Characterization of tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-induced signaling pathways

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Erklärung

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Hiermit erkläre ich ehrenwörtlich, die vorliegende Arbeit in allen Teilen selbständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben. Diese Dissertation hat weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegen. Des Weiteren erkläre ich, dass ich früher weder akademische Grade erworben habe, noch zu erwerben versucht habe.

Würzburg, den _____ _____

(Claudia Roos)

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für Mama und Papa

"TWEAK shall inherit the earth!"

(Vince and Silke, 2006)

Content

1 Introduction

This work is about TNF-like weak inducer of apoptosis (TWEAK) and TWEAK-induced signaling pathways. TWEAK and Fn14 are typical members of the TNF ligand family and the TNF receptor superfamily. To understand the role of TWEAK and its function, the TNF ligand family and the cognate receptor superfamily is introduced first (1.1 and 1.2). Then there will be a brief overview over signaling pathways activated by proteins of the tumor necrosis factor family (1.3) and TNF receptor-associated proteins (1.4). After that the focus is at TWEAK, starting with its structure, expression properties and its cognate receptor (1.5), ending with the state-of-the-art of TWEAK signaling and the aim of this work (1.6).

1.1 Tumor necrosis factor (TNF) superfamily

Pivotal for survival of multicellular organisms is an effective system to ward off pathogens from outside and transformed cells from inside. That is the responsibility of the immune system. Members of the tumor necrosis factor (TNF) superfamily and the corresponding family of receptors (TNFRs) are important effectors/regulator molecules of the immune system and immune cells and are critical for immune homeostasis.

Tumor necrosis factor (TNF) was first described in 1975 as an endotoxin-induced serum factor that triggers necrosis on tumors (Carswell et al., 1975). It became the name giving protein of a group of cytokines with structural and functional homology, the TNF ligand family. Interestingly, sequence identity between the ligand family members is only within the range of 10 – 30 %, but all molecules share a conserved 3-dimensional structure. By now, 19 members of the TNF ligand family and 27 corresponding receptors of the TNF receptor family have been identified. Some of the TNF ligands are able to interact with two or more different TNF receptors e.g. TNFα, LTα and TRAIL and some of the receptors bind to different TNF ligands e.g. TNFR1, TNFR2 and LTβR. (Bodmer et al., 2002; Hehlgans and Pfeffer, 2005) (Figure 1). Different affinities between various binding partners enhance regulatory flexibility and complexity in signaling (Idriss and Naismith, 2000). Until now, TWEAK seems to be one of the ligands that have only one signaling competent receptor within the TNF receptor superfamily, named Fn14.

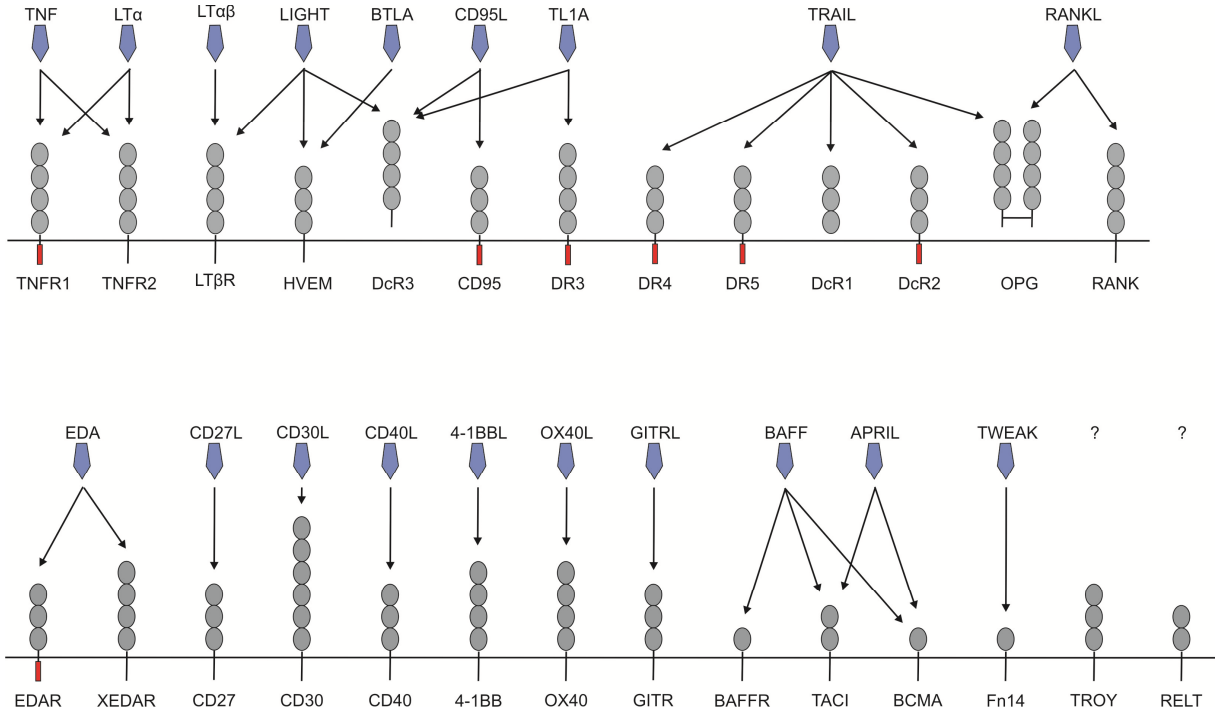

Figure 1 TNF ligands and their receptors
The TNF-superfamily ligands are shown in blue and arrows indicate interactions with their receptors. Extracellular domains of the TNF receptor superfamily members are shown in grey. The number of circles indicates the number of cysteine rich domains (CRDs) in a particular TNF receptor. Cytoplasmic death domains are indicated as red cylinders, soluble receptors lack the transmembrane portion. All other receptors bind TNF receptor-associated factor (TRAF) adaptors for signal transduction.

TNF ligands are expressed as type II transmembrane proteins, thus the N-terminus of these molecules is located in the cytoplasm whereas the C-terminus is extracellular. For some of the ligands it has been shown that the intracellular domain is capable of reverse signaling, meaning the ligand induces a signal within the ligand expressing cell upon binding to its receptor (Eissner et al., 2004; Sun and Fink, 2007).

The structural hallmark of all TNF ligands is a conserved domain in the extracellular region termed the TNF homology domain (THD). This 150 amino acids domain is responsible for trimerisation and receptor binding. Although most of the TNF ligands are initially synthesized as membrane-bound proteins, soluble forms can be generated by alternative splicing or proteolysis via distinct proteases (Blobel, 2002). TNFα for instance is cleaved by the protease TNFα converting enzyme (TACE), which belongs to the adamalysin family (ADAM) of the metzincin metalloproteinases (Black et al., 1997). TWEAK, which is in the focus of this thesis, is effectively processed by proteases belonging to the subtilisin-like furin family (Chicheportiche et al., 1997; Schneider et al., 1999a) (see 1.5.1). The receptor activating capacities of soluble TNF ligands can differ in some cases from their corresponding membrane bound form (Mariani and Krammer, 1998; Tanaka et al., 1998; Wajant, 2003). For example TNFα, FasL and TRAIL are inactive/poorly active on TNFR2, Fas and TRAILR2 as soluble trimers, but activate these receptors in their membrane bound form. Notably, it has

been found that oligomerized soluble trimers often mimic the activity of the corresponding membrane-bound molecules (Wajant et al., 2005).

1.2 TNF receptor superfamily

TNF ligands signal by interaction with cognate members of the TNF receptor superfamily. TNF receptors are expressed as type I transmembrane proteins (intracellular C terminus, extracellular N terminus). Characteristic for this protein family is the presence of one to six pseudorepeats of cysteine rich domains (CRD) in the extracellular region, comprising typically eight cysteine residues. The CRDs are responsible for ligand binding. For some of the TNF receptors, e.g. TNFR1 and 2, TRAIL receptors, Fas and CD40, it has been reported that they form inactive trimers in unstimulated cells via a pre-ligand assembly domain (PLAD) (Chan, 2007; Chan et al., 2000). Pre-ligand assembly of receptors is one mechanism that may have positive regulatory effects on signaling. Upon ligand binding, oligomerization of receptors is possible which may have enhancing effects on signaling. Other mechanisms to stabilize TNF signaling seem to be the formation of supramolecular clusters and raft recruitment of TNF receptors. For negative regulation of TNF receptor signaling at the receptor level, two different mechanisms are known. One mechanism is the internalisation of ligand-receptor-complexes to shut down or to shift the quality of the cellular response (e.g. TNFα). After internalisation, the receptor is not available at the cell surface and signaling is terminated. The other mechanism is cleaving of receptors from the cell surface. Certain stimuli can induce shedding of some of the receptors to soluble forms. These soluble receptors can neutralize their cognate ligands and therefore downmodulate signaling (Hehlgans and Mannel, 2002).

The intracellular domain of TNF receptors has no intrinsic enzymatic activity. Therefore signal transduction is achieved by recruitment of cytoplasmic adaptor proteins. Based upon the adaptor proteins recruited to the cytoplasmic domain, TNF receptors can be classified into three major groups (Dempsey et al., 2003):

1. Receptors containing a cytoplasmic death domain (DD), e.g. TNFR1 or Fas. These receptors recruit DD-containing adaptor proteins like TNF receptor-associated death domain (TRADD) or Fas-associated death domain (FADD). These adaptors cause activation of cysteine proteases, named the caspase cascades and induction of programmed cell death, called apoptosis (see 1.3.1).
2. Receptors containing one or more TNF receptor-associated factor (TRAF)-interacting motifs in their cytoplasmic domain. Their activation leads to recruitment of TNF receptor-associtaed factor (TRAF) proteins and stimulation of multiple pro-survival signaling pathways leading to the activation of NFκB, AKT, JNK, ERK or p38.
3. The third group of receptors does not contain a functional intracellular domain. These so-called decoy receptors compete with signaling receptors for ligand binding or form with their functional counterpart inactive heterocomplexes. Thus, these receptors downmodulate the activation of other TNF receptors.

Members of the TNF superfamily trigger a variety of responses in different cell types and tissues. TNFα, LTα and LTβ for example are responsible for the morphogenesis of secondary lymphoid organs (Fu and Chaplin, 1999; Hehlgans and Pfeffer, 2005; Locksley et al., 2001), The TNF receptors TACI, BCMA and CD40 together with the TNF ligands BAFF and CD40L are responsible for the activation of B-lymphocytes (Gross et al., 2000; Mackay et al., 1999; Vogel and Noelle, 1998), CD27 is a costimulatory molecule for the proliferation of T-lymphocytes (Gravestein and Borst, 1998), CD40 and RANK are responsible for the activation of dendritic cells (Anderson et al., 1997; Bennett et al., 1998), Fas is involved in the activation-induced cell death of immune effectors cells (Krammer, 2000) and, TNFα and LTα have strong inflammatory effects, but are in some scenarios also responsible for the induction of apoptosis and necrosis (Laster et al., 1988). Furthermore, the TNF ligand family members TNFα, EDA and RANKL are responsible for development and differentiation of epithelial structures, endothelial cells and bone resorbing osteoclasts (Locksley et al., 2001).

Not surprisingly, TNF/TNFR superfamily signaling is involved in a variety of diseases, ranging from genetic defects resulting in e.g. cardiovascular disorders (Mellick, 2007) and autoinflammatory diseases to cancer (Dinarello, 2000). TNF/TNFR superfamily members have thus gained increasing interest as targets for therapies against widespread human diseases such as atherosclerosis, osteoporosis, autoimmune disorders, allograft rejection, and cancer (Locksley et al., 2001).

1.3 TNF ligand-induced signaling pathways

Several signaling pathways can be induced by TNF ligands, depending on the respective receptor and cellular context. The most important ones are apoptotic signaling, proinflammatory NFκB activation, and the pro-mitogenic MAP kinase pathways leading to activation of ERK, JNK, and p38 (Figure 2).

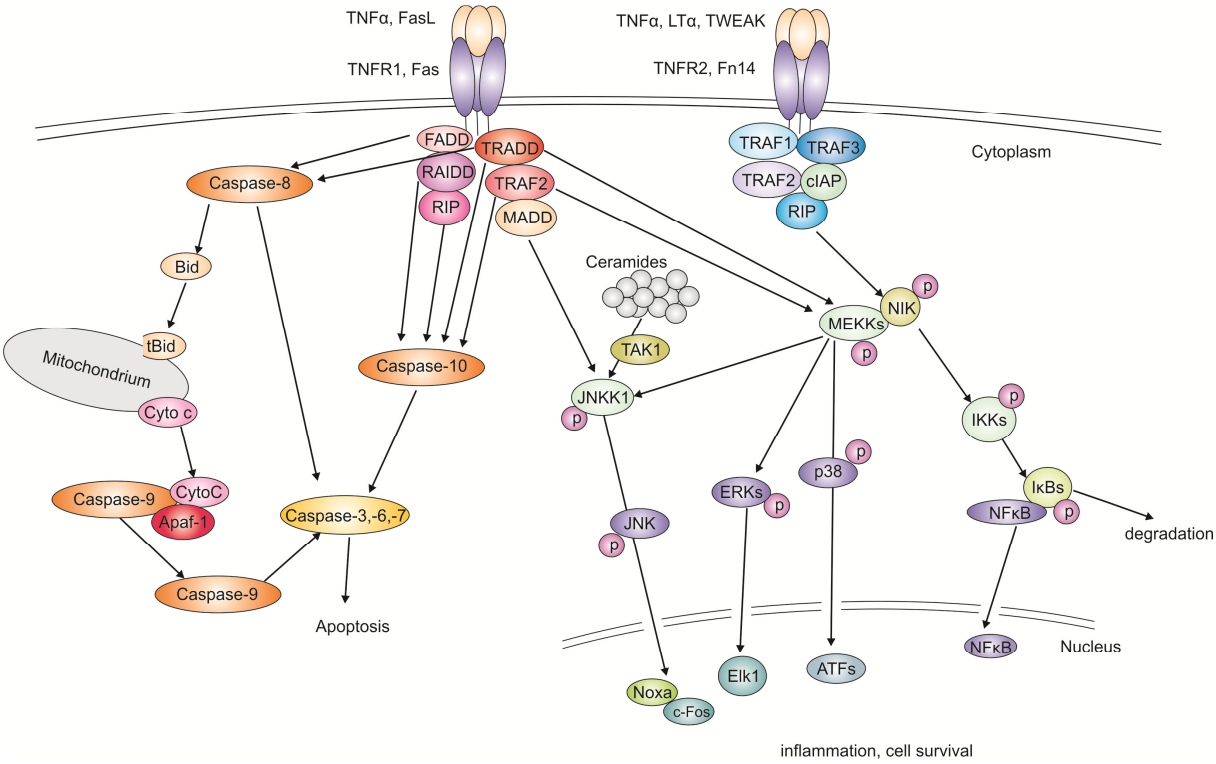

Figure 2 TNF ligand-induced signaling pathways
Death domain receptors (left) of TNF receptor superfamily can induce apoptosis (reddish colors) via the caspase cascade either by extrinsic pathway or intrinsic pathway including cytochrome C release from mitochondria (grey) and building of the apoptosome including caspase-9, Apaf-1 and cytochrome C. Non death domain receptors (right) of TNF receptor superfamily strongly activate the NFκB pathway and the MAP kinase pathways JNK, ERK and p38 (bluish colors). These antiapoptotic pathways are also induced by death domain receptors under defined circumstances.

1.3.1 TNF ligand-induced apoptosis and necrosis

Basically all TNF receptors with an intracellular death domain are able to induce apoptosis. Apoptosis is an important and highly regulated mechanism that regulates the silent and non-immunogenic elimination of millions of damaged or superfluous cells per second in multicellular organisms. Apoptosis also serves as an effector mechanism to destroy potentially dangerous cells, e.g. infected or transformed cells. During apoptosis the integrity of the plasma membrane is retained, preventing inflammatory responses caused by the

release of intracellular molecules. In contrast, during necrosis, the cell is swelling until the plasma membrane bursts and cytoplasmic content is released. This aspect of necrosis induces inflammatory processes. Typically, early apoptotic cells are rapidly phagocytosed by macrophages in an anti-inflammatory manner (Hengartner, 2000). Hallmark features of apoptosis are shrinking of the cell, often release of cytochrome C from mitochondria (Kluck et al., 1997), DNA condensation and degradation (Wyllie et al., 1984).

Key executioner in apoptosis is the protease family of cysteinyl-aspartate-specific proteinases, so-called caspases (Kidd et al., 2000; Thornberry and Lazebnik, 1998), a highly conserved protein family. Caspases possess a cysteine residue within their active core and cleave their substrates after an aspartic acid residue. One can distinguish between initiator (e.g. caspase-8, -10) and executioner caspases (e.g. caspase-3, -7). Initiator caspases are heading the caspase cascade and are activated by the according death signal autocatalytically. Afterwards they can cleave and activate executioner caspases. These in turn cleave many cellular substrates leading to cell death.

Active initiator caspases are obligate dimers of identical catalytic units composed of one large and one small subunit. These subunits are derived from the same precursor molecule by an internal cleavage site. Cleavage is neither required nor sufficient for activation of initiator caspases. The zymogens of the initiator caspases exist within the cell as inactive monomers. These monomeric zymogens require dimerization to assume an active conformation, and this activation is independent of cleavage (Boatright et al., 2003; Donepudi et al., 2003; Stennicke et al., 1999). The dimerization event occurs at multiprotein activating complexes, to which the caspase zymogens are recruited by virtue of their N-terminal recruitment domain. The activating complex involved depends on the origin of the death stimulus, which is classified as being either extrinsic or intrinsic (Boatright and Salvesen, 2003).

In contrast to the initiators, the executioner caspase-3 and -7 zymogens exist within the cytosol as inactive dimers (Boatright et al., 2003). They are activated by limited proteolysis within their interdomain linker, which is carried out by an initiator caspase or occasionally by other proteases under specific circumstances (Boatright and Salvesen, 2003). Caspase-6 is not as extensively studied as caspases-3 and -7, but is usually classified as an executioner caspase on the basis of its lack of a long pro-domain and its presumptive cleavage downstream of the initiators. Additionally, in recombinant form its zymogen is a dimer (Kang et al., 2002).

There are two prototypic signaling pathways that lead to apoptotic cell death. The first is the intrinsic pathway, which is activated from inside the cell by members of the B-cell

leukemia/lymphoma 2 (BCL-2) protein family resulting in the release of proapoptotic factors from the mitochondria. The second is the extrinsic pathway, which is activated from outside the cell by proapoptotic ligands of the TNF superfamily that interact with death receptors (DRs) (Ashkenazi, 2002). Both pathways activate enzymatic caspase cascades, which carries out numerous proteolytic events that mediate the apoptotic cell death program.

The intrinsic pathway of apoptosis is triggered in response to DNA damage, defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of survival factors or other types of severe cell distress. This pathway involves activation of proapoptotic members of the B-cell leukemia/lymphoma 2 (BCL-2) gene family, which, in turn, engage the mitochondria to cause the release of apoptogenic factors such as cytochrome C and SMAC/DIABLO into the cytosol (Adams and Cory, 1998; Green, 2000; Hunt and Evan, 2001). In the cytosol, cytochrome C binds the adaptor protein APAF-1 and ATP, forming a multimeric apoptosome that activates the apoptosis-initiating protease caspase-9 by induced proximity-mediated dimerization. In turn, apoptosome-associated caspase-9 dimers or multimers cleave and activate executioner proteases such as caspase-3, -6 and -7. SMAC/DIABLO promotes apoptosis by binding to inhibitor of apoptosis proteins (IAPs) and preventing these factors from attenuating caspase activation (Du et al., 2000; Verhagen et al., 2000). Most chemotherapy agents and irradiation trigger tumor-cell apoptosis through the intrinsic pathway, as an indirect consequence of causing cellular damage.

The extrinsic pathway is triggered by activation of death receptors, e.g. Fas, TRAILR1 or TRAILR2 (Ashkenazi and Dixit, 1998). Activation of death receptors leads to recruitment of the adaptor protein Fas-associated death domain (FADD) and caspases-8 and -10 whereupon the so-called death inducing signaling complex (DISC) is formed (Kischkel et al., 2000; Kischkel et al., 2001). Knock-out studies showed that these proteins are essential for death receptor-induced apoptosis (Kischkel et al., 1995; Sanchez et al., 1999; Varfolomeev et al., 1998; Yeh et al., 1998; Zhang et al., 1998). Within the DISC, caspase-8 and caspase-10 are autocatalytically cleaved and activated. There are several models for caspase activation, among which proximity-induced activation is currently favored. In this "induced proximity" model, it is assumed that initiator caspases, such as caspase-8, get close-by in supramolecular clusters (Boatright et al., 2003) which induce the activating dimerisation and autoproteolytical cleavage of caspase-8. After self-processing, active caspase molecules were released into the cytoplasm where they cleave and thereby stimulate the effectors caspases-3, -6, and -7, which ultimately execute apoptosis (LeBlanc et al., 2002). The extrinsic pathway is becoming recognized as an important mechanism that is used by narual killer (NK) cells and cytotoxic T-lymphocytes to trigger apoptosis in virus-infected cells and in tumor cells.

Initiator caspase-8 and -10 in turn, activate the same set of executioner caspases that are activated by the intrinsic pathway through caspase-9. This means that the extrinsic and intrinsic pathways converge at the level of the executioner caspases. Notably, cross talk can occur between the two pathways (Lavrik et al., 2005). For example, in cells with low levels of DISC formation, an amplification loop can recruit the intrinsic pathway, and engagement of mitochondria to the death receptors. This cross talk is mediated by caspase 8-dependent cleavage of the BH3-only protein Bid to its active form tBid which in turn acts on mitochondria to trigger the release of apoptogenic factors.

1.3.2 TNF-induced NFκB activation

In vivo TNFα/TNFR1-induced apoptosis seems to be secondary to TNFα/TNFR1-induced inflammatory processes. The reason for this observation is that TNFα/TNFR1-induced apoptosis is suppressed by activation of NFκB.

Inflammatory effects of TNF family ligands are mainly induced by activation of the transcription factor nuclear factor kappa b (NFκB) (Ghosh and Karin, 2002; Karin and Lin, 2002; Luo et al., 2005). The term NFκB covers a small group of dimeric transcription factors that, upon activation (e.g. via TNFα), translocate from the cytoplasm into the nucleus to induce the expression of different inflammatory proteins. In mammals five members are known: NFκB1 (p50), NFκB2 (p52), c-Rel, RelB and RelA (p65). The characteristic common feature of these proteins is the Rel homology domain (RHD) which is responsible for DNA binding, dimerisation and interaction with the inhibitor of κB protein family (IκB) (Verma et al., 1995).

NFκB activation can be stimulated by TNF receptors having a death domain (DD), but primarily by the non-death domain TNF receptors which typically possess one or more TRAF interaction motives (see 1.1). In case of DD receptors, first step is the binding of death domain containing proteins to the receptor e.g. TNF receptor-associated death domain (TRADD) (Hsu et al., 1995). The latter then acts as a platform for recruitment of other proteins involved in activation of NFκB signaling such as TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP) (Hsu et al., 1996a; Hsu et al., 1996b) (Figure 3).

For TNF receptors with TRAF interaction motive, TRAF2 is a direct interaction partner. TRAF2 is a member of the phylogenetically conserved TRAF protein family with a characteristic 180 amino acid spanning C-terminal TRAF domain (see 1.3) (Wajant et al., 2003; Wajant and Scheurich, 2001). Upstream in NFκB signaling, inhibitor of kappa B (IκB)

kinases (IKKs) are essential. The IKKs form a complex consisting of the catalytic subunits IKKα (IKK1) and IKKβ (IKK2) (DiDonato et al., 1997; Mercurio et al., 1997) and the regulatory subunit IKKγ (IKK3, NEMO) (Yamaoka et al., 1998).Studies with TRAF2 and RIP deficient embryonic mouse fibroblasts showed that in context of NFκB activation by TNFR1, TRAF2 is essential for recruitment of the IKK complex whereas RIP is necessary for its activation (Devin et al., 2001). The active IKK complex can phosphorylate IκBs which then are ubiquitinylated and marked for proteasomal degradation (Karin and Lin, 2002). IκB degradation exposes the nuclear localization sequence of NFκB dimers and thus triggers the transport of NFκB dimers into the nucleus (Figure 3).

1.3.2.1 The classical NFκB pathway

The most common form of NFκB activating complex consists of the IKKα and IKKβ catalytic subunits and the IKKγ regulatory subunit (also called NEMO for NFκB essential modulator). In the classical NFκB signaling pathway (Figure 3 left), the activated IKK complex, predominantly acting through IKKβ in an IKKγ-dependent manner, catalyzes the phosphorylation of IκBs (at serine 32 and 36 of IκBα) leading to their polyubiquitination (at lysine 48 for IκBα) and subsequent degradation by the 26S proteasome. The released NFκB dimers translocate to the nucleus, bind DNA and activate gene transcription (Ghosh and Karin, 2002).

It is well established that the classical NFκB pathway, based on IKKβ-dependent IκB degradation, is essential for innate immunity. Deficiencies in RelA and IKKβ are embryonically lethal; but studies with conditional knock-out mice have revealed a marked increase of susceptibility against infections (Alcamo et al., 2001; Senftleben et al., 2001b).

The activation and nuclear translocation of classical NFκB dimers (mostly p50–RelA) is associated with increased transcription of genes encoding chemokines like IL-8, cytokines like IL-6, TNFα and granulocyte-macrophage colony-stimulating factor (GM-CSF), adhesion molecules like intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and endothelial-leukocyte adhesion molecule 1 (ELAM), enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (Elewaut et al., 1999; Ghosh et al., 1998). These chemokines and cytokines are important components of the innate immune response against invading microorganisms and the adhesion molecules are required for migration of inflammatory and phagocytic cells to tissues where NFκB has been activated in response to infection or injury.

1.3.2.2 The noncanonical NFκB pathway

In 2001, a second NFκB activation pathway was discovered, designated the alternative or noncanonical NFκB pathway (Senftleben et al., 2001a). This pathway is activated by certain members of the TNF family e.g. TWEAK, but not by TNFα itself. Current evidence strongly suggests that this noncanonical pathway is especially important for adaptive immunity.

The noncanonical pathway for NFκB activation is strictly dependent on IKKα (Senftleben et al., 2001a) and is independent of IKKβ and IKKγ (Dejardin et al., 2002) (Figure 3 right). NIK phosphorylates p100, the precursor protein for NFκB2 p52, at serines 866 and 870 which is a prerequisite for two molecular events. First, in addition to phosphorylating both serines 866 and 870, NIK serves as a docking molecule for the recruitment of IKKα to p100 (Xiao et al., 2004). Once recruited, IKKα phosphorylates serines at both N-terminal (serines 99, 108, 115 and 123) and C-terminal regions (serine 872) of p100. Second, similar to IκBα, phosphorylated p100 leads to the recruitment of the E3 ligase $SCF^{\beta\text{-}TrCP}$, polyubiquitination of lysine 855 and subsequent degradation or processing of its inhibitory C-terminal half of p100 to p52 (Amir et al., 2004; Fong and Sun, 2002; Liang et al., 2006; Xiao et al., 2004). Once the C-terminal half is degraded, the N-terminal portion of NFκB, the p52 polypeptide that contains the RHD, is released. As the RHD of p100 is most commonly associated with RelB, activation of the noncanonical pathway results in nuclear translocation of p52–RelB dimers (Dejardin et al., 2002).

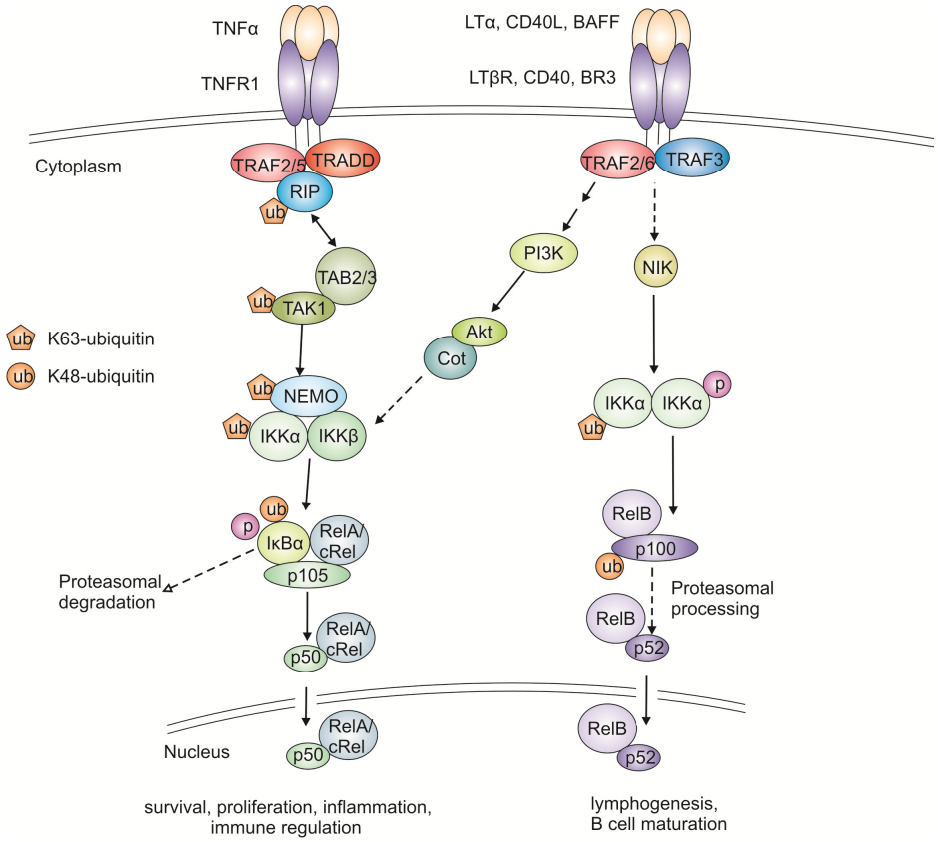

Figure 3 Classical and noncanonical NFκB signaling pathway
The classical NFκB signaling pathway functions via activation of IKKα/β/γ-complex and degradation of IκBα (left). The noncanonical NFκB pathway is strictly dependent on activation of IKKα homodimers and results in processing of p100 precursor protein to the transcription factor p52 (right).

IKKβ is the canonical activator of NFκB in response to infection and inflammation, and IKKα is responsible for activation of a specific NFκB factor required for B-cell maturation and formation of secondary lymphoid organs (Senftleben et al., 2001a). So the noncanonical NFκB pathway has been assigned to a specific aspect of adaptive immunity.

1.3.3 The mitogen activated protein kinase (MAPK) family

Activation of MAP kinases is achieved in several steps. First, an activator such as NGF, EGF, TNFα, IL-1, FasL, TGFβ, reactive ogygen species, lipopolisacharides or small G-proteins stimulates directly or indirectly MAP kinase kinase kinase (MAPKKK, MAP3K), which then phosphorylates and activates a MAP kinase kinase (MAPKK, MAP2K) which finally phosphorylates tyrosine and treonine residues in MAP kinases (MAPK) resulting in their activation. Once activated, MAP kinases accumulate in the nucleus where they activate various transcription factors by phosphorylation. Consequently, transcription of many target genes is initiated (Figure 4). Once dephosphorylated, MAP kinases return to their inactive state, disperse in the cytosol and are ready for another round of activation.

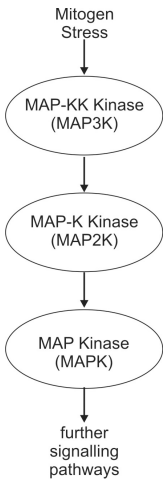

Figure 4 Scheme of MAP kinase cascade
Mitogen stimulation like NGF, EGF, TNFα, IL-1, FasL, TGFβ or other non receptor-mediated stimuli activate MAP kinase kinase kinases (MAPKKK, MAP3K) trough phosphorylation. These in turn activate MAP kinase kinases (MAPKK, MAP2K) and these activate the MAP kinases (MAPK), activating further signaling pathways.

The mitogen activated protein kinase (MAPK) family is divided into three groups: the extracellular signal-related kinases (ERK), the p38 mitogen activated protein kinases (p38) and c-Jun N-terminal kinases (JNK).

- Activation via mitogens of a cascade comprising the MAP3K Raf, the MAP2K MEK-1/2 and ERK-1/2 is called the ERK cascade or ERK signaling pathway and leads to cell growth, proliferation and differentiation. This signaling pathway is hyperactivated in 30 % of all cancers (Garnett and Marais, 2004).
- Activation via stress, TNF or IL-1 of a cascade comprising the MAP3K MLKs/TAK/ASK1, the MAP2K MKK-3/6 and p38/MAPK-α/β leads to inflammatory responses, apoptosis, growth or differentiation. Signaling via p38 has been causally implicated in Sepsis, Ischemic Heart disease, Arthritis, Human Immunodeficiency Virus infection, and Alzheimer's disease (Baan et al., 2006; Kelkar et al., 2005; Lee and Dominguez, 2005).
- Activation of a cascade comprising the MAP3K MLKs/ASK1/MEKK-1/4, the MAP2K MKK-4/7 and SAPK/JNK-1/2/3 via stress, UV light or osmotic shock leads to inflammatory responses, apoptosis, growth or differentiation. There is evidence for elevated JNK phosphorylation in Stroke (Herdegen et al., 1998), Parkinson's (Saporito et al., 2000) and Alzheimer's diseases (Morishima et al., 2001; Reynolds et al., 2000).

The various MAP kinase pathways cannot truly be separated from each other and are often induced together and affect each other in stimulatory or inhibitory ways (Pearson et al., 2001; Seger and Krebs, 1995).

1.3.4 Protein kinase B (PKB)/AKT pathway

One of the most effective antiapoptotic survival pathways in mammals is constituted of phosphoinositide 3-kinase (PI3K) and AKT (Chan et al., 1999). AKT is a serine-threonine protein kinase and acts as a signaling molecule in a Ras-dependent pathway, independent of the MAP kinases (Franke et al., 1995). It was shown that the activation occurs through phosphatidylinositol 3-kinase (PI3K). Active AKT may be able to inhibit phosphorylation of JNK and p38 phosphorylation by inhibition of the upstream kinases MKK 4 and ASK1 (Song and Lee, 2005). Evidence suggests that AKT inhibits apoptosis through multiple mechanisms. These include direct phosphorylation and inactivation of proapoptotic proteins such as procaspase-9, increased expression of antiapoptotic proteins like cellular inhibitor of apoptosis 2 (cIAP2) and cellular FLICE-like inhibitory protein (cFLIP), and down regulation of proapoptotic proteins such as FasL (Brazil and Hemmings, 2001; Nicholson and Anderson, 2002). Therefore AKT signaling is an important player in cancer and modulates TNF receptor signaling. In 2001, some articles have demonstrated that in cell lines derived from prostatic cancers AKT1 protects from apoptosis induced by TRAIL (Beresford et al., 2001; Chen et al., 2001; Thakkar et al., 2001).

1.4 TNF receptor-associated factor (TRAF) family

TRAF proteins form a group of intracellular adaptor proteins which can bind directly or indirectly to many members of the TNF receptor and IL-1/Toll-like receptor family (Dempsey et al., 2003; Leitges et al., 2001; Wajant and Scheurich, 2001). So far, six different TRAFs have been identified in mammalians (TRAF1 to TRAF6).

All TRAF proteins contain a conserved C-terminal TRAF domain which can further be subdivided into a variable N-terminal domain (TRAF-N) and a highly conserved C-terminal domain (TRAF-C). With the exception of TRAF1, all TRAF proteins are further characterized by an N-terminal ring-finger motive (Freemont, 2000; Saurin et al., 1996) and five to seven zinc-finger motives (Chung et al., 2002). The TRAF domain mediates binding to TNF receptors, homo- and heteromerization of TRAF proteins and interaction with a variety of other intracellular proteins. It was shown that the TRAF-C subdomain mediates binding to proteins like TRAF family member-associated NFκB activator (TANK) (Cheng and Baltimore,

1996) and NFκB inducing kinase (NIK) (Malinin et al., 1997) whereas TRAF-N binds the antiapoptotic proteins cIAP1 and cIAP2 (Rothe et al., 1995; Song et al., 1997).

The ring- and zinc-finger motives are essential for TRAF-mediated signal transduction leading to activation of NFκB (Brink and Lodish, 1998; Rothe et al., 1995; Takeuchi et al., 1996) and JNK (Dadgostar and Cheng, 1998). Deletions in that regions result in dominant negative mutations, possibly due to the loss of ability to covalently attach ubiquitin to a lysine on a target protein via an isopeptide bond. Proteins that attach ubiquitin residues to other proteins are called E3-ubiquitin ligases. E3-ubiquitin ligase activity of TRAF proteins depends on these ring- and zinc-finger regions (Chen, 2005; Deng et al., 2000).

TARF1 is a unique member of the TRAF family. It contains a single zinc finger and a TRAF domain. TRAF1 can be recruited to a number of distinct members of the TNFR superfamily, including TNFR2, CD30, CD27, 4-1BB, HVEM and Fn14.

Little is known about the function of TRAF1. The overexpression of TRAF1 in transgenic animals demonstrates an inhibitory role of TRAF1 on the antigen-induced apoptosis of $CD8^+$ T-cells (Speiser et al., 1997). Moreover, it was shown that TRAF1 in concert with TRAF2 and cIAP1 and cIAP2 can inhibit TNF-induced caspase-8 activation (Speiser et al., 1997; Wang et al., 1998).

It is unclear how TRAF1 exerts its antiapoptotic effect. Consistent with the *in vitro* overexpression studies in cell lines, the *in vivo* overexpression of TRAF1 does not affect NFκB or JNK activation. Therefore, TRAF1 could achieve its antiapoptotic effect either via cIAP recruitment, or through the use of some other, unknown pathway.

Activated T-cells from TRAF1-deficient mice exhibited hypersensitivity to TNF, suggesting that TRAF1 is a negative regulator of TNF activity (Tsitsikov et al., 2001). Actually TRAF1 is able to inhibit CD40 and IL-1 signal transduction (Carpentier and Beyaert, 1999; Fotin-Mleczek et al., 2004). One possible mechanism of the TRAF1 inhibition of TNF signaling is that TRAF1 competes with TRAF2 for binding to TNFR2. Another possibility is that TRAF1 forms an inactive heterodimer by binding to TRAF2. Further work is needed to understand the precise biochemical basis of the inhibition of the TNFR2 signaling by TRAF1, and of the role of TRAF1 in the regulation of signaling by other TNFR family members.

Furthermore, TRAF1 is not only capable of regulating the NFκB pathway and apoptosis; it is also regulated by these pathways itself. TRAF1 expression is enhanced after NFκB induction and caspase-8 cleaves TRAF1 specifically (Fotin-Mleczek et al., 2004; Henkler et al., 2003; Irmler et al., 2000; Jang et al., 2001; Schwenzer et al., 1999; Wang et al., 1998). The resulting fragment works as a general inhibitor for NFκB (Henkler et al., 2003).

1.5 TNF-like weak inducer of apoptosis (TWEAK)

TWEAK is the tumor necrosis factor ligand superfamily member 12 (TNFSF12) and was first described in 1997 as a new member of the TNF ligand family. TWEAK is efficiently secreted, able to induce IL-8 synthesis in a number of cell lines and induce apoptosis in IFNγ-stimulated human adenocarcinoma cell line HT29 (Chicheportiche et al., 1997).

Since its discovery, researchers become more and more interested in TWEAK. The number of publications rises every year and discovery of Fn14 as the apparent receptor for TWEAK caused a great leap in the number of publications. TWEAK seems to be involved in development of the immune system and pro-tumoral incidents like angiogenesis and cell migration, putting it at the forefront of autoimmunity and cancer research. The very distinct and sometimes even contradictory effects of TWEAK range from proliferation, induction or inhibition of differentiation, to cell death and more. That fact complicates understanding of the function of TWEAK *in vivo*.

1.5.1 Structure of TWEAK

The relatively basic TWEAK N-terminal cytoplasmic domain is built up of only 18 amino acids and contains a putative serine phosphorylation site.

The TWEAK C-terminal extracellular domain, 206 amino acids in length, has the highest degree of sequence identity to other members of the TNFSF. This domain contains the receptor-binding subdomain. Based on the solved crystal structures of other members of the family, this subdomain is predicted to fold into a β-pleated sheet structure that forms a trimer (Banner et al., 1993). The grooves between the subunits of the trimers serve as binding sites for the receptor.

TWEAK, like many TNF ligands, can be proteolytically cleaved. In the case of TWEAK, the responsible proteases are members of the subtilisin-like furin family (Chicheportiche et al., 1997; Schneider et al., 1999a). Metabolic labeling studies have demonstrated that a soluble form of TWEAK can be found in cell culture medium (Chicheportiche et al., 1997). This observation is consistent with the presence of a consensus sequence motif for furin cleavage in the TWEAK extracellular domain (amino acid residues 90–93). Therefore, TWEAK may be synthesized as a full-length membrane-bound protein but is effectively processed into a smaller, soluble form consisting primarily of the C-terminal receptor-binding subdomain (sTWEAK).

Using gel filtration analysis of soluble recombinant FLAG epitope-tagged TWEAK, it was shown that TWEAK forms homotrimers (Schneider et al., 1999b).

There is a single consensus sequence motif for N-glycosylation in the TWEAK receptor-binding subdomain at position 139, and it has been demonstrated that recombinant TWEAK expressed in transfected cells is indeed a glycoprotein (Schneider et al., 1999b).

1.5.2 Identification of the TWEAK receptor Fn14

Initially, DR3 was reported as the receptor for TWEAK. DR3 was identified using co-immunoprecipitation assay (Marsters et al., 1998), but later reports failed to confirm binding of TWEAK to DR3 (Kaptein et al., 2000). These observation, in combination with other results demonstrating that TWEAK can bind to cells that do not express DR3 (Kaptein et al., 2000; Nakayama et al., 2002; Schneider et al., 1999b), indicated that another TWEAK receptor was likely to exist.

FGF-inducible 14 (Fn14) had previously been cloned as an fibroblast growth factor (FGF)-inducible gene in mice (Meighan-Mantha et al., 1999) and human (Feng et al., 2000). At that time, Fn14 was not recognized and categorized as a TNF receptor family member, since Fn14 has only one cysteine rich domain (CRD) whereas all other TNF receptors known until that date contained three or more CRDs.

The cloning and characterization of a new TNFRSF member with TWEAK-binding activity was initially described in a meeting abstract at the 8th international TNF Congress in June 2000 by Steven R Wiley, and then reported in a journal article published in 2001 (Wiley et al., 2001). Fn14 was identified as the TWEAK receptor using an expression cloning strategy exerting magnetic beads coated with the C-terminal receptor binding domain of TWEAK (Wiley et al., 2001).

1.5.2.1 Structure of Fn14

The human Fn14 gene encodes a 129 amino acid type I transmembrane protein with a predicted molecular mass of 13.911 kDa. The receptor contains a 27 amino acid N-terminal signal peptide sequence, which is proteolytically cleaved (Figure 5). The mature protein is predicted to be 102 amino acids in length, making it the smallest member of the TNFRSF identified to date (Feng et al., 2000; Meighan-Mantha et al., 1999; Wiley et al., 2001). The mature forms of the human and murine Fn14 proteins are closely related, with about 90 % whole sequence identity. As expected, both homologs have additional structural features

characteristic of TNFRSF members (Feng et al., 2000; Meighan-Mantha et al., 1999; Wiley et al., 2001).

The most recognizable characteristic is found in the 53 amino acid extracellular, ligand-binding domain where this protein has the hallmark cysteine rich domain (CRD) shared with all other TNFRs. Inter-cysteine spacing within the CRD predicted that Fn14 would adopt the structure of the 4th CRD of TNFR1 (Bodmer et al., 2002). However, structural analysis of other single-CRD TNF receptors, such as BCMA, TACI, and BAFFR has revealed a surprising cysteine connectivity and structural fold distinct from those found the 4th CRD of TNFR1 (Bodmer et al., 2002; Liu et al., 2003). Thus, a similar distinct structural fold may occur in Fn14, although this needs to be determined yet.

Full-length, unprocessed Fn14 has a highly hydrophobic region with 21 amino acids in length which is predicted to function as a plasma membrane spanning domain.

The human and murine Fn14 proteins contain a highly conserved 29 amino acid cytoplasmic domain that contains a single TRAF-binding site with the amino acid sequence PIEET, flanked by two conserved threonine residues. Interestingly, these threonines are the only potential phosphorylation sites in the entire receptor and may therefore play a role in TRAF association with the cytoplasmic domain of Fn14.

Since Fn14 contains only a TRAF binding site, it is likely to be involved in cellular survival and proliferation (Inoue et al., 2000; Wajant et al., 1999). Exactly which of the TRAFs are involved in Fn14 signaling is not elucidated yet and is part of this study.

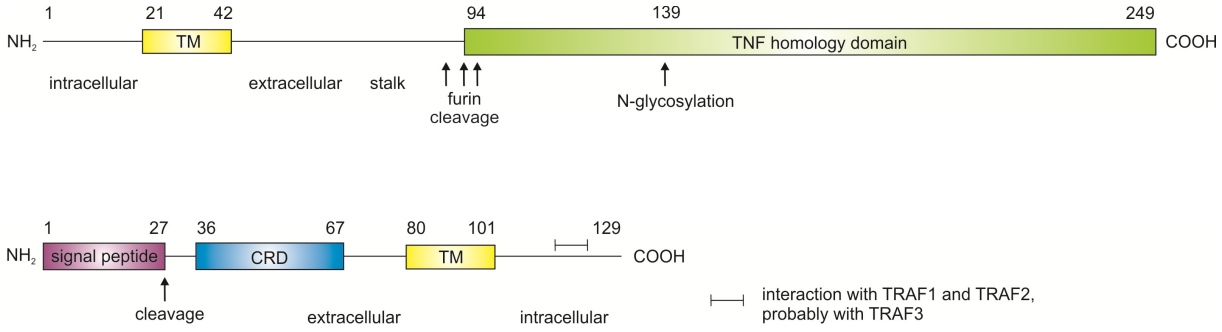

Figure 5 Structure of TWEAK and Fn14
TWEAK (top): N-terminus (left), transmembrane domain (yellow), TNF homology domain (green), C-terminus (right). Fn14 (bottom): N-terminus (left), signal peptide (violet), cysteine rich domain (CRD, blue), transmembrane domain (yellow), TRAF binding site with sequence motive PIEET (thin bar), C-terminus (right).

1.5.3 Expression patterns of TWEAK and Fn14

1.5.3.1 Expression of TWEAK mRNA

TWEAK mRNA is expressed in a variety of tissues and cell types, with relatively high levels found in most major organs including heart, brain, skeletal muscle, and pancreas. Tissues of the immune system also show the presence of TWEAK mRNA including spleen, lymph nodes, and thymus (Chicheportiche et al., 1997; Marsters et al., 1998). TWEAK mRNA expression has also been detected in various human tumor cell lines (Chicheportiche et al., 1997; Marsters et al., 1998; Pradet-Balade et al., 2002), human peripheral blood lymphocytes (Chicheportiche et al., 1997; Marsters et al., 1998; Pradet-Balade et al., 2002), mouse peritoneal macrophages (Chicheportiche et al., 2000), in human fibroblasts (Semov et al., 2002), in astrocytes and microglia (Desplat-Jego et al., 2002), and erythroid lineage precursors (Felli et al., 2005).

Furthermore, TWEAK mRNA was detected in human endothelial cells (EC) and smooth muscle cells (SMC) cultured *in vitro* (Brown et al., 2003). There have been several reports demonstrating TWEAK gene regulation *in vivo*. Chicheportiche and colleques (Chicheportiche et al., 2000) found that lipopolysaccharide injection decreased TWEAK mRNA levels in several mouse tissues (kidney, heart, lung) and in peritoneal macrophages. They also reported that TWEAK mRNA expression was downregulated in mice developing chronic autoimmune diseases like haemolytic anaemia and a lupus-like autoimmune disease. Curiously, lipopolysaccharide treatment of human THP-1 monocytic cells has the opposite effect and induces TWEAK mRNA expression (Chacon et al., 2006). TWEAK levels are also increased in diseases like systemic lupus erythematosus and (Campbell et al., 2004) and multiple sclerosis (Desplat-Jego et al., 2002).

1.5.3.2 Expression of TWEAK protein

Although TWEAK mRNA has been detected in a variety of cell types, membrane TWEAK is only detectable on IFNγ-stimulated monocytes (Nakayama et al., 2000) and monocytes during multiple sclerosis (Desplat-Jego et al., 2009) and in human $CD4^+$ T-cells (Kaplan et al., 2002; Kaplan et al., 2000). TWEAK has also been detected on the surface of various tumor cell lines (Kawakita et al., 2004), in resting and activated monocytes, dendritic cells, and NK cells (Maecker et al., 2005).

Interestingly, TWEAK has not been found on the cell surface of freshly isolated B-cells or NK cells even when these cells are treated with a variety of cytokines (Nakayama et al., 2000). There may be several explanations for that. First there are the findings of Pradet-Balade and

colleques who found that the majority of the TWEAK transcripts in human primary T-cells are not associated with ribosomes, which are responsible for translation of mRNA to proteins (Pradet-Balade et al., 2002). The second being is that TWEAK might be rapidly processed by furin intracellularly or after cell surface exposure. The third and final explanation might be the possibility that the AU-rich sequence element in TWEAK mRNA 3'-untranslated region has translational inhibitory activity similar to that found in TNF mRNA (Burkly et al., 2007; Mijatovic et al., 2000). This AU-rich sequence negatively regulates translation frequency and mRNA stability.

Since, as also described earlier, TWEAK is primarily detected in a soluble secreted form, the definitive cellular sources of TWEAK protein remains technically challenging to determine.

TWEAK expression is significantly increased locally in target tissues in contexts of acute injury and inflammatory disease, which are associated with infiltration of inflammatory cells and/or activation of resident innate immune cell types (Desplat-Jego et al., 2002; Girgenrath et al., 2006; Jakubowski et al., 2005; Perper et al., 2006; Potrovita et al., 2004).

TWEAK expression is also increased in many cancer incidents, resulting in different scenarios. Overexpression of TWEAK in athymic mice promotes tumor growth and angiogenesis in solid tumors (Ho et al., 2004). In mammary epithelial cells TWEAK induces proliferation and morphogenesis (Michaelson et al., 2005) and invasion and migration in invasive lobular and ductal carcinomas of the breast (Zhao et al., 2004).

1.5.3.3 Expression of Fn14 mRNA

Fn14 mRNA has been detected in a variety of adult tissues, with relatively high levels detected in heart, kidney, lung, and placenta (Feng et al., 2000; Meighan-Mantha et al., 1999). Fn14 mRNA is also expressed at high levels in the human aorta and in human vascular endothelial cells and smooth muscle cells cultured *in vitro*. Although Fn14 mRNA is expressed at low levels in normal human liver and brain, significantly higher levels of expression have been noted in multiple liver and brain tumor specimens (Brown et al., 2003; Feng et al., 2000). Also, cDNA microarray hybridization analysis comparing the gene expression profiles of normal pancreas tissue versus pancreatic cancer cell lines has indicated that Fn14 mRNA overexpression may be associated with pancreatic tumorigenesis (Han et al., 2002). In contrast, Fn14 mRNA was not upregulated in a small sample of human breast, ovary, or kidney tumor specimens; therefore, increased expression of Fn14 is not associated with all cancerous tissues (Brown et al., 2003). But Fn14 expression may contribute to the malignancy status of cancerous events as it has been shown for breast cancer. High expression of Fn14 is correlated with high rates of metastasis and poor

prognosis whereas low Fn14 expression in breast cancer tissues stands for less malignant tumors and good prognosis (Willis et al., 2008).

Fn14 mRNA expression is upregulated upon FGF-2, serum, or phorbol ester treatment of quiescent murine NIH 3T3 fibroblasts (Meighan-Mantha et al., 1999), human embryonic lung fibroblasts (Feng et al., 2000), or rat aortic smooth muscle cells (SMC) (Wiley et al., 2001). Elevated Fn14 mRNA levels have also been detected after plating human glioma cell lines on a migration-promoting extracellular matrix preparation (Mariani et al., 2001), and when human dermal fibroblasts are cultured in a mechanically stressed three-dimensional collagen lattice (Kessler et al., 2001). Fn14 mRNA induction has also been noted in two distinct *in vivo* models of tissue repair. Specifically, Fn14 mRNA levels increase during partial hepatectomy-stimulated liver regeneration in the mouse (Feng et al., 2000) and during balloon catheter-stimulated arterial wound repair in the rat (Wiley et al., 2001).

1.5.3.4 Expression of Fn14 protein

There is no evidence for differences in the expression of Fn14 mRNA and Fn14 protein, meaning high mRNA levels result in high protein levels.

The finding that the expression of TWEAK and in particular Fn14, is relatively low in normal tissues but undergoes dramatic upregulation in settings of tissue injury and diseases, coupled with the demonstration that Fn14 is expressed by many tissue-resident progenitor cells (Girgenrath et al., 2006; Jakubowski et al., 2005; Perper et al., 2006), provided critical clues to the biological functions of this TWEAK/Fn14 cytokine-receptor axis which are described in 1.5.5.

1.5.4 Interaction of TWEAK and Fn14

Different approaches were used to demonstrate the interaction between TWEAK and its receptor Fn14, including equilibrium binding and competitive inhibitor assays, which revealed an interaction affinity constant (*K*d) of ~2.4 nM (Wiley et al., 2001). This *K*d value is in the same range as that measured for other TNF-like cytokines and their cognate receptors.

Results to date indicate that homotrimeric TWEAK does not interact with any other receptors of the TNF receptor superfamily. In addition, none of the other members of the TNF ligand family binds to Fn14 (Wiley et al., 2001).

Therefore, TWEAK and Fn14 are widely recognized as exclusive partners. In addition, several studies have suggested that Fn14 can signal in a TWEAK-independent manner (Dogra et al., 2007; Tanabe et al., 2003). Ligand-independent signaling of other TNF receptor family members has been known to occur under conditions of high expression. TWEAK activity independent of Fn14 has also been suggested (De Ketelaere et al., 2004; Polek et al., 2003). In 2007 TWEAK was reported to interact with CD163, a scavenger receptor on macrophages which is responsible for the uptake of circulating haptoglobin-hemoglobin (Hp-Hb) complexes (Bover et al., 2007). Profile analysis suggested that TWEAK mimicked the CD163 natural ligand partner Hp-Hb (Bover et al., 2007). But it is unlikely that TWEAK is functional upon binding to CD163 because the membrane protein is lacking an intracellular domain.

1.5.5 Cellular signaling induced by TWEAK

TWEAK has been shown to activate both, the canonical and the noncanonical NFκB pathways (Brown et al., 2003; Saitoh et al., 2003), as well as the MAPK pathway (Ando et al., 2006), presumably through TRAF-mediated mechanisms. TWEAK binding to Fn14 leads to interaction of the cytoplasmic Fn14 domain with TRAF1, 2, 3, and 5 which has been demonstrated *in vitro* (Brown et al., 2003). The context-specific participation of the various TRAF proteins remains to be elucidated and has turned into an area of investigation scientific scrutiny. As part of the current thesis, TRAF2 and TRAF1 have been investigated in more detail in this respect. The experiments in this work regarding TRAF2 depletion and/or degradation (see 4.5.4 and 4.5.5) and with TRAF1 overexpressing cell lines (see 4.6.1) were done to get some more insights into these interactions. Furthermore, cellular reactions after TWEAK stimulation were investigated in this work regarding cell death induction (see 4.4) and induction of inflammatory responses (see 4.5).

TWEAK has a broad range of functional activities, ranging from induction of cell death to proliferation, differentiation and cell survival signals e.g. in tissue regeneration and wound repair (Burkly et al., 2007; Feng et al., 2000; Vince and Silke, 2006; Zheng and Burkly, 2008). TWEAK has its role in angiogenesis and is important for regulation of immune responses (Aktas et al., 2006; Desplat-Jego et al., 2005; Mas et al., 2008; Mueller et al., 2005) and development of the immune system (Maecker et al., 2005).

1.5.5.1 Proinflammatory activity

The ability of TWEAK to trigger IL-8 production, which is an important proinflammatory cytokine, was originally described with human colon carcinoma, melanoma, and fibrosarcoma cell lines *in vitro* (Chicheportiche et al., 1997). The same group showed that TWEAK induces an array of inflammatory mediators in cultured human dermal fibroblasts and synovial fibroblasts, including PGE2, MMP-1, IL-6, IL-8, RANTES, and IP-10 (Chicheportiche et al., 2002)

In the meantime, many studies employing different cell types showed TWEAK's ability to induce production of chemokines, cytokines, and matrix metalloproteinases (MMPs), establishing proinflammatory signaling as a hallmark function of TWEAK/Fn14 signaling. A variety of cells show proinflammatory signaling after stimulation with TWEAK. Human endothelial cells produce MCP-1, IL-8, ICAM-1, and E-selectin (Harada et al., 2002). Human and murine astrocytes produce IL-6, IL-8, ICAM-1, and MMP-9 (Desplat-Jego et al., 2002; Polavarapu et al., 2005; Saas et al., 2000). Human keratinocytes produce RANTES, but not MCP-1 or IL-8 (Jin et al., 2004). Human gingival fibroblasts produce IL-8 and VEGF (Hosokawa et al., 2006). Human bronchial epithelial cells produce IL-8 and GM-CSF (Xu et al., 2004). Human chondrocytes produce MMP-1, -2, -3, and -9 (Perper et al., 2006).

TWEAK also potently stimulates proinflammatory activity in infiltrating macrophages, as evident from the model cell monocytic cell line THP-1, which produces IL-6, IL-8, MCP-1, and MMP-9 upon treatment with TWEAK and IFNγ (Kim et al., 2004).

Collectively, these studies demonstrate TWEAK's proinflammatory activity on a broad spectrum of cells *in vitro*, though the fine pattern of proinflammatory signaling varies with the particular cell type.

The proinflammatory activity of TWEAK was also demonstrated recently *in vivo* by injection of recombinant TWEAK, which significantly increased kidney expression of MCP-1 and IP-10 in wildtype but not Fn14 deficient hosts (Campbell et al., 2006), as well as in models of disease wherein inhibition of the TWEAK/Fn14 pathway limits inflammatory responses.

Moreover, in humans circulating TWEAK levels are significantly increased in patients with chronic inflammatory diseases such as multiple sclerosis and systemic lupus erythematosus as compared to those found in healthy controls (Desplat-Jego et al., 2009; Schwartz et al., 2006).

An important aspect to note is the 'localized' nature of TWEAK's proinflammatory activity *in vivo*. In contrast to TNF or IL-6, systemic exposure to TWEAK apparently does not lead to an acute phase inflammatory response, either in TWEAK overexpressing mice or after *in vivo*

administration of recombinant TWEAK (Jain et al., 2009), again demonstrating the restricted nature of Fn14 expression in normal resting tissues and underlining the specialized involvement of TWEAK/Fn14 signaling in mediating physiological and pathological changes in injured and/or diseased tissues.

1.5.5.2 Cell death

TWEAK can also induce apoptosis in tumor cells, although cell death induction often requires cosensitizing agents, such as IFNγ, TNFα and IFNγ, cycloheximide (CHX) or actinomycin D. TWEAK-induced cell death can occur via multiple effectors.

In the human HT29 tumor cell line cotreatment with TWEAK and IFNγ leads to cell death that is caspase independent. Here, cell death shows features of apoptosis and necrosis. (Bover et al., 2007; Chicheportiche et al., 1997; Kawakita et al., 2005; Nakayama et al., 2002; Nakayama et al., 2003; Nakayama et al., 2000; Saas et al., 2000; Wilson and Browning, 2002). Human KATO-III tumor cell line also requires IFNγ cotreatment for TWEAK-induced apoptosis (Nakayama et al., 2002; Nakayama et al., 2000).

Induction of cell death on human peripheral blood monocytes does not require IFNγ costimulation (Kaplan et al., 2002) but human NK cells die only if costimulated with TNFα, LPS, or IFNα (Maecker et al., 2005).

Human MCF7 and HeLa tumor cell lines require cycloheximide pretreatment to undergo apoptosis (Marsters et al., 1998) whereas human HSC3 tumor cell line (Nakayama et al., 2002; Nakayama et al., 2000), human SW480 tumor cell line (Kawakita et al., 2005) or human peripheral blood mononuclear cells (Felli et al., 2005) do not need to be sensitized and die after TWEAK stimulation.

Apoptosis of human Kym-1 tumor cell line is an indirect effect mediated by endogenous TNFα which is produced after TWEAK stimulation (Nakayama et al., 2002; Schneider et al., 1999b).

1.5.5.3 Cell survival

In striking contrast to the induction of cell death, TWEAK is also able to prevent cells from dying in certain situations. It is suggested that NFκB-mediated upregulation of BCL-XL and BCL-W expression in glioma cells increases cellular resistance to cytotoxic therapy-induced apoptosis. We propose that the Fn14 protein functions, in part, through the NFκB signaling pathway to up-regulate BCL-XL and BCL-W expression to foster malignant glioblastoma cell

survival. Targeted therapy against Fn14 as an adjuvant to surgery may improve management of invasive glioma cells and advance the outcome of this devastating cancer (Tran et al., 2005).

Further the angiogenic potential of TWEAK is characterized, revealing a dual role for TWEAK as an angiogenic regulator. It is demonstrated that TWEAK is a potent inducer of endothelial cell survival and cooperates with basic fibroblast growth factor to induce the proliferation and migration of human endothelial cells and morphogenesis of capillary lumens. In contrast, TWEAK antagonizes the morphogenic response of endothelial cells to vascular endothelial growth factor (VEGF) without inhibiting VEGF-induced survival or proliferation. Thus, the observations suggest that TWEAK may differentially regulate microvascular growth, remodeling and/or maintenance in vivo, depending upon the angiogenic context (Jakubowski et al., 2002).

1.5.5.4 Differentiation

Regarding to differentiation, TWEAK shows opposed characteristics again. There have been findings showing that TWEAK mediated the differentiation of RAW264.7 monocyte/macrophage cells into multinuclear, functional osteoclasts. It was shown that the TWEAK receptor Fn14 was not responsible for the osteoclastic effect of TWEAK on RAW cells since flow cytometry analysis did not reveal the expression of Fn14 on RAW cells. Moreover, Fn14 neutralizing antibodies did not block TWEAK-induced RAW cell differentiation into osteoclasts. This indicated that a second TWEAKR, TWEAKR2, exists on RAW cells and is responsible for mediating TWEAK-induced differentiation which is not identified yet (Polek et al., 2003).

In other cases TWEAK inhibits differentiation. It was found that treatment of human erythroid precursor cells with IFNγ upregulates the expression of multiple members of the TNF family, including TWEAK, which is able to inhibit erythroid cell growth and differentiation through caspase activation. Treatment of erythroid precursor cells with agents that blocked TWEAK activity was partially able to revert the effect of IFNγ on erythroid proliferation and differentiation. However, the simultaneous inhibition of all involved TNF family members resulted in a complete abrogation of IFNγ inhibitory effects, indicating the requirement of different receptor-mediated signals in IFNγ-mediated hemopoietic suppression. These results establish the role for TWEAK and its receptor in normal and IFNγ-mediated regulation of hematopoiesis and show that the effects of IFNγ on immature erythroid cells depend on multiple interactions between TNF family members and their receptors (Felli et al., 2005).

Further it is demonstrated that TWEAK induced the production of matrix metalloproteinases in human chondrocytes and potently inhibited chondrogenesis and osteogenesis in *in vitro* models. Human osteoblast precursors are not able to form mature osteoblasts and human mesenchymal stem cells cannot differentiate into chondrocytes after TWEAK exposure. These results provide evidence for a novel cytokine pathway that contributes to joint tissue inflammation, angiogenesis, and damage, as well as may inhibit endogenous repair, suggesting that TWEAK may be a new therapeutic target for human rheumatoid arthritis (Perper et al., 2006). Another study showed the effect and the mechanisms by which TWEAK modulates myogenic differentiation. Treatment of C2C12 myoblasts and mouse primary myoblasts with TWEAK inhibited their differentiation and the formation of multinucleated myotubes (Dogra et al., 2006).

1.5.5.5 Proliferation and cell growth

TWEAK binds to all progenitor cells of the mesenchymal lineage and induces NFκB activation and the expression of pro-survival, pro-proliferative and homing receptor genes in the mesenchymal stem cells, suggesting that this proinflammatory cytokine may play an important role in controlling progenitor cell biology. On primary mouse muscle myoblasts, it was demonstrated that TWEAK promoted their proliferation and inhibited their terminal differentiation (Girgenrath et al., 2006). In addition to that it was found that the proliferation of C2C12 myoblasts and the expression of a cell-cycle regulator cyclin D1 were increased in response to TWEAK treatment (Dogra et al., 2006). These results indicate that the TWEAK/Fn14 pathway is a regulator of skeletal muscle precursor cells and illustrate an important mechanism by which inflammatory cytokines influence tissue regeneration and repair (Girgenrath et al., 2006).

Furthermore, it was found that TWEAK promotes kidney infiltration of inflammatory cells, and stimulates proliferation of kidney cells *in vitro* and *in vivo*. Thus, TWEAK may play an important pathogenic role in the development of glomerulonephritis by promoting a local inflammatory environment and inducing kidney cell proliferation. Blocking TWEAK/Fn14 interactions may be a promising therapeutic target in immune-mediated renal diseases (Gao et al., 2009).

Transient TWEAK overexpression leads to a general salivary epithelial cell proliferation (Sugito et al., 2009).

TWEAK treatment of cultured embryonic and postnatal progenitor cells resulted in age-dependent effects on proliferation and on neurite extension by neuronal progeny although embryonic and postnatal day neural progenitor cells both express Fn14. TWEAK treatment

did not alter proliferation of embryonic neural progenitor cells but shifted postnatal progenitor cells toward cell-cycle phases G0 and G1. Conversely, the effects of TWEAK on axon elongation were more prominent in the earlier developmental stage. TWEAK induced extensive neurite outgrowth by the neuronal progeny of embryonic, but not postnatal progenitors. TWEAK/Fn14 receptor activation exerts different effects on neural progenitor cells and their progeny depending on the developmental stage of the cells (Hamill et al., 2007).

There is evidence that TWEAK stimulates oval cell proliferation in mouse liver through its receptor Fn14. Progenitor (oval) cell expansion accompanies many forms of liver injury, including alcohol toxicity and submassive parenchymal necrosis as well as experimental injury models featuring blocked hepatocyte replication. Oval cells can potentially become either hepatocytes or biliary epithelial cells and may be critical to liver regeneration, particularly when hepatocyte replication is impaired (Jakubowski et al., 2005). Moreover, TWEAK significantly promoted cell proliferation and induced NFκB activation in all human hepatocellular carcinoma cells. It was found that TWEAK also promotes cell proliferation and induces the secretion of IL-8 and MCP-1 in human umbilical vein endothelial cells. These results indicate that TWEAK might play a critical role in hepatucellular carcinoma cellular proliferation using both autocrine and paracrine mechanisms, and modulate tumor-related angiogenesis (Kawakita et al., 2004).

1.5.5.6 Proangiogenic activity

As indicated in the paragraph above, cell growth is often directly related to proangiogenic activity, especially in the case of endothelial cells (Brown et al., 2006; Donohue et al., 2003; Harada et al., 2002; Kawakita et al., 2004; Lynch et al., 1999; Nakayama et al., 2003). Positive regulation of angiogenesis includes some different effects like enhanced cell survival, higher proliferation rates, migration activity and tube formation. All of these effects were shown for human endothelial cells under TWEAK treatment (Donohue et al., 2003; Harada et al., 2002; Jakubowski et al., 2002; Lynch et al., 1999; Wiley et al., 2001). Human smooth muscle cells (Lynch et al., 1999) and rat aortic smooth muscle cells (Han et al., 2003) show stronger proliferation after TWEAK exposure, too. In addition to that, increased Fn14 expression levels on human glioma cells promote invasion of these cells which is essential for angiogenesis (Tran et al., 2003).

TWEAK overexpression in HEK293 cells promotes tumor growth and angiogenesis in athymic nude mice. It was found that TWEAK-overexpressing cells form larger and more highly vascularized tumors in athymic mice when compared with control, vector-transfected

cells. This result suggests that the TWEAK/Fn14 signaling system may be a potential regulator of human tumorigenesis. If TWEAK was expressed in human tumors and it could promote tumor growth and angiogenesis *in vivo* (Ho et al., 2004).

Furthermore, it was shown that TWEAK is involved in the angiogenesis in synovial tissue of collagen-induced arthritis mice and therefore contributes for the pathogenesis of the disease. The effect of anti-TWEAK mAb on the angiogenesis in inflamed synovial tissue of collagen-induced arthritis mice was examined by staining of vessels. The density of small vessels was significantly decreased in the anti-TWEAK mAb-treated mice compared with the control IgG-treated mice (Kamata et al., 2006; Perper et al., 2006).

1.5.6 TWEAK in inflammatory disease

Due to its broad range of activity, TWEAK is related to different diseases if disregulated. Upregulation of TWEAK mRNA was shown to be involved in the development of acute and chronic inflammatory pathologies (Chicheportiche et al., 2000) and systemic autoimmunity (Campbell et al., 2004) while inhibition of TWEAK activity is discussed as a new treatment for inflammatory and degenerative diseases (Yepes and Winkles, 2006).

In rheumatoid arthritis TWEAK overexpression leads to angiogenesis and secretion of inflammatory cytokines (Kamata et al., 2006; Kamijo et al., 2008; Park et al., 2008; van Kuijk et al., 2009). In atherosclerosis TWEAK and Fn14 are also involved in pathogenesis (Blanco-Colio et al., 2007). TWEAK expressed by glial cells induces astrocyte proliferation and immune cell infiltration in the central nervous system and increases experimental autoimmune encephalomyelitis severity (Desplat-Jego et al., 2005; Desplat-Jego et al., 2002), whereas inhibited Fn14 signaling protects from chronic relapsing experimental autoimmune encephalomyelitis (Mueller et al., 2005).

In experimental stroke models, both TWEAK and its receptor Fn14 were significantly upregulated. It was shown that TWEAK induces neuronal cell death and is involved in neurodegeneration *in vivo* and therefore contributes to ischemic stroke outcome. (Inta et al., 2008; Potrovita et al., 2004) and cerebral ischemia (Gordon et al., 2005; Hua et al., 2007; Huang et al., 2003; Li et al., 2007; Schwaninger et al., 2006; Webster et al., 2009; Wen et al., 2004; Yepes et al., 2005; Zhang et al., 2007).

Furthermore TWEAK is shown to be a player in autoimmune disorders like autoimmune demyelation (Aktas et al., 2006). It was shown that during multiple sclerosis TWEAK expression appears at the cell surface of monocytes (Desplat-Jego et al., 2009),

TWEAK is involved in systemic lupus erythematosus and lupus nephritis (Kaplan et al., 2002; Kawasaki et al., 2007; Miyagawa et al., 2008; Monrad et al., 2008; Schwartz et al., 2007; Schwartz et al., 2006) and serum level of TWEAK is increased in type 2 diabetes and end-stage renal disease (Kralisch et al., 2008)

1.6 Aim of the study

The aim of this work was to elicit how cellular responses after TWEAK stimulation are regulated and to characterize reactions upon TWEAK stimulation in a few cell lines under different conditions.

These cell lines should have been investigated for reactions after TWEAK stimulation to gain more insights in the outcome of TWEAK-induced signaling. To this end different approaches ought to be used. First cellular reactions should have been measured like apoptosis or necrosis with or without sensitizing agents like IFNγ or CHX or Z-VAD-fmk, and secretion of IL-8 as a hallmark for NFκB activation. Second intracellular responses were intended to be investigated to gain more insights into the underlying molecular pathways responsible for TWEAK signaling. To this end, activation of several signaling pathways like NFκB, JNK, ERK, and p38 shall be detected. For stimulation of cells, recombinant Flag-tagged TWEAK or FC-TWEAK fusion protein should be used. Both TWEAK variants can be secondary crosslinked via tag-binding antibodies or proteins. Crosslinking of trimeric ligand variants mimic activity of membrane bound ligands (Wajant et al., 2005). The present analysis would enable drawing conclusions about the physiological role of TWEAK.

2 Material

2.1 Chemicals, reagents and cell culture media

Table 1: Chemicals, reagents and cell culture media

ABTS di-ammonium	Roche, Mannheim, Germany
acetic acid	J. T. Baker, Leipzig, Germany
acrylamid	Roth, Karlsruhe, Germany
anti-Flag M2 Agarose	Sigma, Deisenhofen, Germany
APS	AppliChem, Darmstadt, Germany
bromphenol blue	AppliChem, Darmstadt, Germany
BSA	Sigma, Deisenhofen, Germany
buffer for ABTS	Roche, Mannheim, Germany
cycloheximide	Sigma, Deisenhofen, Germany
DMSO	Roth, Karlsruhe, Germany
DTT	AppliChem, Darmstadt, Germany
ECL-Kit	Amersham, Braunschweig, Germany
EDTA	AppliChem, Darmstadt, Germany
ethanol	J. T. Baker, Leipzig, Germany
FCS	PAA, Pasching, Germany
Flag-Peptide	Sigma, Deisenhofen, Germany
G418	Gibco, Paisley, UK
glycerol	Roth, Karlsruhe, Germany
glycine	AppliChem, Darmstadt, Germany
methanol	J. T. Baker, Leipzig, Germany
methyl violet	Roth, Karlsruhe, Germany
Na_2CO_3	Roth, Karlsruhe, Germany
Na_2HPO_4	AppliChem, Darmstadt, Germany
NaCl	Sigma, Deisenhofen, Germany
NaH_2PO_4	AppliChem, Darmstadt, Germany
$NaHCO_3$	Roth, Karlsruhe, Germany
nitrocellulose membrane	Schleicher und Schuell, Dassel, Germany
nonfat dried milk powder	Sigma, Deisenhofen, Germany
PageSilver™ Silver Staining Kit	Fermentas, St. Leon-Rot, Germany

Table 1 continued: Chemicals, reagents and cell culture media

PBS	PAA, Pasching, Germany
Penicillin/Streptomycin	PAA, Pasching, Germany
phosphatase-inhibitor I and II	Sigma, Deisenhofen, Germany
prestained protein marker (broad range)	New England Biolabs, Frankfurt am Main, Germany
protease inhibitor cocktail	Roche, Mannheim, Germany
protein G-Agarose	Roche, Mannheim, Germany
recombinant human IFNγ	R&D Systems, Wiesbaden, Germany
RPMI 1640 cell culture medium	PAA, Pasching, Germany
SDS	Roth, Karlsruhe, Germany
TEMED	Sigma, Deisenhofen, Germany
Tris	Roth, Karlsruhe, Germany
Tris base	Roth, Karlsruhe, Germany
Tris HCl	Roth, Karlsruhe, Germany
triton	Roth, Karlsruhe, Germany
trypsin-EDTA	PAA, Pasching, Germany
Tween-20	Roth, Karlsruhe, Germany
Z-VAD-fmk	Bachem, Heidelberg, Germany

2.2 Antibodies and antisera

Table 2: Antibodies and antisera

anti Flag (M2)	mouse monoclonal	Sigma, Deisenhofen, Germany
anti Fn14 (ITEM-4)	mouse monoclonal	eBioscience, Frankfurt am Main, Germany
anti human IgM/G/A (H+L) (AQ503H)	goat monoclonal	Millipore, Temecula, CA, USA
anti human IL-6 (MQ2-13A59)	rat monoclonal	BD Biosciences, Heidelberg, Germany
anti human IL-6 biotinylated	(ELISA detection)	
anti IκBα	rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg, Germany
anti JNK	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA

Table 2 continued: Antibodies and antisera

anti mouse HRP	rabbit polyclonal	Dako-Cytomation, Glostrup, Denmark
anti mouse IgG Phycoerythrin	goat polyclonal	Sigma, Deisenhofen, Germany
anti mouse IRDye 680CW	goat polyclonal	LI-COR Bioscience, Bad Homburg, Germany
anti mouse IRDye 800CW	goat polyclonal	LI-COR Bioscience, Bad Homburg, Germany
anti NFκB p52	mouse monoclonal	Upstate Biotech, Schwalbach, Germany
anti p38	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti ERK P44/42	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti phospho-IκBα	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti phospho-JNK	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti phospho-p38	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti phospho-ERK P44/42	rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti rabbit HRP	goat polyclonal	Dako-Cytomation, Glostrup, Denmark
anti rabbit HRP	goat polyclonal	Cell Signaling Technology, Beverly, MA, USA
anti rabbit IRDye 680CW	goat polyclonal	LI-COR Bioscience, Bad Homburg, Germany
anti rabbit IRDye 800CW	goat polyclonal	LI-COR Bioscience, Bad Homburg, Germany
anti TRAF1 (H-132)	rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg, Germany
anti TRAF2 (H249)	rabbit monoclonal	Santa Cruz Biotechnology, Heidelberg, Germany
anti Tubulin (DM1A)	mouse monoclonal	Dunn Labortechnik, Ansbach, Germany
anti TWEAK	goat polyclonal	R&D Systems, Wiesbaden, Germany

Table 2 continued: Antibodies and antisera

anti β-Actin (AC-15)	mouse monoclonal	Sigma, Deisenhofen, Germany
IL-8 ELISA Kit		BD Biosciences, Heidelberg, Germany
streptavidin-HRP		BD Biosciences, Heidelberg, Germany
TWEAK ELISA-Kit		Antigenix America, NY, USA

2.3 Cell lines

The human embryonic kidney cell line HEK293 was obtained from the American Type Cultrure Collection (ATCC), Rockville, MD, USA. The TNFR2 expressing cervical cancer cell line HeLa-TNFR2 has been generated in the group of Prof. Wajant and has been described in detail before (Weiss et al., 1997). Keratinocyte cell line KB was provided by M. Kracht from Rudolf-Buchheim Institute of Pharmacology, Gießen, Germany. Human fibrosarcoma cell line HT1080, rhabdomyosarcoma cell line KYM-1 and the colon adenocarcinoma cell line HT29 were present at the workgroup. The 95.1 vector control and TRAF1 expressing transfectants were generated by me and members of the workgroup.

2.4 TWEAK fusion proteins

A cDNA fragment encoding the TNF homology domain of TWEAK (amino acids 106 to 249) lacking all putative furin cleavage sites was obtained by PCR using a pCR3.1 construct containing full length human TWEAK as template. The amplicon was processed with restriction enzymes and inserted in frame into pCR3.1 vectors encoding either the 8 amino acids Flag-tag or the FC-portion of human IgG1 (amino acids 244 to 469). Assembling of vectors for Flag-TWEAK and FC-TWEAK was done by assistants of the group. Nucleotide and amino acid sequences of TWEAK and its fusion proteins are given in the attachments.

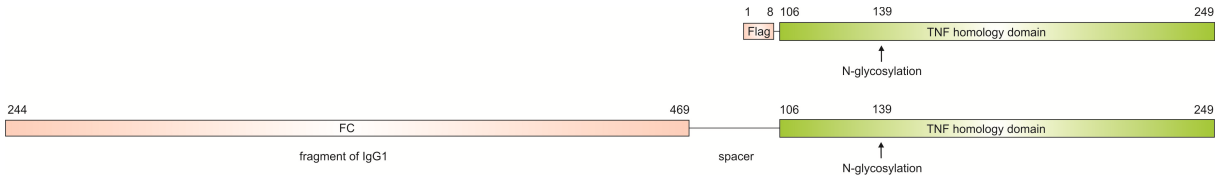

Figure 6 Structure of Flag-TWEAK and FC-TWEAK
Flag-TWEAK consists of the Flag-tag (8 amino acids, pink) a short spacer and the TWEAK TNF homology domain (amino acids 106 to 249, green). FC-TWEAK consists of 225 amino acids of IgG1 FC-portion (amino acids 244 to 469, pink), a short spacer and again the TWEAK TNF homology domain (amino acids 106 to 249, green).

2.5 Buffers and solutions

Table 3: Buffers and solutions

ABTS-solution	1.8 mM ABTS, 1 mg/ml buffer for ABTS
acid elution buffer	100 mM glycine, pH 2.7
blot buffer	0.025 M Tris, 0.192 M glycine, 20 % (v/v) methanol, pH 8.3
coating Buffer	0.1 M carbonate, pH 9.5
methyl violet solution	20 % methanol, 0.5 g methyl violet
neutralization buffer	1 M Tris-HCl, pH 9
PBS-T	PBS, 0,05 % Tween-20
PBS-T milk	PBS, 0.05 % Tween-20, 5 % nonfat dried milk powder
phosphate buffered saline (PBS)	0.02 M Na-phosphate, 0.7 % NaCl, pH 7.2
protein G-agarose starting buffer	20 mM sodium phosphate, pH 7
protein G-agarose washing buffer	20 mM sodium phosphate, 150 mM sodium chloride, 2 mM EDTA, pH 7
resolving gel buffer (SDS-PAGE)	1.5 M Tris, 0.015 M SDS, pH 8.8
running buffer (SDS-PAGE)	0.05 M Tris, 0.38 M glycine, 0.004 M SDS, pH 8.3
sample buffer (SDS-PAGE, 4fold)	8 % SDS, 0.1 M DTT, 40 % glycerol, 0.2 M Tris, bromphenol blue, pH 8.0
stacking gel buffer (SDS-PAGE)	0.5 M Tris, 0.015 M SDS, pH 6.8
TBS-T	TBS, 0.05 % Tween-20
TBS-T milk	TBS, 0.05 % Tween-20, 5 % nonfat dried milk powder
Tris buffered saline (TBS)	0.02M Tris, 8 % NaCl, pH 7.6
triton X-100 cell lysis buffer	0.03 M Tris-HCl, pH 7.5, 1 % triton X-100, 10 % glycerol, 0.12 M NaCl

3 Methods

3.1 Cultivation of eukaryotic cell lines

All cells were cultured under standard conditions (5 % CO_2, 37 °C) in standard medium (RPMI 1640, 10 % heat inactivated FCS). Adherent cells were harvested with trypsin (0.025 %) and EDTA (10 mM). Cells were counted in a haemocytometer.

3.2 Transfection of cells

To obtain transient or stable expression of Flag-TWEAK or FC-TWEAK, HEK293 cells were transfected with plasmid DNA by electropermeabilisation of the cell membrane. To this end, $4x10^7$ cells were resuspended in one ml standard medium with 40 µg of plasmid DNA. After electroporation at 250 V, 1800 µF and maximum resistance, (EquiBio Easyject Plus, Peqlab, Erlangen, Germany) cells were seeded in a 15 cm cell culture plate with standard medium and Pen/Strep. The next day, medium was changed to low serum concentration (0.5 % FCS) and cultured for 3 to 4 days, after which supernatant was collected. For isolation of stable transfectants, cells were selected with G418 (0.5 µg/ml) for at least 3 weeks before changing the medium to low serum conditions. Recombinant protein levels in the culture supernatant were determined by SDS-PAGE using a Flag-tagged protein with known concentration as a mass standard and a prestained protein marker as size standard.

3.3 Purification of TWEAK proteins

TWEAK proteins were purified by affinity chromatography using anti-Flag M2 agarose for Flag-TWEAK and protein G agarose for FC-TWEAK according to instructions of the corresponding manufacturers. In brief, supernatants with Flag-TWEAK or FC-TWEAK were allowed to pass the agarose column slowly twice. Bound Flag-TWEAK was eluted with 100 µg/ml Flag-peptide in PBS while FC-TWEAK was eluted by acidic buffer (100 mM glycine, pH 2.7) and immediately neutralized with 1 M Tris-HCl, pH 9. Recombinant TWEAK proteins were finally dialyzed against PBS. Again, protein levels were determined by SDS-PAGE using a Flag-tagged protein with known concentration as a mass standard and a prestained protein marker as size standard.

3.4 Cytotoxicity assay

Cells were seeded into 96-well flat bottom plates ($2x10^4$ cells per well) and the next day treated with Flag-TWEAK or FC-TWEAK. To this end, the protein was serially diluted in presence or absence of constant concentrations of the cross linking antibody anti-Flag mAb M2 or protein G respectively and applied to the cells. HT29 cells were pretreated with IFNγ (20 ng/ml) 24 hours prior stimulation, other cells were pretreated 1 hour with CHX (2.5 µg/ml) to sensitize them for cell death induction. 24 or 48 hours after stimulation living cells were stained with methyl violet solution (20 % methanol, 0.5 g methyl violet). To this end, medium from the plates was discarded, 50 µl of methyl violet solution was added to each well and incubated at room temperature for 20 minutes. Then plates were washed gently three times with water and dried one hour at room temperature. Methyl violet then was solved in methanol (200 µl per well) and plates were analysed by measuring the absorbance in an ELISA reader (Anthos Lucy 2) at 595 nm.

3.5 Determination of IL-8 production

$2x10^4$ cells per well were seeded into 96-well flat bottom plates and stimulated the next day with TWEAK proteins in serial dilutions with or without cross linking antibodies. Cell culture medium was exchanged prior stimulation to minimize the IL-8 background. To prevent apoptosis in sensitive cells, 10 µM Z-VAD-fmk was added 30 minutes prior stimulation. After 24 hours of incubation, IL-8 levels in cell culture supernatants were determined by using a conventional IL-8 ELISA Kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. Plates were analysed by measuring the absorbance in an ELISA reader (Anthos Lucy 2) at 405 nm.

3.6 Preparation of cell lysates

Cell lysates for western blot analysis with phosphoprotein-specific antibodies were prepared with 4x sample buffer (8 % SDS, 0.1 M DTT, 40 % glycerol, 0.2 M Tris, bromphenol blue, pH 8.0). Shortly before use, phosphatase inhibitors I and II were added. Cells were harvested with a cell scraper and washed once with three ml of ice cold PBS. Cell pellets were resolved in 4x sample buffer with phosphatase inhibitors added, sonicated with 10 pulses of ultrasound and heated 5 minutes at 95 °C. After 10 minutes centrifugation at 14000 rpm, lysates were stored at -20 °C until use.

Cell lysates for detection of other cytoplasmic proteins were prepared in triton X-100 cell lysis buffer (0.03 M Tris-HCl, pH 7.5, 1 % triton X-100, 10 % glycerol, 0.120 M NaCl) supplemented with protease inhibitor cocktail. Cell pellets were resolved in triton X-100 cell lysis buffer (0.03 M Tris-HCl, pH 7.5, 1 % triton X-100, 10 % glycerol, 0.12 M NaCl) and incubated 20 minutes on ice. After two times of centrifugation (5000 rpm, 5 min, 4 °C and 14000 rpm, 20 min, 4 °C), supernatant was supplemented with the corresponding amount of 4x sample buffer, heated 5 minutes at 95 °C and then stored at -20 °C until use.

3.7 Western blot

3.7.1 SDS-polyacrylamide gel electrophoresis and silverstaining

Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The resolving gel was prepared by using a resolving gel buffer (0.375 M Tris pH 8.8, 0.00375 M SDS, 0.1 % APS) and 12 % acrylamide. Polymerisation was started by TEMED (0.1 %). The resolving gel was overlaid with stacking gel (6 % acrylamide in 0.125 M Tris pH 6.8, 3.75 mM SDS, 0.1 % APS, 0.1 % TEMED) with pockets for the protein probes. The electrophoretic separation was performed for 90 minutes at 90 to 130 volts. After that, polyacrylamide gel was washed with water and further treated with solutions of the PageSilver™ Silver Staining Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions.

3.7.2 Electrotransfer of proteins to nitrocellulose membranes

The transfer of proteins was achieved in a semi-dry blot chamber (Pequlab, Erlangen, Germany). Nitrocellulose membrane and whatman paper were soaked in blot buffer (0.025 M Tris pH 8.3, 0.192 M glycine, 20 % (v/v) methanol) and stacked in the following order: anode – 2 sheets whatman-paper – nitrocellulose membrane – polyacrylamide gel – 2 sheets whatman paper – cathode. The proteins were transferred to the nitrocellulose membrane with a current of 1.5 mA per cm^2 gel for 100 to 120 minutes.

3.7.3 Immunostaining of western blots

All following steps were performed on a shaker. Saturation of nitrocellulose membrane was achieved by incubation with blocking buffer (PBS or TBS, 0.05 % Tween-20, 5 % nonfat dried milk powder) at room temperature for 1 to 3 hours. Each incubation step was followed

by three washes with PBS-T or TBS-T. Membranes were incubated with primary antibodies for 2 hours at room temperature or at 4 °C over night; and with secondary antibodies for 1 hour at room temperature. Secondary antibodies were either conjugated with horseradish-peroxidase and detected with the ECL-system or were labelled with fluorescent dyes for detection with the LI-COR Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

3.8 FACS analysis

FACS analysis was used to detect expression of TWEAK receptor Fn14 or cell bound TWEAK proteins.

To this end, 1 x 10^5 cells were incubated for 30 minutes at 4 °C with 2 µg/ml of anti-Fn14 antibody ITEM-4 or IgG2b as isotype control and then washed 3 times with PBS-BSA (PBS, 0.5 % BSA). After incubation with 2 µg/ml of the second PE-labelled antibody cells were washed again and cell bound antibodies were detected by FACS (FACScalibur, BD Biosciences, Heidelberg, Germany).

For detection of TWEAK binding, cells were incubated 30 minutes at 4 °C with different concentrations of Flag-TWEAK together or successively with 5 µg/ml of the FITC-labelled Flag-specific antibody mAb M2. Different concentrations of FC-TWEAK were detected by using 5 µg/ml of PE-labelled anti-human IgM/G/A (H+L) $F(ab')_2$ fragments. After washing, the amount of cell-bound TWEAK protein was again measured by FACS.

To prove receptor-specificity of TWEAK binding, cells were incubated one hour at 4 °C with 2 µg/ml of anti-Fn14 antibody ITEM-4. Then Flag-TWEAK or FC-TWEAK was added at a concentration of 2 µg/ml for another hour without washing the cells. Staining of Flag-TWEAK was achieved with 5 µg/ml FITC-labelled anti Flag mAb M2, FC-TWEAK was detected with 5 µg/ml PE-labelled anti-human IgM/G/A (H+L) $F(ab')_2$ fragments. After washing the cells, receptor bound Flag-TWEAK or FC-TWEAK was detected by FACS.

3.9 High performance liquid chromatography (HPLC)

Affinity purified TWEAK proteins (100 µg/ml, 50 µl) were applied to a BioSep-SEC-S3000 (300 x 7.8) column (Phenomenex, Aschaffenburg, Germany). The column was equilibrated with PBS and proteins were eluted in 0.5 ml/min. Fractions were collected for further

applications and stored at -20 °C until use. Size of eluted proteins was determined by comparison with the Phenomenex standard.

4 Results

4.1 Characterization of TWEAK fusion proteins

To study TWEAK signaling, soluble forms of the molecule were made that contain the THD which is responsible for trimerization and receptor binding. Since the Flag-TWEAK and FC-TWEAK fusion proteins used in this work spare the intracellular and transmembrane domain of the molecule they are directly secreted into the surrounding media upon expression in HEK293 cells. For Flag-TWEAK a trimeric appearance was expected and FC-TWEAK should assemble into hexameric molecules as the FC-portion of IgG builds disulfide bonded dimers.

To characterize recombinant TWEAK proteins for purity, molecular organization, receptor binding and activity, different approaches were used. To check affinity purified TWEAK proteins for purity and size, size exclusion chromatography and SDS-PAGE analyses were performed.

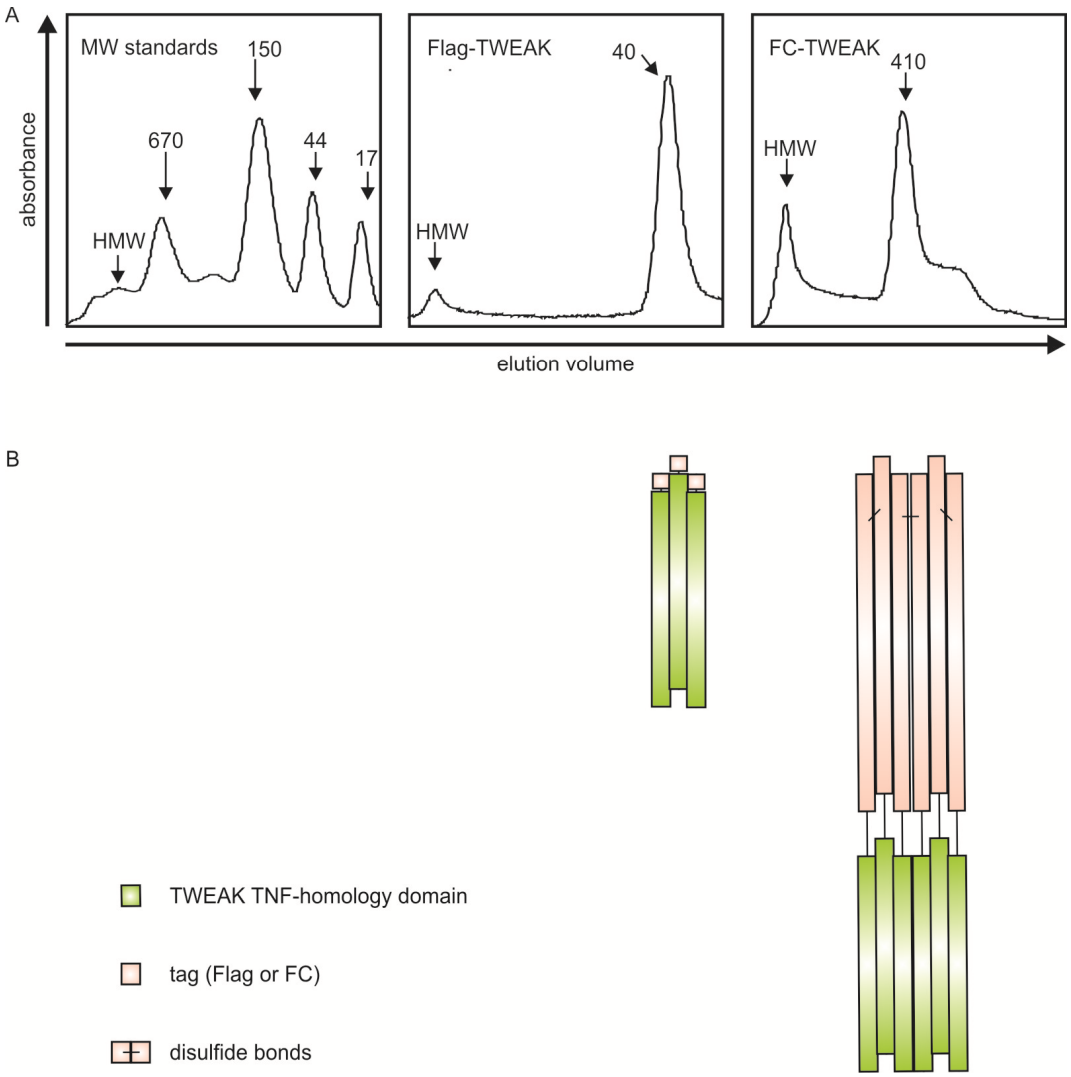

Figure 7 Gel filtration analysis of TWEAK fusion proteins
A: 50 µl of affinity purified TWEAK proteins were applied on HPLC column BioSep-SEC-S3000 at a concentration of 100 µg/ml in PBS and eluted with 0.5 ml/min. Protein content was detected by UV absorption. B: Scheme of trimeric Flag-TWEAK (left) and hexameric FC-TWEAK (right) with disulfide bonds between two of the FC-domains (small black bar). Tag (pink) and TWEAK TNF homology domain (green).

Flag-TWEAK eluted at a calculated size of 40 kDa. Therefore a trimeric form of the 154 amino acid protein was concluded. FC-TWEAK eluted at a calculated size of 410 kDa, indicating a hexameric organization of the 400 amino acid protein. A schematic illustration of the appearance of both TWEAK proteins is given in figure 7B. High molecular weight peaks appeared in preparations of both proteins (Figure 7A). In order to check affinity purified and gel filtration purified TWEAK proteins for purity, corresponding samples were analyzed by SDS-PAGE and silver staining or by western blot with anti-TWEAK antibody.

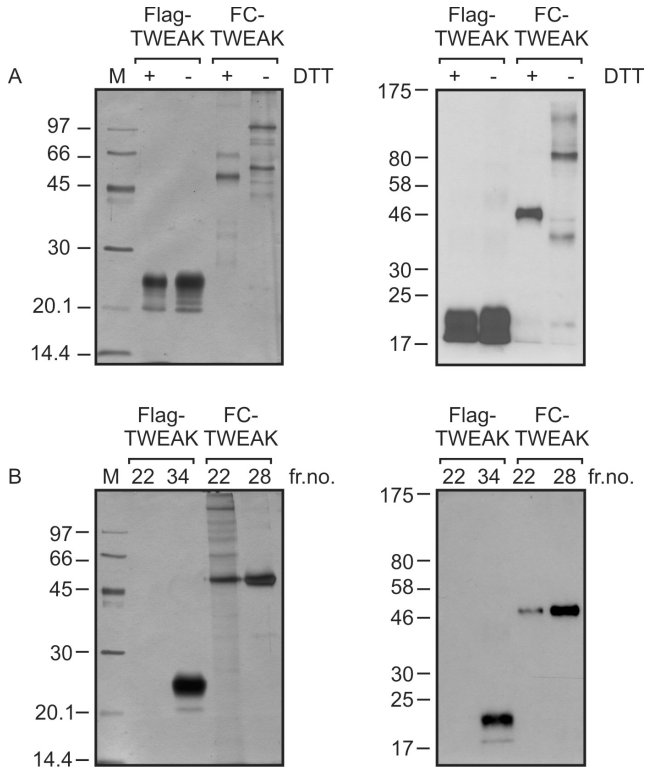

Figure 8 Analysis of TWEAK fusion proteins by silverstaining and western blotting
A: Affinity purified TWEAK proteins were separated in SDS-PAGE under reducing (+ 0.1 M DTT) or nonreducing (without DTT) conditions and were detected by silverstaining (left box) or in western blotting with anti-TWEAK antibody (right box). B: Gel filtration fractions of TWEAK proteins corresponding to the peaks shown in figure 7A were separated in SDS-PAGE under reducing conditions and detected with silverstaining (left box) or in western blot with anti-TWEAK antibody (right box). Fraction no. 22 corresponds to the high molecular weight fractions of both TWEAK proteins. Fraction no. 34 corresponds to the 40 kDa peak of Flag-TWEAK and fraction no. 28 corresponds to the 410 kDa peak of FC-TWEAK.

Affinity purified Flag-TWEAK preparations migrate in SDS-PAGE under reducing (with 0.1 M DTT) and nonreducing conditions (without DTT) as a range of bands of 20 to 25 kDa, which was considered to represent the monomeric form of Flag-TWEAK. The appearance of TWEAK with this pattern may be due to variable glycosylation of the protein. Affinity purified FC-TWEAK is being detected in silverstaining mainly in a band with 45 kDa under reducing conditions which closely resembles the expected molecular weight of the monomeric form of this molecule (Figure 8A left). In anti-TWEAK western blot analysis, solely the 45 kDa band appears under reducing condititions, representing the FC-TWEAK monomers (Figure 8A right). Under nonreducing conditions, the electrophoresis pattern for FC-TWEAK changes. The sizes of the bands shift and the major band appears at about 90 kDa, indicative for a FC-TWEAK connected by a disulfide bond between two FC-tags (Figure 8A). As seen in high performance liquid chromatography, affinity purified TWEAK proteins contain a high molecular weight protein fraction. Analysis of the corresponding fractions showed that the impurities in the high molecular weight fraction of FC-TWEAK (fraction number 22) contain higher aggregates of FC-TWEAK (Figure 8B).

To probe the aggregated TWEAK molecules for activity, they were tested for the ability to induce cell death on Kym-1 cells and IL-8 secretion on HT1080 cells. For comparison, active Flag-TWEAK trimers and FC-TWEAK hexamers were used.

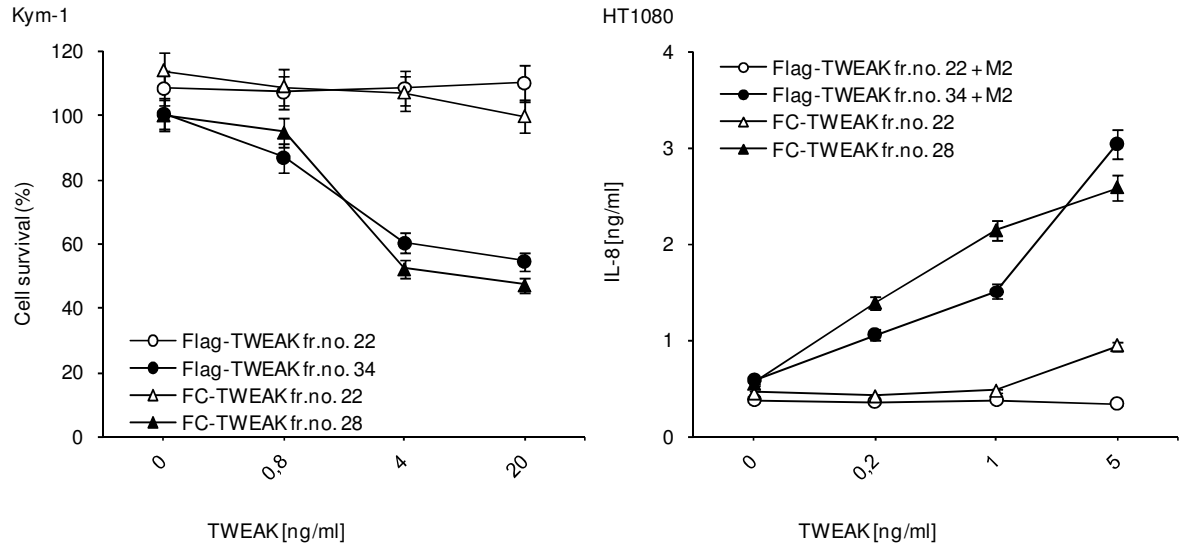

Figure 9 Activity of peak fractions of TWEAK proteins
Protein from high molecular weight peaks (Flag-TWEAK and FC-TWEAK fraction number 22. Open circles and open triangles), trimeric Flag-TWEAK (fraction number 34, filled circles) and hexameric FC-TWEAK (fraction number 28, filled triangles) were applied on Kym-1 cells at indicated concentrations (Flag-TWEAK fr.no. 22: undiluted) in triplicates and incubated 24 hours under standard conditions. After that, cell viability was measured by staining surviving cells with methyl violet (left picture). On HT1080 cells, Flag-TWEAK and FC-TWEAK fractions were added at indicated concentrations in triplicates, Flag-TWEAK fractions were secondary crosslinked with anti-Flag mAb M2 (0.5 µg/ml). After 24 hours of incubation, supernatants were collected and probed for IL-8 content (right picture).

Kym-1 cells are known to be very sensitive for TWEAK-induced apoptosis (Schneider et al., 1999b). Cell viability was measured after 24 hours by staining surviving cells with methyl violet. With a TWEAK concentration of 4 ng/ml, 50 % of Kym-1 cells died within 24 hours. Only the HPLC fractions containing trimeric or hexameric TWEAK (fraction number 34 or 28) were able to induce apoptosis in Kym-1 cells while the high molecular weight fractions (fractions number 22) which contain higher aggregates of the molecules failed to induce apoptosis (Figure 9 left).

Human fibrosarcoma cell line HT1080 respond with strong IL-8 secretion to TWEAK stimulation, if TWEAK is hexameric or aggregated via secondary crosslinking of Flag-TWEAK with anti-Flag M2 antibody. Like in cell death induction, only aggregated trimeric and hexameric TWEAK are able to induce IL-8 secretion on HT1080 cells, while the high molecular weight fractions failed to induce this effect. HT1080 cells produce up to 3 ng/ml of

IL-8 within 24 hours when stimulated with 5 ng/ml of trimeric or hexameric TWEAK (Figure 9 right).

Together, these results show that the impurities and aggregated TWEAK molecules present in the affinity purified TWEAK preparations are not responsible for TWEAK-induced cellular effects. Therefore, for simplicity, affinity purified TWEAK proteins were used in this work.

4.2 Characterization of cell lines regarding Fn14 expression

Until now, Fn14 is the only definite receptor for TWEAK binding and is expressed by a broad range of cells (see 1.5.3.3). This gives the rationale for looking at the cell lines used in this work regarding cell surface expression of Fn14 by flow cytometry.

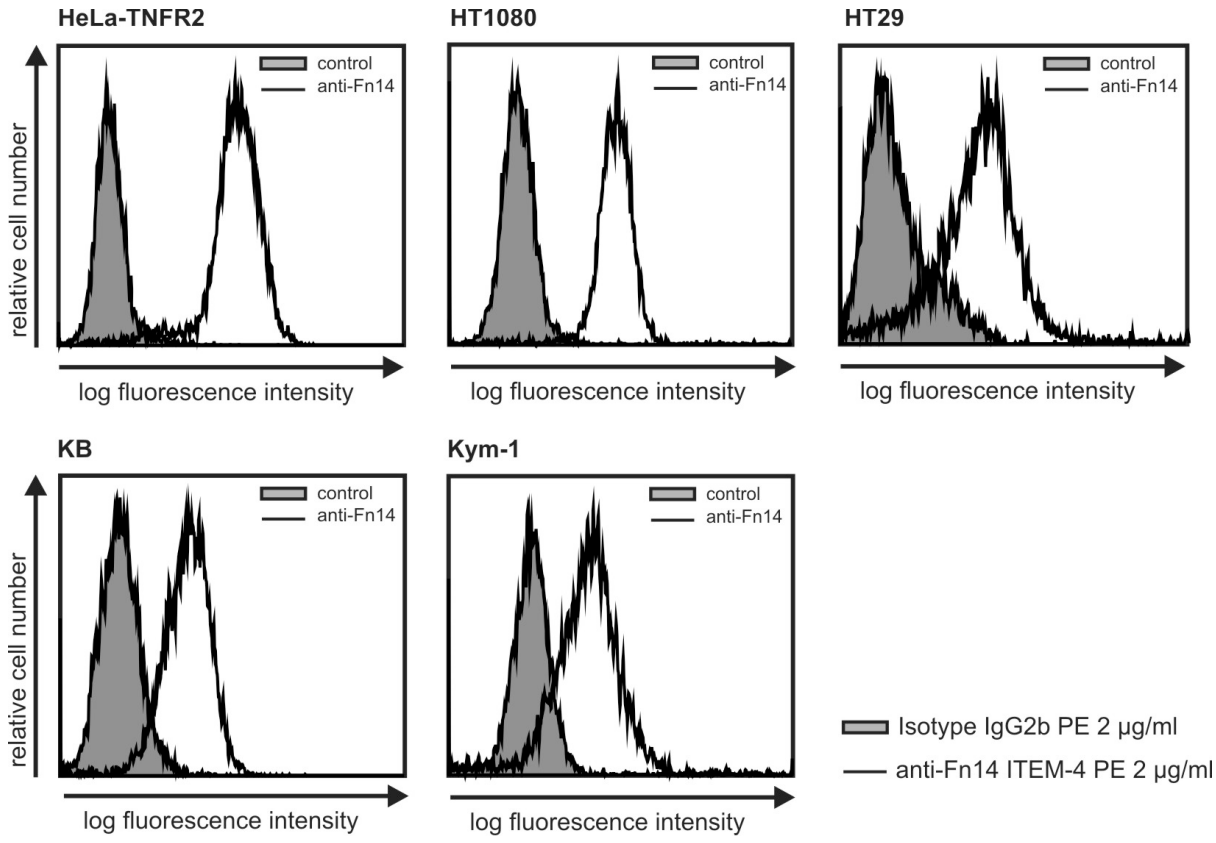

Figure 10 FACS analysis for expression of TWEAK receptor Fn14
Different cell lines were incubated 30 minutes at 4 °C with 2 µg/ml PE-labelled anti-Fn14 antibody ITEM-4 (blank area) or, as a control, with the corresponding Isotype, PE-labelled IgG2b (filled area). After washing, cell bound antibodies were detected by FACS.

As expected based on literature, all cells used in this work showed strong expression of the TWEAK receptor Fn14. Compared to the control IgG stained cells, anti-Fn14 ITEM-4 PE-labelled cells show strong fluorescence intensities (Figure 10). Additional experiments were carried out to see how these Fn14 expressing cells bind the TWEAK fusion proteins used in this work.

4.3 Binding studies for TWEAK proteins

4.3.1 Binding of TWEAK proteins to Fn14 expressing cell lines

FACS analysis was performed to verify that the constructed TWEAK proteins used in this work bind to Fn14 expressing cells. To this end, different concentrations of Flag-TWEAK, oligomerized Flag-TWEAK or FC-TWEAK were applied to the cells and ligand binding was measured by FACS analysis.

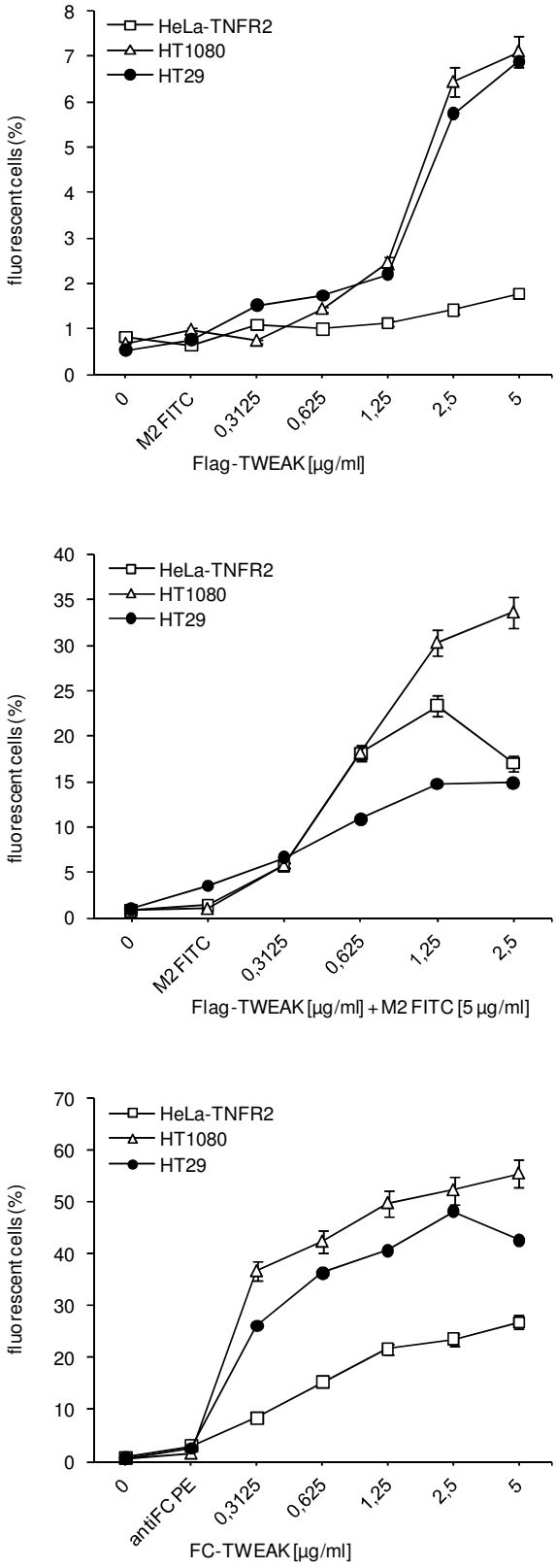

Figure 11 Binding of TWEAK proteins
HeLa-TNFR2, HT1080 and HT29 cells were incubated with the indicated concentrations of trimeric Flag-TWEAK (top), anti-Flag M2 FITC oligomerized Flag-TWEAK (middle) or hexameric FC-TWEAK (bottom). After washing remaining cell bound TWEAK proteins were detected with FITC-labelled anti-Flag M2 antibody (5 µg/ml) for Flag-TWEAK or PE-labelled anti-FC antibody (5 µg/ml) for FC-TWEAK. After another washing step, number of fluorescent cells was measured by FACS.

All used TWEAK proteins were shown to bind to Fn14 expressing cells in a dose depended manner. Interestingly the TWEAK variants exhibit different abilities in binding to the cells. While Flag-TWEAK stained up to 8 % of cells at the highest concentration, compared to unstained cells, anti-Flag M2 oligomerized Flag-TWEAK stains about 35 % of cells at the maximum concentration. FC-TWEAK even causes staining of over 60 % of cells at this concentration. Furthermore, FC-TWEAK shows the best ED50 value and reaches high binding efficiencies at low concentrations at a dose of 0.3 µg/ml, while Flag-TWEAK shows an ED50 value at a dose of about 1.25 µg/ml if oligomerized and at about 2.5 µg/ml as trimeric molecule (Figure 11).

4.3.2 Fn14-specific antibody inhibits TWEAK binding

To elicit if TWEAK binding to cell lines was via interaction with Fn14, the Fn14-specific antibody ITEM-4 was used (Nakayama et al., 2002). This mouse monoclonal, antagonistic antibody has been used for inhibition of TWEAK-induced effects before by our as well as other working groups.

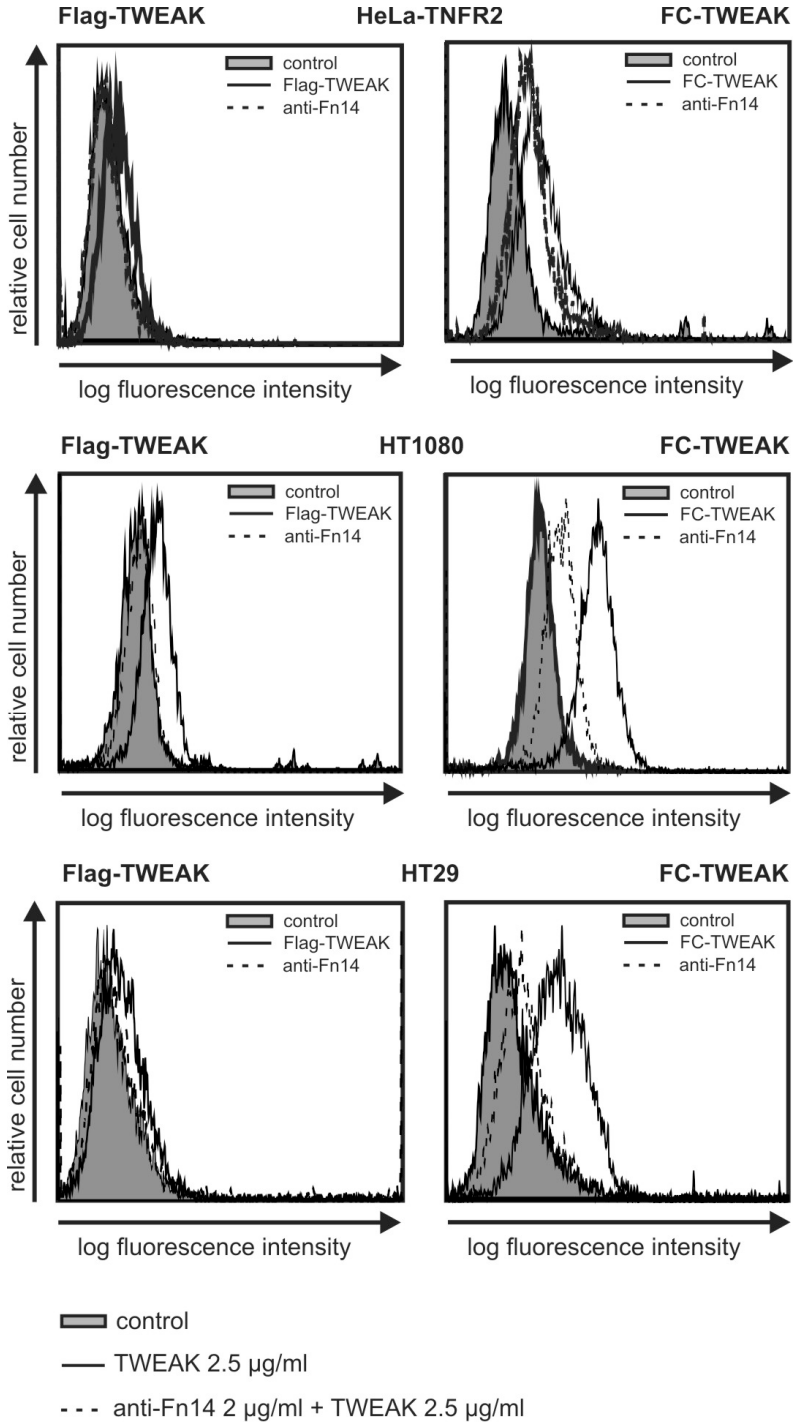

Figure 12 Anti-Fn14 antibody inhibits binding of TWEAK proteins to cells
The indicated cell lines were incubated with 2.5 µg/ml Flag-TWEAK (left) or 2.5 µg/ml FC-TWEAK (right) and some of the groups were pretreated with anti-Fn14 antibody ITEM-4 (2 µg/ml). After washing the cells, Flag-TWEAK was stained with FITC-labelled anti-Flag M2 antibody (5 µg/ml) and FC-TWEAK with PE-labelled anti-FC antibody (5 µg/ml). After another washing step, the number of fluorescent cells was measured by FACS.

In the histogram view, relative weak binding of TWEAK proteins compared to the Fn14-specific antibody (Figure 10) is obvious. Once again, the difference between binding of trimeric Flag-TWEAK and hexameric FC-TWEAK was considerable. The right row in figure

12 shows binding of FC-TWEAK and maximum of fluorescent cells is 7 to more than 20 times higher than fluorescence of cells stained with Flag-TWEAK (left row).

Most important, on all tested cell lines, preincubation with anti-Fn14 antibody ITEM-4 reduced the cellular binding capacity for TWEAK proteins from 50 % (HeLa-TNFR2 and HT1080 with FC-TWEAK) to almost 100 % (HT1080 with Flag-TWEAK) (Figure 12).

4.4 Cell death induction by trimeric and oligomerized TWEAK

Soluble TNF ligands can exhibit different receptor activating capacities in comparison with their membrane bound forms (Mariani and Krammer, 1998; Tanaka et al., 1998; Wajant, 2003). So, some TNF ligands that are inactive or poorly active as soluble trimers were found to mimic the activity of the corresponding membrane-bound molecule upon oligomerization (Wajant et al., 2005).

TWEAK can induce different types of cell death in variable ways, e.g. via endogenous TNFα and TNFR1 on rhabdomyosarcoma cell line KYM-1 (Schneider et al., 1999b) or direct induction of apoptosis or necrosis on IFNγ sensitized colon adenocarcinoma cell line HT29 (Nakayama et al., 2002). Cosensitizing agents are often required to induce TWEAK-mediated cell death on human tumor cell lines. HeLa cervical cancer cell line requires cycloheximide pretreatment to undergo TWEAK-induced apoptosis (Marsters et al., 1998).

Additionally, the non tumor keratinocyte cell line KB was used. Keratinocytes were described to show inflammatory responses upon TWEAK stimulation before (Jin et al., 2004).

All studies to date on cell death induction by TWEAK have used either oligomerized TWEAK constructs or bacterially produced and therefore likely aggregated TWEAK. To correctly assess the activity of trimeric versus oligomerized TWEAK in this work cell lines were treated with trimeric Flag-TWEAK and oligomerized TWEAK.

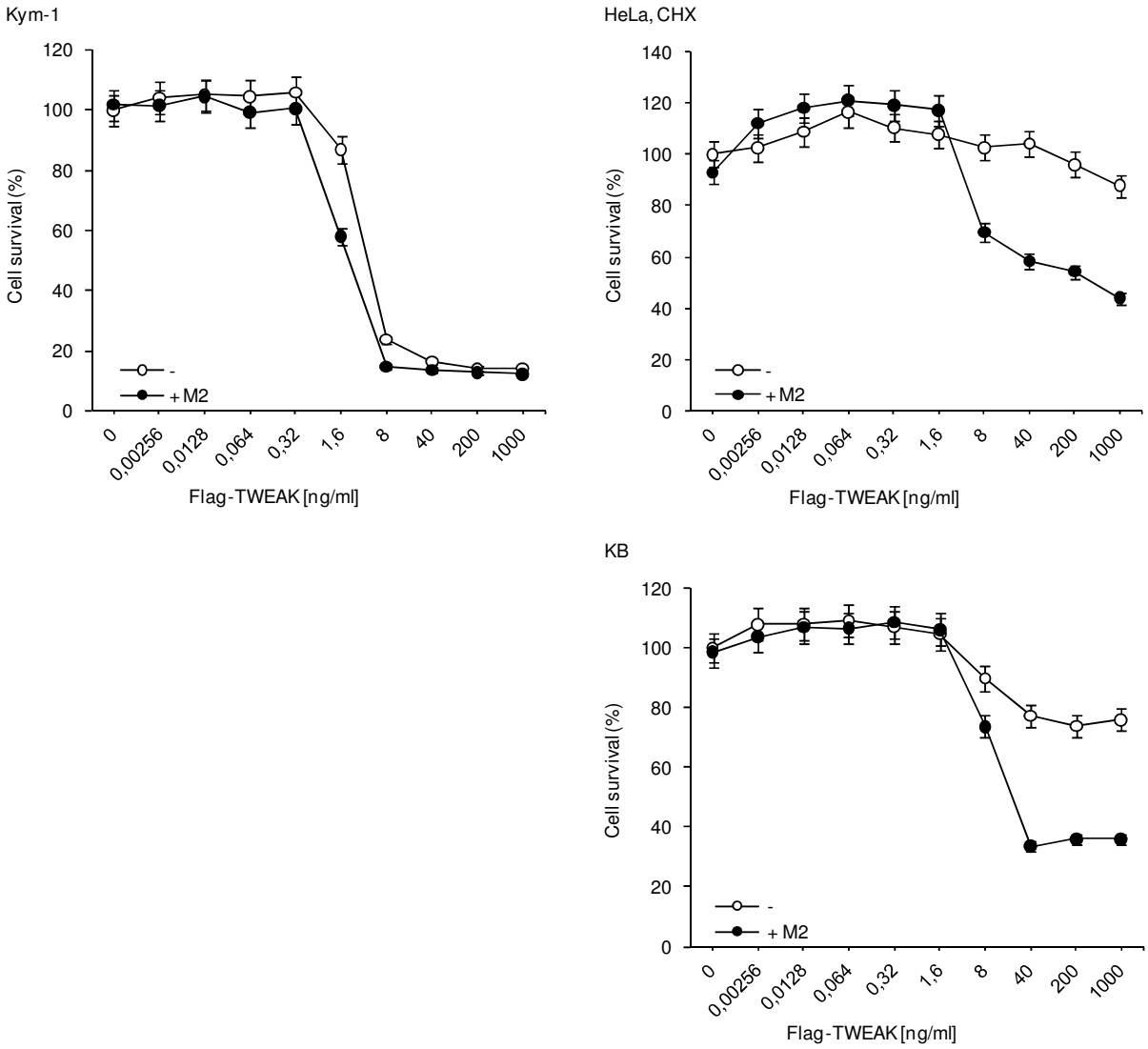

Figure 13 TWEAK induced apoptosis in various cell lines
Different cell lines were stimulated with indicated concentrations of Flag-TWEAK with (filled circles) or without (open circles) crosslinking antibody mAb M2 (0.5 µg/ml) 48 hours in triplicates. HeLa cells were treated with CHX (2.5 µg/ml) 30 minutes prior stimulation. After that, cell viability was measured by methyl violet staining.

Kym-1 cells, treated with oligomerized or trimeric TWEAK, died within 48 hours in a dose dependent way without significant difference between trimeric and oligomerized TWEAK. HeLa cells need to be sensitized by incubation with the protein biosynthesis inhibitor cycloheximide (CHX) to prevent synthesis of antiapoptotic proteins after TWEAK stimulation. HeLa and KB cells both undergo apoptosis if stimulated with oligomerized TWEAK. The ED50 value for apoptosis induction on these two cell lines for stimulation with oligomerized Flag-TWEAK is reached with a dose of 8 ng/ml. However, apoptosis induced by trimeric TWEAK was shown to be very weak for these two cell lines (Figure 13).

In order to reveal differences in the ability of TWEAK to induce cell death, cell lines were treated for 24 or 48 hours with oligomerized or trimeric TWEAK in the presence or absence of the cosensitizing agent CHX.

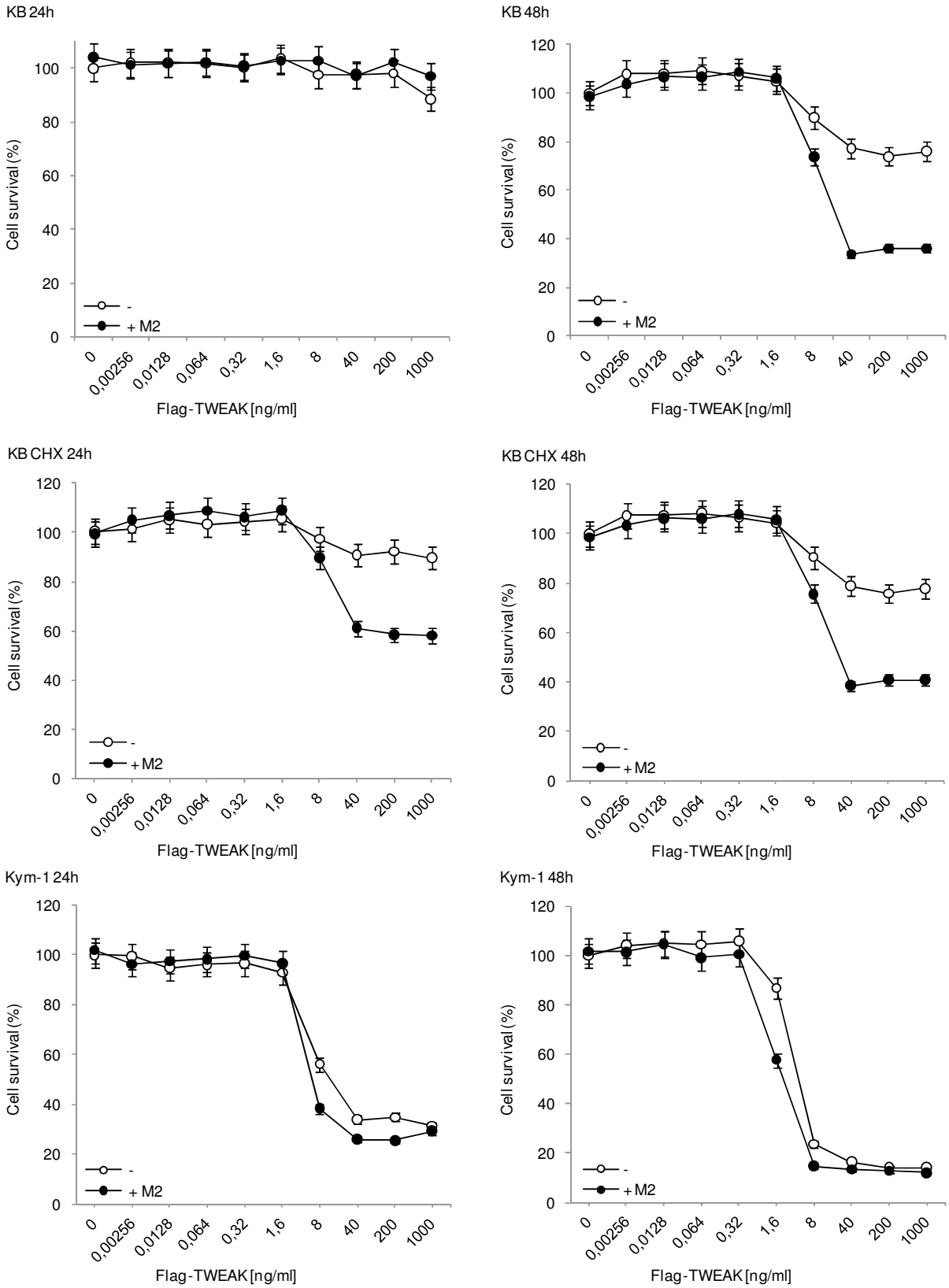

Figure 14 Differences in TWEAK-induced apoptosis in rapidness and strength
Cell lines were stimulated with indicated concentrations of Flag-TWEAK with (filled circles) or without (open circles) crosslinking antibody mAb M2 (0.5 µg/ml) for 24 hours (left) or 48 hours (right) in triplicates. KB cells were treated with CHX (2.5 µg/ml) 30 minutes prior and during stimulation (middle row) or left untreated until Flag-TWEAK stimulation (upper row). After that, cell viability was measured with methyl violet staining.

Keratinocyte cell line KB was shown to be sensitized by CHX for faster induction of apoptosis. If pretreated with CHX, cytotoxic effects of TWEAK on KB cells were measured after 24 hours. However, these cells do not need necessarily to be sensitized by CHX for apoptosis. If incubated 48 hours with TWEAK, both sensitized and untreated cells showed the same rate of TWEAK-induced apoptosis. In any case oligomerization of TWEAK is essential to obtain full activity in apoptosis induction (Figure 14).

Kym-1 cells are an exception. Here, apoptosis induction is achieved by stimulation with trimeric and oligomerized TWEAK within 24 hours and is not significantly enhanced by longer incubation (48 hours) (Figure 14).

HT29 cells are more complex with respect to TWEAK-induced cell death. They can be sensitized by IFNγ for apoptosis induction and by the pan caspase inhibitor Z-VAD-fmk for necrosis.

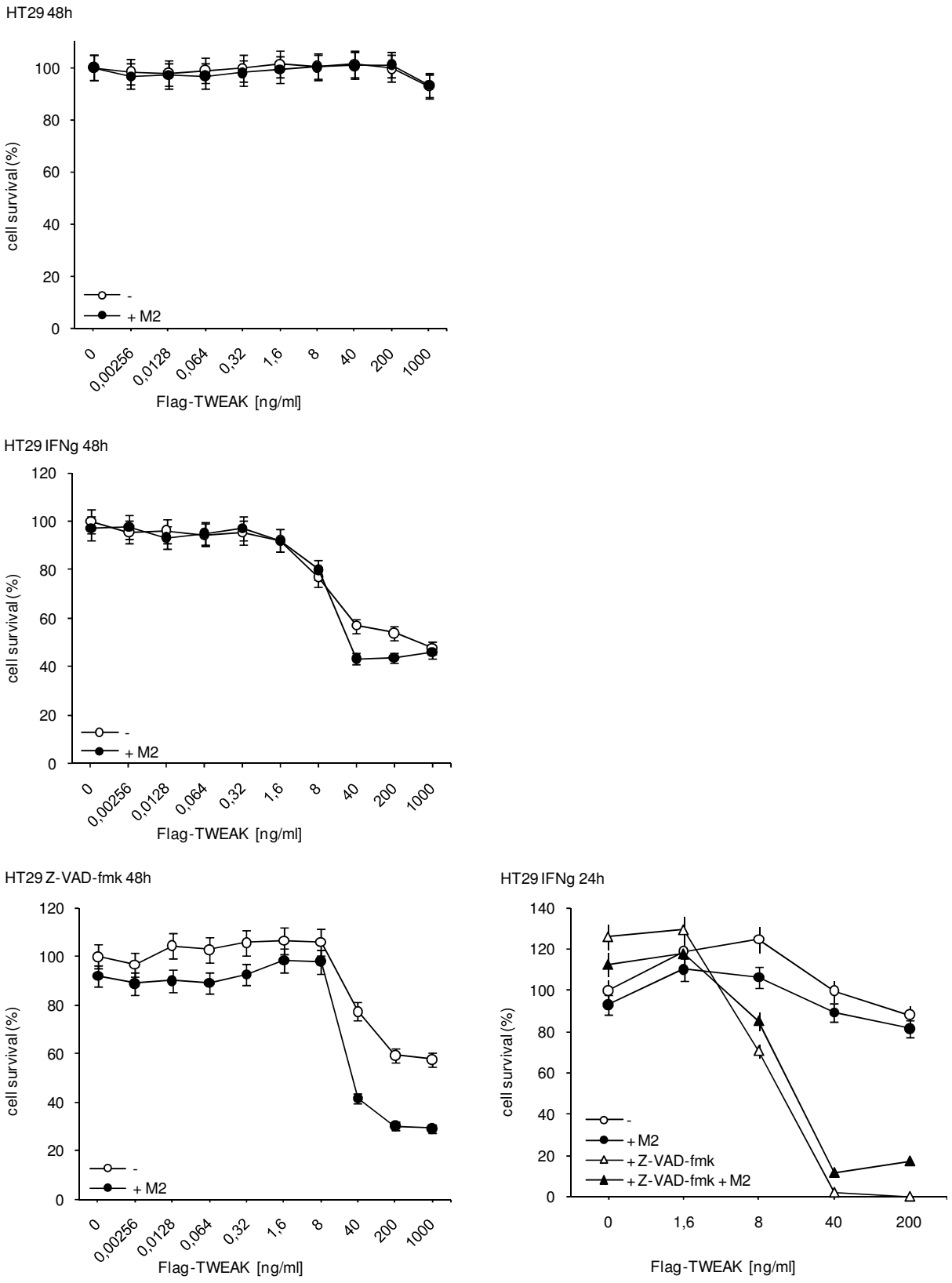

Figure 15 HT29 cells can be sensitized for cell death with IFNγ or Z-VAD-fmk
A: HT29 cells were stimulated with indicated concentrations of Flag-TWEAK with (filled circles) or without (open circles) crosslinking antibody mAb M2 (0.5 µg/ml) for 48 hours in triplicates. To sensitize cells, they were pretreated with IFNγ (20 nM) 16 hours prior stimulation. After that, cell viability was measured with methyl violet staining. B: HT29 cells were pretreated with IFNγ (20 nM) 16 hours and partially pretreated with Z-VAD-fmk (40 µM) (triangles). After that, cells were stimulated with the indicated concentrations of

Flag-TWEAK with (filled symbols) or without (open symbols) crosslinking antibody mAb M2 (0.5 µg/ml) for 24 hours in triplicates. Then, cell viability was measured with methyl violet staining.

HT29 cells are completely resistant to TWEAK-induced cell death, whereas incubation with IFNγ sensitizes the cells for apoptosis so that up to 60 percent die after 48 hours. In this case there is no difference between oligomerized and trimeric TWEAK (Figure 15 top). Incubation with Z-VAD-fmk leads to necrosis in up to 70 percent of the cells after 48 hours of stimulation with oligomerized TWEAK, while trimeric TWEAK induces necrosis in about 40 percent of the cells. Both sensitizing reagents IFNγ and Z-VAD-fmk combined lead to almost 100 percent killing of cells after only 24 hours in case of oligomerized TWEAK and trimeric TWEAK. As apoptosis and necrosis occur side by side, effects are much stronger and clearly detectable after 24 hours (Figure 15 bottom).

4.4.1 Fn14 blockade inhibits TWEAK-induced cell death

To determine whether TWEAK-induced cell death on the various cell lines investigated is induced via Fn14, the Fn14-specific blocking antibody ITEM-4 was used. Induction of cell death and potential blocking effects of antagonistic anti-Fn14 mAb ITEM-4 were assessed in HT29 cells and KB cells. To rule out agonistic, death inducing effects of the anti-Fn14 antibody, untreated cells were compared to Fn14-blocked cells without TWEAK stimulation.

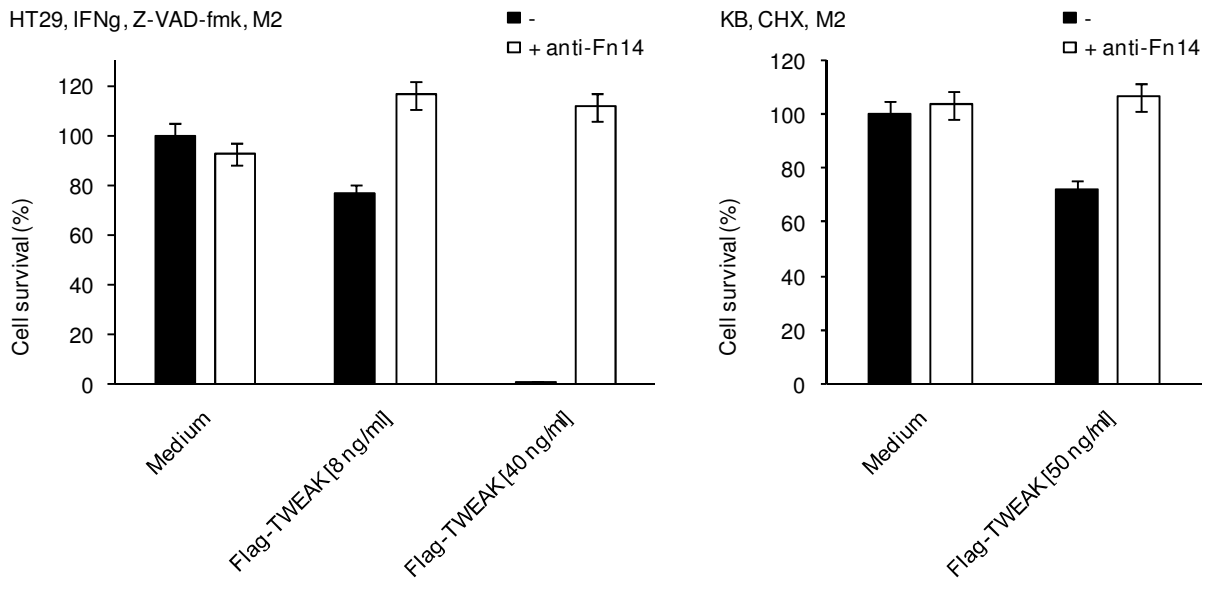

Figure 16 Inhibition of TWEAK-induced cell death by anti-Fn14 antibody
HT29 cells were pretreated with IFNγ (20 nM) 16 hours prior stimulation and were sensitized with Z-VAD-fmk (40 µM) 30 minutes prior stimulation with oligomerized Flag-TWEAK in indicated concentrations in triplicates. In some groups, Fn14 was blocked by anti-Fn14-specific antibody ITEM-4 (1.25 µg/ml) (white bars), others left unblocked (black bars). KB cells were sensitized with CHX (2.5 µg/ml) 30 minutes prior stimulation with oligomerized Flag-TWEAK (50 ng/ml) in triplicates. Half the groups were blocked with anti-Fn14-specific antibody ITEM-4 (1 µg/ml) (white bars), others left unblocked (black bars). After 24 hours, cell viability was measured by methyl violet staining.

Cells treated with the antagonistic anti-Fn14 antibody ITEM-4 alone did not undergo apoptosis, with similar survival as untreated cells, which excludes death inducing activity of the anti-Fn14 antibody.

Interestingly, on HT29 cells both apoptosis and necrosis can be inhibited by the use of the antagonistic anti-Fn14 antibody. Whereas HT29 cells were killed to a 100 percent by 40 ng/ml oligomerized TWEAK within 24 hours, cell death was totally inhibited with anti-Fn14 antibody ITEM-4. KB cells, if sensitized with CHX, underwent apoptosis within 24 hours up to 40 percent after stimulation with 50 ng/ml oligomerized TWEAK. This effect was also completely blocked by anti-Fn14 antibody ITEM-4, too. (Figure 16)

HT29 and KB cells both stay viable in the presence of cytotoxic concentrations of oligomerized TWEAK if the Fn14 receptor is blocked by the antagonistic antibody ITEM-4. Thus, although Fn14 does not have a death domain on its intracellular portion, it seems to be responsible for induction of death signaling.

4.5 Proinflammatory effects of trimeric or oligomerized TWEAK

Physiological even more relevant than induction of cell death is the ability of TWEAK to induce inflammatory responses on various cell types for different reasons (Chicheportiche et al., 2002; Chicheportiche et al., 2000; Saas et al., 2000) (see 1.5.5.1). Because of the differences in the ability of cell death induction between trimeric and oligomerized TWEAK (see 4.4), I looked for differences in the induction of inflammatory responses after stimulation with trimeric and oligomerized TWEAK.

4.5.1 Induction of IL-8 and IL-6 by trimeric, hexameric and oligomerized TWEAK

IL-8 is one of the target genes of NFκB, so secretion of IL-8 occurs as a consequence of the activation of the classical NFκB pathway. Indeed, several cell lines react with IL-8 secretion after TWEAK stimulation (Chicheportiche et al., 2002).

TWEAK's ability to trigger IL-8 production was originally described for human colon carcinoma, melanoma, and fibrosarcoma cell lines *in vitro* (Chicheportiche et al., 1997). A few years later, the same working group showed that human fibroblasts produce a variety of proinflammatory cytokines after TWEAK stimulation, including IL-6 (Chicheportiche et al., 2002).

In this work different tumor cell lines were tested for inflammatory responses after TWEAK stimulation, namely the cervical cancer cell line HeLa-TNFR2, human fibrosarcoma cell line HT1080, and colon adenocarcinoma cell line HT29. HeLa were transfected for TNFR2 expression before (Weiss et al., 1997), but this was shown to have no effect on TWEAK-induced responses (Wiley et al., 2001) (See 1.5.4). Since it is known that these cell lines react with IL-8 secretion, potential differential responses ot these cell lines in terms of NFκB activation by trimeric and oligomerized TWEAK were analyzed.

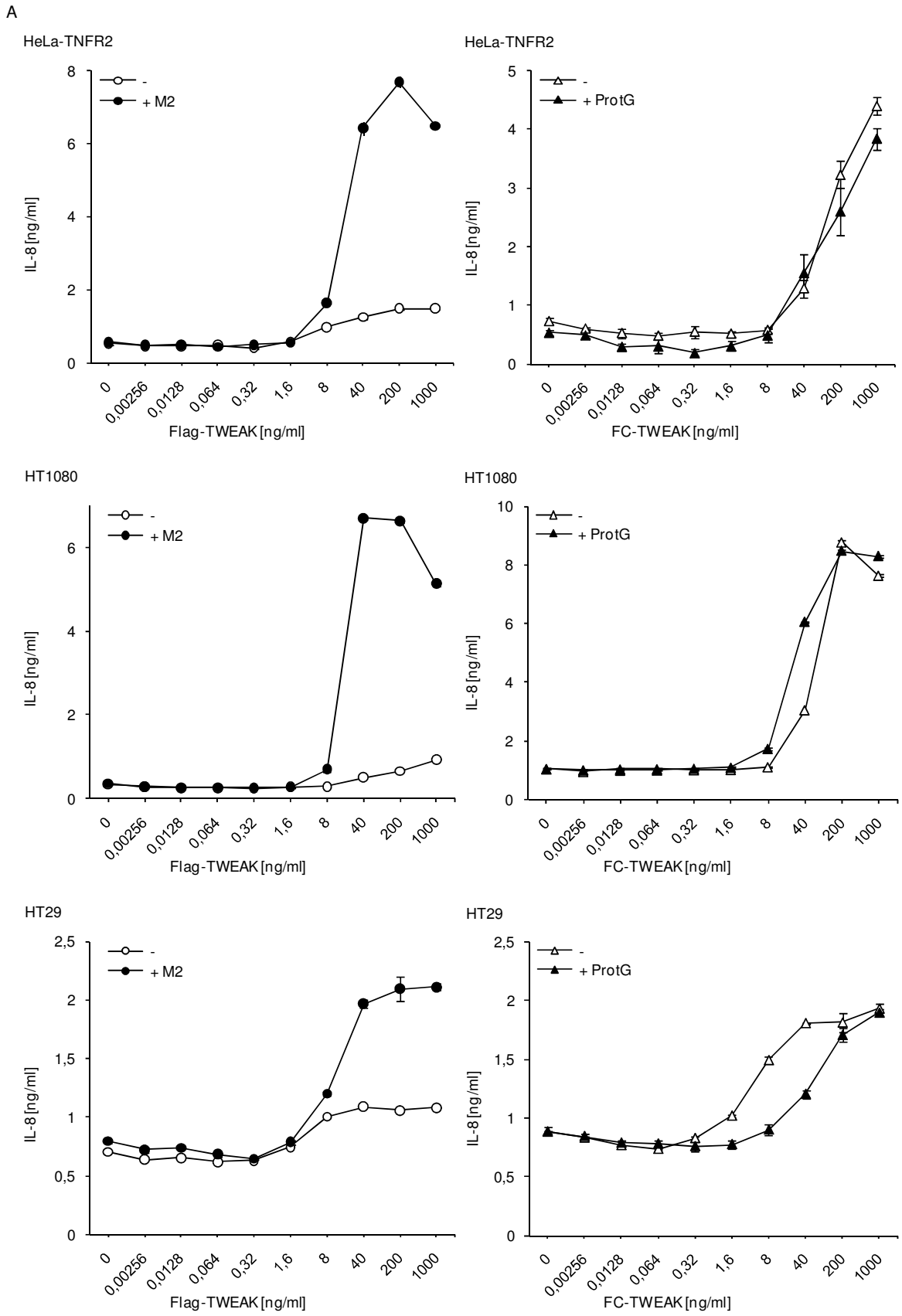

Figure 17 IL-8 and IL-6 secretion after TWEAK stimulation
A: HT1080, HeLa-TNFR2 and HT29 cells were stimulated with trimeric (open circles) or with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK (filled circles) (left column) or hexameric (open triangles) or with protein G (0.5 µg/ml) oligomerized FC-TWEAK (filled triangles) (right column) with the indicated concentrations in triplicates. After 24 hours, supernatants were collected and probed for IL-8 by ELISA.

B

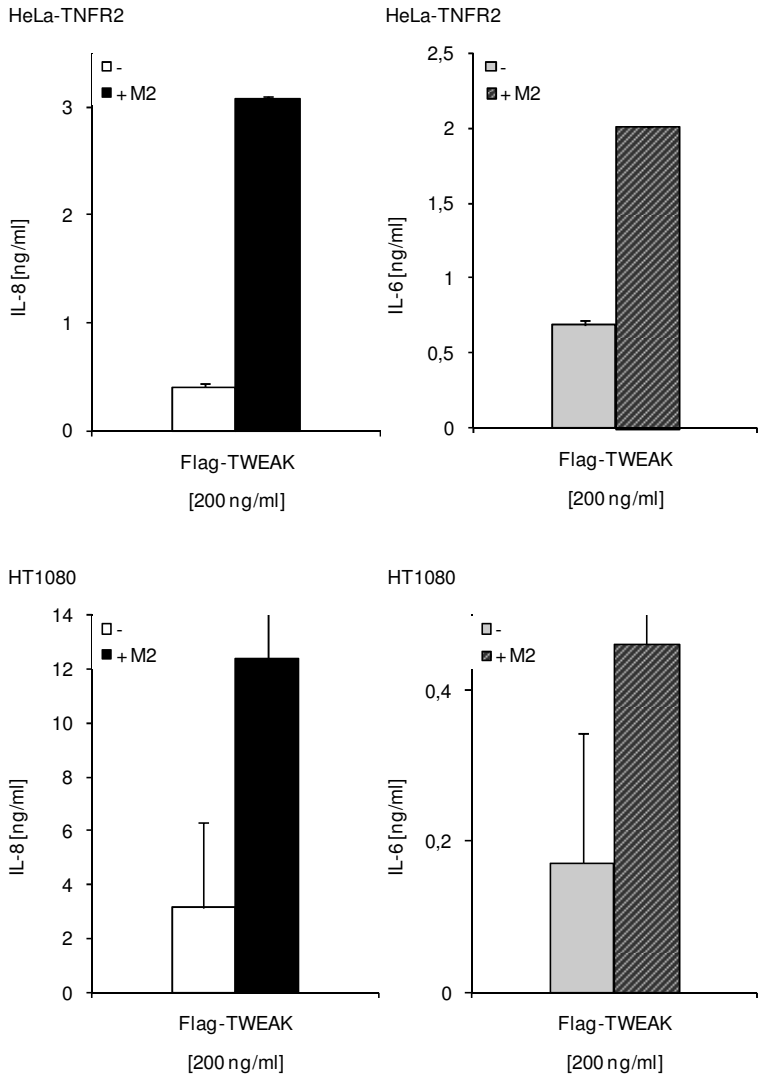

Figure 17 IL-8 and IL-6 secretion after TWEAK stimulation
B: HeLa-TNFR2 (upper row) and HT1080 (lower row) cells were stimulated with 200 ng/ml trimeric (lighter bars) or with anti-Flag mAb M2 (0.5 µg/ml) oligomerized (darker bars) Flag-TWEAK in triplicates. After 24 hours, supernatants were collected and probed for IL-8 (left column) or IL-6 (right column) content.

On all tested cell lines, oligomerized Flag-TWEAK induces IL-8 secretion at concentrations about 8-40 ng/ml, while trimeric TWEAK is not able to induce strong NFκB activation and IL-8 secretion. These findings are totally consistent with the ability of TWEAK in cell death induction (see 4.4).

FC-TWEAK, which is naturally hexameric, shows already full activity regarding IL-8 induction, comparable to that of oligomerized Flag-TWEAK. The activity of FC-TWEAK cannot be enhanced by protein G oligomerization. On HT29 cells hexameric TWEAK even loses some of its ability to induce IL-8 secretion by secondary crosslinking (Figure 17A).

In direct comparison between trimeric and oligomerized TWEAK, oligomerized TWEAK induces four to six times more IL-8 secretion than trimeric TWEAK at a concentration of 200 ng/ml. In IL-6 induction, similar effects can be observed. IL-6 secretion can be increased with two to three fold higher rates by oligomerization of TWEAK at that concentration (Figure 17B).

4.5.2 Phosphorylation of IκBα by trimeric, oligomerized and hexameric TWEAK

Since I detected IL-8 secretion upon TWEAK stimulation, I further analyzed another hallmark of classical NFκB pathway activation, namely phosphorylation and degradation of IκBα.

In the classical NFκB signaling pathway (see 1.3.2.1, Figure 3), the activated IKK complex catalyzes the phosphorylation of IκBα, followed by polyubiquitination at lysine 48 and subsequent proteasomal degradation. This results in the release of NFκB dimers, mostly consisting of p50 and RelA, that translocate into the nucleus, bind DNA and activate gene transcription (Ghosh and Karin, 2002). This process is associated with increased expression of chemokines like IL-8, cytokines like IL-6, and enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (Elewaut et al., 1999; Ghosh et al., 1998).

Regarding the results in 4.5.1 (Figure 17), one would expect different efficiencies for trimeric and oligomerized or hexameric TWEAK to induce IκBα phosphorylation.

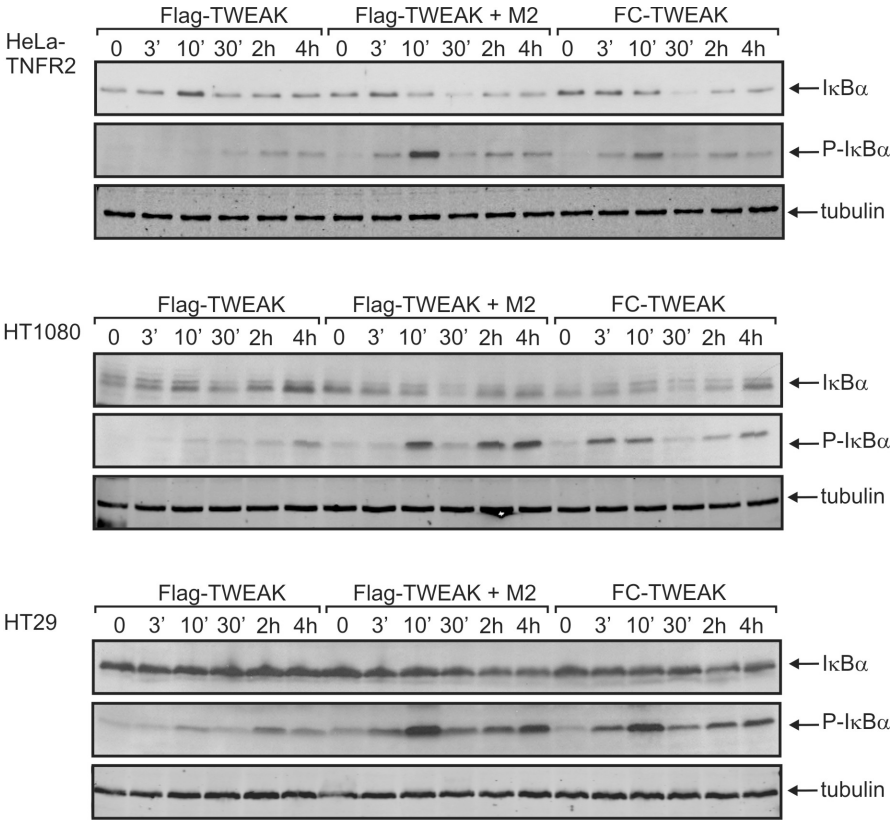

Figure 18 Phosphorylation and degradation of IκBα is induced by oligomerized TWEAK
Different cell lines were stimulated for the indicated time with 200 ng/ml trimeric Flag-TWEAK, with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or hexameric FC-TWEAK. After that, cells were harvested and lysed for analysis in western blot. Blots were probed with antibodies against IκBα and phospho-IκBα. A tubulin-specific antibody was used to control loading.

Indeed, on all investigated cell lines, TWEAK had to be oligomerized or hexameric to properly induce phosphorylation of IκBα. In cells stimulated with oligomerized or hexameric TWEAK phosphorylated IκBα was detectable after 3 to 10 minutes. Accordingly, degradation of IκBα was detectable after 30 minutes of stimulation. Phosphorylation of IκBα is sustained and/or reached another apex after two to four hours of stimulation with oligomerized or hexameric TWEAK (Figure 18). Trimeric TWEAK induced only weak phosphorylation of IκBα after two to four hours of stimulation which is responsible for weak IL-8 induction I saw before (Figure 17).

4.5.3 Phosphorylation of other signaling molecules by oligomerized TWEAK

Besides induction of cell death and NFκB pathways, TWEAK has been shown to activate mitogen activated protein kinase (MAPK) pathways (Ando et al., 2006), presumably through TRAF-mediated mechanisms. There are three major mitogen activated protein kinase

(MAPK) signaling pathways: the extracellular signal-related kinases (ERK), the p38 mitogen activated protein kinases (p38) and c-Jun N-terminal kinases (JNK) (see 1.3.3).

AKT is a serine-threonine protein kinase and acts as a signaling molecule in a Ras-dependent pathway, independent of the MAP kinases (Franke et al., 1995) (see 1.3.4). Active AKT may be able to inhibit phosphorylation of JNK and p38 phosphorylation by inhibition of upstream kinases MKK 4 and ASK-1 (Song and Lee, 2005).

The human cervix carcinoma cell line HeLa-TNFR2, colon adenocarcinoma cell line HT29 and the keratinocyte cell line KB were stimulated with oligomerized TWEAK and examined for activation of the afore mentioned pathways.

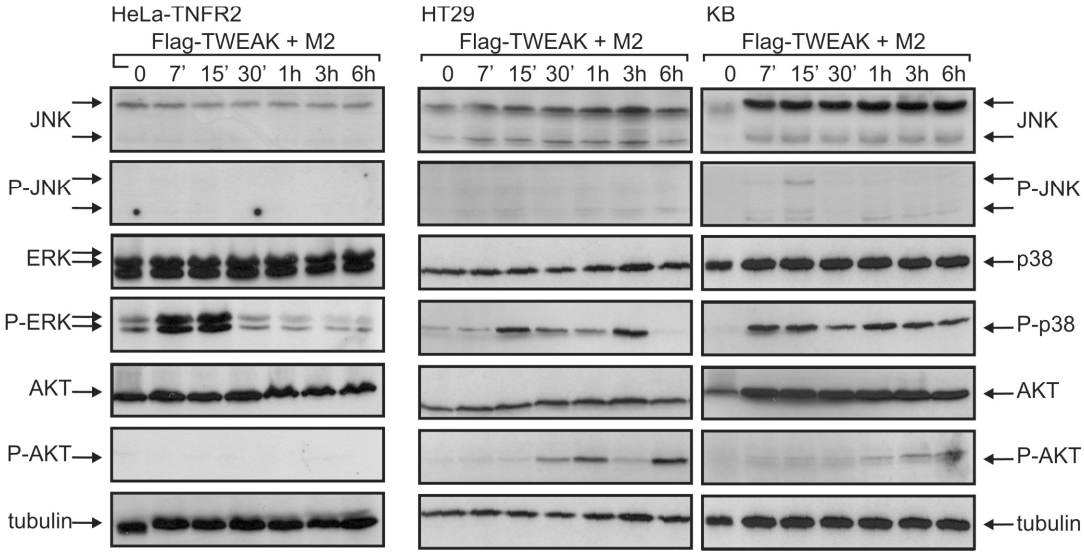

Figure 19 TWEAK induced phosphorylation of kinases
Different cell lines were incubated with 200 ng/ml Flag-TWEAK or with anti-Flag mAb M2 (0.5 µg/ml) for the indicated time. After that, cells were harvested and lysed for analysis in western blot. Blots were probed with antibodies against JNK and phospho-JNK, ERK and phospho-ERK, p38 and phospho-p38, AKT and phospho-AKT. Tubulin was detected as a load control.

None of the three tested cell lines showed strong activation of the JNK signaling pathway. Only a slight signal could be detected in the keratinocyte cell line KB after 15 minutes of stimulation with oligomerized TWEAK. However, there is strong activation of ERK signaling pathway in HeLa-TNFR2 cells after 7 to 15 minutes and strong activation of p38 signaling pathway in HT29 and KB cells after 7 to 15 minutes. p38 activation is sustained in these two cell lines, whereas ERK signaling is terminated in HeLa-TNFR2 cells after 15 to 30 minutes (Figure 19).

HT29 is the only cell line showing clear and strong AKT phosphorylation and activation with two activity peaks after 1 and 6 hours of TWEAK stimulation. Since AKT is known to have the ability to inhibit phosphorylation of JNK and p38 phosphorylation, this may contribute to the decreasing phosphorylation of p38 in HT29 cells at exactly these points in time of TWEAK stimulation (Figure 19).

4.5.4 Hexameric and oligomerized TWEAK induces TRAF2 degradation

The cytoplasmic domain of human Fn14 protein contains a single, putative TRAF-binding site. Recruitment of one or more of the six known TRAFs to these sites result in the activation of signaling pathways that generally function to promote cellular survival and proliferation (Inoue et al., 2000; Wajant et al., 1999). Direct association between the cytoplasmic domain of Fn14 and TRAF1, 2, 3, and 5 has been demonstrated *in vitro* (Brown et al., 2003).

TRAF2 is a crucial component of almost the entire TNFR superfamily-induced signaling pathways. It initiates important downstream signaling events, such as the activation of the NFκB family of transcription factors and activation of MAP kinase cascades (Song et al., 1997).

Furthermore, TRAF2 is known to get degraded during signaling (Duckett and Thompson, 1997). To elicit if Fn14-mediated TWEAK signaling has an effect on TRAF2 levels in the cell, lysates of TWEAK-stimulated cells were tested in western blot analysis.

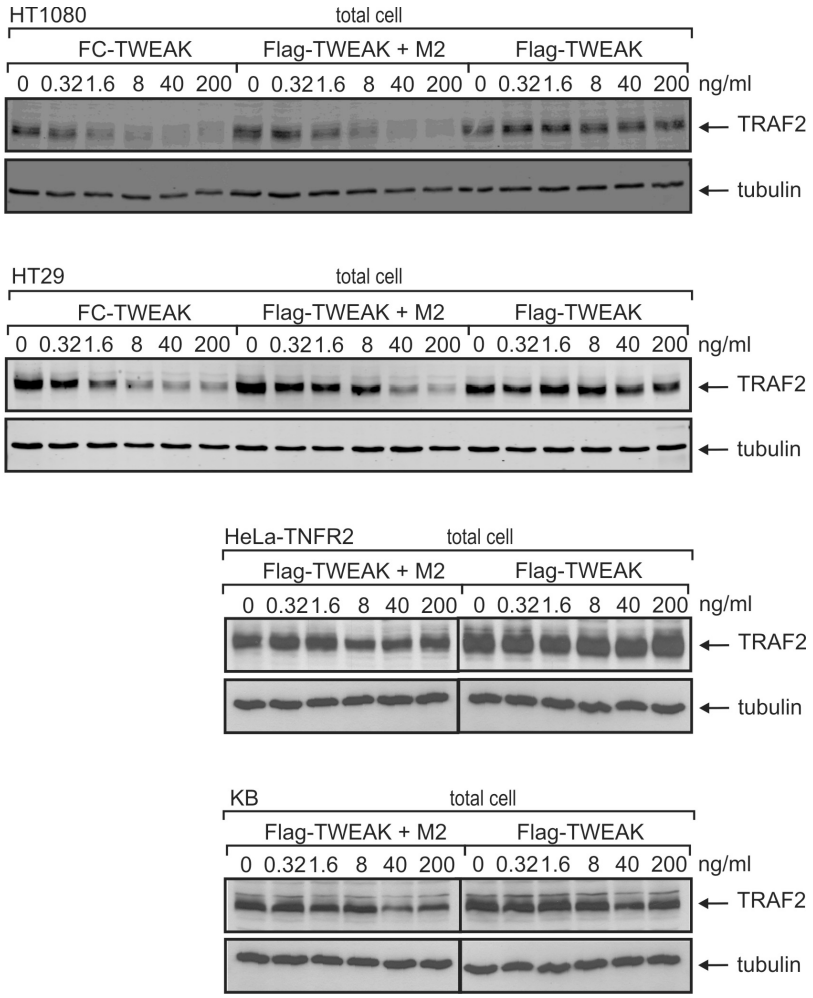

Figure 20 TRAF2 is degraded upon stimulation with oligomerized TWEAK
Different cell lines were incubated with indicated concentrations of hexameric FC-TWEAK, anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or trimeric Flag-TWEAK for 18 hours. After that, cells were harvested and lysed for analysis in western blot. Blots were probed with antibodies against TRAF2 and tubulin as a load control.

In all investigated cell lines, TRAF2 was degraded after stimulation with hexameric or oligomerized TWEAK in a dose dependent manner after 18 hours. These findings provide evidence for involvement of TRAF2 in TWEAK-induced signaling pathways on these cell lines (Figure 20).

Since trimeric TWEAK was shown to have no or only very weak signaling competence until now, it was expected that there is no TRAF2 degradation after stimulation with trimeric TWEAK. Indeed, the investigation of total cell lysates reveals no change in TRAF2 levels after 18 hours of stimulation with trimeric Flag-TWEAK (Figure 20).

4.5.5 Trimeric TWEAK induces TRAF2 depletion from cytoplasm

Before degradation, TRAF2 is recruited to the intracellular domain of stimulated receptors (Duckett and Thompson, 1997). Since trimeric TWEAK showed only weak abilities in induction of both apoptotic and inflammatory effects (Figure 13, 14, 17 and 18), I wondered weather TRAF2 was recruited to the Fn14 receptor upon treatment with trimeric TWEAK. To this end, cell lysates containing only cytoplasmic components were prepared. All membrane bound particles are triton X insoluble and were precipitated. Triton X soluble supernatants containing cytoplasmic proteins were tested for TRAF2 content.

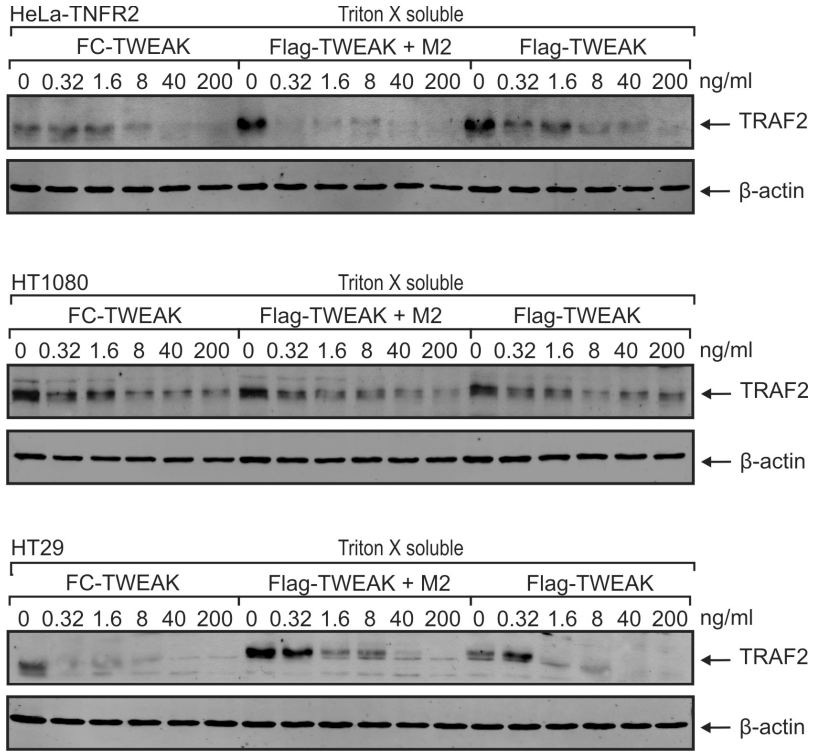

Figure 21 TRAF2 is depleted by trimeric and oligomerized TWEAK stimulation
Different cell lines were incubated with indicated concentrations of hexameric FC-TWEAK, anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or trimeric Flag-TWEAK for 18 hours. After that, cells were harvested and lysed for analysis of cytoplasmic proteins in western blot. Blots were probed with antibodies against TRAF2 and tubulin as a load control.

These results showed that even trimeric, soluble TWEAK depletes TRAF2 from cytoplasm. TRAF2 is recruited and bound to the intracellular portion of Fn14 and therefore disappearing from the cytoplasmic fraction of cell lysates. Thus, both trimeric and hexameric/oligomerized TWEAK recruits TRAF2 from cytoplasm (Figure 21). The difference between stimulation with trimeric and oligomerized or hexameric TWEAK is that trimeric TWEAK does not induce TRAF2 degradation.

Depletion of TRAF2 from cytoplasm and recruitment to Fn14 was a hint for possible signaling activity of soluble TWEAK. The activation of the classical NFκB by oligomerized or hexameric TWEAK was concluded upon the observation of IL-8 and IL-6 secretion as well as phosphorylation and degradation of IκBα (Figure 17 and 18).

4.5.6 Activation of noncanonical NFκB by trimeric and oligomerized TWEAK

Another property of TWEAK is the activation of the noncanonical NFκB pathway (Brown et al., 2003; Saitoh et al., 2003). Therefore, differences or similarities between trimeric and oligomerized TWEAK regarding the activation of the noncanonical NFκB pathway was examined.

Activation of the noncanonical NFκB pathway includes NFκB2/p100, which is phosphorylated (Senftleben et al., 2001a), polyubiquitinylated and proteasomal processed to a p52 form. Detection of processing of p100 to p52 was used in this work to monitor activation of the noncanonical NFκB pathway.

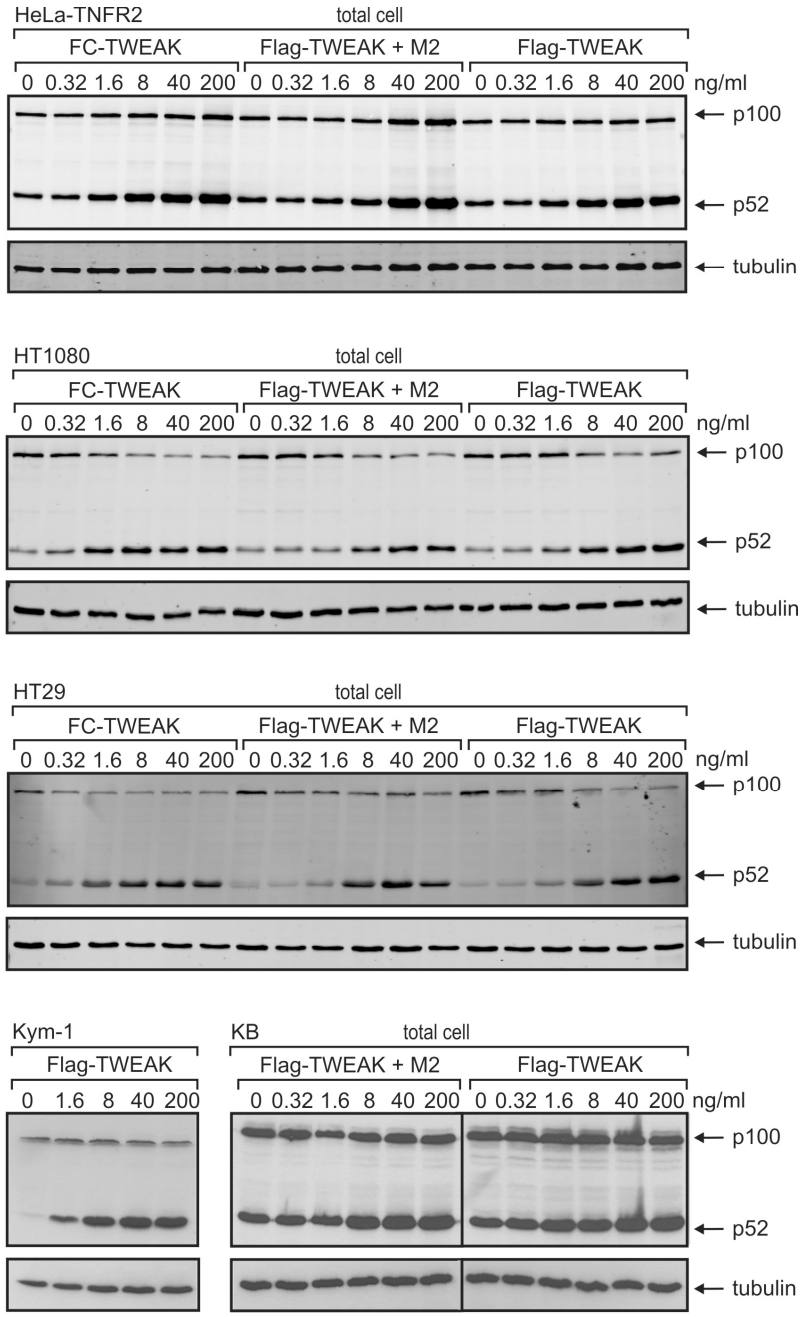

Figure 22 p100 processing is induced by trimeric and oligomerized TWEAK stimulation
Different cell lines were incubated with indicated concentrations of hexameric FC-TWEAK, anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or trimeric Flag-TWEAK for 18 hours. After that, cells were harvested and lysed for analysis in western blot. Blots were probed with antibodies against p52 and tubulin as a load control.

On all tested cell lines, the noncanonical NFκB pathway is activated after TWEAK stimulation in a dose dependent manner. This is shown in decreasing signals for p100 precursor protein and increasing amounts of p52 protein (Figure 22).

The cell lines used in this study react with classical NFκB activation, measured in IL-8 secretion and phosphorylation of IκBα (Figure 17 and 18) and noncanonical NFκB activation.

The keratinocyte cell line KB, which does not react with classical NFκB activation upon TWEAK stimulation, shows clear reaction with noncanonical NFκB activation after TWEAK stimulation.

Importantly, trimeric Flag-TWEAK showed the same ability in induction of p100 procession as oligomerized Flag-TWEAK or hexameric FC-TWEAK (Figure 22).

4.5.7 Fn14-specific antibody inhibits TWEAK-induced inflammatory responses

To determine if the TWEAK receptor Fn14 was required for induction of both NFκB signaling pathways, the antagonistic antibody ITEM-4 against Fn14 was used and responses to TWEAK stimulation were detected.

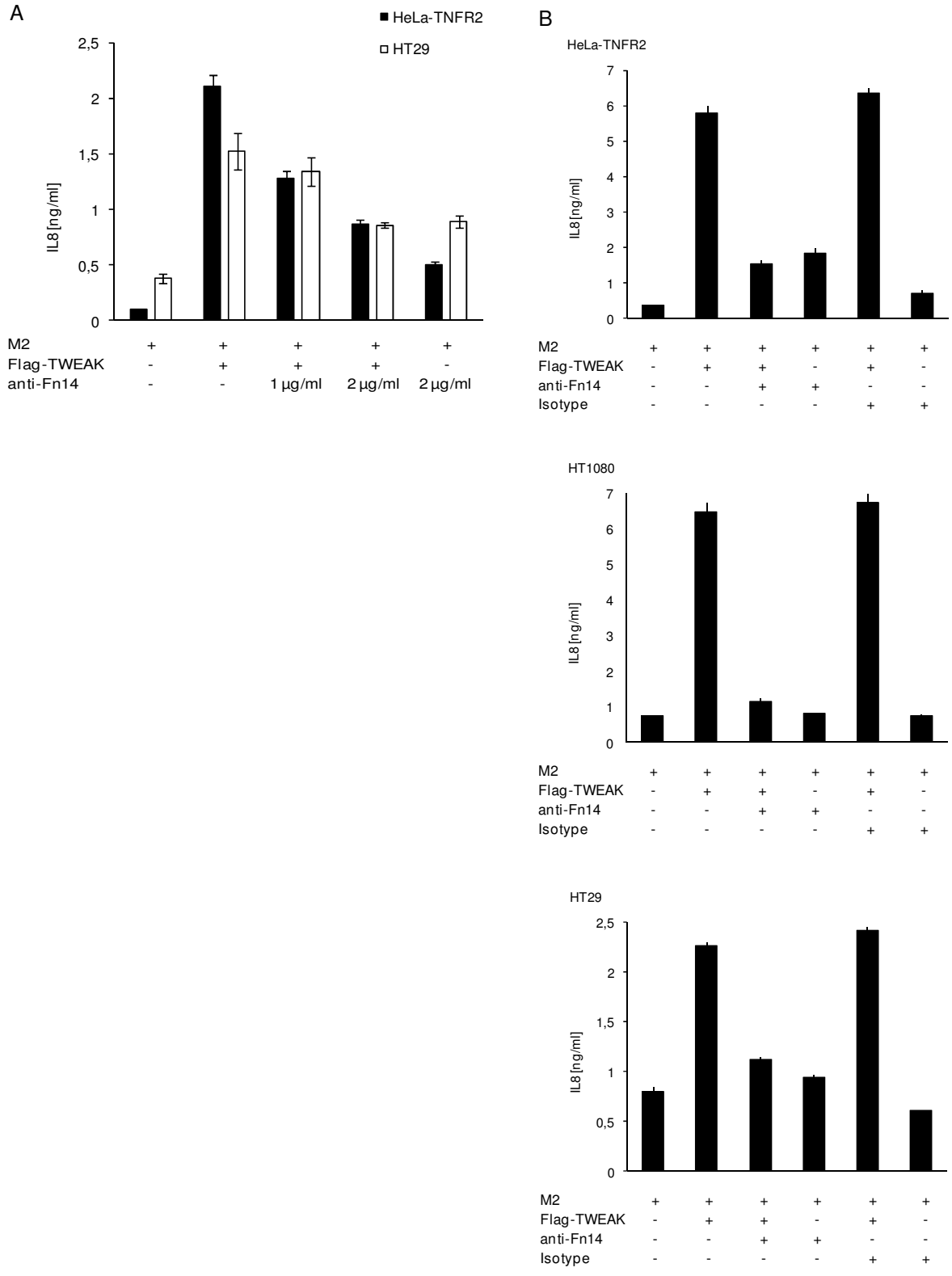

Figure 23 Blocking of Fn14 with ITEM-4 is dose dependent and Fn14-specific
A: Different cell lines were stimulated with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK (40 ng/ml) in triplicates. Some of the groups were pretreated with the indicated concentrations of Fn14-specific antibody ITEM-4. After 24 hours supernatants were collected and probed for IL-8. B: Different cell lines were stimulated with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK (40 ng/ml) in triplicates. Some of the groups were treated with Fn14-specific antibody ITEM-4 (2 µg/ml) or the appropriate Isotype IgG2b (2 µg/ml) alone or prior stimulation. After 24 hours supernatants were collected and probed for IL-8.

IL-8 secretion induced by oligomerized Flag-TWEAK was inhibited by anti-Fn14 antibody ITEM-4 in a dose dependent manner, despite the fact that antagonistic Fn14 antibody ITEM-4 induced weak IL-8 secretion on both cell lines in the highest concentration (2 µg/ml). Indeed, ITEM-4 reduced TWEAK-induced IL-8 secretion to 50 to 75 % (Figure 23A). On HT1080 cell line, reduction of IL-8 secretion by blocking Fn14 with ITEM-4 antibody even reached more than 80 % (Figure 23B).

To verify that this effect was mediated by specific blocking of Fn14 and not by any unspecific, antibody-mediated activity, an isotypic antibody was used in the same concentration as the Fn14-specific antibody (Figure 23B). This isotype control antibody showed no inhibiting effect on TWEAK-induced IL-8 secretion on all tested cell lines.

In order to determine whether activation of the noncanonical NFκB pathway is also mediated by Fn14, human colon adenocaorcinoma cell line HT29 and fibrosarcoma cell line HT1080 were stimulated with trimeric Flag-TWEAK, oligomerized Flag-TWEAK or hexameric FC-TWEAK. All of these TWEAK variants are able to induce p100 processing (Figure 22). To prove the involvement of Fn14 in this effect, too, again ITEM-4 was used.

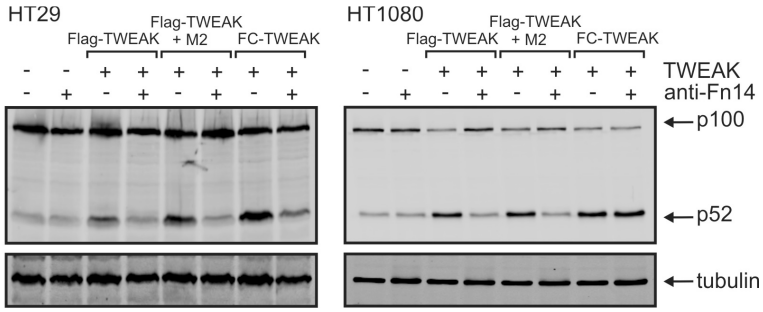

Figure 24 Blocking of Fn14 receptor inhibits TWEAK-induced p100 processing
Different cell lines were incubated with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK (200 ng/ml) for 4.5 hours. Some of the groups were treated with anti-Fn14 antibody ITEM-4 (2 µg/ml) 30 minutes prior stimulation. After that, cells were harvested and lysed for analysis in western blot. Blots were probed with an antibody against p52. A tubulin-specific antibody was used to control loading.

It was shown that TWEAK-induced Fn14 signaling can be effectively inhibited by an Fn14-specific antibody. In case of trimeric and oligomerized TWEAK Fn14 blocking leads to total inhibition of p100 processing like seen in untreated cells or cells incubated with Fn14 antibody alone. So in this case the Fn14 antibody exhibits no agonistic effects. Hexameric TWEAK-induced p100 processing was markedly reduced when Fn14 was blocked. However, stimulation with hexameric FC-TWEAK causes still some p100 processing even in Fn14 blocked cells (Figure 24).

4.6 The role of TRAF1 in TWEAK signaling

TRAF1 is known to have both enhancing and inhibiting effects regarding TNF-induced NFκB activation (Schwenzer et al., 1999; Tsitsikov et al., 2001). Moreover, it was shown that TRAF1 interacts with TRAF2 and inhibits TNF-induced caspase-8 activation (Speiser et al., 1997; Wang et al., 1998). One possible mechanism of TRAF1-mediated inhibition of TNF signaling is that TRAF1 competes with TRAF2 for binding to TNFR2. Another possibility is that TRAF1 binding to TRAF2 forms an inactive heterodimer (Lee and Lee, 2002).

Furthermore, TRAF1 is not only capable of regulating the NFκB pathway and apoptosis; it is also regulated by these pathways itself. TRAF1 expression is enhanced after NFκB induction and caspase-8 cleaves TRAF1 specifically (Fotin-Mleczek et al., 2004; Henkler et al., 2003; Irmler et al., 2000; Schwenzer et al., 1999; Wang et al., 1998). The resulting fragment works as a general inhibitor for NFκB (Henkler et al., 2003).

Since activation of both classical and noncanonical NFκB signaling was observed, I wanted to bring to light which effect enhanced TRAF1 expression in these cell lines may have on TWEAK-induced responses. To this end, transfectants of human adenocarcinoma cell line HT29 and cervix carcinoma cell line HeLa-TNFR2 expressing enhanced levels of endogenous TRAF1, comparable to the TRAF1 level of TNF stimulated cells, were used.

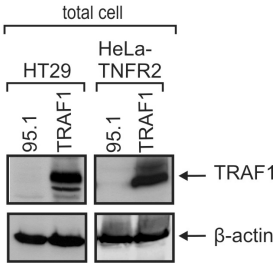

Figure 25 Expression level of TRAF1 in TRAF1 transfectants
Empty vector control cells (95.1) and transfectants with enhanced TRAF1 levels (TRAF1) of different cell lines were harvested and lysed for analysis in western blot. Blots were probed with antibodies against TRAF1 and tubulin as a load control.

The enhanced expression of TRAF1 in these two cell lines was tested in western blot. While TRAF1 is not detectable in untransfected, unstimulated cells, TRAF1 transfected cells show massive expression of the protein (Figure 25).

4.6.1 TWEAK-induced effects on TRAF2 in TRAF1 expressing cells

TRAF2 depletion from cytoplasm after TWEAK stimulation was seen before (Figure 21). To see if enhanced TRAF1 expression has an effect on TRAF2 depletion, cytoplasmic lysates were prepared from the human tumor cell line HeLa-TNFR2. To this end, empty vector control cells HeLa-TNFR2 95.1 were compared with transfectants expressing enhanced TRAF1 levels regarding the reaction upon TWEAK stimulation.

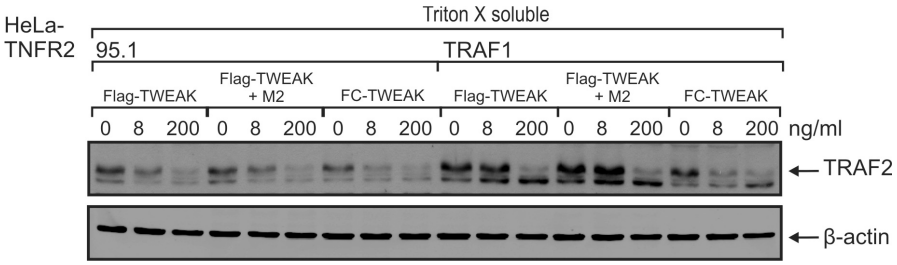

Figure 26 TRAF2 is stabilized in TRAF1 expressing cells
HeLa-TNFR2 vector control cells (left) and transfectants expressing enhanced TRAF1 levels (right) were stimulated with indicated concentrations of trimeric Flag-TWEAK, anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or hexameric FC-TWEAK for 18 hours. After that, cells were harvested and lysed for analysis of cytoplasmic proteins in western blot. Blots were probed with an antibody against TRAF2. A β-actin-specific antibody was used to control loading.

Cells expressing enhanced levels of TRAF1 showed higher TRAF2 levels than in empty vector control. In addition, TWEAK-induced TRAF2 depletion from cytoplasm was delayed and did not reach the level of depletion in empty vector control cells. With a concentration of 8 ng/ml TWEAK, in empty vector control cells TRAF2 already disappeared from the cytoplasm, whereas no effect was observed in TRAF1 expressing cells stimulated with this concentration of TWEAK. High concentrations of TWEAK, however, induced TRAF2 depletion also in TRAF1 expressing cells (Figure 26).

Since TRAF2 is involved in the activation of both, the classical and noncanonical NFκB pathway, TRAF2 stabilisation in cells expressing enhanced TRAF1 levels (Figure 26) is likely to have an impact on TWEAK-mediated NFκB activation. So, further experiments were performed to resolve if TRAF1-mediated TRAF2 stabilization affects NFκB activity.

4.6.2 Effects of TRAF1 expression on TWEAK-induced NFκB activation

Firstly, activation of noncanonical NFκB pathway after TWEAK stimulation in empty vector control cells and cells expressing enhanced TRAF1 levels was investigated. For that

purpose, cells were stimulated with trimeric, oligomerized or hexameric TWEAK proteins and the processing of p100 to p52 was detected subsequently.

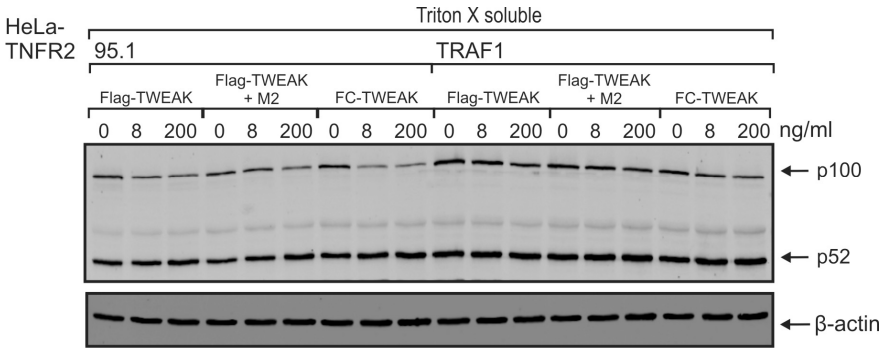

Figure 27 TWEAK-induced p100 processing is slightly affected by enhanced TRAF1 expression
HeLa-TNFR2 transfectants were stimulated with indicated concentrations of trimeric Flag-TWEAK, anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or hexameric FC-TWEAK for 18 hours. After that, cells were harvested and lysed for analysis of cytoplasmic proteins in western blot. Blots were probed with antibodies against p52 and β-actin was used as a load control.

Empty vector transfected human cervical cancer cell line HeLa-TNFR2 95.1 was compared with transfectants expressing enhanced TRAF1 levels with respect to activation of noncanonical NFκB signaling after TWEAK stimulation. There seems to be a slight difference between empty vector control cells and TRAF1 expressing cells in activation of noncanonical NFκB pathway after stimulation with trimeric, oligomerized or hexameric TWEAK. In HeLa-TNFR2 TRAF1 expressing cells there seems to be slightly more p100 and p52 in general. However, this effect is very weak (Figure 27). TRAF1 expression and resulting TRAF2 stabilization might have an enhancing effect on noncanonical NFκB activation after TWEAK stimulation.

Besides the noncanonical NFκB pathway TRAF2 is also involved in the classical NFκB pathway. Therefore it was interesting to see whether TRAF1-mediated stabilization of TRAF2 affects activation of classical NFκB pathway in a stimulatory or inhibitory way. To this end, phosphorylation and degradation of IκBα was detected as a hallmark for activation of the classical NFκB pathway after stimulation with trimeric, oligomerized or hexameric TWEAK.

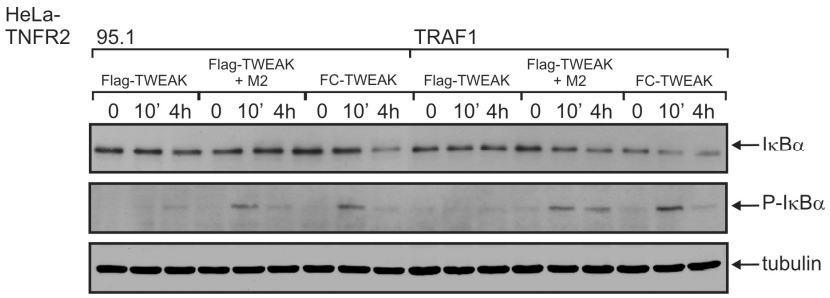

Figure 28 Enhanced phosphorylation of IκBα in TRAF1 expressing cells after TWEAK stimulation
HeLa-TNFR2 transfectants were stimulated for the indicated time with 200 ng/ml trimeric Flag-TWEAK, anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK or hexameric FC-TWEAK. After that, cells were harvested and lysed for analysis of phosphorylated proteins in western blot. Blots were probed with antibodies against IκBα and phospho-IκBα. A tubulin-specific antibody was used to control loading.

In this analysis cells expressing enhanced TRAF1 levels show clear differences compared to their empty vector control cells. Since trimeric Flag-TWEAK does not activate the classical NFκB pathway, no phosphorylation of IκBα is detectable in this stimulation in all cells. However, stimulation with oligomerized Flag-TWEAK leads to sustained phosphorylation of IκBα in TRAF1 expressing HeLa-TNFR2 cells whereas in empty vector control cells the signal is terminated. Stimulation with hexameric FC-TWEAK results in stronger phosphorylation of IκBα compared to that in empty vector control cells and consequently, stronger degradation of IκBα is detectable in TRAF1 expressing HeLa-TNFR2 cells (Figure 28).

4.6.3 TRAF1 expression leads to stronger IL-8 secretion after TWEAK stimulation

The finding that enhanced TRAF1 expression leads to stronger phosphorylation and degradation of IκBα means stronger activation of the classical NFκB pathway. So cells expressing TRAF1 should react with stronger IL-8 secretion after TWEAK stimulation compared to their empty vector transfectants.

In order to prove this suggestion, cervix carcinoma cell line HeLa-TNFR2 and colon adenocarcinoma cell line HT29 empty vector transfectants and TRAF1 expressing transfectants were stimulated with trimeric Flag-TWEAK, oligomerized Flag-TWEAK and hexameric FC-TWEAK and levels of IL-8 secretion were compared in IL-8 ELISA.

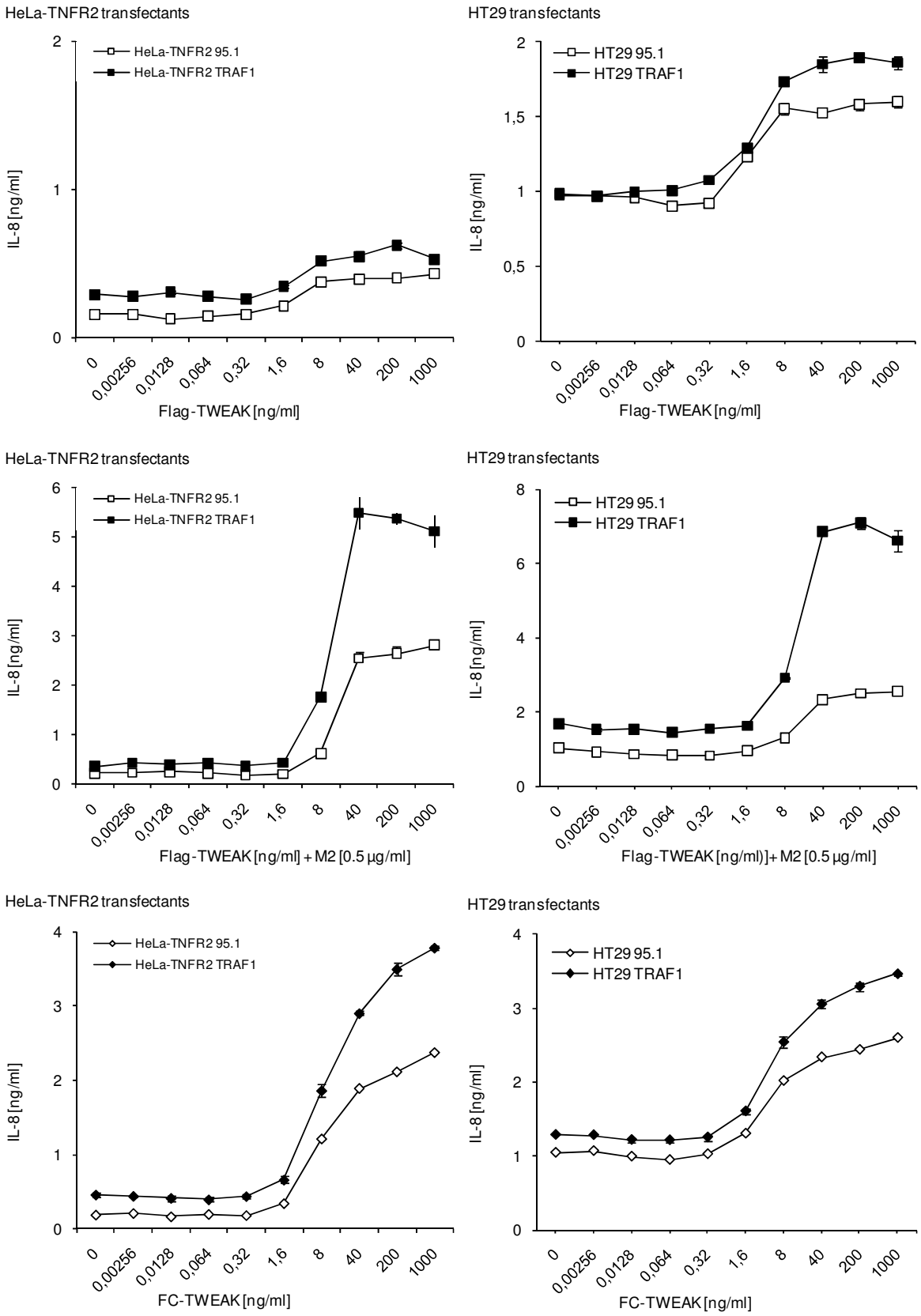

Figure 29: IL-8 secretion is amplified in TRAF1 expressing cells after TWEAK stimulation
HeLa-TNFR2 and HT29 vector control cells (95.1, empty symbols) and TRAF1 expressing cells (filled symbols) were stimulated with trimeric (left column) or with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK (middle column) or hexameric FC-TWEAK (right column) in the indicated concentrations in triplicates. After 24 hours, supernatants were collected and probed for IL-8.

Measurement of IL-8 concentrations in cultivation media provided evidence for this suggestion. TRAF1 overexpressing cells produce two to three times more IL-8 when stimulated with oligomerized Flag-TWEAK. In the case of stimulation with hexameric FC-TWEAK the difference between TRAF1 expressing and empty vector control cells is not that strong, but significant. Trimeric Flag-TWEAK is not able to induce IL-8 secretion on HeLa-TNFR2 cells, but there is weak induction of IL-8 secretion on HT29 cells. The enhancing effect of TRAF1 expression is weak but still visible (Figure 29).

4.6.4 TRAF1 expression attenuates TWEAK-induced cell death

Enhanced expression of TRAF1 showed enforcing effects regarding TWEAK-induced activation of classical NFκB pathway. Activation of the classical NFκB pathway is associated with increased transcription of genes encoding proinflammatory and antiapoptotic mediators (Elewaut et al., 1999; Ghosh et al., 1998). As a conclusion, cells with enhanced TRAF1 expression may be protected from TWEAK-induced cell death due to their stronger inflammatory responses. In order to test this suggestion, TRAF1 expressing and empty vector control transfectants of human colon adenocarcinoma cell line HT29 were sensitized for cell death induction and stimulated with trimeric and oligomerized Flag-TWEAK or hexameric and oligomerized FC-TWEAK.

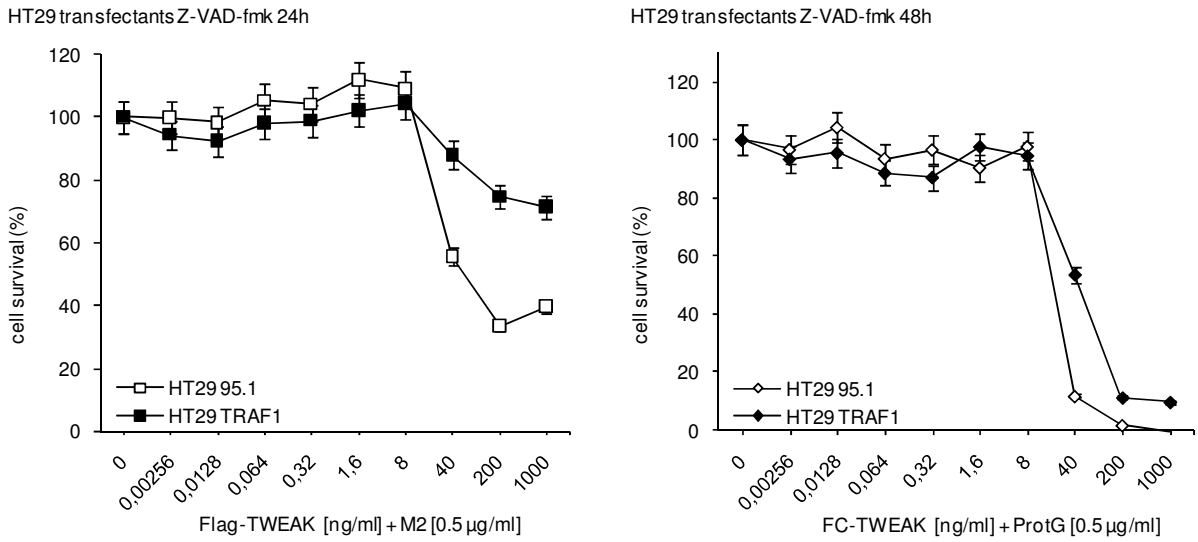

Figure 30 TWEAK induced cell death is inhibited in TRAF1 expressing cells
HT29 vector control cells (95.1, empty symbols) and TRAF1 expressing cells (filled symbols) were stimulated with anti-Flag mAb M2 (0.5 µg/ml) oligomerized Flag-TWEAK (left picture) and with protein G (0.5 µg/ml) oligomerized FC-TWEAK (right) in the indicated concentrations in triplicates. To sensitize cells, they were pretreated with Z-VAD-fmk (40 µM) 30 minutes prior stimulation After 24 or 48 hours, cell viability was measured by staining surviving cells with methyl violet (left)

The results indeed show that TRAF1 expression has an inhibiting effect on cell death induction after TWEAK stimulation on HT29 cells. Empty vector control cells died to a comparable extent regarding the parental HT29 cell line (Figure 15) if stimulated with oligomerized Flag-TWEAK. Cell survival rate on cells expressing enhanced TRAF1 levels under stimulation with oligomerized Flag-TWEAK is about 70 %, whereas only 30 % of 95.1 empty vector transfected cells survive that treatment. With oligomerized FC-TWEAK, the TRAF1 protective effect was lower but still detectable. The latter may be due to the longer incubation time used in this experiment (Figure 30).

5 Discussion

5.1 Activities of trimeric and oligomerized TWEAK

Ligands of the TNF family occur as transmembrane molecules, but also as soluble variants (Bodmer et al., 2002; Locksley et al., 2001). Generally, the transmembrane and the soluble forms of the ligands both interact with their corresponding receptor(s). The receptors engaged by ligands of the TNF family are structurally related and are grouped together in the TNF receptor superfamily (Locksley et al., 2001). Few of the TNF receptors (e.g. TNFR1, BAFFR1 and TRAILR1) are robustly activated by both, their soluble and membrane bound ligand (Grell et al., 1995; Wajant et al., 2001). However, some members of the TNF receptor superfamily are not or only poorly activated upon binding of the soluble, trimeric form of their ligand, whereas the corresponding transmembrane ligand causes strong activation of the receptor. This behaviour was shown for the ligand-receptor pairs TNF and TNFR2, TRAIL and TRAILR2, FasL and Fas, OX40L and OX40, CD27L and CD27, 4-1BBL and 4-1BB and APRIL and TACI (Bossen et al., 2008; Grell et al., 1995; Muller et al., 2008; Samel et al., 2003; Wajant et al., 2001; Wyzgol et al., 2009). Interestingly, most of the inactive or poorly active soluble TNF ligand trimers gain high activity when oligomerized. Oligomerization can be achieved by the use of cross-linking antibodies or by genetic fusion with an appropriate multimerization domain, e.g. the FC portion of immunoglobulin-G (Wajant et al., 2005).

The question whether a particular TNF receptor is activated by its corresponding soluble ligand is of relevance for the development of recombinant TNF ligand variants. Though, differences in activation of the corresponding receptor by soluble or membrane bound ligands are even more important for the understanding of the biological function of this pair. Therefore, this issue was addressed for TWEAK and its receptor Fn14 in this work.

5.1.1 Cell binding of different TWEAK variants

A study for TWEAK/Fn14 interaction revealed an interaction affinity constant (*K*d) of ~2.4 nM (Wiley et al., 2001). This *K*d value is consistent with the measured receptor-binding affinities of other TNF-like cytokines. This study was done with full length human TWEAK expressed on CV1/EBNA monkey kidney epithelial cells on which then binding capacity for Fn14-FC protein was measured (Wiley et al., 2001). In the present study, Fn14 expressing cells were tested for the binding of trimeric, oligomerized or hexameric TWEAK. These tests revealed different binding affinities for the TWEAK proteins to Fn14. The weakest binding occours with trimeric Flag-TWEAK which can be enhanced upon oligomerization and the strongest binding

is given with hexameric FC-TWEAK (Figure 11). These findings correlate with the differences between trimeric and aggregated TWEAK in activation of cellular responses and might be part of the explanation for these differences.

5.1.2 Differences in cell death induction

It was shown before that TWEAK has different abilities to induce cell death on different cell lines (see 1.5.5.2). In some cases, these differences may contribute to the organisation of TWEAK, meaning whether it is soluble, trimeric or oligomerized and mimicking the membrane bound form. So in this work, some cell lines were investigated towards their sensitivity for TWEAK-induced cell death.

The organisation of the proteins determined in HPLC implies that Flag-TWEAK efficiently formed trimers whereas FC-TWEAK assembled into hexameric molecules or inactive or weakly active HMW complexes (Figure 6, 7 and 8). Testing these proteins for cell death induction showed that trimeric Flag-TWEAK is not or only poorly active while oligomerization enhances the ability of TWEAK to induce cell death. Hexameric FC-TWEAK is as active as oligomerized Flag-TWEAK in cell death induction (Figure 12 to 15). Blocking Fn14 with the antagonistic antibody inhibited cell death induction and proved that these effects are Fn14-mediated (Figure 16).

The signaling mechanisms underlying TWEAK-induced apoptosis are completely unexplored except for one case wherein TWEAK induces apoptosis indirectly through secondary activation of the TNF/TNFR1 pathway (Schneider et al., 1999b). Given the absence of death domain in Fn14, it is tempting to speculate that TWEAK may also utilize the recently described TRAF3-mediated apoptosis-inducing complex formation that is responsible for the LTβ receptor triggered cell death (Kuai et al., 2003).

5.1.3 Differences in activation of classical NFκB signaling

The recent demonstration that TWEAK, but not TNF, is capable of inducing prolonged NFκB activation through temporally regulated biphasic activation of canonical and noncanonical NFκB pathways provides a concrete example of the complexity of Fn14 signaling potential (Saitoh et al., 2003). The ability of TWEAK/Fn14 to trigger sustained NFκB activation may also have important implications for the pathway's contribution to the pathogenesis of chronic inflammatory diseases.

Furthermore, *in vivo* studies suggest that TWEAK and Fn14 have a central role in the regulation of innate immunity and wound repair, but might also contribute to a variety of autoimmune diseases. I therefore focused on the question whether trimeric TWEAK on the one hand and oligomerized or hexameric TWEAK on the other hand differ in their capability to activate the proinflammatory NFκB pathways. To this end, the NFκB-inducing activities of recombinantly produced Flag-tagged TWEAK (Flag-TWEAK), anti-Flag monoclonal antibody M2 oligomerized Flag-TWEAK aggregates and hexameric FC-TWEAK were analysed and compared.

Interestingly, the various TWEAK forms had different capabilities to stimulate the classical NFκB pathway. Anti-Flag mAb M2 oligomerized Flag-TWEAK and FC-TWEAK efficiently induced production of IL-8, which is controlled by the classical NFκB pathway, at low concentrations. However, trimeric Flag-TWEAK failed or only modestly stimulated IL-8 production even with the highest concentrations tested (Figure 17). Moreover, the oligomerized TWEAK variants, but not the trimeric TWEAK molecules, induced robust IκBα phosphorylation after a short time (Figure 18). This is in accordance with the concept that oligomerized or immobilized variants of inactive soluble TNF ligands mimic the activity of their highly active transmembrane counterpart. Blocking of Fn14 inhibited these effects, proving that they are mediated by Fn14 (Figure 23).

5.2 No differences in induction of noncanonical NFκB

IKKα is responsible for activation of a specific NFκB factor required for B-cell maturation and formation of secondary lymphoid organs. So the noncanonical NFκB pathway has been assigned to a specific aspect of adaptive immunity (Senftleben et al., 2001a).

In this study all TWEAK proteins induced p100 processing, which is a hallmark of the noncanonical NFκB pathway, starting at low concentrations in a dose dependent manner. Moreover, oligomerization showed no significant effect on the maximal amplitude or the dose-response relationship of p100 processing. As expected, hexameric or oligomerized TWEAK also induced efficient p100 processing (Figure 22). This effect is Fn14-mediated too, since blocking of the receptor results in inhibition of p100 processing (Figure 24). Thus, while classical NFκB signaling is differentially induced by trimeric and oligomerized or membrane TWEAK, activation of the alternative NFκB pathway is comparably accomplished by all TWEAK variants.

Until now, all soluble trimeric TNF ligands analysed were either able to stimulate all receptor activities investigated or were inactive or poorly active in all assays (Bossen et al., 2008;

Grell et al., 1995; Muller et al., 2008; Samel et al., 2003; Wajant et al., 2001). In the latter cases, oligomerization restored all activities of these TNF ligands. Here for TWEAK, it is observed for the first time that different variants of the same TNF ligand induce qualitatively different activities of the corresponding TNF receptor.

This finding raises the question, how the different activity states of Fn14 induced by trimeric TWEAK or hexameric and oligomerized TWEAK is realized at the molecular level. TRAF2 might be crucial in this respect and its role could be elucidated in future studies.

5.3 The role of TRAF2 in NFκB activation

The fact that the Fn14-interacting adapter protein TRAF2 plays a role in classical and noncanonical NFκB signaling brought it into focus for this work (Hayden and Ghosh, 2008; Sun and Ley, 2008).

Actually, trimeric and oligomerized TWEAK induce disappearance of TRAF2 from the cytosolic compartment suggesting interaction of Fn14 with TRAF2 (Figure 21). This happens with a similar dose response-relationship as found before for the induction of p100 processing (Figure 22).

This is in accordance with the current model of activation of the noncanonical NFκB pathway. This model says that TRAF2 associates with high efficacy with cIAP1 and cIAP2 and recruits these E3 ligases to a complex of TRAF3 and the kinase NIK, which can activate IKK1, which in turn triggers processing of p100. The resulting complex of cIAP1, cIAP2, TRAF2, TRAF3 and NIK leads to the ubiquitination of NIK at lysine 48 and thus its constitutively degradation by the proteasome (Figure 3) (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). As a consequence, TRAF2 depletion, e.g. by recruitment to a liganded member of the TNF receptor superfamily allows newly synthesized NIK to escape from degradation and thus enhances p100 processing (Vallabhapurapu et al., 2008; Zarnegar et al., 2008). Therefore, in noncanonical NFκB signaling TRAF2 seems to be activated, but not marked for degradation in cells stimulated with soluble, trimeric TWEAK. In context of activation of the classical NFκB pathway, however, TRAF2 and its associated cIAPs requires complex receptor-associated activating events, e.g. oligomerization, modification or interaction with other proteins to fulfill its signal transducing role (Chen, 2005). So, it appears possible that trimeric TWEAK recruits TRAF2 to Fn14, but fails to stimulate receptor-associated activation and degradation of TRAF2. The latter might be achieved by oligomerized TWEAK and hexameric TWEAK (Figure 31).

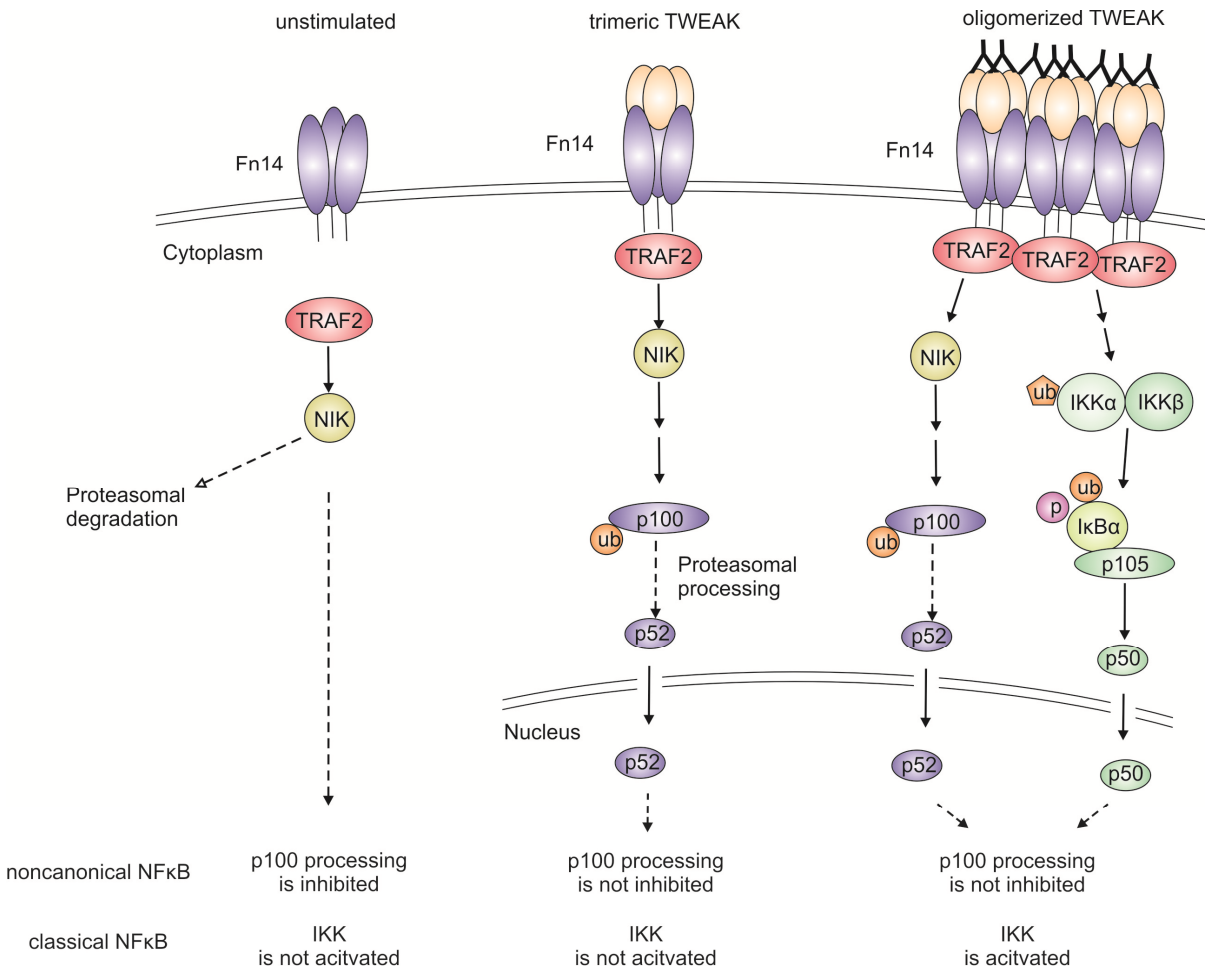

Figure 31 Model of the role of TRAF2 in NFκB activation
TRAF2 is involved in the activation of classical and noncanoncial NFκB signaling upon TWEAK stimulation. TRAF2 promotes the degradation of NIK if it is not recruited to the Fn14 receptor e.g. in unstimulated cells (left) and therefore inhibits the activation of noncanonical NFκB signaling. If recruited to Fn14 e.g. upon the stimulation with soluble, trimeric TWEAK, TRAF2 is no longer able to induce NIK degradation and the noncanonical NFκB signaling pathway is activated (middle). Only upon binding to oligomerized Fn14, e.g. after stimulation with oligomerized or membrane bound TWEAK, TRAF2 is able to promote IKK activation leading to phosphorylation of IκBα and therefore activation of the classical NFκB pathway. Besides, the noncanonical NFκB pathway is not inhibited because of TRAF2 recruitment to Fn14 (right).

5.3.1 Effects of TRAF1 on TRAF2 and activation of NFκB

TRAF1 expression is enhanced after NFκB induction (Schwenzer et al., 1999; Wang et al., 1998) and TRAF1 is known to interact with TRAF2 (Lee and Lee, 2002; Speiser et al., 1997; Wang et al., 1998). To gain more insight into the interaction between TRAF1 and TRAF2 in TWEAK-induced cellular responses, cells expressing enhanced TRAF1 levels were analyzed.

In TRAF1 expressing cells, TRAF2 levels were increased and depletion upon TWEAK stimulation was delayed in comparison to control cells (Figure 26). So TRAF1 might have a stabilizing effect on TRAF2 in this scenario.

Stabilized TRAF2 might affect the activation of NFκB upon TWEAK stimulation. Actually, the phosphorylation of IκBα is stronger and prolonged in TRAF1 expressing cells (Figure 28), consequently, IL-8 secretion in these cells was markedly increased after stimulation with TWEAK (Figure 29). So the classical NFκB pathway is positively affected by TRAF1-mediated TRAF2 stabilization upon TWEAK stimulation. For noncanonical NFκB signaling the effects are just slightly visible. There seems to be more p100 and p52 present in TRAF1 expressing cells, but this result might be not significant (Figure 27).

Furthermore, enhanced classical NFκB signaling in TRAF1 expressing cells might affect TWEAK-induced cell death. In fact, TRAF1 expressing cells are partially protected from TWEAK-induced cell death (Figure 30). Accordingly, TWEAK signaling might use this feedback loop including increased TRAF1 expression and TRAF2 stabilization to amplify NFκB signaling and to prevent cells from cell death.

5.4 Physiological role of soluble and membrane TWEAK

The cytokine TWEAK has overlapping signaling functions with TNFα, but displays a much wider tissue distribution. It is tempting to speculate that the differences in activation of NFκB signaling of trimeric TWEAK and oligomerized TWEAK via Fn14 are of relevance for the spatial and temporal orchestration of immune reactions.

The TWEAK producing, thus potentially membrane TWEAK expressing immune cells themselves might elicit a rather strong, but local proinflammatory response in an autocrine/paracrine fashion by activation of the classical NFκB pathway. More distant cells will respond only to soluble, trimeric TWEAK and thus should show an NFκB response biased towards the noncanonical pathway.

Moreover, we and others have recently shown that trimeric TWEAK-induced TRAF2 depletion reduces the responsitivity of cells towards TNFR1-induced activation of the classical NFκB pathway and as a consequence sensitizes cells for TNFR1-induced cell death (Vince et al., 2008; Wicovsky et al., 2009). So, soluble TWEAK released from the focus of an infection might have the role to desensitize peripheral cells for the proinflammatory cytokines as TNF, produced by activated immune cells in the center of the infection. Therefore, the membrane TWEAK-driven proinflammatory response might keep its local character by the help of soluble TWEAK.

Studies with knockout or transgenic mice showed that the activity of the TWEAK/Fn14 system can be of crucial relevance in a variety of physiological and pathophysiological processes reaching from muscle cell and liver regeneration over angiogenesis and cancer

progression to autoimmune paradigms such as experimental autoimmune encephalomyelitis and systemic lupus erythematosus (Burkly et al., 2007; Winkles, 2008). Future studies could show whether these TWEAK/Fn14-related scenarios can be assigned to the prevalent action of soluble, trimeric TWEAK or membrane TWEAK.

Besides the classical and noncanonical NFκB pathways some other signaling pathways are utilized by Fn14 (Figure 19). It will also be important to learn whether these and other Fn14-associated pathways are also differentially activated by trimeric TWEAK and oligomerized TWEAK and how they contribute to the various functions of TWEAK and Fn14.

6 Summary

TWEAK is a typical member oft he TNF ligand family. Therefore it is initially expressed as a type II transmembrane protein, but a soluble variant can be released by proteolytic processing.

In this work it is shown that oligomerized TWEAK is more competent than soluble, trimeric TWEAK regarding the activation of classical NFκB signaling pathway. However, both TWEAK variants are able to induce depletion of TRAF2 and processing of p100, which are hallmarks for the activation of the noncanonical NFκB pathway. Like other solube TNF ligands with no or poor activity on their corresponding receptor, TWEAK gains high activity upon oligomerization resembling the activity of the transmembrane ligand.

TRAF2 has a key role in TWEAK-induced NFκB signaling. Depletion or degradation of TRAF2 is crucial for activation of the noncanonial or both, the classical and the noncanonical NFκB pathway.

Blocking the TWEAK receptor Fn14 inhibits the activation of NFκB signaling, irrespective of the TWEAK form used for stimulation. This indicates that the different activities of the two TWEAK variants in activation of classical and noncanonical NFκB signaling are not caused by the use of different receptors. Therefore this study on TWEAK is the first reported case where one TNF ligand in different variants induces qualitatively different activities of the corresponding TNF receptor.

7 Zusammenfassung

TWEAK ist ein typischer Vertreter der TNF Ligandenfamilie. TWEAK wird als Typ II Transmembranprotein exprimiert, kann jedoch durch proteolytische Prozessierung auch als lösliches Protein freigesetzt werden.

In dieser Arbeit wird gezeigt, dass oligomerisiertes TWEAK in Hinblick auf die Aktivierung des klassischen NFκB Signalweges deutlich aktiver ist als lösliches, trimeres TWEAK. Jedoch sind beide TWEAK-Varianten in der Lage, die Depletion von TRAF2 und die Prozessierung von p100, beides Kennzeichen für die Aktivierung des alternativen NFκB Signalweges, zu induzieren. Ebenso wie andere lösliche TNF-Liganden, die ihren entsprechenden Rezeptor nur schwach aktivieren, erlangt lösliches TWEAK durch Oligomerisierung vergleichbare Aktivität zum membrangebundenen Liganden.

TRAF2 spielt eine Schlüsselrolle in der TWEAK-vermittelten NFκB Aktivierung. Durch Depletion oder Degradation von TRAF2 fällt die Entscheidung, ob lediglich der alternative oder beide, der klassische und der alternative NFκB Signalweg aktiviert werden.

Die Blockade des TWEAK-Rezeptors Fn14 inhibiert die Aktivierung der NFκB Signalwege, ungeachtet welche Form von TWEAK zur Stimulation genutzt wird. Das weist darauf hin, dass die unterschiedlichen Aktivitäten der beiden TWEAK-Varianten in der Induktion des klassischen und alternativen NFκB Signalweges nicht durch die Nutzung verschiedener Rezeptoren verursacht sind. Damit wird in dieser Arbeit anhand von TWEAK zum ersten mal gezeigt, dass ein TNF Ligand in unterschiedlichen Varianten qualitativ unterschiedliche Aktivitäten des entsprechenden TNF Rezeptors auslöst.

8 Literature

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9 Attachments

9.1 Sequences

Full length human TWEAK

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21   L  V  P  L  A  L  G  L  G  L  A  L  A  C  L  G  L  L  L  A    40
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41   V  V  S  L  G  S  R  A  S  L  S  A  Q  E  P  A  Q  E  E  L    60
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61   V  A  E  E  D  Q  D  P  S  E  L  N  P  Q  T  E  E  S  Q  D    80
    cctgcgcctttcctgaaccgactagttcggcctcgcagaagtgcacctaaaggccggaaa
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201  S  S  L  G  P  Q  L  R  L  C  Q  V  S  G  L  L  A  L  R  P   220
    gggtcctccctgcggatccgcaccctcccctgggcccatctcaaggctgccccccttcctc
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Flag-TWEAK

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FC-TWEAK

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```

9.2 Abbreviations

Table 4: Abbreviations

ABTS	2,2'-Azinobis-(3-ethylbenzthiazolin-6-sulfonsäure) di-ammonium salt
°C	degree Celsius
µF	micro farad
µg	microgram
µl	microliter
µM	micromolar
4-1BB	ligand of the TNF superfamily
ADAM	adamalysin
AKT	serine-threonine protein kinase
APAF-1	apoptotic peptidase activating factor 1
APRIL	a proliferation-inducing ligand
APS	ammoniumpersulfat
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AU-rich	Adenylate Uridylate rich
BAFF	B-cell activating factor
BAFFR	B-cell activating factor receptor
BCL-2	B-cell leukemia/lymphoma
BCL-W	antiapoptotic BCL-2 family member
BCL-XL	antiapoptotic BCL-2 family member
BCMA	B-cell maturation protein
BH	BCL-2 homology domains
BH3-only protein	proteins containing only the BH3 domain
Bid	BH3 interacting domain death agonist, proapoptotic member of the BCL-2 family
BSA	bovine serum albumin
C2C12	mouse myoblast cell line

Table 4 continued: Abbreviations

caspases	cysteinyl-aspartate-specific proteinases
CD163	scavenger receptor on macrophages
CD27	T-cell activating antigen, TNF receptor family member
CD30	Lymphocyte activating antigen, TNF receptor family member
$CD4^+$ T-cells	cluster of differentiation 4 positive T-cells
CD40	costimulatory protein on antigen presenting cells, TNF receptor family member
CD40L	CD40 ligand expressed from T-cells, TNF ligand family member
$CD8^+$ T-cells	cluster of differentiation 8 positive T-cells
cDNA	complementary DNA
cFLIP	cellular FLICE-like inhibitory protein
CHX	cycloheximide
cIAP1/2	cellular inhibitor of apoptosis protein 1/2
cm	centimeter
cm^2	square centimeter
CO_2	carbon dioxide
CRD	cysteine rich domain
c-Rel	proto-oncogene, NFκB family member
CV1/EBNA	monkey kidney epithelial cells
cyclin D1	cell-cycle regulator
DD	death domain
DISC	death inducing signaling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DR3	death receptor 3
DRs	death receptors
DTT	dithiothreitol
EC	endothelial cells

Table 4 continued: Abbreviations

ECL	enhanced chemiluminescence
ED50	effective dose for 50 % response
EDA	ectodysplasin-A, TNF ligand family member
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ELAM	endothelial-leukocyte adhesion molecule 1
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-related kinases
ERK-1/2	MAP2K, extracellular signal-related kinases 1/2
E-selectin	cell adhesion molecule expressed on endothelial cells
FACS	fluorescence activated cell sorting
FADD	Fas-associated death domain
Fas	TNF receptor family member
FasL	Fas ligand, TNF ligand family member
FC-portion	fragment crystallizable portion of IgG
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FLAG	Flag epitope
Fn14	fibroblast growth factor inducible 14 kDa protein
G0, G1	cell-cycle phase gap0, gap1
G418	Geneticin
GM-CSF	granulocyte-macrophage colony-stimulating factor
HCl	hydrogen chloride
HEK293	human embryonic kidney cell line
HeLa	human cervical cancer cell line
HMW	high molecular weight
Hp-Hb	haptoglobin-hemoglobin complexes

Table 4 continued: Abbreviations

HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSC3	human tumor cell line
HT1080	human fibrosarcoma cell line
HT29	human colon adenocarcinoma cell line
HVEM	herpesvirus entry mediator, TNF receptor family member
IAPs	inhibitor of apoptosis proteins
ICAM-1	intercellular adhesion molecule 1
IFNα	interferon alpha
IFNγ	interferon gamma
IgG	immunoglobulin G
IKKs	inhibitor of kappa B kinases
IKKα (IKK1)	inhibitor of kappa B kinase alpha/1
IKKβ (IKK2)	inhibitor of kappa B kinase beta/2
IKKγ (IKK3, NEMO)	inhibitor of kappa B kinase gamma/3, NFκB essential modulator
IL-1/6/8	interleucine 1/6/8
IP-10	interferon gamma induced 10 kDa protein
IκB	inhibitor of kappa B protein
IκBα	inhibitor of kappa B alpha
JNK	c-Jun N-terminal kinase
KATO-III	human tumor cell line
KB	human keratinocyte cell line
*K*d	interaction affinity constant
kDa	kilo Dalton
Kym-1	human rhabdomyosarcoma cell line
LPS	lipopolysaccharide
LTα	lymphotoxin alpha
LTβ	lymphotoxin beta

Table 4 continued: Abbreviations

M	molar
mA	milliampere
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
MAPKK/MAP2K	mitogen activated protein kinase kinase
MAPKKK/MAP3K	mitogen activated protein kinase kinase kinase
MCF7	human tumor cell line
MCP-1	monocyte chamotactic protein 1
MEK-1/2	MAP2K, MAP/ERK kinase 1/2
min	minutes
MKK-3/6	MAP2K 3/6
MKK-4/7	MAP2K 4/7
ml	milliliter
MLKs/ASK1/MEKK-1/4	MAP3K5, apoptosis signal-regulating kinase 1
MLKs/TAK/ASK1	MAP3K, TGF-beta activated kinase
mM	millimolar
mm	millimeter
MMP-1/2/3/9	matrix metalloproteinase 1/2/3/9
mRNA	messenger-ribonucleic acid
Na_2CO_3	sodium carbonate
Na_2HPO_4	disodium phosphate
NaCl	sodiumchloride
NaH_2PO_4	monosodium phosphate
$NaHCO_3$	sodium hydrogen carbonate
NFκB	nuclear factor kappa B
NFκB1 (p50)	nuclear factor kappa B 1 50 kDa protein
NFκB2 (p52)	nuclear factor kappa B 2 52 kDa protein
ng	nanogram

Table 4 continued: Abbreviations

NGF	nerve growth factor
NIH 3T3	murine fibroblast cell line
NIK	NFκB inducing kinase
NK cells	natural killer cells
nM	nanomol
OX40	TNF receptor family member, expressed on activated antigen presenting cells
OX40L	OX40 ligand, costimulatory protein on dentritic cells, TNF receptor family member
p100	NFκB2 (p52) 100 kDa precursor protein
p105	NFκB1 (p50) 105 kDa precursor protein
p38	p38 mitogen activated protein kinases
p38/MAPK-α/β	MAP2K 38 kDa protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
Pen/Strep	Penicillin/Streptomycin
PGE2	prostaglandin E2
PI3K	phosphoinositide 3-kinase
PKB	protein kinase B
PLAD	pre-ligand assembly domain
Raf	MAP3K, proto-oncogene serine/threonine-protein kinase
RANK	receptor activator of NFκB
RANKL	receptor activator of NFκB ligand
RANTES	regulated on activation normal T-cell expressed and secreted
Ras	rat sarcoma protein superfamily involved in signal transduction
RAW264.7	mouse monocyte/macrophage cell line
RelA (p65)	NFκB family member, 65 kDa protein

Table 4 continued: Abbreviations

RelB	NFκB family member
RHD	Rel homology domain
RIP	receptor interacting protein
rpm	rounds per minute
RPMI 1640	Roswell Park Memorial Institute medium no. 1640
SAPK/JNK-1/2/3	MAP2K, stress-activated protein kinase, c-Jun N-terminal kinase 1/2/3
$SCF^{\beta\text{-}TrCP}$	E3 ubiuquitin ligase
SDS	sodium dodecyl sulfate
SMAC/DIABLO	second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI
SMC	smooth muscle cells
sTWEAK	soluble TWEAK
SW480	human tumor cell line
TACE	TNF alpha converting enzyme
TACI	TNF receptor family member, transmembrane activator and CAML interactor
TANK	TRAF family member-associated NFκB activator
tBid	truncated BH3 interacting domain death agonist
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethyl-ethane-1,2-diamine
TGFβ	transforming growth factor beta
THD	TNF homology domain
THP-1	human monocytic cell line
TNF	tumor necrosis factor
TNFR1/2	tumor necrosis factor receptor 1/2
TNFRSF	TNF receptor superfamily
TNFRSF12A	TNF receptor superfamily member 12 A
TNFSF	tumor necrosis factor superfamily

Table 4 continued: Abbreviations

TNFα	tumor necrosis factor alpha
TRADD	TNF receptor-associated death domain
TRAF	TNF receptor-associated factor
TRAF1	TNF receptor-associated factor 1
TRAF2	TNF receptor-associated factor 2
TRAF3/5/6	TNF receptor-associated factor 3/5/6
TRAF-C	conserved C-terminal TRAF domain
TRAF-N	variable N-terminal TRAF domain
TRAIL	TNF-ralated apoptosis-inducing ligand
TRAILR1/2	TNF-ralated apoptosis-inducing ligand receptor 1/2
TWEAK	TNF-like weak inducer of apoptosis
TWEAKR2	TWEAK receptor 2
Tween-20	Polyoxyethylen(20)-sorbitan-monolaurat
V	volt
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
Z-VAD-fmk	carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone

9.3 List of figures

9.4 List of tables

9.5 Publications

TNF-like weak inducer of apoptosis inhibits proinflammatory TNF receptor-1 signaling.
Wicovsky A, Salzmann S, Roos C, Ehrenschwender M, Rosenthal T, Siegmund D, Henkler F, Gohlke F, Kneitz C, Wajant H.
Cell Death Differ. 2009 Nov;16(11):1445-59. Epub 2009 Jun 26.
PMID: 19557010

Soluble and transmembrane tumor necrosis factor-like weak inducer of apoptosis (TWEAK) differentially activate the classical and noncanonical NF-kappaB pathway.
Claudia Roos, Andreas Wicovsky, Nicole Müller, Tina Rosenthal, Holger Kalthoff, Anna Trauzold, Christian Kneitz and Harald Wajant.
(Journal of Immunology, submitted)

9.6 Curriculum vitae

Personal data

Name	Claudia Roos
Date of birth	January 22nd 1979
Place of birth	Marktheidenfeld, Germany

Education

09/1985	**Primary and Secondary General School**, elementary school Bischbrunn
07/1994	Certificate of Secondary Education
09/1994	**Commercial College**, education center Wertheim-Bestenheid
07/1996	Secondary School Level I Certificate
09/1996	**Commercial Grammar School**, education center Wertheim-Bestenheid
07/1999	General Qualification for University Entrance

Study

10/1999	Begin of studies of Biology at the University of Kassel, Germany
09/2002	**Intermediate exams**
02-04/2003	**Work placement at the Institute for Molecular Infection Biology**, workgroup Heuner, Analysis of *Legionella pneumophila* specific mobilizable DNA-elements, Research Center for Infectious Diseases, Wuerzburg, Germany
02-04/2004	**Work placement at the Institute for Molecular Infection Biology**, workgroup Bruhn, Analysis of von pathogenithity factors of *Entamoeba histolytica* using mutagenesis and activity tests, Research Center for Infectious Diseases, Wuerzburg, Germany
10/2004	**Diploma exams**
12/2004	**Diploma thesis** at the Institute for Molecular Infeciton Biology, workgroup Bruhn, Research Center for Infectious Diseases, Wuerzburg, Germany.
08/2005	Titel: Analysis of Saposin-like proteins of *Entamoeba histolytica* using siRNA
08/2005	**Diploma certificate**
10/2005	**Dissertation** at the clinical research group of Molecular Inner Medicine of University hospital Wuerzburg, Germany. Supervisor Prof. Dr. Wajant.
03/2009	Titel: Characterization of tumor necrosis factor-like weak inducer of apoptosis (TWEAK)-induced signaling pathways.