much more protein, showed very low and low specific activity, respectively (Figure 8 B&D closed symbols). An efficient way to activate the FasL fusion proteins is to induce multimerization through cross-linking of the

Flag-tag. Cross-linking of the various receptor-FasL fusion proteins via their internal Flag-tag resulted surprisingly in a decrease in activities for TNFR1-Flag-FasL, TNFR2-Flag-FasL and 4-1BB-Flag-FasL (Figure 8 A&C open symbols and data not shown for TNFR2-Flag-FasL). The activity of RANKed-Flag-FasL could be increased by a factor of 50-100 depending on the experimental conditions upon cross-linking. Under these experimental conditions, on the other hand, the cell death-inducing capacity of CD40-Flag-FasL was virtually unchanged by artificial cross-linking (Figure 8 B&D open symbols). The fusion proteins used here were generated from the extracellular domain of FasL (AA 139-281) which spans the trimerization domain of FasL (see chapter 3.1). The recombinant proteins were thus expected to have a homotrimeric organization and therefore able to bind Fas but not to induce efficient activation. As mentioned earlier, HT1080 cells express no specific target structures that could lead the various fusion proteins to behave like membrane bound entities. The high specific activities observed for TNFR1-Flag-FasL, 4-1BB-Flag-FasL and TNFR2-Flag-FasL is thus likely the result of the presence of significant amounts of higher MW aggregates of homotrimers in the supernatants. RANKed-Flag-FasL is probably mostly non-aggregated and is therefore activated upon cross-linking. CD40-Flag-FasL behaves like soluble trimeric FasL in that it has low specific activity and the lack of any M2-mediated effect is probably not indicative of a defect in the FasL part of the protein but might rather reflect, for example, steric hindrance of the M2 antibody. RANKed-Flag-FasL and CD40-Flag-FasL were thus subsequently further analysed with respect to their capacity for target-dependent activation.

To establish that cell death was due to Fas/FasL interaction, HT1080 cells sensitized for cell death were treated with supernatants of the fusion proteins 4-1BB-Flag-FasL, TNFR1-Flag-FasL, or with fusion proteins that had been pre-incubated with Fas-Comp, a soluble high avidity chimaeric receptor assembled on a pentameric scaffold derived from the coiled-coil domain of cartilage oligomeric matrix protein (Comp). As is evident from Figure 8 E, Fas-Comp almost completely inhibited cell death induced by the fusion proteins, confirming that the cell death induced by the various reagents is due to Fas activation.

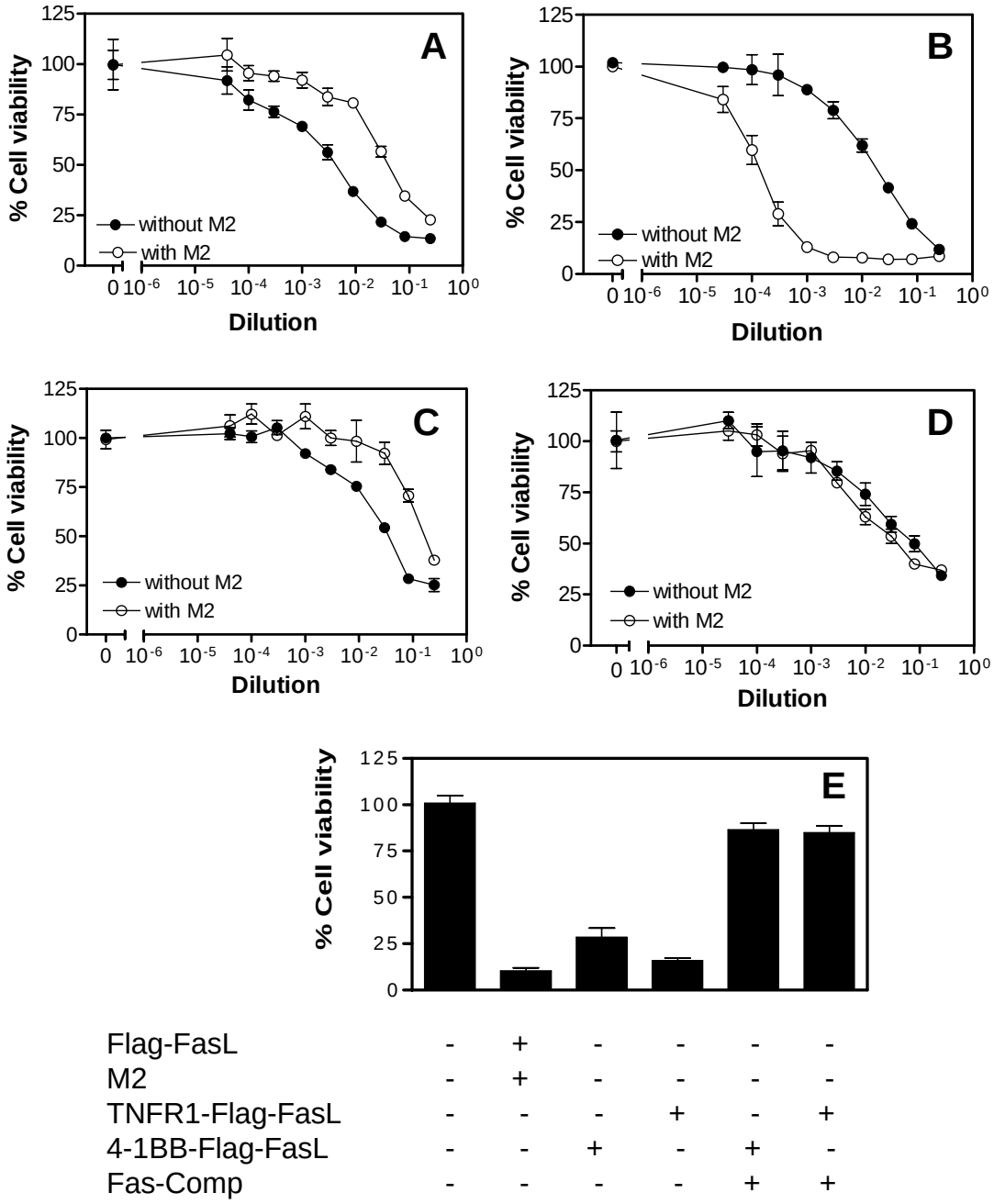

Figure 8. Target-independent cell death. HT1080 cells were cultured in 96-well plates for at least 5 hours and then treated with CHX (2.5 μg/ml) for 45 min. The supernatants of fusion proteins **A**: TNFR1-Flag-FasL±M2: **B**: RANKed-Flag-FasL±M2 **C**: 4-1BB-Flag-FasL±M2, **D**: CD40-Flag-FasL±M2, were titrated 4-fold starting with a 1:4 dilution. **E**: Fas dependency of cell death was established by treating cells either with untreated fusion protein supernatants or with supernatants pre-incubated for 1 h with Fas-Comp (5 μg/ml) to neutralize the FasL moiety. Cells were also treated with cross-linked Flag-FasL (200 ng/ml) as a positive control. Flag-FasL or the supernatants were cross-linked with anti-Flag M2 (1μg/ml). Assays were developed after 12-13 h via crystal violet staining. In panels A, B, C & D open & closed symbols

correspond to cross-linked & non-cross-linked supernatants, respectively. Graphs show the means and standard deviations of triplicate experiments and are representative of three independent experiments.

5.4 Production and purification of CD40-Flag-FasL

In order to obtain an amount of protein sufficient to better characterize CD40-Flag-FasL, the expression construct PCR3CD40FasL was transfected into HEK293 cells and after 2-3 days, the cells were treated with G418 for 2-3 weeks. The supernatant from the resulting polyclonal population was assayed for expression of CD40-Flag-FasL by Western blotting (WB). CD40-Flag-FasL was successfully expressed, and comparative WB analysis with a Flag-FasL of known concentration allowed us to estimate that the stably transfected cells produced between 1-2 μg/ml of protein. The cells were then expanded in RPMI containing 2.5% FCS and 3-4 liters of supernatant were collected over 3 weeks. Prior to purification the supernatant was reduced to 1/5 of its original volume by dialysis with PEG 6000.

For purification, the supernatant, cleared of cellular debris, was loaded onto an M2-agarose column and allowed to run through the column by gravity flow. CD40-Flag-FasL bound to M2-agarose beads was eluted by competition with a Flag-peptide and finally the excess Flag-peptide was removed by dialysis against PBS. The protein concentration was determined through the bicinchonic acid method. Figure 9 A shows the purification process. The purity of the protein was also assessed by SDS-PAGE followed by silver staining and, as Figure 9 B shows, affinity-purified CD40-Flag-FasL was free from any contaminant. We also determined the bioactivity of the CD40-Flag-FasL supernatant prior to concentration and that of the affinity-purified protein. Affinity purification did not cause any change in the bioactivity of CD40-Flag-FasL (data not shown). We analyzed the purified protein in native conditions by gel filtration to control homogeneity of the protein. CD40-Flag-FasL eluted with a sharp peak between fractions 22-25 (apparent MW 471 kDa). When 30 μl of fractions 17-33 were analyzed by SDS-PAGE under reducing conditions followed by Western blotting, the protein could be detected in fractions 20 through to fraction 26; the protein could not be detected in fractions corresponding to dimers and monomers. In conclusion, analysis of the native protein

showed that CD40-Flag-FasL elutes with an apparent MW of 471kDa, corresponding to a 9.4 mer (Figure 9 C). Two trimers of FasL brought in close proximity represent the minimal ligand structure required to signal apoptosis (Holler et al, 2003). One would thus predict that a nonamer would be highly toxic to HT1080 cells. Since that is not the case the gel filtration analysis results are likely an overestimate. In fact gel filtration chromatography is most useful for globular proteins and does tend to overestimate the molecular masses of asymmetric proteins. Analyzing the protein under non-reducing conditions gave no evidence of intermolecular disulphide bonds (data not shown).

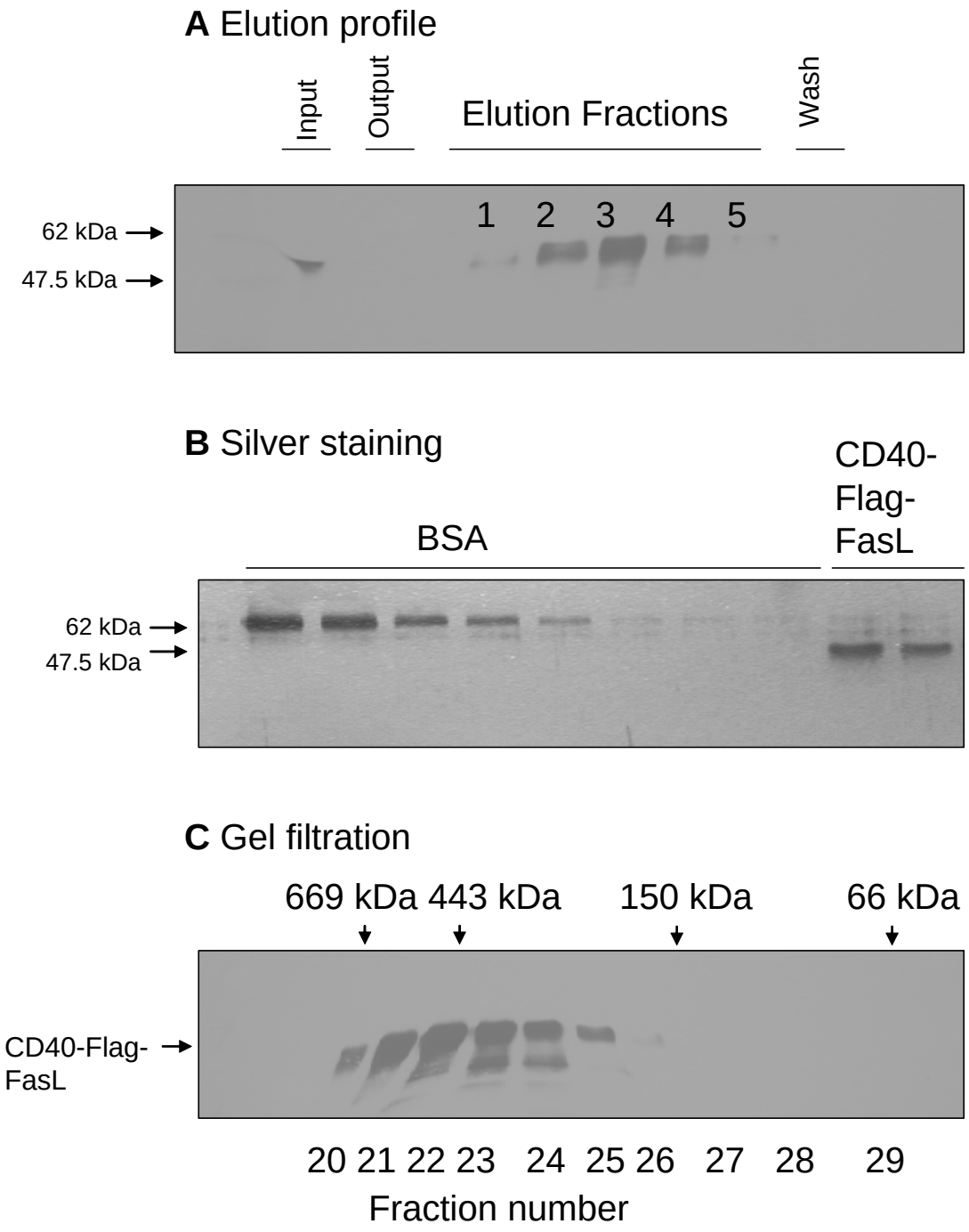

Figure 9. Purification of CD40-Flag-FasL by affinity chromatography. A: Western blotting: The input 20 µl, the output 20 µl and 5 µl of each of the fractions were analyzed under reducing conditions with a mouse anti-Flag antibody followed by an anti-mouse IgG–AP secondary antibody. **B: 13.5 % SDS-PAGE & silver staining**: BSA (100-50-25-10-5-2.5-1-0.5 ng) or CD40-Flag-FASL (100-50 ng) were analyzed **C: Gel filtration analysis:** Affinity purified CD40-Flag-FasL was concentrated in dialysis bags on PEG6000 then 325 µg of protein were fractionated on a Superdex-200 column. 30 µl of the eluted fractions were analyzed by Western blotting under reducing conditions and CD40-Flag-FasL was detected with the M2

antibody. For MW estimation, the column was calibrated with the standard proteins: tyroglobulin (669 kDa), apoferritin (443 kDa), ADH (150 kDa), BSA (66 kDa), carbonic anhydrase (29 kDa).

5.5 Establishment of CD40L positive stable cell lines for the characterization of CD40-Flag-FasL

In order to be able to further characterize the recombinant CD40-Flag-FasL protein it was necessary to create cell lines stably expressing CD40L, the ligand for CD40. For this purpose the expression plasmid pEYFPC1-CD40L containing full length, i.e. membrane-bound, CD40L was transfected into the epithelial cell line KB and the fibrosarcoma cell line HT1080. After 3 weeks of selection, the polyclonal population expressing the protein CD40L-YFP was sorted three times by FACS to obtain cells expressing high levels of CD40L, and CD40L cell surface expression was determined (Figure 10). The stable cell lines established are referred to as HT1080-CD40L and KB-CD40L. Live cell microscopy analysis showed that the CD40L expressed by the two stable cell lines is functional as co-culture of HT1080-CD40L cells with HeLa-CD40 cells transfected with TRAF2-CFP induced recruitment of TRAF2 to the site of interaction of CD40 with CD40L (data not shown).

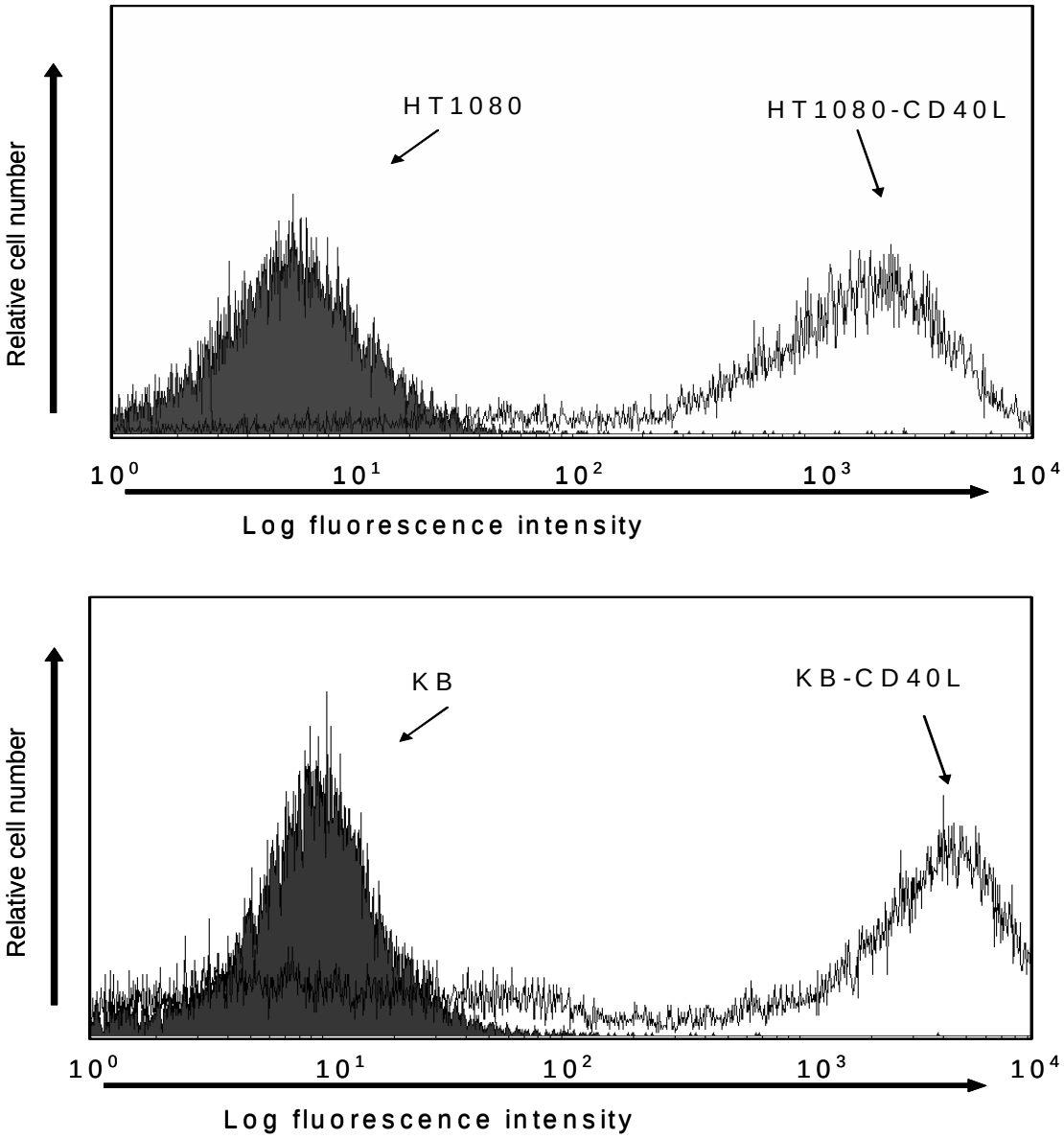

Figure 10. FACS analysis of HT1080 and KB-CD40L-YFP transfectants. Parental HT080, KB cells or the CD40L-expressing transfectants were incubated with a mouse anti-human CD40L and detection was done with a PE-labeled goat anti-mouse IgG.

5.6 CD40-Flag-FasL binds specifically to CD40L positive cells and induces clustering of Fas

The ability of CD40-Flag-FasL to bind CD40L was assessed by immunofluorescence flow cytometry using the two established CD40L expressing cell lines (HT1080-CD40L, KB-CD40L) and the parental cell lines as well as the M2 anti-flag antibody.

Specific binding was detected on both transfected cell lines but not on the parental HT1080 and KB cells (Figure 11). Binding through the FasL part of the protein can not be excluded but since both parental and transfected cell lines express similar amounts of Fas both are similarly shifted due to Fas detection so the way this experiment has been performed allows only the discrimination of binding through the CD40 part of the protein.

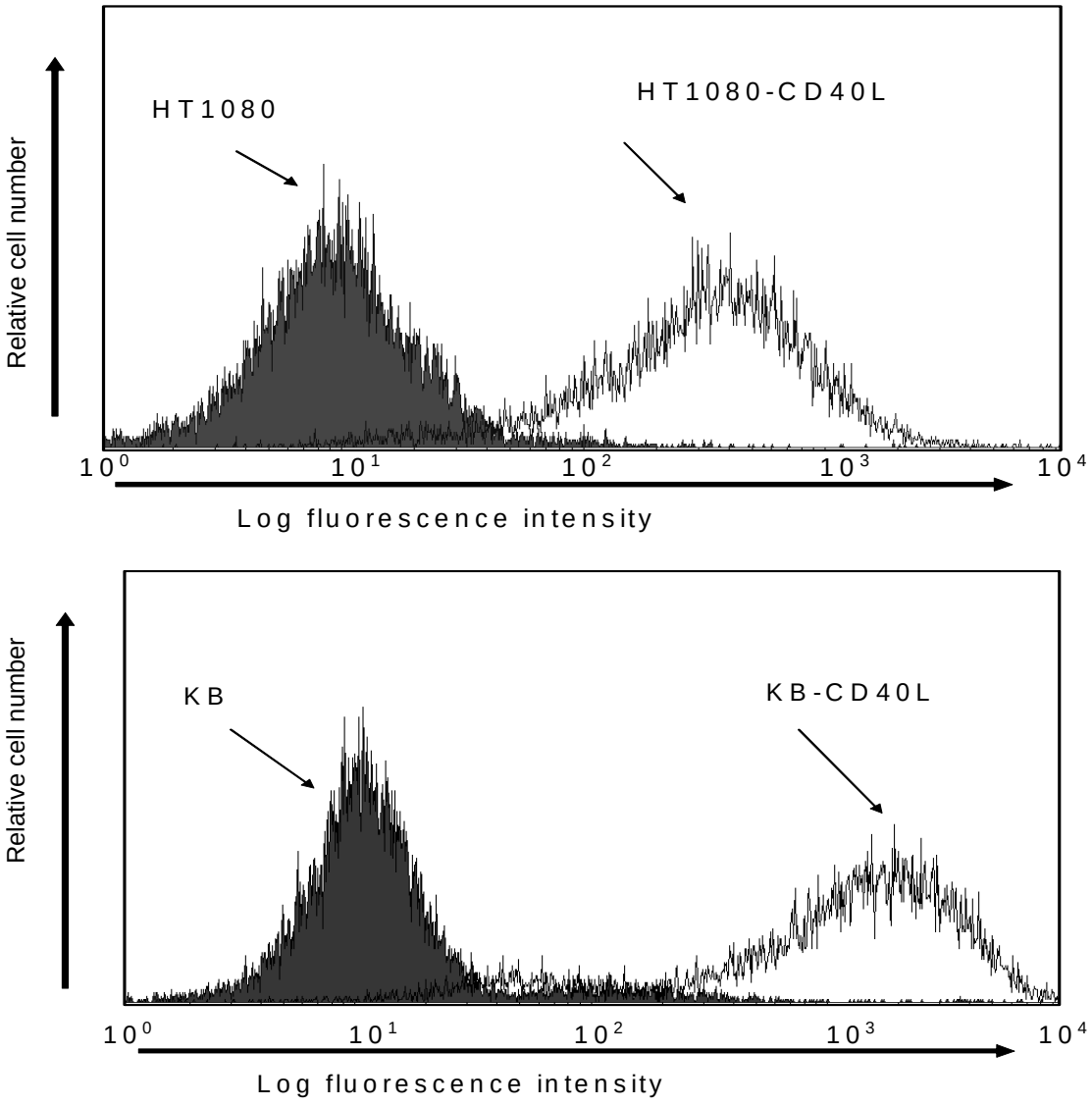

Figure 11. Binding of CD40-Flag-FasL to cell surface-expressed CD40L. Recombinant CD40-Flag-FasL was incubated with parental HT080, KB cells or with the CD40L-expressing transfectants. Specific binding was detected with a mouse anti-Flag antibody followed by a PE-labeled goat anti-mouse IgG.

Fas has been reported by several authors to form clusters at the cell surface in a ligand-dependent fashion. Algeciras-Schminch and colleagues (2002) have proposed a five step model of signal initiation following Fas ligation: first Fas resides on the plasma membrane in a pre-associated form due to its PLAD domain and forms microaggregates after ligation that, at this stage, cannot be detected as receptor clusters by immunofluorescence microscopy. The third step involves actin filament-dependent formation of the DISC which in turn allows activation of caspase-8; active caspase-8 in association with other factors is important for the fourth step that comprises formation of Fas surface clusters. The final step of the events at the plasma membrane involves internalization of the activated DISC. Taking advantage of the stable cell lines established, we investigated the ability of cell surface CD40L-immobilized CD40-Flag-FasL to to induce Fas clustering on neighbouring cells. HT1080-CD40L cells were co-cultured with HeLa cells previously transfected with a construct encoding Fas-CFP. CD40-Flag-FasL was added directly to the living cells at a final concentration of 1 μg/ml and the cells monitored using confocal microscopy. Immobilized CD40-Flag-FasL induced a punctate pattern at the cell to cell contact site indicating clustering of Fas on adjacent HeLa cells (Figure 12). Punctate aggregations could be observed as early as 10 min post stimulation. Receptor clustering was confirmed to be due to the CD40-CD40L interaction since co-cultures of HT1080 and HeLa cells transfected with vectors encoding YFP and Fas-CFP, respectively, did not lead to receptor clustering following stimulation with CD40-Flag-FasL (data not shown). Taken together, these data suggest that immobilized CD40-Flag-FasL induces Fas receptor clustering, a phenomenon considered to be part of the initiation of Fas signaling.

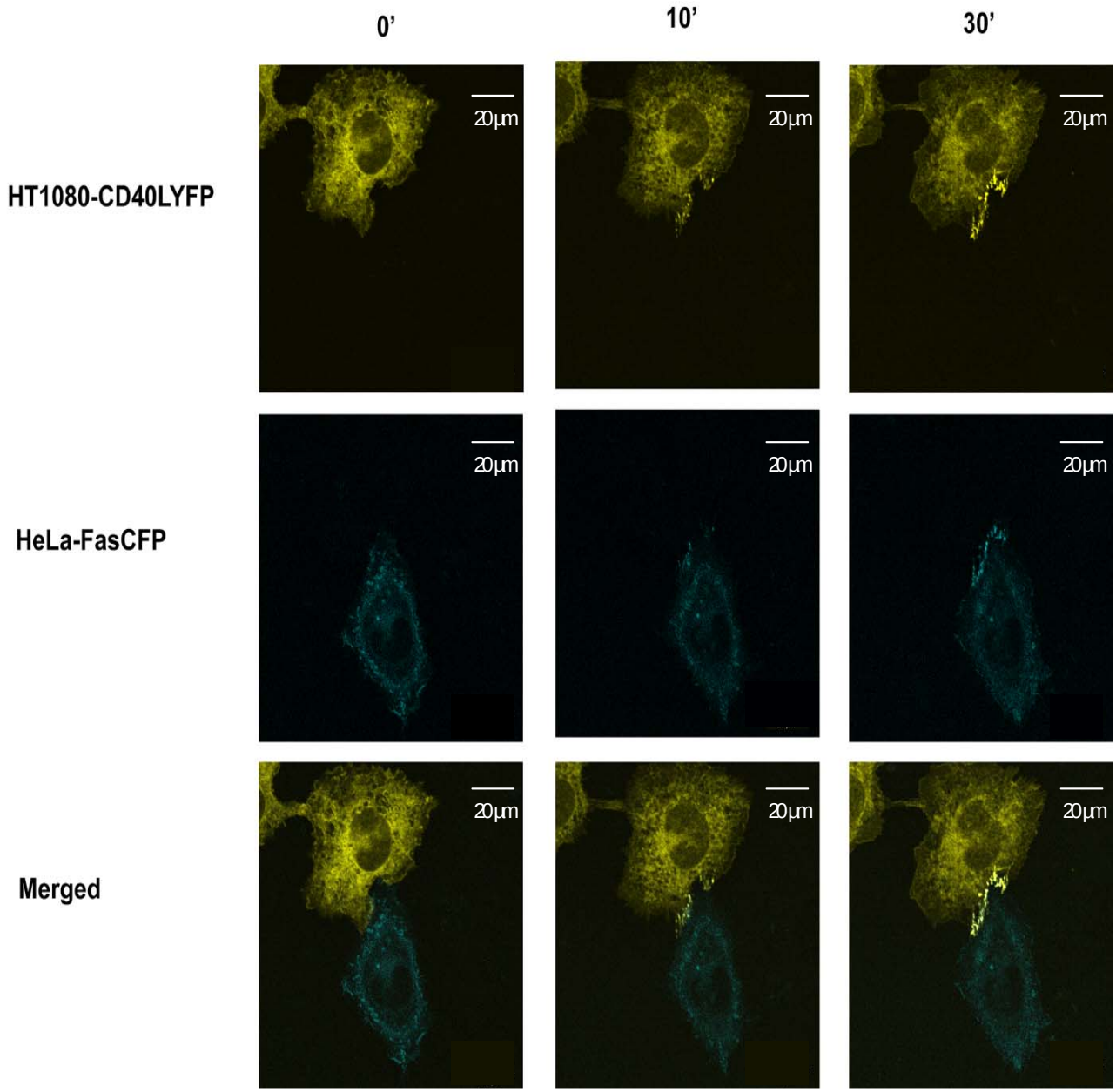

Figure 12. CD40-Flag-FasL induces clustering of Fas expressed on HeLa cells. HT1080-CD40LYFP cells were co-cultured with HeLa cells transfected with Fas-CFP overnight. The following day the supernatant was removed and cells were supplemented with fresh medium. CD40-Flag-FasL was added to the cells to a final concentration of 1 µg/ml and a section of the cells was analysed by live cell imaging with a TCS SL confocal microscope from Leica. Objective: HCX PL APO CS 63.0X1.32 OIL.

5.7 CD40-Flag-FasL induces selective cell death of CD40L-expressing cells

As mentioned earlier, the aim of the work presented here was to develop soluble pro-apoptotic FasL derivatives that would induce death in a cell surface antigen-restricted manner. Of the various FasL-containing fusion proteins tested in the course of this study the CD40-Flag-FasL construct induced mild apoptosis in cells that do not express CD40L. As described above, this construct binds specifically to cells expressing CD40L and upon cell surface immobilization it induced clustering of Fas on the surface of neighboring cells. We next decided to investigate whether these initial steps in Fas-mediated signaling would actually lead to apoptotic removal of cells.

In the first experiments performed, we wanted to preclude any bias in the outcome due to differences in sensitivity to FasL-mediated cell death between the parental and the CD40L transfected cell lines, we analysed cell surface expression of Fas on all cells and also assessed the sensitivity of the different cell lines to apoptosis induced by cross-linked Flag-FasL. Figure 13 shows equivalent levels of cell surface expression of Fas on all cell lines and equal sensitivity to Fas-mediated apoptosis.

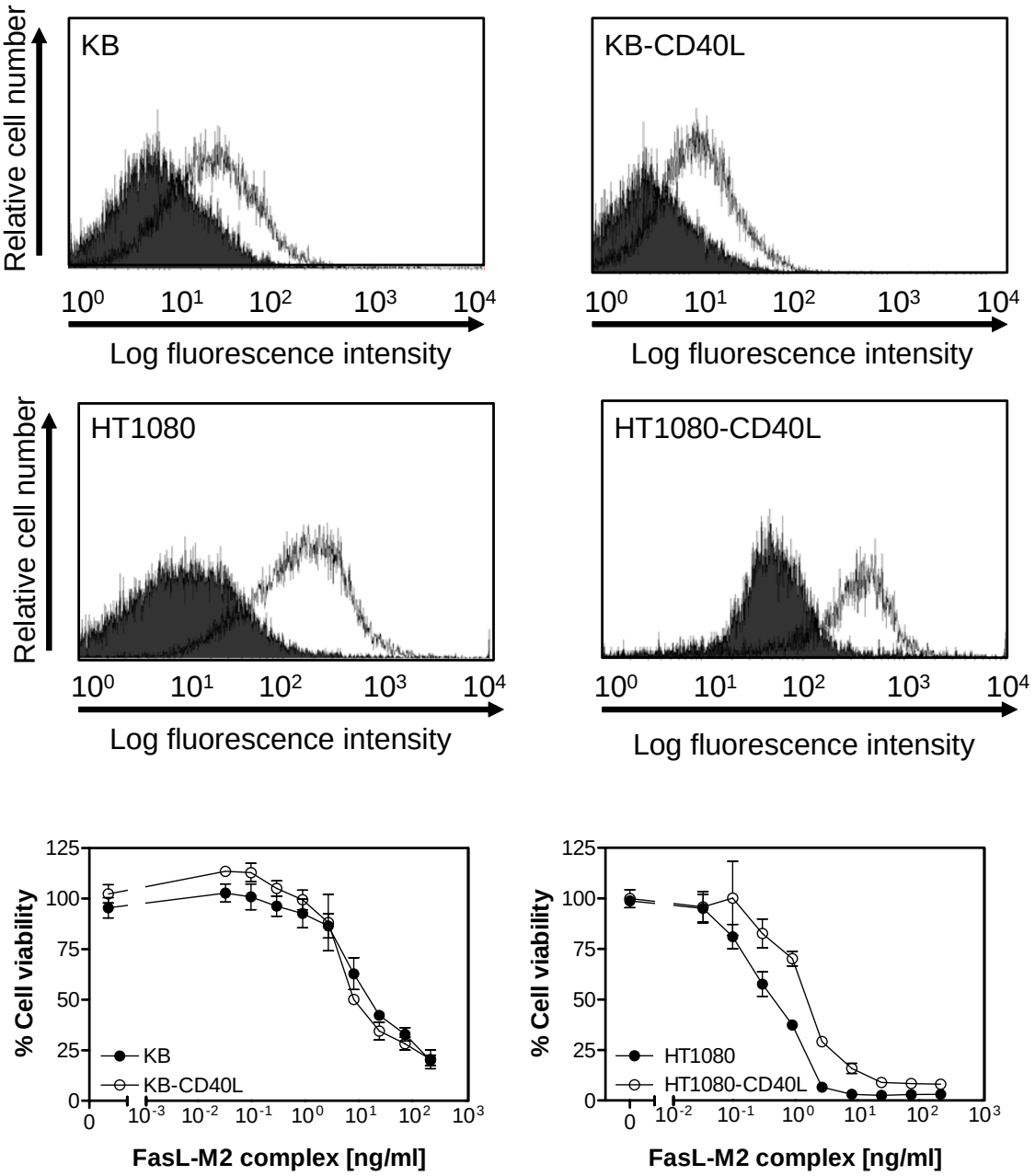

Figure 13. Fas expression and induction of apoptosis by cross-linked Flag-FasL. Flow cytometry: HT1080/HT1080-CD40L cells or KB/KB-CD40L cells were pre-incubated with a mouse anti-human Fas monoclonal antibody (clear) or with a mouse IgG1isotype match control (gray curves). A PE-labeled goat anti-mouse IgG second antibody was used for detection. **Cytotoxicity assay**: Cells were seeded in 96-well plates and subsequently treated with CHX (2.5 µg/ml) for 45 min. Flag-FasL was cross-linked with the M2 anti-Flag antibody (used at 1 µg/ml) for 45 min at 37°C prior to being titrated onto the cells. The assay was developed after 12-13 h via crystal violet staining. Graphs show means and standard deviations of triplicate experiments.

In the next step, parental HT1080 and KB cells and their CD40L transfectants previously sensitized for cell death with cycloheximide (CHX) were treated with different doses of CD40-Flag-FasL. The protein induced cell death in a dose-dependent manner specifically in CD40L transfectants, with negligible cell death detectable in parental HT1080 or KB cells (Figure 14).

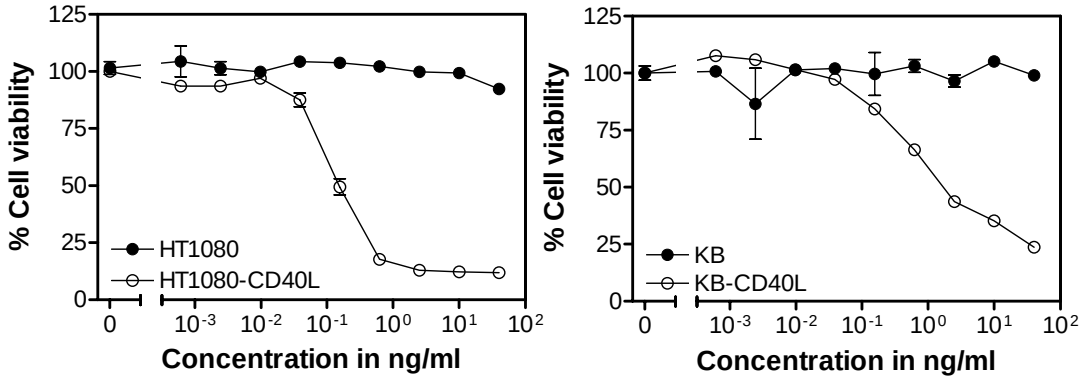

Figure 14. Bioactivity of CD40-Flag-FasL. HT1080, HT1080-CD40L, KB, and KB-CD40L cells were seeded in 96-well plates for at least 5 h. The cells were treated with CHX (2.5 μg/ml) for 45 minutes and CD40-Flag-FasL was titrated at the indicated concentrations. The assay was developed after 12-13 h via crystal violet staining. Graphs show the means and standard deviations of triplicates and are representative of three independent experiments.

CD40L-independent, FasL-dependent cell death of parental HT1080 or KB cells was observed when the protein is used at high concentrations (> 2 μg/ml), most probably due to traces of high molecular weight aggregates with activity similar to that of cross-linked soluble FasL (data not shown). The estimated EC_{50} (the concentration of protein required to induce death of 50 % of the cells) for parental HT1080 cells is more than 3 logs (between 1000 and 10 000 times) higher than that for transfected HT1080-CD40L cells, clear evidence of the selectivity of the protein (data not shown). Cell death induced by the purified CD40-Flag-FasL fusion protein could be completely inhibited by the pan-caspase inhibitor zVAD, and, furthermore, pre-incubation of cells with an anti-human CD40L antibody completely abrogated CD40-Flag-FasL-induced cell death. Neutralization of the FasL domain by inhibitor (Fas-Comp) also led to complete rescue

from cell death (Figure 15). Taken together, these data show that the cell death observed is CD40L- and FasL- dependent and that, moreover, cell death in this system involves induction of caspase activity.

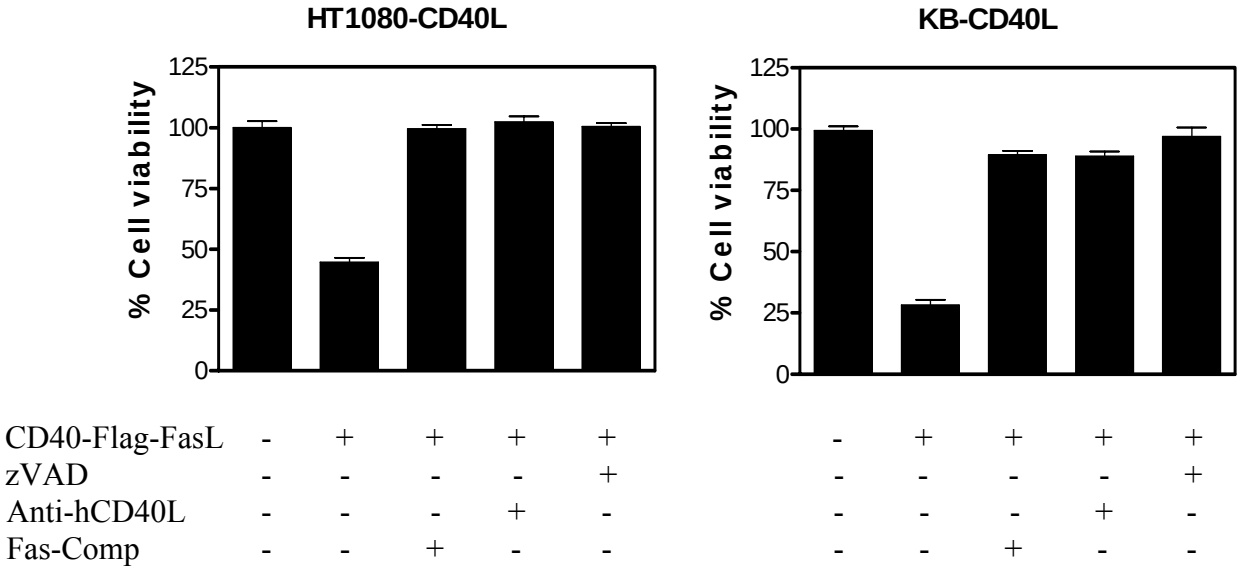

Figure 15. Neutralization assays. HT1080-CD40L and KB-CD40L cells were seeded in 96-well plates for at least 5 hours and then treated with CHX (2.5 μg/ml) for 45 min. CD40-Flag-FasL was used alone or after pre-incubation with Fas-Comp (4 μg/ml) for 1 h, while cells were either untreated or pre-incubated with either anti-h CD40L(1μg/ml) or zVAD (20 μM) for 1 h. The assay was developed after 12-13 h through crystal violet staining. The graphs show the means and the standard deviations of triplicates and are representative of three independent experiments.

5.8 CD40-Flag-FasL induces apoptotic cell death

The Fas/FasL system has been shown to mediate both apoptotic cell death and necrotic cell death (Matsumura et al, 2000). To better characterize the type of cell death involved in the system, the two pairs of cell lines were treated with CD40-Flag-FasL in the presence of CHX for the indicated time (Figure 16).

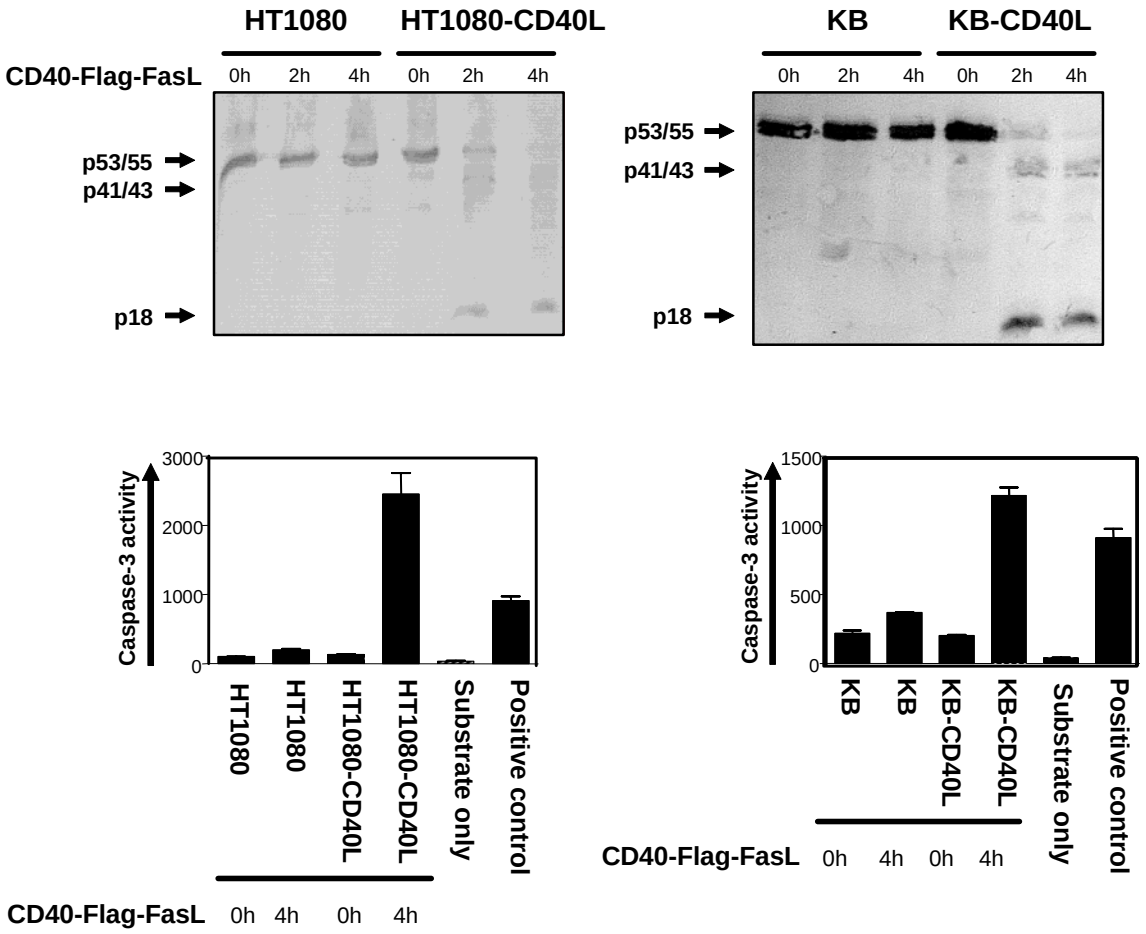

Figure 16. Caspase activity. KB, KB-CD40L, HT1080 and HT1080-CD40L cells were cultured in a 6cm petri dish for at least 5 hours and treated with CHX (2.5 µg/ml) for 45 min. The cells were then stimulated with CD40-Flag-FasL (40 ng/ml) for the indicated times and subsequently lysed. Pro-caspase-8 processing was detected by Western blotting using a caspase-8 specific antibody and the activity of caspase-3 was determined as explained in the material and methods section. The experiment includes a positive control consisting of lysates of HT1080 cells treated with cross-linked Flag-FasL. Incubating the caspase substrate without lysate does not lead to caspase-3 activity. Caspase-3 activity was measured after 30 min incubation with its substrate.

The cells were then lysed and processed procaspase-8 was detected by WB with a caspase-8-specific monoclonal antibody. As illustrated in Figure 15 CD40-Flag-FasL induces efficient processing of pro-caspase-8 to generate the partial cleavage fragments p43/41 and the p18 fragment resulting from full processing, but only in the CD40L transfected cell lines and not in the parental cells.

Processing of pro-caspase-8 is the initial step of the apoptotic process, in fact the apoptotic destruction of the cell is due to the action of effector caspases such as caspases -3, -6 & -7. In the experimental setting described above, the activity of caspase-3 was measured through an *in vitro* caspase-3 activity assay. For this purpose cell lysates used to determine caspase-8 activity were incubated with Ac-DEVD-AMC, the caspase-3 specific substrate. CD40-Flag-FasL induced a greater than 3-fold increase in caspase-3 activity in KB-CD40L cells and an almost 300- fold increase in such activity in HT1080-CD40L cells relative to untreated cells, thus arguing for apoptotic rather than necrotic cell death.

5.9 CD40-Flag-FasL induces production of IL-8, an NF-κB target gene

Relatively new reports provide compelling evidence of the role of Fas in non-apoptotic signaling pathways, such as the NF-κB signaling pathway. Under certain circumstances Fas-mediated NF-κB activation has been reported to be as strong as that resulting from TNF stimulation. Fas-mediated NF-κB activation and Fas-induced apoptosis are tightly correlated: the NF-κB pathway negatively regulates the apoptotic program by up-regulating anti-apoptotic molecules and activated caspases cleave several molecules important for the NF-κB pathway. Thus the capacity of Fas to strongly induce apoptosis generally masks its ability to signal through NF-κB (reviewed by Wajant et al, 2003a). Through RPA analysis and an IL-8-specific ELISA, Kreuz and others noticed that in KB and HT1080 cells Fas-mediated NF-κB activation led to robust up-regulation of IL-8 especially when apoptosis induction was blocked by zVAD or Bcl-2 (Kreuz et al, 2004). In light of these observations, we assessed the capacity for CD40-Flag-FasL,

immobilized by its interaction with CD40L expressed on transfected KB and HT1080 cells, to induce NF-κB through quantification of IL-8 production. In a first round of pilot experiments, we determined the conditions under which IL-8 was induced in our system. In parental HT1080 and CD40L transfectant HT1080-CD40L cells, cross-linked FasL or CD40-Flag-FasL induced IL-8 production did not depend on inhibition of protein synthesis through CHX but was critically dependent on inhibition of the apoptotic pathway with zVAD. Treatment of the cells with both CHX and zVAD did not lead to a further increase in IL-8 production. For KB cells however, IL-8 induction was observed when the cells were treated with zVAD and CHX whereas for KB-CD40L treatment of the cells with zVAD alone was sufficient (data not shown). As can be seen from Figure 17, cross-linked Flag-FasL induced IL-8 in a dose dependent manner in HT1080 and HT1080-CD40L cells. Consistent with earlier observations, CD40-Flag-FasL led to IL-8

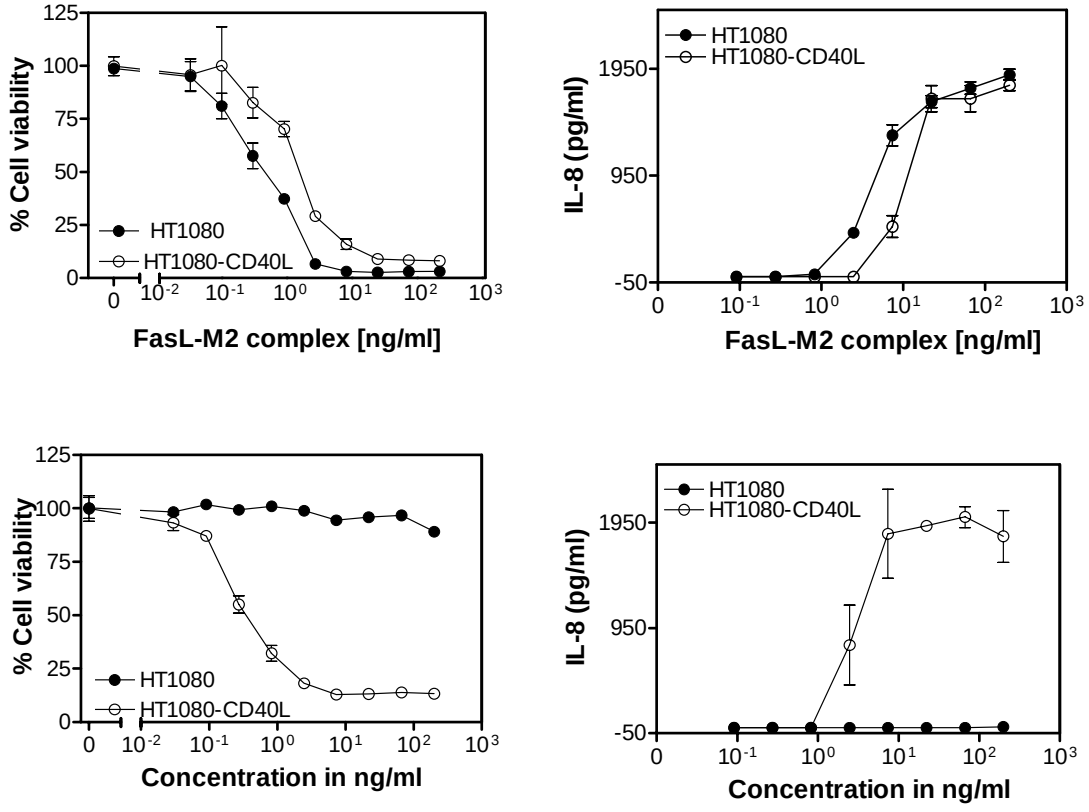

Figure 17. CD40-Flag-FasL induces IL-8 production in HT1080-CD40L cells. **Cytotoxicity assay.** Performed as described earlier in the presence of CHX (2.5 μg/ml). **IL-8 ELISA:** HT1080, HT1080-CD40L cells were seeded in 96 well plates overnight. The following day, the supernatant was removed and

the cells were treated with cross-linked Flag-FasL or CD40-Flag-FasL for 6 h after which the supernatant was collected and the amount of IL-8 produced was determined using a commercial IL-8 ELISA kit. Before stimulation, the cells were treated with zVAD (20 μM) for 30 min. Flag-FasL & M2 anti-Flag antibody were used at 200 ng/ml and 1 μg/ml, respectively. CD40-Flag-FasL was used at 200 ng/ml. Starting concentrations were as illustrated and were diluted in 4-fold steps. Graphs show the results of triplicates (means & standard deviations) and are representative of three independent experiments.

production by HT1080-CD40L cells whereas HT1080 cells, that do not express the immobilizing structure (CD40L), did not. In both cases the cells were pre-treated with zVAD. In KB and KB-CD40L cells treated with both CHX and zVAD and then stimulated with CD40-Flag-FasL, IL-8 was produced in a dose-dependent manner only by KB-CD40L cells (Figure 18 B). It is interesting to note that transfection of KB cells with CD40L was sufficient to abrogate the dependency on treatment with CHX in addition to zVAD for cross-linked FasL induction of NF-κB as determined by IL-8 production. For HT1080/HT1080-CD40L and KB/KB-CD40L transfectants, induction of apoptosis was dependent on inhibition of protein synthesis with CHX (Figures 17&18), whereas the NF-κB pathway is activated when the apoptotic pathway is down regulated.
The RANKed-Flag-FasL construct was analysed in a similar way taking advantage of the B16-C151 cell line, a stable transfectant of the mouse melanoma cell line B16 that expresses RANKL, and the B16-C91 stable cell line that expresses only the empty plasmid vector. HT1080 cells were co-cultured with B16-C91 or B16-C151 RANKL-expressing cells. The B16 cell line does not express Fas and therefore serves only as a way of immobilizing RANKed-Flag-FasL so that the immobilized molecule may mimic membrane FasL and thereby efficiently interact with Fas on neighboring HT1080 cells. IL-8 production was quantified. Figure 18 shows the capacity of RANKed-Flag-FasL to induce IL-8 only in co-cultures with RANKL positive cells (Figure 18 C).

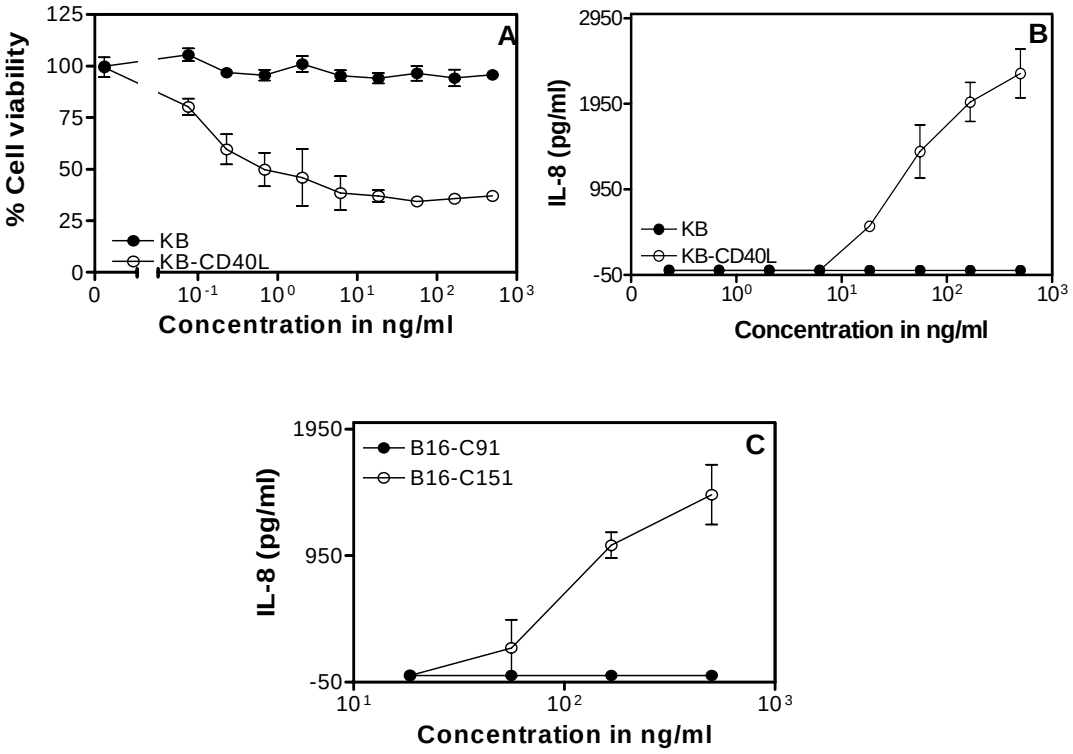

Figure 18. CD40-Fag-FasL induces IL-8 production in KB-CD40L cells.. **IL-8 ELISA.** KB/KB-CD40L cells were seeded in 96 well plates overnight. The following day, the supernatant was removed and the cells were treated with cross-linked Flag-FasL or CD40-Flag-FasL for 6h after which the supernatant was collected and the amount of IL-8 determined by ELISA. HT1080 cells were c-cultured overnight with either B16-C91 or B16-C151 cells in round bottom 96-well plates. The following day, the supernatant was removed and the cells were treated with RANKed-Flag-FasL. Before stimulation, the co-cultures were treated with zVAD (20 μM). KB/KB-CD40L cells were treated with zVAD (20 μM) and CHX (2.5 μg/ml) for 30 min. Flag-FasL & M2 anti-flag antibody were used at 200 ng/ml & 1 μg/ml, respectively. CD40-Flag-FasL or RANKed-Flag-FasL were used at 500 ng/ml. Starting concentrations were diluted in 4-fold steps. Graphs show the results of triplicate experiments (means & standard deviations). **Cytotoxicity assay:** was performed as explained earlier in the presence of only CHX (2.5 μg/ml). All these graphs are representative of three independent experiments.

5.10 CD40-Flag-FasL bound to its target antigen induces cell death in adjacent cells

To assess whether CD40-Flag-FasL targeted to the cell membrane via CD40L binding has the ability to induce cell death in a paracrine manner, we performed co-culture experiments in which 25 % of the CD40L-positive transfectants were co-cultured with 75 % of HT1080 cells. Figure 19 shows that CD40-Flag-FasL-treated HT1080 cells can only be killed when mixed with the CD40L-expressing cell lines KB-CD40L or HT1080-CD40L; co-culture with the parental cell lines did not elicit any cell death. At higher concentrations, CD40-Flag-FasL mediated cell death in more than 50 % of the total cell population, consistent with the idea that immobilized CD40-Flag-FasL exerts its action both on the 25 % target- expressing cells as well as on the bystander HT1080 population (Figure 19). In a similar experimental setting HT1080 cells were replaced with the cell line SV80. SV80 cells are not sensitive to Fas-mediated apoptosis as is evident from treatment with cross-linked Flag-FasL that does not lead to cell death (data not shown). As seen in Figure 19, high concentrations of CD40-Flag-FasL in SV80/HT1080-CD40L co-culture no longer induces 50 % cell death, cell death is restricted only to the CD40L positive population. Taken together, these data argue for paracrine killing or by-stander elimination of neighboring cells. Several experimental settings have described the instability of tumor markers (Samel et al, 2003). In the context of the study described here we performed a pilot experiment in which KB-CD40L cells, more than 98 % of which expressed CD40L as determined by flow cytometric analysis, were injected subcutaneously into nude mice. The development of a local tumor was monitored over 3 weeks, after which time the xenograft was excised and cell surface marker expression was analyzed. CD40L expression was observed in less than 5 % of cells (data not shown). Theoretical considerations thus suggest that a construct with the properties of the CD40-Flag-FasL described here could be of use in such settings due to the ability to induce ‘by-stander’ cell death following interaction with the small fraction of cells expressing the appropriate target cell surface marker.

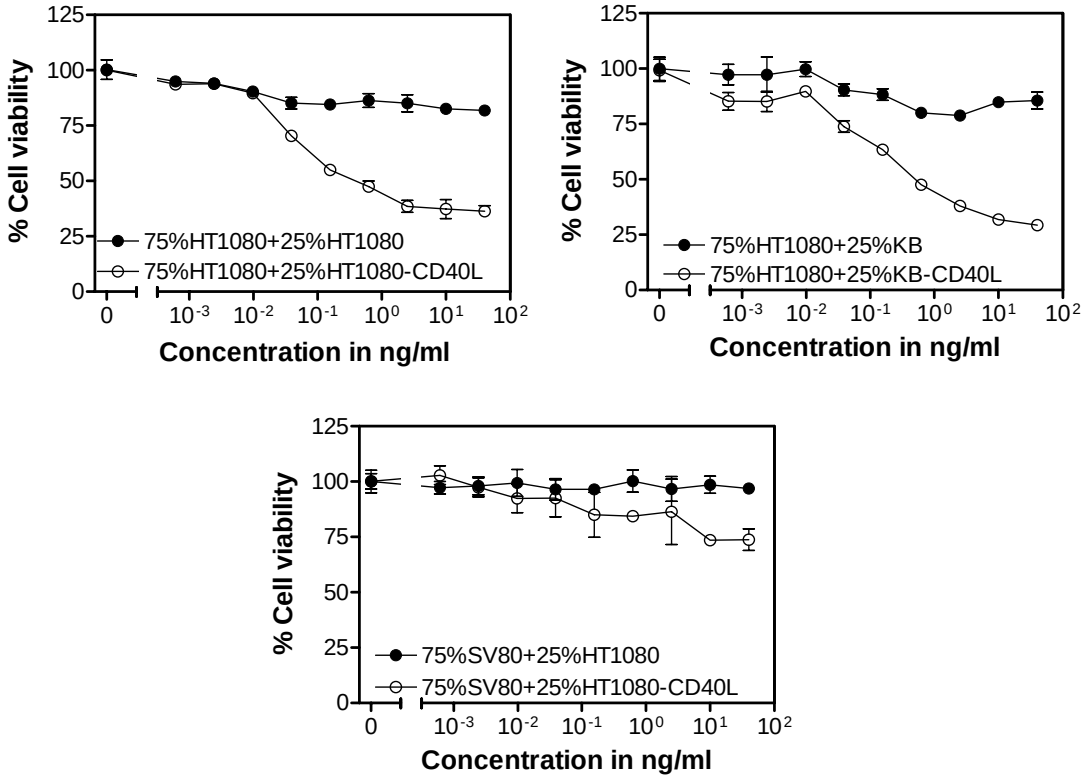

Figure 19. By-stander elimination of cells. Cells were co-cultured in the proportions indicated in 96-well plates for at least 5 hours then treated with CHX (2.5 µg/ml). CD40-Flag-FasL was titrated in 4-fold dilutions from an initial concentration of 40 ng/ml. Cell viability was determined 12-13 h later via crystal violet staining. Graphs are representative of three different experiments and illustrate the results of triplicate experiments with means and standard deviations.

FasL-sensitive cells are classified as type I or type II cells according to their dependence on the mitochondrion for efficient activation of caspase-3: Type I cells form a reasonable amount of DISC and pro-caspase-8 is efficiently processed to yield sufficient amounts of active caspase-8 which in turn activates caspase-3. Type II cells are characterized by a poor DISC formation via which very little active caspase-8 is generated and effective activation of caspase-3 depends on the small amount of caspase-8 yielded cleaving the BH3-only protein Bid which in turn leads to the formation of the apoptosome platform where pro-caspase-9 is activated (Scaffidi et al, 1998). Type II cells are recognized experimentally by the fact that apoptosis is inhibited by over-expression of Bcl-2. The ability of FasL to induce apoptosis can also be influenced at the level of the DISC by $FLIP_S$ and $FLIP_L$. These two proteins have a structure similar to pro-caspase-8 and thus

efficiently interact with pro-caspase-8 and through different mechanisms can impede its full processing. In this context, co-culture experiments performed with HT1080-$FLIP_L$ cells or HT1080-Bcl-2-expressing cells show that CD40-Flag-FasL-induced apoptosis is limited to around 25 % of the cells so cell death is probably limited to the cells expressing CD40L whereas cells expressing FLIP or Bcl-2 are no longer killed in a paracrine manner (Figure 20). HT1080 cells behave thus like type II cells.

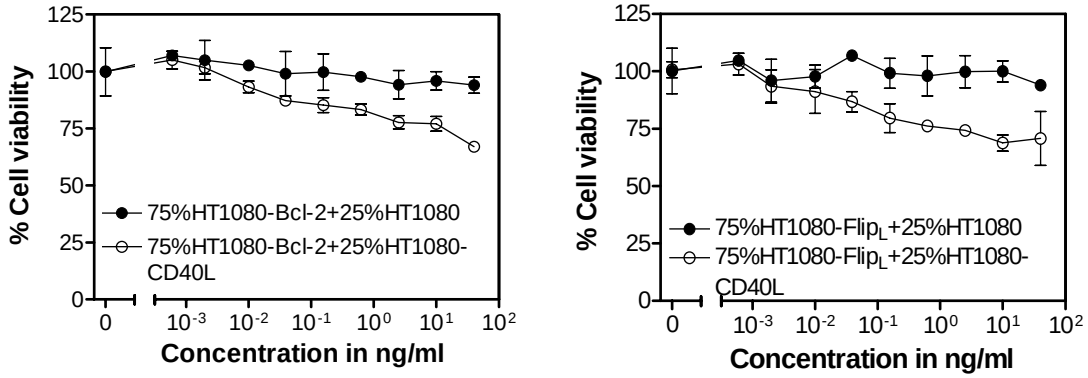

Figure 20. $FLIP_L$ and Bcl-2 interfere with CD40-Flag-FasL-induced cell death. Cells were co-cultured in the proportions indicated in 96-well plates for at least 5 hours then treated with CHX (2.5 µg/ml). CD40-Flag-FasL was titrated in 4-fold dilutions from an initial concentration of 40 ng/ml. Cell viability was determined 12-13 h later via crystal violet staining. Graphs are representative of three different experiments and illustrate the results of triplicate experiments with means and standard deviations.

5.11 CD40-Flag-FasL is devoid of *in vivo* toxicity

The promising results obtained *in vitro* led to assessment of the *in vivo* toxicity of CD40-Flag-FasL. In a first round of experiments, separate groups of 3 *Balb/c* mice were injected intravenously either with CD40-Flag-FasL (alone or cross-linked via a second injection of M2), with cross-linked Flag-FasL, with M2 antibody or with PBS alone (Table 1 a & b).

Table 1a. CD40-Flag-FasL shows no systemic toxicity I

I. v. injections 1st	2nd*	Mouse strain	Surviving animals	Animals dead within 1 hour
30 µg Flag-FasL	10 µg M2	Balb/c	0	3
70 µg CD40-Flag-FasL	PBS	Balb/c	3	0
20 µg Flag-FasL	10 µg M2	C57BL/6xCBA/J	0	3
100 µg CD40-Flag-fasL	PBS	C57BL/6xCBA/J	3	0

Table 1b. CD40-Flag-FasL shows no systemic toxicity II

I.v. injections 1st	2nd*	Mouse strain	Surviving animals	Animals dead within 6 hours
70 µg CD40-Flag-FasL	10 µg M2	Balb/c	3	0
70 µg CD40-Flag-FasL	PBS	Balb/c	3	0
10 µg M2	PBS	Balb/c	3	0
PBS	PBS	Balb/c	3	0
100 µg CD40-Flag-FasL	10 µg M2	C57BL/6xCBA/J	0	3
100 µg CD40-Flag-FasL	PBS	C57BL/6xCBA/J	3	0
10 µg M2	PBS	C57BL/6xCBA/J	3	0
PBS	PBS	C57BL/6xCBA/J	3	0

*The second injection was within 30 minutes of the first.

30 µg of cross-linked Flag-FasL were sufficient to cause the death of all 3 animals within 1 hour, while all the animals that received 70 µg CD40-Flag-FasL alone survived. Animals injected with 70 µg cross-linked CD40-Flag-FasL survived up to the 6 hour time-point when they were sacrificed. M2 antibody or PBS alone caused no fatalities.

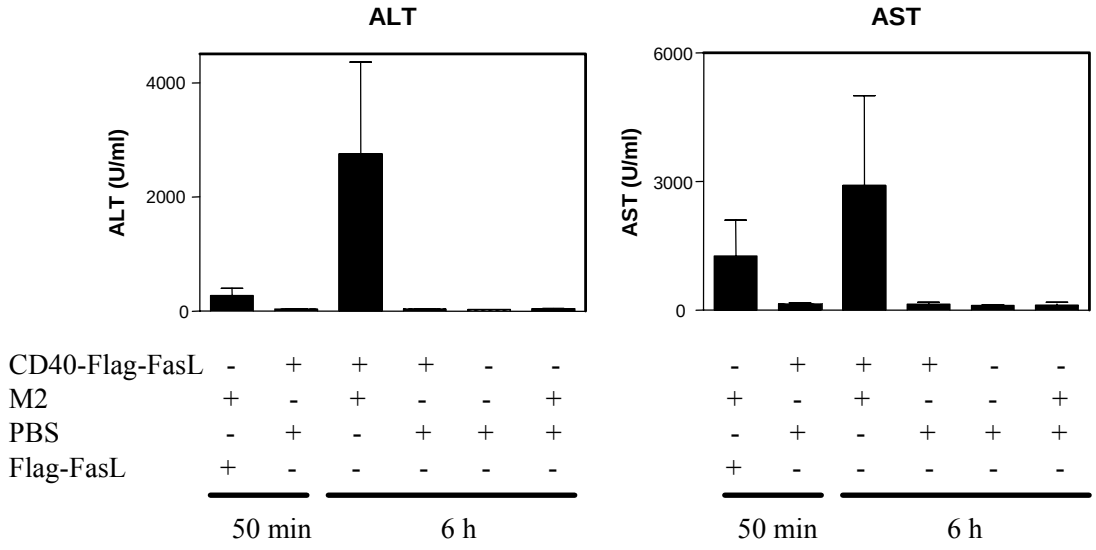

Figure 21. CD40-Flag-FasL is devoid of systemic toxicity. Groups of 3 Balb/c mice were injected i.v. with CD40-Flag-FasL±M2, Flag-FasL±M2, PBS or PBS+M2 as indicated in Table 1 a & b. After 50 min or 6 h mice were sacrificed and serum ALT & AST activity determined. Bar graphs illustrate means and standard deviations of enzyme activity obtained for mice in each group.

The levels of both serum alanine and aspartate amino transferase (ALT/AST), indicative of hepatic and systemic damage respectively, were elevated in the mice that received cross-linked Flag-FasL. In contrast to *in vitro* data demonstrating that cross-linking was associated with virtually no cell death, mice that received cross-linked CD40-Flag-FasL had elevated serum ALT and AST levels whereas injection of CD40-Flag-FasL alone was well tolerated (Figure 21). Since systemic administration of Fas agonists or recombinant FasL has been shown to lead to liver failure we also determined intra-hepatic caspase-3 activity. Similar to other Fas agonists, the M2-cross-linked Flag-FasL induced liver cell apoptosis as determined by a sharp rise in the activity of intra-hepatic caspase-3. M2-cross-linked CD40-Flag-FasL complexes were also hepatotoxic but to a lesser extent. CD40-Flag-FasL alone, M2 antibody or PBS were not associated with liver damage measured in this way (Figure 22).

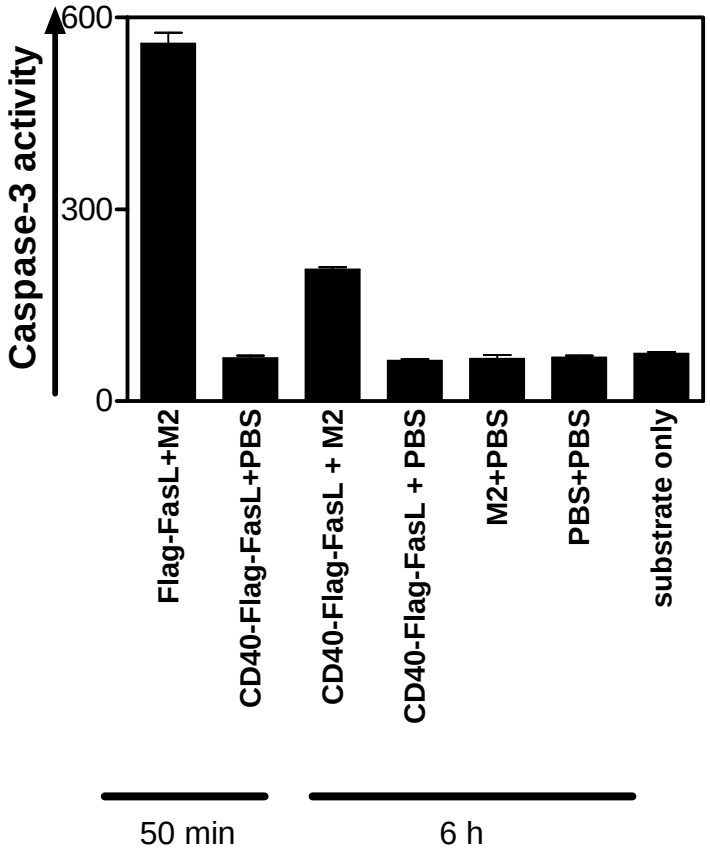

Figure 22: CD40-Flag-FasL is not hepatotoxic. I. Balb/c mice were injected i.v. with CD40-Flag-FasL±M2, Flag-FasL±M2, PBS or PBS+M2 as indicated in Table 1. After 1 or 6 h the mice were sacrificed and intra-hepatic caspase-3 activity was determined using liver homogenates. Bar graphs for each experimental condition represent the mean and standard deviation of the caspase-3 activity value for each group.

The results obtained with cross-linked CD40-Flag-FasL prompted us to perform another round of experiments to improve our understanding of the dose-dependent toxicity of CD40-Flag-FasL (Table 1 a & b). For that purpose the *C57BL/6xCBA/J* mouse strain was used as macroscopic haemorrhagic changes in the liver are more easily visualized. The doses of CD40-Flag-FasL were also increased, and acute toxicity was measured up to 7 hours post-injection. Intravenous injection of 100 µg of CD40-Flag-FasL alone gave similar outcomes to injection of either M2 antibody or PBS alone i.e. no acute toxicity was observable with respect either to haemorrhagic liver changes or to increased intra-hepatic caspase-3 activity (Figure 23 A, B lower panels and 23 C). Injection of cross-

linked Flag-FasL was lethal within 1 hour and mice injected with 100µg of cross-linked CD40-Flag-FasL died within 5 hours post injection. In both cases high intra-hepatic caspase-3 activity was detected and this was corroborated by the macroscopic evidence of haemorrhagic changes in the livers (Figure 23 A, B upper panels). Thus, systemic administration of CD40-Flag-FasL alone was not associated with acute toxicity: no increase in either serum ALT, AST or of intra-hepatic caspase-3 was seen in *Balb/c* mice. Doses of up to 100 µg of protein were also well tolerated by *C57BL/6xCBA/J* mice. Cross-linking the protein, however, increased its bioactivity and was associated with fatal outcomes.

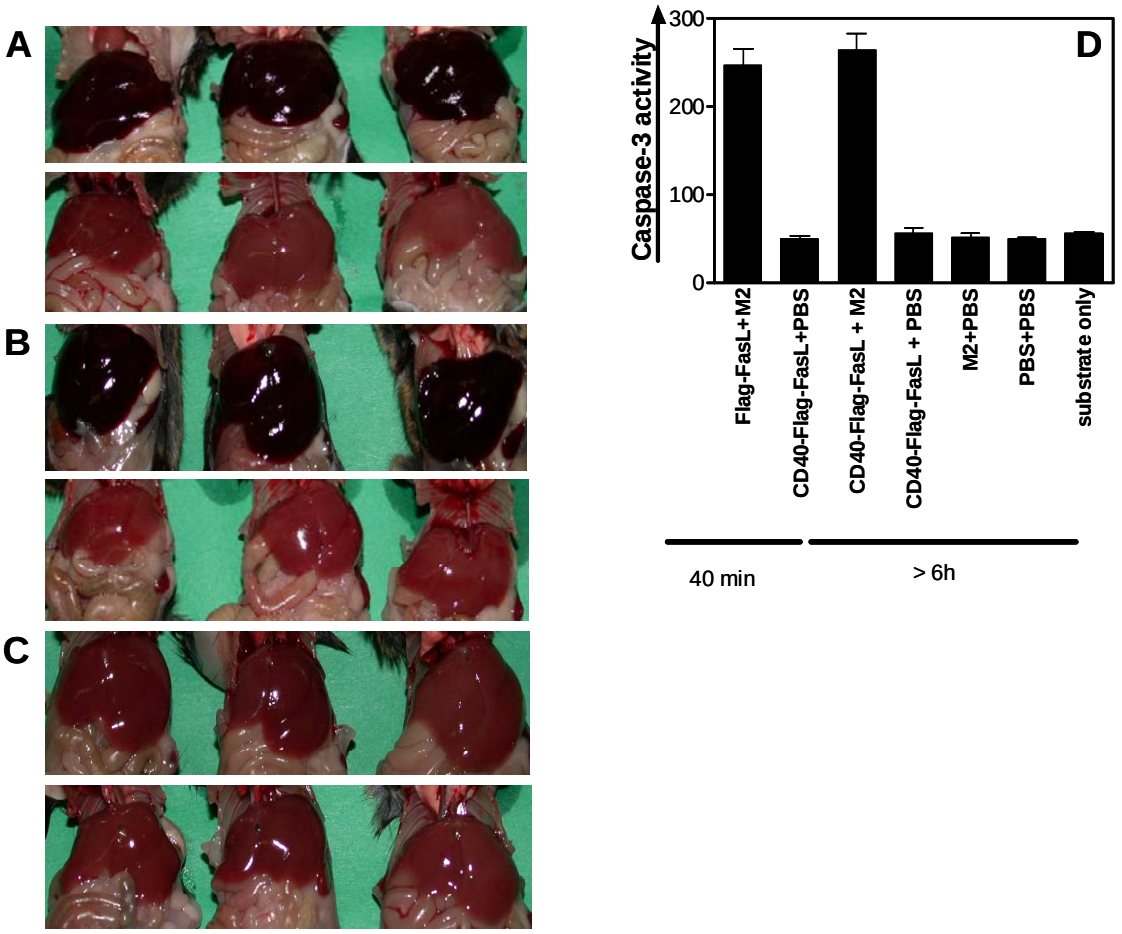

Figure 23: CD40-Flag-FasL does not induce liver toxicity. II. C57BL/6xCBA/J mice were treated intravenously as described in Table 1. After ~40 min or ~7 h the mice were sacrificed and the appearance of their livers was photographically documented. Mice which received cross-linked CD40-Flag-FasL died within 5 h. **Caspase-3 activity:** mice were injected i.v. with CD40-Flag-FasL±M2, Flag-FasL±M2, PBS or PBS+M2 as indicated in Table 1. After 1 or 6 h the mice were sacrificed and intra-hepatic caspase-3

activity was determined using liver homogenates. Bar graphs for each experimental condition represent the mean and standard deviation of the caspase-3 activity value for each group. Caspase-3 activity was measured after 30 min incubation with the substrate of caspase-3.

5.12 CD40-Flag-TRAIL shows target-specific activity

We also analysed the activity of CD40-Flag-TRAIL, a protein obtained by fusing the extracellular domain of CD40 to that of human TRAIL (see section 5.1 for more details). CD40-Flag-TRAIL was transiently expressed in chinese hamster ovary (CHO) cells. WB analysis showed a protein which is reasonably expressed, and under non-reducing conditions CD40-Flag-TRAIL had a homotrimeric organization that was further reinforced by intermolecular disulphide bonds (data not shown). CD40-Flag-TRAIL was analyzed for specific binding and bioactivity on CD40L-positive and CD40L-negative HT1080 and KB cells. Indirect immunofluorescence FACS analyses showed specific binding only to CD40L-positive cells (data not shown).

Prior to testing on parental HT1080, KB and HT1080-CD40L, KB-CD40L the sensitivity of the cells to cross-linked Flag-TRAIL was determined. Figure 24 shows that both pairs of cell lines were equally sensitive to cross-linked Flag-TRAIL.

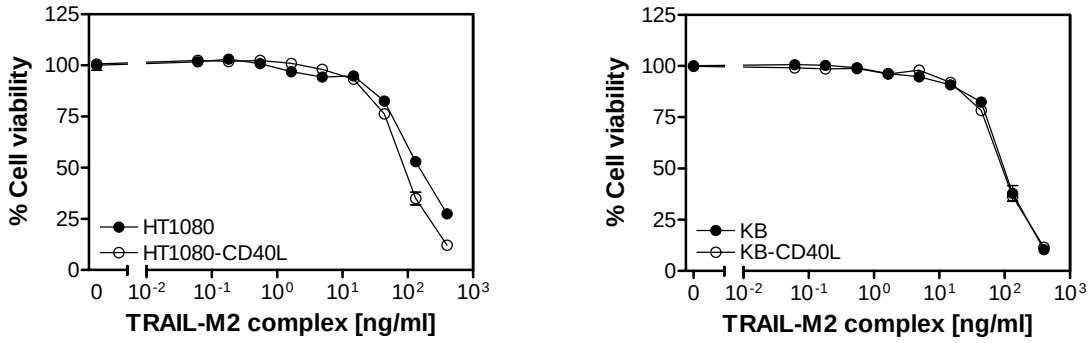

Figure 24. Bioactivity of crosslinked Flag-TRAIL. HT1080, HT1080-CD40L, KB and KB-CD40L cells were seeded in 96-well plates for at least 5 h and treated with CHX (2.5 µg/ml) for 45 min. The cells were treated with cross-linked Flag-TRAIL diluted 4-fold from a starting concentration of 500 ng/ml. M2 anti-flag antibody was used at 1 µg/ml. Assays were developed after 12-13 h via crystal violet staining. Graphs are representative of three different experiments and data points illustrate the results of triplicates with means and standard deviations.

Titration of CD40-Flag-TRAIL on HT1080/HT1080-CD40L and KB/KB-CD40L led to dose-dependent cell death induction only of CD40L positive cells. CD40-Flag-TRAIL treatment of KB-CD40L and HT1080-CD40L led to cell death via the apoptotic pathway as demonstrated by the induction of caspase-3 activity in these cells (Figure 25).

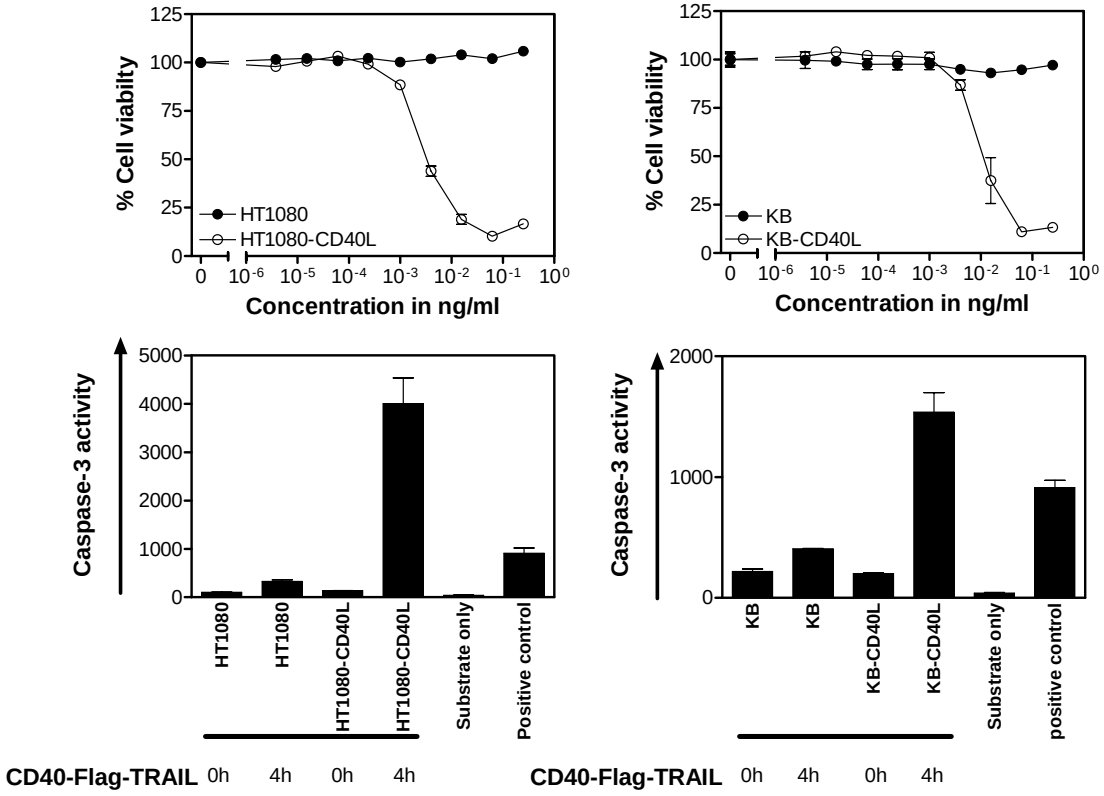

Figure 25: CD40-Flag-TRAIL induces cell death specifically in CD40L-positive cells. Cytotoxicity assay. HT1080, HT1080-CD40L, KB, and KB-CD40L cells were seeded in 96-well plates for at least 5 h and treated with CHX (2.5 µg/ml) for 45 min. The cells were treated with 4-fold dilutions of CD40-Flag-TRAIL starting at 250 ng/ml. The assay was developed after 12-13 h via crystal violet staining. Each data point represents the mean and standard deviation of triplicates. **Caspase-3 activity**: HT1080, HT1080-CD40L, KB and KB-CD40L cells were seeded in 6cm Petri dishes for at least 5 h and treated with CHX (2.5 µg/ml) for 3 h then with CD40-Flag-TRAIL at 125 ng/ml. Cells were lysed as described in materials and methods and caspase-3 activity was determined. Bar graphs for each experimental condition represent the mean and standard deviation of the caspase-3 activity value of triplicates. Caspase-3 activity shown is obtained after incubation for 30 min with the substrate

Apoptotic cell death induced in CD40L positive cells could be shown to be due to the interaction between CD40 and CD40L because pre-treatment of the cells with an anti-human CD40L antibody led to abrogation of apoptosis induced by CD40-Flag-TRAIL. As expected, cell death was inhibited by treatment with zVAD and was TRAIL-specific as it was neutralized by pre-incubation with anti-TRAIL monoclonal antibodies. Moreover, as reported for other soluble TRAIL derivatives designed to be activated upon target dependent immobilization, CD40-Flag-TRAIL signals cell death mainly through TRAIL-R2 in both HT1080-CD40L and KB-CD40L (Wajant et al, 2001) (Figure 26). As well as inducing apoptosis, TRAIL, like FasL, efficiently activates the NF-κB pathway (see section 3-3-2-a of the introduction). CD40-Flag-TRAIL induced NF-κB activation in HT1080-CD40L and KB-CD40L and not in HT1080 or KB cell lines after they have been treated with CHX and zVAD (data not shown).

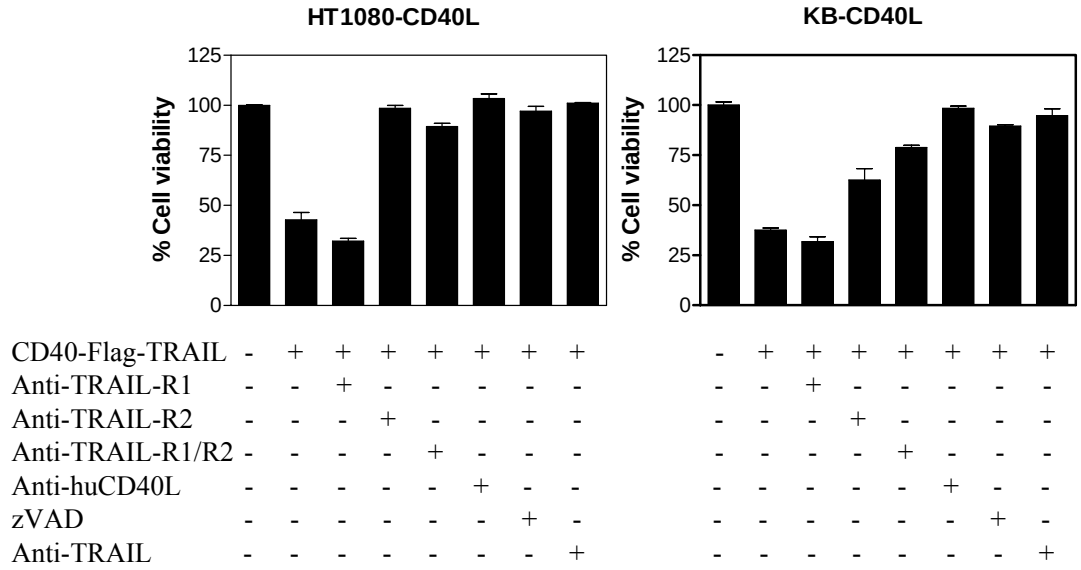

Figure 26. Neutralization assay. HT1080-CD40L and KB-CD40L cells were seeded in 96-well plates for at least 5 h and treated with CHX (2.5 µg/ml) for 45 min. Prior to addition of CD40-Flag-TRAIL, the cells were incubated with either anti-TRAIL-R1 (25 µg/ml), anti-TRAIL-R2 (25 µg/ml), anti-TRAIL-R1 plus anti-TRAIL-R2 (25 µg/ml each) anti-human CD40L(1 µg/ml) or zVAD (20µM) for 1 h. CD40-Flag-TRAIL was pre-incubated with the anti-TRAIL mAbs (E11, 2G2, anti-APO2-L 1 µg/ml each) for 1 h. The assay was developed after 12-13 h via crystal violet staining. Graphs are representative of three different experiments and illustrate the results of triplicates with means and standard deviations.

6 Discussion and conclusion

6.1 Expression of fusion proteins

Plasmids encoding several targeting structures in frame with a Flag-epitope and a COOH-terminal portion of the extracellular domain of human Fas Ligand (AA 139-281) were transfected into the human embryonic kidney cell line HEK293. Secreted soluble fusion proteins in supernatants of transfected cells were used as such or affinity purified using immobilized anti-Flag antibodies. The theoretical molecular masses of the encoded recombinant proteins were (~38 kDa, ~41 kDa, ~46 kDa, ~39 kDa, 38 kDa) for 4-1BB-Flag-FasL, TNFR1-Flag-FasL, TNFR2-Flag-FasL, CD40-Flag-FasL, RANKed-Flag-FasL respectively. As can be seen from Figure 7, there is a discrepancy between predicted molecular masses and observed molecular masses which is especially striking for RANKed-Flag-FasL and CD40-Flag-FasL. One explanation for these differences in molecular masses could be the presence of carbohydrate residues. In fact, we and others have found that when soluble Flag-tagged FasL (AA 139-281) is analyzed by Western blotting under reducing conditions, a total of four evenly spaced bands can be detected with molecular masses ranging between ~18 kDa- ~30 kDa. As unglycosylated soluble human FasL has been reported to have a molecular mass of 18 kDa and possess three potential N-glycosylation sites (Asn-184, Asn-250, Asn 260), the four bands detected correspond to unglycosylated, mono-, di- and tri-N-glycosylated soluble FasL monomers (Schneider et al, 1997, and data not shown). Glycosylation of the FasL part could therefore account for the high observed molecular masses of the chimaeric proteins. For FasL and some other members of the TNF family N-glycosylation appears to be important for their activity. Indeed glycosylated recombinant CD40L is readily expressed at the cell surface of eukaryotic cells but transport to the cell surface is blocked in the presence of the N-glycosylation inhibitor tunicamycin (Jumper et al, 1995). FasL can be efficiently expressed in a variety of eukaryotic systems, (Jumper et al, 1995; Mariani et al, 1996, Tanaka et al, 1996) but forms inclusion bodies when expressed in bacteria. Tanaka and colleagues tried to produce soluble human FasL in *E. coli* using the sequence of alkaline phosphatase. The *E. coli* harboring the FasL expression plasmid secreted a

protein carrying the extracellular region of human FasL. However, the purified secreted protein had no FasL activity. On the other hand, the glycosylated human FasL produced in *P. pastoris* was functional (Tanaka et al, 1997). We also observed that the higher the degree of glycosylation of FasL, the more it is secreted; this could explain the fact that RANKed-Flag-FasL and CD40-Flag-FasL have the highest observed molecular masses and are also the best-secreted soluble fusion proteins (data not shown).

6.2 Target-independent activities of fusion proteins

Any reagent that binds to Fas without inducing optimal signaling could potentially protect cells against the effects of a more active Fas agonist. Trimeric human FasL, for example, protects peripheral blood T cells or Hela cells against active versions of FasL (Suda et al, 1997; Tanaka et al, 1998). Moreover the aggregation state of FasL trimers and the intrinsic capacity of a target cell to cope with weak Fas signals, which is related to the ratio between Fas and intracellular inhibitors of the pathway such as FLIP (Kataoka et al, 2002), are two main factors determining Fas sensitivity. Thus the suboptimal signals induced by trimeric FasL leads to killing of certain cell types but not others. Membrane bound FasL, on the other hand, kills all types of Fas-sensitive cells. These properties of soluble FasL were used to create recombinant soluble FasL chimaeras, the bioactivity of which is reconstituted only upon binding to defined cell-surface molecules. When the various molecules were assayed on Fas-sensitive HT1080 cells for their soluble FasL-like activities, CD40-Flag-FasL behaved in a manner similar to soluble trimeric FasL, with only limited toxicity at high concentrations. Other constructs, including RANKed-Flag-FasL, 4-1BB-Flag-FasL, TNFR1-Flag-FasL, TNFR2-Flag-FasL, all exhibited comparatively greater toxicity to HT1080 cells. As mentioned earlier, Flag-tagged soluble FasL is rendered active by cross-linking with an anti-Flag antibody. Cross-linked RANKed-Flag_FasL displayed enhanced cytotoxicity, while cross-linked CD40-Flag-FasL displayed no enhanced activity and cross-linking 4-1BB-Flag-FasL, TNFR2-Flag-FasL, TNFR1-Flag-FasL actually decreased their apoptosis-inducing capacities. These observations were somewhat unexpected but could be explained by the aggregation status

of the various fusion proteins. We and others have noticed that the functionality of TNF, TRAIL or FasL fusion proteins is hard to predict. Certain scFv targeting modules, or TNF receptor family member targeting structures, lead spontaneously to the formation of higher MW aggregates of otherwise homotrimeric molecules, thus giving rise to molecules with enhanced activities when compared to soluble FasL or even to cross-linked soluble FasL. Obtaining a functionally useful antibody-mediated activation of apoptotic zytokine AMAZE (sc40-Flag-FasL) or a NAMAZE (CD40-Flag-FasL) molecule might therefore require extensive screening (Samel et al, 2003; Mueller et al, submitted).

The enhanced cytotoxicity of soluble cross-linked FasL is not observed at high FasL to antibody ratios, probably because cross-linked trimers are either not formed (antibody might bind to two epitopes in the same trimer) or because the action of cross-linked trimers is antagonized by the excess of non-cross-linked FasL (Holler et al, 2003). However this is unlikely to explain the reduced cytotoxicity of cross-linked 4-1BB-Flag-FasL, TNFR2-Flag-FasL, TNFR1-Flag-FasL. Most probably, in these cases, the cross-linking antibody disrupts an optimal aggregation state leading therefore to the reduction of the observed apoptotic activity.

6.3 Target-dependent activation

The observation that soluble FasL is not at all or is only poorly toxic, but that cross-linked FasL is toxic suggests that specific induction of soluble FasL aggregation on a target cell would lead to killing of the cell. This was achieved by fusing soluble, trimeric, inactive FasL to a single chain antibody or a receptor as exemplified in this study. When the single chain antibody or the receptor recognizes its cognate ligand, FasL shifts from its soluble to a membrane-bound-like form, allowing specific killing of the target cell. The single chain targeted FasL was able to kill antigen-positive tumour cells in a live animal without inducing liver toxicity inherent to other Fas agonists (Samel et al, 2003). The study presented here demonstrates that CD40-Flag-FasL and CD40-Flag-TRAIL induced cell death in CD40L-positive cells as well as on antigen-negative (that is non-

target) cells by non-specific mechanisms i.e. by-stander effects. The concept developed here was applied not only to soluble FasL and TRAIL but also to soluble TNF. The feasibility of targeting TNF to tumour sites by the use of TNF-Selectokine prodrugs has been described, offering the possibility of avoiding the lethal inflammatory and septic shock-like symptoms associated with systemic application of TNF. In addition to its direct action on targeted cells CD40-Flag-FasL exerted a juxtatropic FasL action as has also been observed for the TNF-Selectokine (Wuest et al, 2002). One of the factors determining the choice of a ligand (targeting structure) used to target a drug or an effector molecule in cancer therapy is that the ligand should have a receptor or antigen with a high density on the surface of the targeted cells and, also, target cells should not show a high degree of heterogeneity in their antigen expression. However if the targeted treatment leads to paracrine killing, as seen with CD40-Flag-FasL, then some degree of antigen heterogeneity might be tolerated.

The results obtained with antibody derivatives such as scFv40-Flag-FasL, as in the study cited above, or in a previous study applying the same concept to obtain MB0S4TRAIL - a recombinant antibody derivative made of the minibody MBOS4 (recognizing the tumour stroma marker fibroblast activation protein: FAP) and soluble TRAIL - can thus now be expanded to receptor-ligand interactions. CD40-Flag-TRAIL, as MBOS4TRAIL, induced apoptosis in HT1080 cells expressing their respective ligands via TRAIL-R2 that is known to be activated only by membrane-bound TRAIL (Wajant et al, 2001).

In addition to the apoptotic pathway, CD40-Flag-FasL and CD40-Flag-TRAIL can activate non-apoptotic pathways like NF-κB. Activation of this pathway could be observed only in a target-dependent manner. NF-κB activation was enhanced by treating KB-CD40L and HT1080-CD40L cells with zVAD to inhibit the apoptotic pathway. Parental KB cells, however, required treatment with both CHX and zVAD for efficient NF-κB signaling, so in the case of KB/KB-CD40L cells the experiment included pretreatment of the cells with both zVAD and CHX, the latter being a means of down-regulating the expression of the NF-κB pathway inhibitor FLIP (data not shown, Wajant et al, 2000; Kreuz et al, 2004). Interestingly one of the applications of such pro-apoptotic reagents is to target the devastating action of FasL to tumour cells. We have shown that such reagents could induce both the apoptotic deletion of tumour cells as well as the

production of the NF-κB target gene IL-8, in a manner similar to cross-linked soluble FasL. Fas stimulation is known to lead to the production of chemokines such as MCP-1 and IL-8 in a variety of cell types. The properties of constructs like CD40-Flag-FasL and CD40-Flag-TRAIL could thus extend beyond their apoptosis-inducing capacity to recruitment of granulocytes/neutrophils to the tumour site either by direct induction of chemokine production or by other mechanisms, thus leading to tumour rejection (Wajant et al, 2003a).

The mechanism underlying the superior signaling capacity of membrane FasL compared to that of soluble FasL is not fully understood, but a study in which two trimeric FasL were brought together by fusion to adiponectin or to the Fc portion of an IgG1 shed some light on this question. According to Holler and colleagues (2003), two FasL trimers must act in close proximity to induce apoptosis, a condition that might be achieved in the case of membrane-bound FasL by augmentation of the local concentration of FasL. However the stalk of FasL - the portion between the transmembrane and the TNF homology domain - may have some self-aggregating properties, which would help maintain clusters of FasL within the membrane. As the stalk region is absent from the constructs we engineered, their enhanced activity might reflect a capacity of the membrane to locally increase the concentration of the chimaeric constructs, thus leading to optimal aggregation of Fas. According to the model for the interaction of Fas-FADD proposed in the above mentioned publication, three DDs of Fas may recruit three DDs of FADD in their center. The three DEDs of FADD could thus form a trimer ready to recruit one of the DEDs of procaspase-8. By analogy with pro-caspase-9, pro-caspase-8 is likely to exist as a monomer at physiological concentrations and is activated by dimerization. Were FADD to have a trimeric structure it might not provide sufficient docking sites to recruit two procaspase 8 molecules. These requirements would be met only with two closely packed trimers of FADD DED, as would be possible with hexameric FasL. A similar mechanism may apply to membrane-bound FasL or the fusion proteins described here that mimic membrane-bound FasL, with the membrane itself playing the role of a cross-linker (Weber et al, 2001; Holler et al, 2003). Also worthy of mention is the fact that neither soluble CD40-Flag-FasL nor soluble CD40-Flag-TRAIL were able to induce

cell death or NF-κB activation in KB or HT1080 cells, as efficient induction of both the apoptotic and the non-apoptotic pathways depends on optimal aggregation of Fas.

6.4 CD40-Flag-FasL is devoid of systemic toxicity

Attempts to exploit Fas agonists for cancer therapy have revealed their severe systemic toxicity, liver failure being the major determinant of lethality (Ogasawara et al, 1993). However, prevention of the systemic action of FasL, either by the use of mice lacking a functional Fas/FasL system (glp/lpr) or through the use of human Fas-specific antibodies and xenotransplants, has demonstrated that the principle of Fas activation as an anti-tumoural strategy is feasible (Trauth et al, 1989). Moreover, locally applied FasL kills tumour cells very efficiently without systemic toxicity (Rensing-Ehl et al, 1995). Strategies aimed at directing the toxicity of TNF family members such as FasL and TNF to specific targets have been explored and have been shown to be associated with acceptable or no systemic toxicity (Bulfone-Paus et al, 2000; Elhalel et al, 2003; Samel et al, 2003; Bauer et al, 2004). In particular Samel and colleagues reported that sc40-Flag-FasL, a fusion protein comprising a FAP-recognizing scFv and FasL (AA 139-281), was very well tolerated by mice even after i.v. injection of up to 90 µg of protein per animal. On the other hand cross-linking of doses of 4-8 µg of the protein via its internal Flag-tag was sufficient to induce death through liver failure of the animals. In accordance with these findings, we have shown here that CD40-Flag-FasL was totally devoid of acute toxicity in mice in which up to 100 µg per animal was administered by i.v. injection. However CD40-Flag-FasL, like sc40-Flag-FasL, retained its capacity to induce cell death through cross-linking, but, in comparison with sc40-Flag-FasL, high doses of cross-linked CD40-Flag-FasL (> 70 µg/animal) were required to cause death. CD40-Flag-FasL should thus be a particularly safe molecule *in vivo* as it has strong pro-apoptotic features and the doses required to delete target cells should be low; small doses of CD40-Flag-FasL are unlikely to be activated in a non-specific way by any potential *in vivo* cross-linker of the cytokine derivative.

6.5 Conclusion and perspectives

In a physiological context the co-stimulatory properties of the CD40-CD40L interaction are crucial for T cell-dependent antibody production. Indeed in germinal centers, antigen stimulation followed by engagement of CD40 by CD40L expressed on T cells causes somatic hypermutation of antigen receptor genes in B cells; those producing antibody displaying higher antigen avidity are selected and can undergo heavy chain class-switching to produce different antibody subclasses. A mutation in the CD40L gene induces hyper IgM syndrome: IgM-expressing cells cannot undergo isotype conversion to IgG expression. The CD40-CD40L interaction has also been implicated in several pathological settings including cancer (Younes et al, 2003). The use of the CD40-CD40L interaction pathway in cancer therapy is quite controversial because of the added immunological function of that system, but CD40 and CD40L can nevertheless be co-expressed in several types of B cell malignancies leading to an autocrine/paracrine CD40-CD40L survival loop that has been proposed to play a role in the pathogenesis and survival of some B cell neoplasms. A similar survival loop was recently proposed for cutaneous T cell lymphoma and large B cell lymphoma (Younes and Kadin, 2003). One intervention strategy involves therapeutic interruption of the CD40-CD40L interaction, with the aim of depriving malignant cells of this survival and chemotherapy resistance loop. This could be achieved by anti-CD40L or anti-CD40 blocking antibodies or by molecules such as CD40-Flag-FasL and CD40-Flag-TRAIL. Alternatively CD40-Flag-FasL or CD40-Flag-TRAIL could be used to target activated T cells. Since Medawar demonstrated 50 years ago that immunological tolerance can be induced in newborn mice (Billingham and Medawar, 1953), the challenge to immunologists has been to achieve therapeutic tolerance in the areas of transplantation, autoimmune disease and allergy. Although the underlying mechanisms are not well understood, it is generally believed that antigen recognition by T cells in the absence of costimulation may alter the immune response, leading to anergy or tolerance. Further support for this concept comes from animal models of autoimmunity and transplantation, where treatments based on costimulatory blockade, in particular with CD40L-specific antibodies, have been highly effective (Larsen et al, 1996; Kirk et al, 1999). Peripheral anergy has been accomplished

by blocking costimulatory signals with a soluble form of CTLA-4 that prevents the interaction of APC-expressed B7 molecules with T cell-expressed CD28. Antibody-mediated targeting of CD25, the α-subunit of the IL-2 receptor, is one of the immunosuppressive methods currently in clinical use. Moreover novel strategies like the use of IL-2-IgG-FasL have proven beneficial in a murine model of delayed-type hypersensitivity (Bulfone-Paus et al, 2000). Another fusion protein, CTLA-4·FasL, integrates two functional elements within a single protein, similar to the above-mentioned fusion protein and CD40-Flag-FasL or CD40-Flag-TRAIL: a costimulatory blocking moiety and FasL that triggers the inhibitory Fas receptor on T cells. This CTLA-4·FasL construct has been shown to mediate hyporesponsiveness to primary and secondary alloantigens in mixed lymphocyte reactions, with a mechanism involving induction of anergy, clonal deletion via apoptosis and induction of cell-mediated immunoregulation (Elhalel et al, 2003). Finally, murine acute graft-versus-host disease can be prevented by depletion of alloreactive T lymphocytes using activation-induced cell death (AICD) (Hartwig et al, 2002). FasL and TRAIL are two mediators of T cell AICD. Since CD40L is expressed on activated T cells, the CD40-Flag-FasL and CD40-Flag-TRAIL constructs described here could thus prove beneficial in the above-mentioned disease condition or for diseases in which excessive activation of T cells is either causal or contributes to pathogenesis.

7 Zusammenfassung

Es wurde kürzlich gezeigt, dass lösliche *single chain* Antikörper-Fusionsproteine, die Teile der extrazellulären Domäne aus TNF, FasL oder TRAIL enthalten, funktionelle Eigenschaften ähnlich der entsprechenden membrangebundenen Form nach Interaktion mit der Antigen-exprimierenden Zelloberfläche der Zielzelle, tragen (Wajant et al, 2001; Wuest et al, 2001, Samel et al, 2003). In der hier dargelegten Arbeit wurde dieses Prinzip auf andere selektiv ausgewählte Protein-Protein Interaktionen ausgeweitet. Es wurden mehrere lösliche Fusionsproteine, die die N-terminale extrazelluläre Domäne von humanem CD40 (AA1-192), 4-1BB (AA1-186), TNFR1 (AA 1-211), TNFR2 (AA 1-257), RANK (AA 29-213) und die C-terminale extrazelluläre Domäne von humanem FasL (AA 139-281) umfassen, hergestellt. Außerdem wurde CD40 an die extrazelluläre Domäne von humanem TRAIL (AA 95-281) fusioniert. Um die Detektion und Aufreinigung der Fusionsproteine zu erleichtern, wurden in allen Konstrukten die zwei Domänen durch die Flag Sequenz getrennt. Desweiteren besitzen alle Konstrukte eine *leader sequence*, um die Sekretion der Proteine zu gewährleisten. Das Molekulargewicht der Fusionsproteine wie es sich im Western Blot darstellte war etwas höher als das errechnete. Dies war besonders deutlich für RANKed-Flag-FasL und CD40-Flag-FasL und ist wahrscheinlich auf post-translationale Modifikationen wie Glykosylierungen zurückzuführen. Die Proteine wurden nachfolgend auf ihre lösliche FasL-ähnliche Aktivität auf HT1080 Zellen analysiert. Trotz einer nur mittleren bzw. einer kaum detektierbaren Expression von TNFR1-Flag-FasL, 4-1BB-Flag-FasL und TNFR2-Flag-FasL wiesen diese Protein eine hohe spezifische Aktivität auf. Im Gegensatz dazu, zeigten Überstände von CD40-Flag-FasL bzw. RANKed-Flag-FasL, die beide höhere Expressionen aufwiesen, lediglich eine sehr niedrige bzw. schwache spezifische Aktivität. Da die Aktivität von löslichem FasL durch Multimerisierung erhöht werden kann (Schneider et al, 1998) wurden die Proteine über den internen Flag tag vorvernetzt. Überaschenderweise führte dies zu einer Abnahme der Aktivität bei TNFR1-Flag-FasL, TNFR2-Flag-FasL und 4-1BB-Flag-FasL Die Aktivität von RANKed-Flag-FasL hingegen konnte um das 50-100fache erhöht werden, während die Zelltod-induzierende Wirkung von CD40-Flag-FasL durch die Vorvernetzung nahezu unbeeinflusst blieb Die hohe spezifische Aktivität, die für TNFR1-Flag-FasL, 4-1BB-Flag-FasL and TNFR2-

Flag-FasL beobachtet werden konnte, resultiert höchstwahrscheinlich aus der Präsenz von signifikanten Mengen an hochmolekularen Aggregaten der Homotrimere in den Zellkulturüberständen.. RANked-Flag-FasL liegt wahrscheinlich in einem nicht aggregiertem Zustand vor, und konnte daher durch Vorvernetzung aktiviert werden. Die Beobachtung, daß bei CD40-Flag-FasL kein Antikörpervermittelter Effekt durch Vorvernetzung mit M2 erzielt werden konnte, muss nicht für einen Defekt im FasL-Teil des Fusionsproteins sprechen, sondern könnte viel eher, z.B. eine sterische Hinderung des M2 Antikörpers reflektieren. RANKed-Flag-FasL und CD40-Flag-FasL wurden daher weiter auf ihre Kapazität hin zur Zielstruktur-abhängigen Aktivierung untersucht.

CD40-Flag-FasL wurde stabil in HEK293 Zellen exprimiert und aus dem Überstand der stabilen Zellen affinitätsaufgereinigt. Western Blot Analysen zeigten, dass CD40-Flag-FasL unter reduzierenden Bedingungen bei einem Molekulargewicht von ~50 kDa migriert. Untersuchungen am nativen Protein mittels Gelfiltration zeigten, dass CD40-Flag-FasL bei einem wahrscheinlichen MW von 471 kDa eluiert. Dies würde einem 9,4mer bzw. drei Homotrimeren entsprechen, was wahrscheinlich jedoch eine Überschätzung darstellt, da sich CD40-Flag-FasL in Aktivitätsmessungen wie ein homotrimeres Molekül verhält. Bindungsexperimente zeigen, dass CD40-Flag-FasL spezifisch durch CD40L-exprimierende Zellen immobilisiert und außerdem, nach Bindung die Ausbildung von Fas-Rezeptorclustern auf benachbarten Zellen induziert. CD40-Flag-FasL induziert in CD40L-transfizierten HT1080 und KB, nicht jedoch in parentalen Zellen, trotz ähnlicher Sensitivität dieser Zellen gegenüber Antikörper-vernetztem FasL, konzentrationsabhängig Apoptose und die Aktivierung von NF-κB.

Bei Untersuchungen von RANKed-Flag-FasL unter Bedingungen, die die Zielantigen-vermittelte Immobilisierung zuließ, führte RANKed-Flag-FasL zu einer RANKL-abhängigen Geninduktion. Der EC_{50}-Wert von CD40-Flag-FasL ist vier 10er Potenzen geringer in HT1080-CD40L im Vergleich zu HT1080 Zellen. Der induzierte Zelltod in CD40L-positiven Zellen beruht auf der Spaltung von Pro-caspase-8 und der Aktivierung von Caspase-3 und kann vollständig durch zVAD, Fas-Comp und einen humanen CD40L Antikörper inhibiert werden. Die CD40-Flag-FasL -induzierte Apoptose über einen parakrinen Weg in *bystander* Zellen gilt aufgrund Untersuchungen *in vivo* in Mäusen auf akute Toxizität für sicher, wohingegen artifizielles Vernetzen von CD40-Flag-FasL

konzentrationsabhängig Leberversagen induziert. Die vielversprechenden Resultate mit CD40-Flag-FasL führte zu der Klonierung von CD40-Flag-TRAIL Expressionskonstrukten, die CD40L-abhängig sowohl Apoptose als auch NF-κB induzieren. Es konnte nicht nur gezeigt werden, dass die CD40-Flag-TRAIL-vermittelte Apoptose TRAIL-spezifisch ist, sondern auch, dass sie aus der Interaktion von CD40 und CD40L resultiert und von der Interaktion von TRAIL-R2 in sowohl KB-CD40L als auch HT1080-CD40L Zellen abhängig ist. Diese vielversprechenden Ergebnisse stellen die Basis dar für die relativ einfache Entwicklung von sichereren, d.h. weniger toxischen FasL und TRAIL Derivaten für die Behandlung verschiedener humaner Pathologien.

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9 Appendix

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I hereby confirm that I performed the present work independendtly without further help or other materials than stated.

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