Enhancement of Toll-like receptor3 (TLR3)-induced death signaling by TNF-like weak inducer of apoptosis (TWEAK)

Verstärkung der Toll-like receptor3 (TLR3)-induzierten Todessignalisierung durch TNF-like weak inducer of apoptosis (TWEAK)

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Dedicated to my beloved parents, my lovely wife and my children

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1. Introduction

1.1. Innate immunity.

The immune system can recognize and eradicate exogenous pathogens, which attack humans and lead to infection and diseases. Moreover, it can discriminate and eliminate endogenous cellular debris, oncogenic cells, and other toxic substances. In sum the immune system is keeping the human organism in a healthy and stable condition (Beck and Habicht, 1996; Saitoh et al., 2003; Gasteiger et al., 2017). Nevertheless, pathogens can protect itself from detection and neutralization via the immune system through quickly evolving and adapting to the surrounding environment. The innate immune system functions as the first barrier to rescue organisms against pathogens; even a unicellular organism has a primary immune system that can resist bacteriophage infection using enzymes. Furthermore, insects and plants have a basic innate immune system made up of phagocytes, and the complement system (Beck and Habicht, 1996; Bryant and Monie, 2012; Buchmann, 2014).

In mammals, the innate immune system includes almost all tissues, mainly barrier surfaces such as the skin or the mucosal surfaces of the gastrointestinal and respiratory tract, effector cells, and specialized lymphoid and myeloid sensor (Galli et al., 2011; Gasteiger et al., 2017). Indeed, nonhematopoietic cells, can launch and exert innate defense mechanisms and respond to infection, tissue damage, or genotoxic stress. The innate immune system can detect such situations through germline-encoded receptors, e.g., pattern recognition receptors (PRRs) such as toll-like receptors (TLRs). The responses of the innate immune system are based on cell-dependent mechanisms including phagocytosis and cytotoxicity, and secreted factors, like antimicrobial peptides (AMPs) (Bevins and Salzman, 2011; Hilchie et al., 2013; Lai and Gallo, 2009).

In humans, the innate immune cells consist of macrophages, natural killer cells, dendritic cells, natural killer T cells, basophils, eosinophil, and neutrophils. All of these cells can produce pro-inflammatory cytokines, chemokines, or interferons immediately when they semse exogenous pathogens (Gasteiger et al., 2017). Macrophages are responsible for phagocytosis, which can clean aged cells and dead cellular debris in the late stage of infection. Additionally, macrophages play an essential role in antigen presentation, which links the innate and the adaptive immune system. Because after ingesting and digesting a pathogen, macrophages can present antigens derived of a pathogen to T cells that can induce the adaptive immune system (Bevins and Salzman, 2011). Other than macrophages, dendritic cells are existing in tissues which are in interaction with the external atmosphere, such as skin and lining of the nose, lungs, stomach, and intestines. After being activated by the pathogens, dendritic cells migrate to the lymph node where they present the antigen to T cells or B cells to initiate and

shape the adaptive immune response. In the meantime, specific dendritic cells can produce a high amount of interferon after the detection of viruses (Gasteiger et al., 2017). Eosinophils are essential for combating multicellular parasites and certain infection in vertebrates (Gasteiger et al., 2017).

Furthermore, mast cells and basophils are responsible for allergic reactions (Gasteiger et al., 2017). Neutrophils are the most abundant type of polymorphonuclear cells in mammals. They play a crucial role in innate immunity. Because of their high migration capability, neutrophils can rapidly assemble at the center of an infection at the early phase of inflammation, especially for those caused by a bacterial infection and some cancers. Also, neutrophils can detect chemokines gradients of interferon gamma (IFN-gamma), and interleukin-8 (IL-8) which are essential for neutrophils migration. Also, neutrophils can express and release cytokines, which in turn increase the inflammatory reaction. As well, neutrophils play an essential role in the removal of the microorganisms and particles via phagocytosis, because they have Fc-receptors for opsonin (Gasteiger et al., 2017).

The initial innate immune response to pathogen invasion by sensing viral and bacterial conserved pathogen-associated molecular pattern (PAMP) via pattern recognition receptor (PRR) is rucial for eradicating the pathogen. In the meantime, the immune system produces certain types of suppressive cytokines such as interferons, interleukins, chemokines, lymphokines, and tumor necrosis factor to balance the immune response. Altogether, the different components of the innate immune system cooperate to keep the homeostasis of the organism (Gasteiger et al., 2017).

1.2. Pattern recognition receptors

Pattern recognition receptors (PRRs) are a crucial part of the innate immune system and play an essential role in the detection of viral or bacterial pathogens by sensing PAMPs. PAMPs are pathogen-associated molecular patterns not present in humans and include bacteria-specific compounds such as lipopolysaccharides (LPS) but also nucleic acids as well such as viral DNA which is exposed to sensors localized in the cytoplasm or to signatures existing in viruses such as 5" triphosphate RNA, which is not typically found in host RNA (Thompson et al., 2011). The name of PRRs traced back to their ability to recognize a different high number of pathogens via recognition of their nucleic acids or genomes which form during pathogen replication. Moreover, PRRs are also sensing molecules from non-pathogenic molecular pattern and identify also endogenous molecules which are released from damaged cells, thus named as damage-associated molecular patterns (DAMPs) (Piccinini and Midwood, 2010). It is well known that innate immunity is not only responsible for the initial pathogen defence, but also for originating, checking and shaping the adaptive immune system (Palm and Medzhitov, 2009). Nevertheless, not all PRRs are equal in terms of their ability to activate adaptive immunity. Consequently, this phenomenon invigorated research on the mechanism of each type of PRRs. There are different classes of PRRs including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), intracellular DNA sensors such as Nod-like receptors (NLRs), AIM2-like receptors (ALRs), and C-type lectin receptors (CLRs) (Akira et al., 2006, Cai et al., 2014).

1.3. Toll-like Receptors

Toll-like receptors (TLRs) are expressed as type1 transmembrane proteins. They are essential for sensing PAMPs in the extracellular environment. The name giving Toll protein was first identified for its function in the dorsal-ventral patterning of Drosophila embryos. Later results found it to be crucial for the immune response in the adult fly to fungal and bacterial infections. To date, 13 TLRs have been reported in mice, 10 in human with TLRs 1-9 mutual to both (Figure 1). TLR1, TLR2, TLR4, TLR5, and TLR6 are situated on the plasma membrane while TLR3, TLR7, TLR8, and TLR9 are endosomal. Those found on the plasma membrane are usually specific for hydrophobic proteins and lipids while those present in endosomes sense nucleic acids (Brubaker et al., 2015; Thompson et al., 2011). This isolation seems intentional, permitting innate cells to respond to components of the viral envelope such as fusion machinery at their surface. Contradictory, the endosome detects the nucleic acids where many viruses uncoated their genomes and pass in the cytoplasm (Thompson et al., 2011). Each TLR is formed of an ectodomain with leucine-rich repeats (LRRs) that mediate PAMP detection, a transmembrane domain, and a cytoplasmic Toll/IL-1 receptor (TIR) domain that mediate downstream signaling. The ectodomain shows a horseshoe-like structure, and TLRs bind with their DAMPs or PAMPs as a homo- or heterodimer along with a co-receptor or

accessory molecule (Botos et al., 2011). Upon detection of DAMPs and PAMPs, TLRs recruit TIR domain-containing adaptor proteins such as TRIF and MyD88, which trigger signal transduction pathways leading to the activation of IRFs, NF-κB trabscriptuin factors, or MAP kinases to regulate the expression of type I IFNs, cytokines, and chemokines that eventually defend the host from microbial infection (Brubaker et al., 2015; Kawasaki and Kawai, 2014).

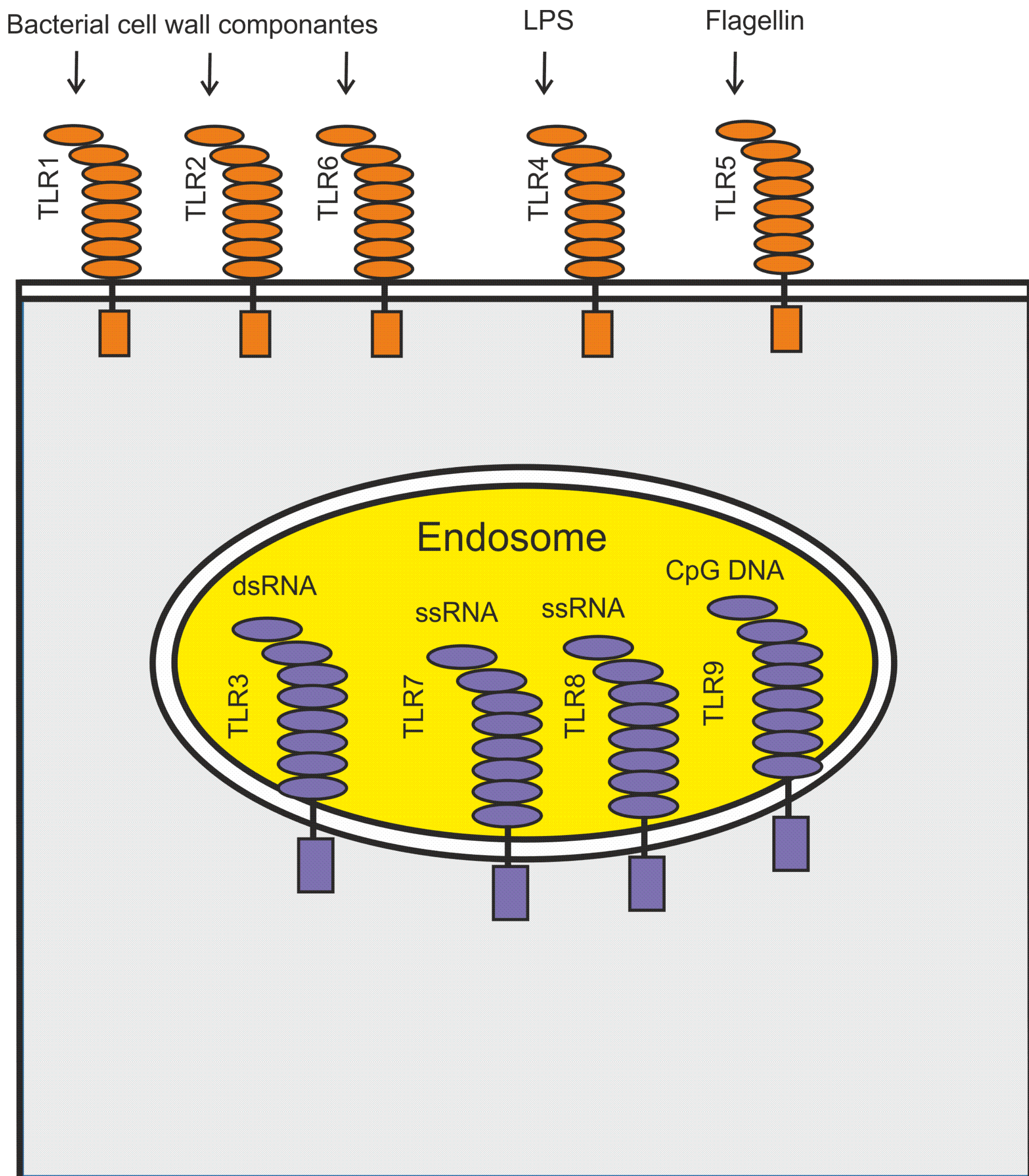


Figure 1: Toll-like receptors and their ligands.

1.4. Toll-Like Receptor 3

TLR3 is highly expressed in innate immune cells except in neutrophile and pDCs, where it is localized to the endosomal compartment (Hayashi et al., 2003; Schroder and Bowie, 2005). Furthermore, It has been shown that some non-immune cells express TLR3 such as epithelial cells (Estornes et al., 2012; Leung, 2004;Feoktistova et al., 2011; Jiang et al., 2008; McAllister et al., 2013; Weber et al., 2010). TLR3 plays an essential role in the recognition of double-strand RNA which is produced during viral infection, as a replication consequence or as a by-product of symmetrical transcription in DNA viruses (Alexopoulou et al., 2001; Thompson et al., 2011). After binding its ligand, TLR3 can trigger the production of the type I interferon (IFNs) and NF-κB activation (Alexopoulou et al., 2001; Thompson et al., 2011). Moreover, the crystal structure of TLR3 shows that two ectodomains homodimerize and create a glycosylation-free area which binds polyinosinic-polycytidylic acid (poly(I:C), a synthetic dsRNA analog (Alexopoulou et al., 2001; Brubaker et al., 2015; Thompson et al., 2011).

1.4.1. TRIF signaling

The ectodomain of TLR3, comprising 23 LRRs, is situated in the endosomal lumen, whereas the cytoplasmic domain of TLR3 forms of the linker region (LR) and the toll/interleukin-1 receptor (TIR) domain. The LR controls the subcellular presence of TLR3, while the TIR domain is crucial for the recruitment of signaling molecules. The adaptor protein MyD88 is essential in all TLRs except TLR3; it uses TRIF as its downstream adaptor molecule (Chattopadhyay and Sen, 2014; Yamamoto et al., 2003). TRIF encompasses a TIR domain that interacts with the TIR domain of TLR3. The interaction between TRIF and TLR3 is considered to be the key event for triggering downstream signaling. Engagement of TLR3 leads to oligomerization of TRIF and TBK1 or IKKe recruitment, which controls the phosphorylation of IRF-3 (Fitzgerald et al., 2003; Yamamoto et al., 2003). The NF-kB signalling axis of TLR3 is stimulated by TRIF-dependent recruitment of RIP1, IKK, and TAK1. A recent study showed that unlike other TLRs, TLR3 needs to be tyrosine phosphorylated after dsRNA-binding but before the recruitment of TRIF (Chattopadhyay and Sen, 2014). The cytoplasmic domain of TLR3 possesses five tyrosine residues. The phosphorylation of at least Tyr759 and Tyr858 is crucial for TLR3 signaling (Sarkar et al., 2004); mutation of either of these two tyrosine residues causes the formation of a protein that cannot signal. Src and EGFR, are two protein tyrosine kinases, act as the enzymes responsible for TLR3 phosphorylation (Yamashita et al., 2012). dsRNA binds to the TLR3 ectodomain in the endosomal lumen which affects the confirmation of its cytoplasmic domain and leads to exposure of EGFR-binding site to its LR. EGFR binds to TLR3, but not its enzymatic activity is required to recruit Src to the complex. The two kinases phosphorylate the two Tyr residues of TLR3 with high specificity; EGFR phosphorylates Tyr858 and Src phosphorylates Tyr759. TRIF can ligand only to the

dual phosphorylated TLR3 and activate transcriptional signaling. Another kinase, PI3 kinase (PI3K), binds to stimulated TLR3 and is essential for the full activation of IRF-3 and NF-kB (Sarkar et al., 2007; Sarkar et al., 2004). This shows an unexpected linking between EGFR and Src and TLR3-mediated innate immunity and, which control cell growth and potential oncoproteins (Chattopadhyay and Sen, 2014).

1.4.2. Role of TLR3 in Viral Pathogenesis

TLR3 identifies extracellular dsRNA produced from virus-infected cells or damaged tissues. dsRNA mediates the activation of TLR3 and direct so induction of antiviral genes in virus-infected cells. Although a variety of virus activates TLR3 signaling, its role in viral pathogenesis is complicated, with both pro- and anti-viral properties. Indeed, TLR3 signaling also controls the response of innate and adaptive immune system such as regulation of dendritic cells (DCs) maturation by dsRNA, which is responsible for the promotion of antigen-specific T-cell responses (Kumar et al., 2008). Previous studies showed mice lacking TLR3 or has a lethal mutation in TRIF tends to be very sensitive to the infection by mouse cytomegalovirus in the spleen, with 1,000-fold higher viral titers, compared to the wild type mice (Tabeta et al., 2004). It has been approved the highly susceptible to HSV-2- induced CNS infection in the mice with a dominant negative mutant of TLR3 (Reinert et al., 2012). Likewise, TLR3 protects the CNS from HSV viral infection (Zhang et al., 2013). Invariant to the antiviral role of TLR3 in HSV-2 infection, it has been approved that TLR3 plays a role in the promotion of West Nile virus (WNV) pathogenesis (Wang et al., 2014). Compared to the wild type mice, the deficiency of TLR3 leads to resistance to WNV-induced encephalitis. Also, TLR3 - / - mice show a significant reduction in brain inflammation and the viral load of WNV (Chattopadhyay and Sen, 2014).

1.4.3. Role of TLR3 in nonviral diseases.

TLR3 plays a crucial role in several pathogeneses such as cancer, diabetes mellitus, and colitis. A significant anti-angiogenic role of TLR3 has been approved in animal models and clinical study (Kleinman et al., 2008). Consequent genetic studies showed that the antiangiogenic function of TLR3 is dependent on the activity of NF-κB but independent on the activity of IRF-3. Moreover, it has been approved that TLR3 signaling is related to progression of age-related macular degeneration (AMD), the main reason of irreversible visual impairment. A variant of TLR3 (L412F) defends against AMD in human patients, maybe by defeating the death of retinal pigmented epithelial cells (Allikmets et al., 2009). Recently it has been revealed that TLR3-stimulated hMSCs trigger the expression of migration-responses-related genes and inflammatory genes. Also, TLR3-stimulation in hMSCs leads to the activation of forkhead box protein O1 (FOXO1), which stimulates the migration of cells (Hwa Kim et al., 2019). Also, previous studies showed that TLR3 signaling reduces the induction of colitis by dextran sulfate

sodium (DSS), an experimental model for Crohn's disease and inflammatory bowel disease. Injection of dsRNA defends the subcutaneous but not intragastric of mice against DSS-induced colitis and this protective activity of dsRNA was ablated in TLR3 knock out mice (Vijay-Kumar et al., 2007). A further study revealed that dsRNA of commensal bacteria triggers TLR3 signaling and so production of IFN-β which acts against the induction of colitis via DSS (Kawashima et al., 2013;Chattopadhyay and Sen, 2014). TLR3 also plays a crucial role in the protection against type I diabetes mellitus, which is triggered by the selective obliteration of islet beta cells which are responsible for the secretion of insulin (Castano and Eisenbarth, 1990). A viral infection can cause type I diabetes; in hematopoietic cells, TLR3 can bound the infection of beta cell infection. Chimeric mice contain WT hematopoietic cells and TLR3- / - stroma cells shows resistant to the disease, while those with TLR3- / - hematopoietic cells and WT stroma cells are sensitive to encephalomyocarditis virus strain D (EMCV-D)-induced diabetes (McCartney et al., 2011). TLR3 signaling has also been implicated in protection against cancer via the induction of cellular apoptosis using both extrinsic and intrinsic apoptotic pathways (Sun et al., 2011). Indeed, Poly(A:U) works as an effective adjuvant, plays a crucial role in breast cancer treatment, which expresses TLR3 (Conforti et al., 2010). A recent study indicated that CD103+cDC1 and, to a much lesser extent CD11b+ cDC2 express TLR3 at the tumor site, the treatment of these cells with poly(A:U) leads to their activation and effective changes in the structure of the tumor immune penetration, switching the immune resistant tumor environment to anti-tumor immunity (Roselli et al., 2019).

1.5. TNF ligands and receptors

Ligands of the Tumor necrosis factor (TNF) superfamily (TNFSF) and their receptors (TNFRSF) are vital effectors and regulators of the immune system and are crucial for immune homeostasis, cell death, cell proliferation and morphogenesis (Aggarwal, 2003; Bodmer et al., 2002; Locksley et al., 2001). Because of these essential roles, the TNFSF ligands and TNFRSF receptors are attractive targets for the treatment of different diseases, such as cancer, graft-versus-host disease, autoimmunity and infectious diseases (Vanamee and Faustman, 2018).

1.5.1. TNF ligands

Tumor necrosis factor (TNF), the name giving ligand of the TNFSF, was first defined in 1975 as an endotoxin-induced serum factor that prompts necrosis on tumors (Carswell et al., 1975). The mammalian TNFSF consists of 19 ligands and the TNFRSF of 29 receptors (**Table 1**) (Aggarwal, 2003; Bodmer et al., 2002). The ligands include, but are not limited to, TNF, lymphotoxin-α (LTα), LTβ, B cell activating factor of the tumor necrosis factor family (BAFF), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor weak inducer of apoptosis (TWEAK), which were also partially described in chicken. Following the discovery of TNF and LTβ, more advanced molecular biological techniques have also been used to identify other members, confirming the existence of a TNFSF. The ligands of TNFSF show similarities in their structure, their biological activity and also in their immunostimulating functions (Aggarwal, 2003; Aggarwal et al., 2012; Carswell et al., 1975; Messer et al., 1991; Granger et al., 1969; MacEwan, 2002). The characteristic feature of the TNFSF ligands is the "TNF homology domain" (THD), which comprises the extracellular C-terminus of the ligands and is responsible for binding to the receptors and the formation of trimeric molecules (Fesik, 2000). The THD is highly conserved and has approximately 20-30% sequence homology between family members (Bodmer et al., 2002; Hehlgans and Pfeffer, 2005; Idriss and Naismith, 2000). Except for LTα, TNFSF ligands are initially expressed as trimeric membrane-bound molecules. However, proteolysis of membrane TNFSF ligands by metalloproteases such as furin also generates soluble trimeric forms (Hehlgans and Pfeffer, 2005). TNFRSF receptors differ in their ability to be activated by membrane-bound and soluble ligands. For example, TNF, both as a membrane-bound and as a soluble molecule, can bind to TNFR1 and leads to its activation (Grell et al., 1995). On the other hand, a membrane-bound form of CD95L has much higher bioactivity than the soluble variant (Schneider et al., 1998). However, soluble trimeric TNF ligands which can bind their corresponding receptor without activating it may forcefully act as receptor activators upon oligomerization or immobilization on the cell surface, similar to the membrane-bound form (Kontermann et al., 2008; Wyzgol et al., 2009).

1.5.2. Receptors of the TNFRSF

The TNFRSF members are type I transmembrane proteins that made up of an ectodomain, a transmembrane domain, and an intracellular domain which recruits signal transduction proteins inside the cell. The ectodomains of TNFRSFs consist of several cysteine-rich domains (CRDs), which are responsible for ligand binding and form elongated structures. The TNFRSF can be categorized into three groups: (i) death receptors (DRs) such as DR3, TRAILR1/DR4, TRAILR2/DR5, DR6, nerve growth factor receptor (NGFR), and tumor necrosis factor receptor -1 (TNFR1) which have a death domain (DD) in their intracellular part and trigger apoptosis by TNFR1-associated death domain (TRADD), Fas-associated death domain (FADD), or other DD-binding partners (Bridgham and Johnson, 2003; Aggarwal, 2003); (ii) TNFR-associated factor (TRAF)–interacting receptors that act together with members of the TRAF family, these receptors are characterized by the activation of signaling cascades that contribute to the survival and growth of the cells. This group includes Tumor Necrosis Factor Receptor-2 (TNFR2), Fibroblast growth factor-inducible-14 (Fn14), B cell activating factor of the tumor necrosis factor family receptor (BAFFR), Receptor Activator of NF-κB (RANK), and glucocorticoid-induced tumor necrosis factor receptor (GITR) (Aggarwal, 2003); and finally, (iii) decoy receptors (DcRs) such as DcR1, DcR3, and osteoprotegerin (OPG) that characterized by lacking of intracellular interacting partners and which function as inhibitors of other TNFRSF receptors. As well as the DcRs scheduled in **Table 1**, the cleaved soluble forms of other TNFRSFs can also act as inhibitors (Hehlgans and Pfeffer, 2005; Tansey and Szymkowski, 2009).

Table 1. TNFRSF receptors, their ligands, and their intracellular binding partners

TNFRSF receptor (TNFRSF#, other names)	Intracellular binding partner	TNFSF ligand (TNFSF#, other names)
I) Death Receptors		
TNFR1(1a, CD120a)	TRADD, FADD, RIP	TNF (2, TNF-α), LTα (1, TNF-β), LTβ (3)
Fas (6, CD95)	FADD	FasL (6, CD178)
TRAILR1 (10A, DR4, CD261)	FADD, TRADD, RIP	TRAIL/Apo2L (10, CD253)
TRAILR2 (10B, DR5, CD262)	FADD, TRADD, RIP	TRAIL/Apo2L (10, CD253)
NGFR (16, p75NTR, CD271)	NADE	NGF (not a TNFSF member)
DR3 TL1A (15, VEGI)	TRADD, FADD	(25 or 12, TRAMP), TWEAK (12)
DR6 (21, CD358)	TRADD, RIP	N-APP (not a TNFSF member)
EDAR	EDARADD	EDA-A1
II) TRAF-interacting Receptors		
TNFR2 (1b, CD120b)	TRAF1–3	TNF (2, TNF-α), LTα (1, TNF-β), LTβ (3)
LTβR (3)	TRAF2, TRAF3, TRAF5	LTβ (3), $LT\alpha\beta_2$
OX40 (4, CD134)	TRAF1–3, TRAF5, TRAF6	OX40L (4, CD252)
CD40 (5)	TRAF1–3, TRAF5, TRAF6	CD40L (5, CD154)
CD27 (7)	TRAF2, TRAF3, TRAF5	CD27L (7, CD70)
CD30 (8)	TRAF1–3, TRAF5	CD30L (8, CD153)
4-1BB (9, CD137)	TRAF1–3	4-1BBL (9, CD137L)
RANK (11A, CD265)	TRAF1–3, TRAF5, TRAF6	RANKL (11, TRANCE, D254)
Fn1 (12A, TWEAKR; CD266)	TRAF2, TRAF6	TWEAK (12)
TACI (13B, CD267)	TRAF2–3, TRAF5, TRAF6	APRIL (13, CD256)
BAFFR (13C, BR3, CD268)	TRAF2, TRAF3, TRAF6	BAFF (13B/20, BLys, THANK, CD257)
HVEM (14, CD270)	TRAF1–3, TRAF5	LIGHT (14, CD258), LTα (1, TNF-β)
BCMA (17, CD269)	TRAF1–3, TRAF5, TRAF6	APRIL (13, CD256), BAFF (13B/20, BLys, THANK, CD257)
GITR (18, AITR, CD357)	TRAF1–5	GITRL (18, AITRL, TL6)
TROY (19, TAJ)	TRAF2, TRAF5, TRAF6	?
RELT (19L)	TRAF1	?
XEDAR (27)	TRAF3, TRAF6	EDA-A2
III) Decoy receptors		
TRAILR3 (10C, DcR1, D263)	n/a	TRAIL/Apo2L (10, CD253)
TRAILR4 (10D, DcR2, D264)	n/a	TRAIL/Apo2L (10, CD253)
OPG (11B)	n/a	TRAIL/Apo2L (10, CD253), RANKL (11, TRANCE, CD254)
DcR3 (6B)	n/a	FasL (6), TL1A (15, VEGI), LIGHT (14, CD258)

1.6. The TWEAK-Fn14 system

Tumour necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is expressed as a 249 amino acid (aa) type II transmembrane protein and can be proteolytically cleaved into a soluble, biologically active 156 aa cytokine (Bodmer et al., 2002; Chicheportiche et al., 1997). Similar to other ligands of the TNFSF, membrane TWEAK consists of 3 parts. (i) a C-terminal TNF homology domain (THD), which is responsible for the formation of homotrimeric protein and binding of TWEAK with its receptor. (ii) a stalk region plus a transmembrane part at which occurs the recognition site for serine proteases of the furin family, helped in the processing of membrane TWEAK to be in a soluble form (**Figure 2**a). (iii) The short intracellular N-terminal part of TWEAK is highly preserved like the rest of the molecule; nevertheless, its role is still unclear (Wajant, 2013). TWEAK expression has been reported at the mRNA level in different cell lines and cell types. In contrast, cell surface expression of TWEAK has so far only be identified in certain cells such as interferon-γ stimulated NK cells, dendritic cells, monocytes (Nakayama et al., 2000; Felli et al., 2005; Maecker et al., 2005), patient-derived monocytes, multiple sclerosis (Desplat-Jego et al., 2009), hepatocellular carcinoma cell line (Kawakita et al., 2004), a few colonic adenocarcinoma (Kawakita et al., 2005), and the MDA-MB-231 breast cancer cell line (Willis et al., 2008). A few studies indicated that hypoxia and tissue damage induce the expression of TWEAK, in accordance with this, the hypoxia-controlled interferon-γ and forkhead transcription factor FOXO3a have been involved in upregulation of TWEAK (Baxter et al., 2006). Otherwise, the transcriptional regulation of TWEAK has been poorly studied (Wajant, 2013).

Fibroblast growth factor-inducible 14 (Fn14) is expressed as a 129 amino acid type I transmembrane protein and is the smallest member of the TNFRSF with a molecular weight of 14 kDa (**Figure 2**b) (Wiley et al., 2001). Fn14 has been firstly identified as a transcriptional target of fibroblast growth factor-1 in NIH 3T3 fibroblasts (Meighan-Mantha et al., 1999). Afterwards, Fn14 has been documented as a TNFRSF family member responsible for the transduction of TWEAK signals (Wiley et al., 2001). In addition to Fn14, CD163, a cysteine-rich haemoglobin scavenger receptor and the TNFRSF receptor DR3 expressed on macrophages and monocytes, have been claimed to be TWEAK receptors (Marsters et al., 1998; Bover et al., 2007). However, both the TWEAK-CD163 interaction as well as the TWEAK-DR3 interaction were not reproducible in other studies (Schneider et al., 1999; Kaptein et al., 2000; Fick et al., 2012). All extracellular parts of TNF receptors contain two to six copies of cystein-rich domain in their ectodomain except Fn14 and B cell maturation, BaffR and the short isoform of transmembrane activator and calcium-modulator, and cytophilin ligand interactor have only a single copy of this particular motif (Locksley et al., 2001). Fn14 has been identified as a typical TRAF-interacting TNFRSF receptor because its cytoplasmic domain,

which encompasses 28 amino acid residues, is armed with a single TRAF binding motif. Fn14 is highly expressed on most tumour cell lines of non-lymphoid origin. However, immunohistochemical investigation of human biopsy specimens and murine tissue samples showed a more differentiated expression pattern of Fn14 *in vivo*. Low expression of Fn14 has been described for healthy homeostatic tissue in epithelial, fibroblasts, and endothelial cells. Contradictory, high Fn14 expression was often occurred on mesenchymal and epithelial progenitor cells and especially in context of tissue damage triggered by various insults such as oxidative stress, hypoxia, chemical, and mechanical injuries, inflammation and tumour growth (Winkles, 2008; Burkly et al., 2011). Therefore, It is not really surprising that Fn14 expression has been documented for various immune diseases, including glomerulonephritis, rheumatic arthritis, hepatitis, bowels disease, and multiple sclerosis but also after stroke and heart attack, and in atherosclerosis. Moreover, up-regulation of Fn14 has been reported in liver, breast, melanoma, prostate, and glioma and non-small cell lung cancer (Chao, 2013; Feng et al., 2000; Sanz et al., 2012; Tran et al., 2006; Whitsett et al., 2012; Zhou et al., 2013). In accordance with the tissue damage-induced expression pattern of Fn14, numerous growth factors which are associated with tissue repair processes including VEGF and EGF, as well as cytokines, such as TNF, TGF-β, and IFN-γ, have been reported to induce Fn14 expression (Donohue et al., 2003; Ebihara et al., 2009; Hosokawa et al., 2006; Sanz et al., 2012; Whitsett et al., 2012).

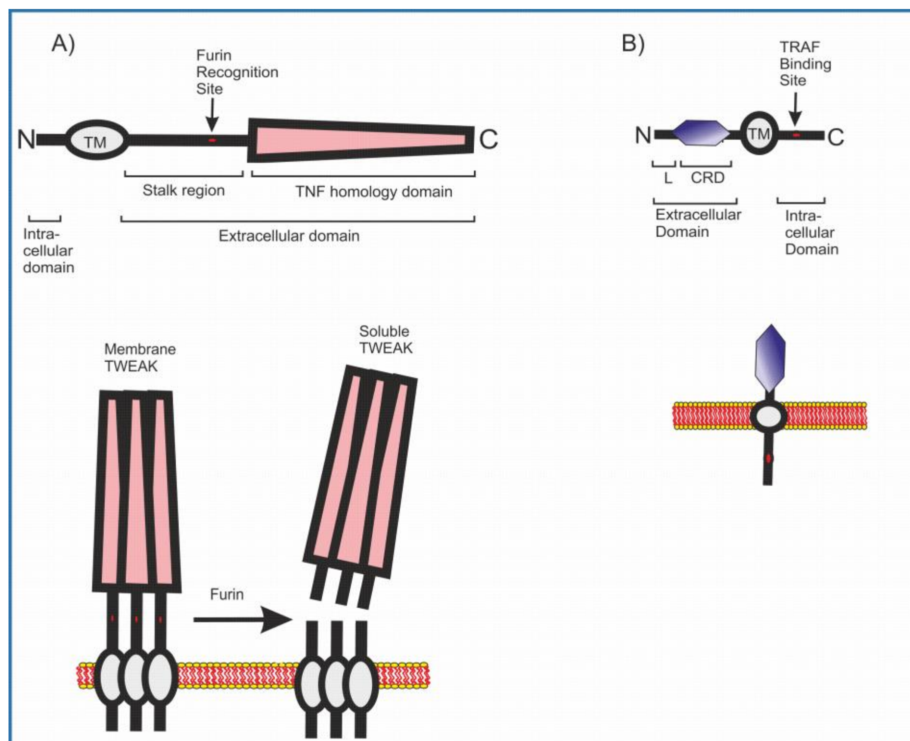


Figure 2: Domain architecture of TWEAK (A) and Fn14 (B).

1.6.1. Fn14-induced cell death.

TWEAK received its name because of its ability to induce cell death in IFN-γ sensitized HT29 cells but not in other cell lines (Chicheportiche et al., 1997). Later studies showed that TWEAK also efficiently induces cell death in a very few other cell lines such as SKOV-3, Kym-1, and OVCAR-4, however, the ability of TWEAK/Fn14 system to mediate cell death remains still the rare exception (Wajant, 2013). Other ligands of the TNFSF stimulate vigorous cell death responses through activation of death receptors such as TNFR1, Fas, and the TNF-related apoptosis-inducing ligand (TRAIL) receptors-1 and 2. Activation of cell death via the death domain of death receptors occurs via the activation of caspase-8 (Dickens et al., 2012). Death receptors also engage the death domain-containing serine/threonine kinase RIP1, which permits them to activate an alternative necrotic form of programmed cell death under well-defined conditions (Dickens et al., 2012).

It was reported that Fn14 without having a death domain can induce cell death by the concerted action of two mechanisms; Namely induction of TNF and subsequent stimulation of the prototypic death receptor TNFR1 and depletion of TRAF2-cIAP1/2 complexes (Dickens et al., 2012; Vince et al., 2008; Wicovsky et al., 2009). In studies where TNFR1 stimulation has been blocked, TWEAK-induced apoptosis has been blocked (Schneider et al., 1999). TNF-induced cell death is furthermore strongly boosted via TWEAK/Fn14 system also in such cell lines resistant to the effect of TWEAK proposing that this sensitizing mechanism shows a common feature of Fn14 stimulation (Wicovsky et al., 2009).For these reason, TWEAK-mediated apoptosis not only depends on the induction of TNF but also from a second such non-toxic sensitizing mechanism. Induction of apoptosis by TNFR1-related TRAIL death receptors is not or only poorly boosted by TWEAK treatment, indicating that Fn14 signaling affects an apoptosis relevant issue of particular relevance in TNFR1 signaling (Wicovsky et al., 2009). Recently, enhancement of TNFR1-induced apoptosis by TWEAK has been traced back to the ability of stimulated Fn14 to recruit TRAF2-cIAP1/2 complexes.

TRAF2 is a characteristic member of the TRAF adapter protein family and has a RING domain with E3 ligase activity (Gonzalvez et al., 2012). Cellular inhibitor of apoptosis-1 (cIAP1) and cIAP2 are also RING domain E3 ligases which have besides the RING domain a caspase recruitment domain and three baculovirus inhibitor of apoptosis (IAP) repeat domains involved in substrate recognition. Because of the expression of TRAF2-cIAP1/2 complexes are relatively low in relation to the expression of Fn14 in most cells, TWEAK-induced recruitment of TRAF2-cIAP1/2 complexes to Fn14 leads to exhaustion of the cytosolic pool of these proteins and limits so their availability for TNFR1 (Vince et al., 2008; Wajant, 2013; Wicovsky et al., 2009). In fact, enhancement of TNFR1-mediated apoptosis and necroptosis by depletion of TRAF2 has also been confirmed for TNFR2 and CD30 and CD40 (Duckett and Thompson,

1997; Weiss et al., 1997; Weiss et al., 1998; Grell et al., 1999; Fotin-Mleczek et al., 2002; Siegmund et al., 2018). Furthermore, SMAC mimetics, a group of anticancer drugs presently under examination in clinical trials enhance TNFR1 signaling better than TWEAK and Fn14 through triggering proteasomal degradation of cIAP1 and cIAP2 and also prevent the anti-apoptotic xIAP protein (Fulda and Vucic, 2012). A few publications also showed that TWEAK/Fn14-induced cell death in normal human keratinocytes and cortical neurons is TNF-independent. However, the underlying signaling mechanisms have been poorly investigated so far (Haile et al., 2010; Sabour Alaoui et al., 2012).

1.6.2. Fn14-induced NFkB signaling

Depletion of the cytoplasmic TRAF2, cIAP1, and cIAP2 complexes by the TWEAK-Fn14 system plays not only a crucial role in heightening of TNFR1-induced caspase-8 activation but also in the regulation of NFkB signaling pathways stimulated by members of the TNF receptor family and pattern recognition receptors (Wajant, 2013). These molecules act as critical players in the stimulation of numerous transcription factors of the NFκB family via the TWEAK/Fn14 system (Vince et al., 2008). The activation of NFκBs by TWEAK/Fn14 system is comparably well addressed and considered as a reason for many of the proinflammatory effects (Winkles, 2008; Burkly et al., 2011). Indeed, NFκBs function as homo- and heterodimers which formed by the five members of the Rel transcription factor family (p50, p52, RelA, RelB, and c-Rel) (Hoffmann et al., 2003; Leung, 2004; Sanjabi et al., 2000; Sanjabi et al., 2005; Tsui et al., 2015). NFκBs significantly vary in the mode they become activated and also in their target genes. There are two prototypic signaling pathways that cause activation of different NFκB dimers, the canonical and the non-canonical NFκB pathways (**Figure 3**) (Hayden and Ghosh, 2012; Mitchell et al., 2016). TWEAK/FN14 system can stimulate both the canonical and the non-canonical NFκB pathways. The critical step in the non-canonical NFκB pathway is phosphorylation of the p100 precursor form of the p52 by the inhibitor of kappaB kinase (IKK)1; this leads to K48-ubiquitination and proteasomal processing of p100 to p52 and nuclear translocation of p52-containing NFκB dimers (Mordmuller et al., 2003). The activation of IKK1 needs phosphorylation by NIK, a cytosolic MAP3-kinase that is constitutively degraded in unstimulated cells by a TRAF2-cIAP1/2 complex-dependent mechanism. Whereas TRAF2 recruits NIK by interaction with TRAF3, which binds to NIK and subjects it so to K48-ubiquitination via the TRAF2-associated IAPs and consequent proteasomal degradation (Mitchell et al., 2016; Sun et al., 2011). In the framework of the non-canonical NFκB pathway, cytosolic TRAF2-cIAP1/2 complexes achieve an inhibitory role blocked by their TWEAK-induced recruitment to membrane-bound Fn14. On the other side, stimulation of the canonical NFκB pathway by TWEAK and several other ligands of the TNF family needes stimulatory activities of TRAF2 and the cIAPs (Mitchell et al., 2016). In the cytoplasm of unstimulated cells,

the activated NFκB dimers of the canonical NFκB pathway are retained by forming a ternary complex with inhibitory proteins, the IκBs. The activation of the IKK complex is the crucial step in the canonical NFκB pathway, which includes the scaffolding protein NEMO, IKK1 and IKK2 whereby the IKK1 seems mostly unessential for the role of the IKK complex in classical NFκB signaling (Wajant, 2013). The IKK complex phosphorylates IκB proteins causing their proteasomal degradation and nuclear translocation of the released NFκB dimers. The TRAF2-cIAP1/2 complex has a dual role in stimulation of the canonical NFκB pathway. Whereas TRAF2 acts as an adapter protein responsible for the recruitment of the IKK complex to receptors of the TNFRSF, the cIAPs K63-ubiquitinate the IKK subunit NEMO, and also RIP in the framework of TNFR1 signaling, generating docking sites for a diversity of ubiquitin-binding proteins. The latter play a crucial role in activation of IKK, for instance, the linear ubiquitin chain assembly complex (LUBAC) and the TAK1-TAB2-TAB3 complex, which causes formation of additional docking sites for ubiquitin-binding proteins, but also recruits two deubiquitinating enzymes, Cyld and A20 (Cheong et al., 2006; Niu et al., 2012; Inoue et al., 2015). The significance of TRAF2, IKK2, TAK1, and the cIAPs for Fn14-mediated activation of the canonical NFκB pathway has been recently proved by help of siRNA experiments, knockout mice derived fibroblasts, IAP antagonists and dominant-negative ubiquitin-binding domains (Saitoh et al., 2003; Sims et al., 2012; Varfolomeev et al., 2012).

Noticeably, membrane TWEAK expressing cells activate both canonical and non-canonical pathways efficiently, while soluble TWEAK only activates weak and delayed activation of the canonical NFκB pathway but leads to intense stimulation of the non-canonical NFκB pathway (Wajant, 2013). Similarly, artificial immobilization on a cell surface converts soluble TWEAK from a weak to a potent stimulator of canonical NFκB signaling while having no significant effect on the efficacy of activation of the non-canonical NFκB pathway (Roos et al., 2010; Wajant, 2013). Consequently, the boosted efficiency with which membrane-bound TWEAK activates canonical NFκB signaling gives the impression to be principally associated to the spatially restricted way by which membrane TWEAK is presented to Fn14 (Wajant, 2015). Moreover, intense stimulation of the canonical NFκB pathway can also be attained with dimerized or oligomerized trimers of soluble TWEAK. However, this has no significant effect on Fn14 occupation by TWEAK or on the dose dependency of TWEAK-induced activation of the non-canonical NFκB pathway (Fick et al., 2012). It is consequently attractive to speculate that the secondary interaction of two or more initially formed TWEAK-Fn14-TRAF2-cIAP1/2 complexes is essential for stimulation of the canonical NFκB pathway while the single recruitment of the TRAF2-cIAP1/2 complex is totally enough for prompting of non-canonical NFκB signaling (Saitoh et al., 2003; Varfolomeev et al., 2012; Wajant, 2015).

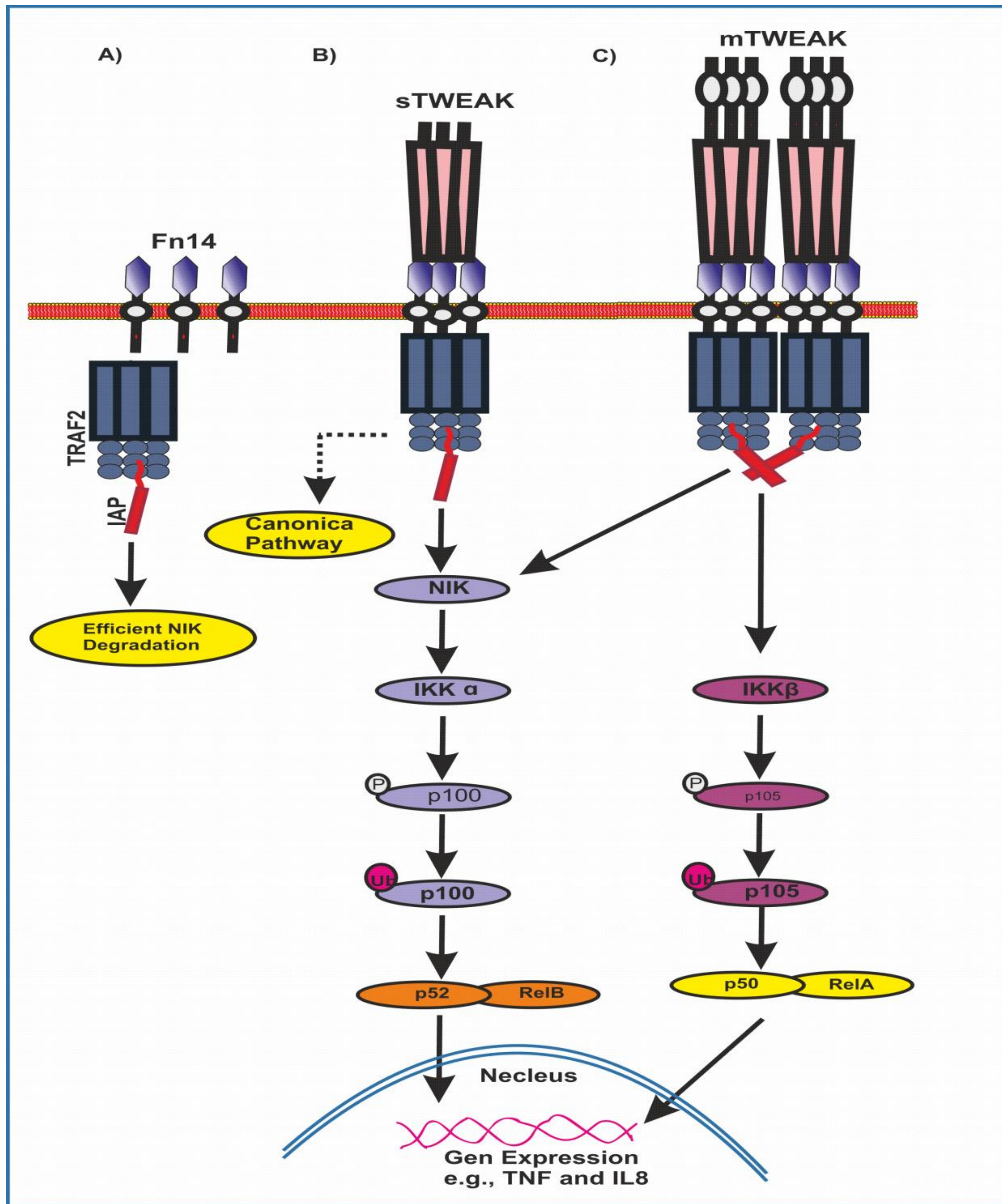


Figure 3: Induction of canonical and non-canonical NF by soluble and membrane TWEAK.

A) In unstimulated cells TRAF2-cIAP1/2 are restricted in the cytoplasm and activate degradation of NIK. **B)** Soluble TWEAK triggers the non-canonical NFκB pathway. Binding of soluble TWEAK trimers to Fn14 leads to recruitment and depletion of cytosolic TRAF2-cIAP1/2 but not in the transactivation of the single inhibitor of apoptosis (IAP) molecules linked with a TRAF2 trimer. **C)** Membrane TWEAK triggers both canonical and non-canonical NFκB pathway. Binding of mTWEAK (or oligomeric sTWEAK or immobilized Fn14 antibodies) to Fn14 not only leads to recruitment and depletion of cytosolic TRAF2-cIAP1/2 but also in the transactivation of TRAF2-associated IAP molecules.

1.6.3. Role of the TWEAK/Fn14 system in cancer

Oncogenesis is often strongly related to chronic inflammation and characterized by repetitive tissue damage and the corresponding repair processes. As above mentioned the TWEAK/Fn14 system is highly upregulated during chronic inflammation and tissue damage. Therefore, the tumour microenvironment typically delivers a situation governing the upregulation of Fn14 expression. Moreover, tumour penetrating immune cells act as a natural source of TWEAK. Actually, in tumour tissue such as breast cancer, hepatocellular carcinoma, nonsmall cell lung carcinoma, pancreatic cancer, glioblastoma multiforme as well as malignant , the expression of Fn14 is often found to be strongly boosted compared to non-transformed tissue and is also regularly detectable on tumour cell lines (Feng et al., 2000; Tran et al., 2006; Willis et al., 2008; Kwon et al., 2014; Zhou et al., 2013).

The upregulation of TWEAK at the mRNA level has been confirmed in numerous tumour cell lines and tumour entities as well as in tumour tissue by immunohistochemistry, but expression of membrane TWEAK on cell surface has only be stated for a very few breast cancer and hepatocellular carcinoma cell lines, so far (Kawakita et al., 2004).

The vast majority of cellular effects prompted by the TWEAK/Fn14 system are apparently of potential advantage for tumor development (Wajant, 2013). Indeed, TWEAK/Fn14-mediated cell migration and invasion have been confirmed for glioblastoma cells, ovarian cancer cells, non small cell lung cancer, androgen-independent prostate cancer cells and breast cancer cell lines (Tran et al., 2006; Willis et al., 2008; Huang et al., 2011). Moreover, TWEAK expressing matrigel encapsulated Hek293 cells presented boosted angiogenesis in nude mice (Ho et al., 2004) and Fn14 overexpressing A549 adenoma carcinoma cells show enhanced lung metastasis upon tail vein injection in mice (Whitsett et al., 2012). Many clinical studies showed a correlation between the induction of tumor and TWEAK/Fn14 system. High expression of Fn14 associates with clinical evidence of poor prognosis in breast cancer patients, glioma specimens, and gastric cancer (Kwon et al., 2014; Tran et al., 2006; Willis et al., 2008).

There are other studies showing that high levels of TWEAK and Fn14 might not always be linked to poor patient outcome. In colorectal cancer, high TWEAK expression associates with improved overall and disease-free survival, and this resembles an inhibitory effect of TWEAK on the invasiveness of colon cancer cell lines *in vitro* (Lin et al., 2012; Di Martino et al., 2016).

1.6.4. TWEAK and Fn14 as a potential therapeutic target for cancer.

The TWEAK/Fn14 system is an attractive anti-tumour target due to its extensive and high tumour-associated expression and its multiple protumoral roles. It has been published that a multi-functional fusion protein formed of the extracellular domain of Fn14 and TRAIL can bind to TRAIL receptor triggering apoptosis in malignant cells. The main functions of the developed Fn14–TRAIL fused protein to prevent TWEAK/Fn14 signaling and to induce TRAIL signaling (Aronin et al., 2013). Moreover, the developed Fn14–TRAIL fused protein leads to growth inhibition of hepatocellular carcinoma both in vitro and in vivo and is well tolerated by mice without changing liver histology (Aronin et al., 2013). TWEAK can oligomerize Fn14–TRAIL primes formation of a stable complex that displays higher efficiency in malignant lymphoblasts-induced apoptosis (Prigozhina et al., 2017).

Fn14 antibodies show antitumor effect depending on the stimulation of antibody effector functions, such as the activity of antibody conjugated drugs, or on antibody-dependent cellular cytotoxicity (ADCC) (Culp et al., 2010; Michaelson et al., 2011;Trebing et al., 2014; Zhou et al., 2011; 2013). In several conditions, the crosstalk between TWEAK and TNF leads to induction of apoptosis, so it can also be used for cancer treatment by promoting tumour cell death using agonistic Fn14-antibodies or recombinant TWEAK (Wajant, 2013). Therefore, dependent on the circumstances agonistic or antagonistic recombinant proteins and antibodies can be exploited for targeting the TWEAK/Fn14 system in the future cancer therapy (Guanglei et al., 2017; Wajant 2013; 2015).

1.7. Programmed cell death

1.7.1. Apoptosis

Apoptosis is a well-defined programmed cell death pathway and is well conserved between higher multicellular organisms. This pathway has evolved to keep the number of cells tightly regulated by controlling the rate of cell death as well as by controlling the rate of cell proliferation. Apoptosis is morphologically characterized by cell shrinkage, chromatin condensation and fragmentation of the cell (Kroemer et al., 2009) and is regulated by different stimuli coming from within a cell (Intrinsic cell death pathway) (**Figure 4**)**,** such as the production of reactive oxygen species (ROS) (Brumatti et al., 2010; Czabotar et al., 2014; Nunez et al., 1990; Pihan et al., 2017; Roos et al., 2016; Vitale et al., 2017)or from its environment (Extrinsic cell death pathway) e.g., death receptor ligands (Steller, 2008).The death receptors include tumor necrosis factor (TNF) receptors, Fas receptors, and TNF-related apoptosis-inducing ligand (TRAIL) receptors. As a surface receptor, for example, TNF receptor-1 (TNF-R1), it will bind with TNF to induce the recruitment of adaptor proteins such as Fas-associated protein with death domain (FADD) and Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), which recruits a series of downstream factors, including Caspase-8, which is a critical mediator of the extrinsic pathway, resulting eventually in cell apoptosis (Flusberg and Sorger, 2015; Galluzzi et al., 2018).

The intrinsic apoptosis pathway is initiated by, for example, chemotherapy and/or radiotherapy. It is activated by a range of exogenous and endogenous stimuli, such as DNA damage, ischemia, and oxidative stress. Moreover, it plays an important function in development and in the elimination of damaged cells (Loreto et al., 2014). In the intrinsic pathway, the functional consequence of pro-apoptotic signaling is mitochondrial membrane perturbation and release of cytochrome c in the cytoplasm, where it forms a complex or apoptosome with apoptotic protease activating factor 1 (APAF1) and the inactive form of caspase-9. This complex hydrolyzes adenosine triphosphate to cleave and activate caspase-9. The initiator caspase-9 then cleaves and activates the executioner caspases-3/6/7, resulting in cell apoptosis. It's totally different from the extracellular signals, which are usually generated by cytotoxic cells of the immune system and trigger apoptosis mainly through the extrinsic pathway (Brumatti et al., 2010; Galluzzi et al., 2018; Loreto et al., 2014; Vitale et al., 2017).

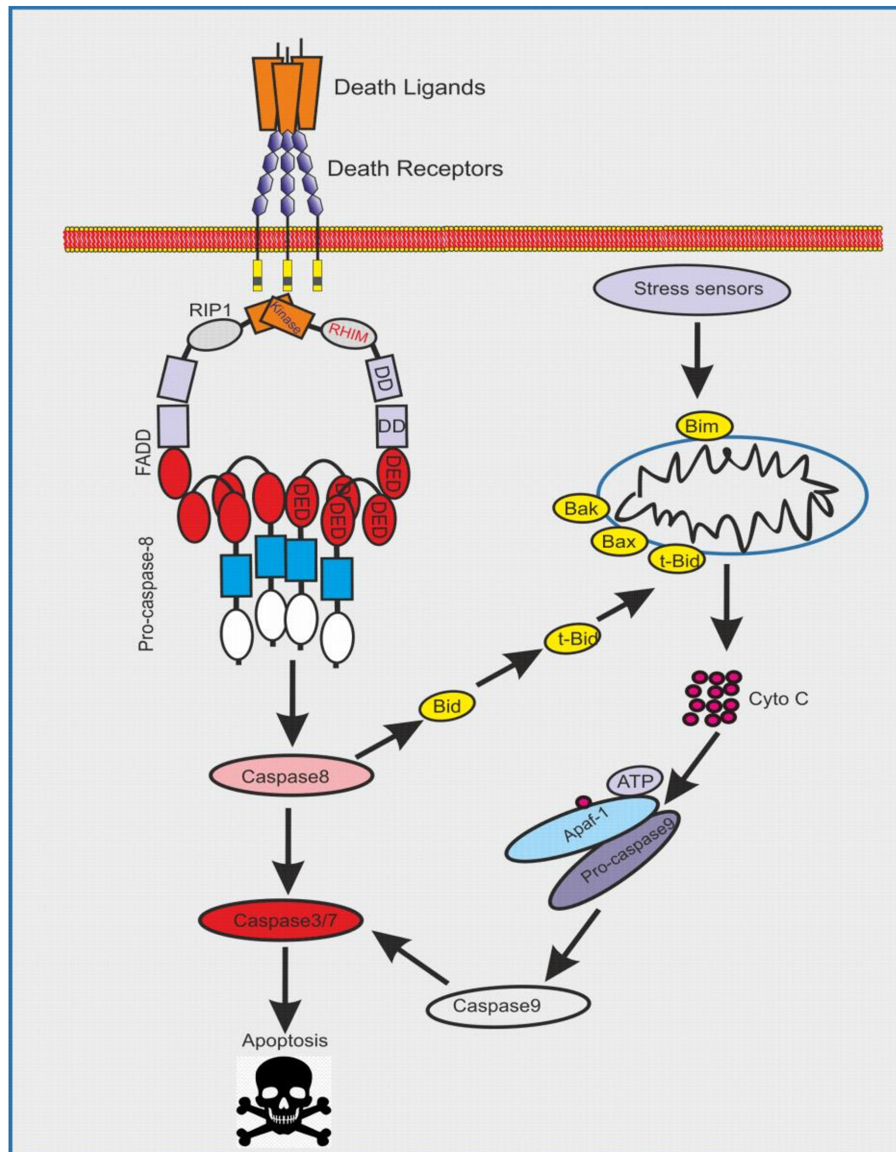


Figure 4: Schematic representation of the signaling pathway to Intrinsic and extrinsic pathways of apoptosis.

1.7.2. Necroptosis

Necroptosis is an alternative mode of controlled cell death in which receptor interacting protein (RIP) kinases play critical roles. In 1988，Scott M. Laster showed that TNF-α could induce both apoptotic and necrotic forms of cell death depending on the cell type, thus questioning the purely unregulated nature of necrosis (Laster et al., 1988). Afterward, it has confirmed that necrosis can indeed be regulated (Scaffidi et al., 2002; Zong et al., 2004; Zong and Thompson, 2006). A new form of regulated necrosis was introduced, termed programmed necrosis (Necroptosis) (Vandenabeele et al., 2010). Recent publications have been concentrated on TNFα, caspase-8, and RIPK3 to recognize the molecular mechanism of necroptosis (Dhuriya and Sharma, 2018). Necroptosis can be mediated by receptors of the TNFRSF, interferon receptors, and Toll-like receptors (TLR3 and TLR4). Depending on the stimulating factors,

necroptosis is categorized into three classes: (i) Extrinsic programmed necrosis is enthused by TNFα, (ii) Intrinsic programmed necrosis is enthused by reactive oxygen species (ROS), and (iii) Ischemia mediated-necroptosis (Dhuriya and Sharma, 2018).

TNFα-induced necroptosis, similar to other TNFR1 responses, starts with the formation of a short-lived membrane signaling complex (called complex I) including RIPK1, TRADD, TRAF2/TRAF5, and cIAP1/cIAP2. TNFR1 recruits RIPK1 and TRADD (Hanahan and Weinberg, 2011; Micheau and Tschopp, 2003). Consequently, TRAF2/5 and cIAPs are engaged to complex I (Vandenabeele et al., 2010), which in turn leads to ubiquitination of RIPK1 and stable complex I formation which initiates antiapoptotic pathways leading to the activation of (Newton et al., 2014). NF-κB signaling plays a crucial role in offsetting the cytotoxic effect of TNFα, and the prosurvival effect of NF-κB are due to induction of cFLIPL (cellular FLICE-like inhibitory protein) and cIAP1/2 (Kreuz et al., 2001; Papa et al., 2004). Usually, the formation of caspase-8 and cFLIPL heterodimer leads to inactivation of caspase-8 and inhibition of apoptosis. It is well known that caspase-8 mediates the exogenous apoptosis and prevents the necroptosis by impeding the activity of RIPK1 and RIPK3 (Dhuriya and Sharma, 2018). Contradictory, inhibition or removal of caspase-8 causes activation of RIPK1 through deubiquitination induced by cylindromatosis (CYLD) hence destabilizing complex I (O'Donnell et al., 2011). Inhibition of linear and K63 RIPK1 ubiquitination causes its interaction with TRADD, FADD, caspase-8, and RIPK3, which further give rise to the formation of complex II. RIPK1 binds RIPK3 through the RIP homotypic interaction motif (RHIM) leading to the formation of the necrosome, which in turn triggers the activation of the necroptotic execution molecules such as MLKL (Zhu et al., 2019). Both RIPK1 and RIPK3 play an essential role in the induction of necroptosis; However, RIPK3 can alone induce necroptosis when it is overexpressed in cells. RIPK3 phosphorylates the pseudokinase MLKL (mixed lineage kinase domain-like protein) (Dondelinger et al., 2013). Several groups have researched the mechanisms of how MLKL induces membrane rupture in the final stages of necroptosis. Wang et al. and Dondelinger et al. gave evidence that MLKL can puncture the membrane by forming pores. When MLKL becomes phosphorylated, it undergoes conformational changes, leading to the formation of oligomers and translocation to the plasma membrane, where its N-terminal domain binds directly to negatively charged phosphatidylinositol phosphates (PIPs) and cardiolipin (CL) leading to the formation of pores **(Figure 5)** (Dondelinger et al., 2014; Hildebrand et al., 2014; Quarato et al., 2016; Murphy et al., 2013; Wang et al., 2014). Another model suggests that MLKL may regulate ion channels at the plasma membrane during programmed necrosis to mediate osmolysis (Chen et al., 2014; Hildebrand et al., 2014; Huang et al., 2017).

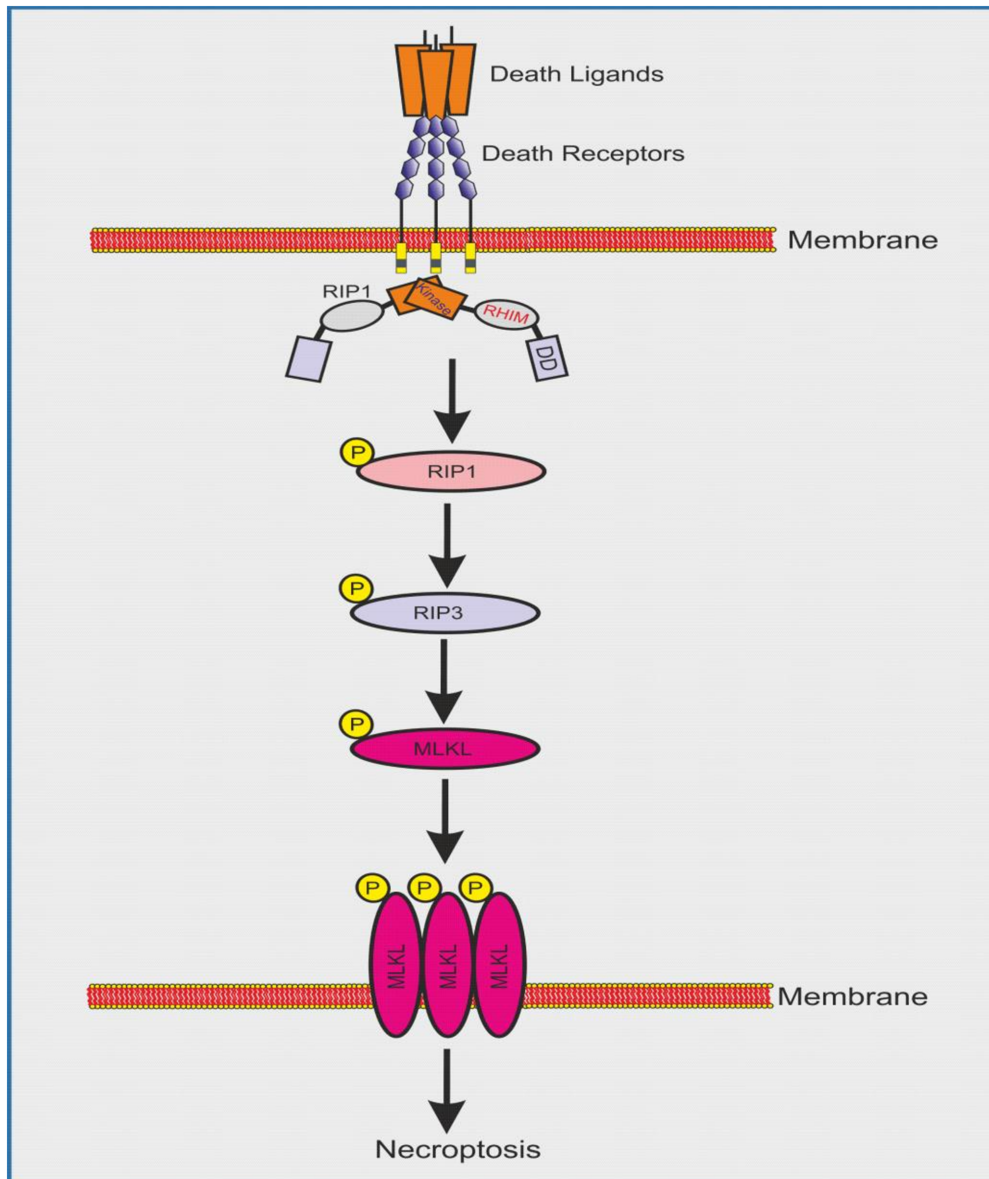


Figure 5: Molecular mechanism of necroptosis.

Details are described above in the text.

1.8. Aim of the study

The TWEAK/Fn14 system plays a crucial role in different cellular activities such as inflammation, proliferation, differentiation and in rare cases cell death induction. The upregulation of Fn14 expression in non-hematopoietic cells and release of DAMPs/PAMPs such as double-stranded RNA and lipopolysaccharide (LPS) were observed after tissue injury. On the other side, it has been shown that both immune and non-immune cells can detect PAMPs and DAMPs by the help of membranous and cytosolic pattern recognition receptors (PRRs). One of the PRRs is TLR3 which can detect double-stranded RNA and which stimulate both proinflammatory and cell death signaling. Consequently, it seems that the TWEAK/Fn14 system and TLR3 control togethersome cellular activates. Recently our group showed that TWEAK sensitizes cells for death receptor-induced cell death. RIPK1, TRADD, caspase-8, and FADD are responsible for the transduction of death and proinflammatory signaling by death receptors and TLR3. Indeed, both TWEAK and the TLR3 ligand poly(I:U) are used in different clinical trials for the treatment of different diseases such as cancer. This project aimed to investigate whether TWEAK can prime the cells for poly(I:C)-induced cell death. This goal was further divided into five specific research aims:

1) To investigate whether TWEAK enhances in the HeLa-RIPK3 and HaCaT cell line models poly(I:C)-induced apoptosis and/or necroptosis.
2) To evaluate the mechanism by which TWEAK sensitizes ells for poly(I:C)-induced cell death.
3) To study possible differences between soluble and membrane TWEAK in poly(I:C)-induced cell death.
4) To analyze the role of RIPK1, TRADD, caspase-8, and FADD in poly(I:C)-induced proinflammatory and death signaling.
5) To address whether short and long isoforms of cFLIP induce or inhibits poly(I:C)-induced necroptosis.

The overall aim of this research was aimed at identifying the crosstalk between the TWEAK/Fn14 system and TLR3 ligand poly(I:C) signaling, with a particular emphasis on programmed necrotic cell death.

2. Materials

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

Table 2: Chemicals, reagents and cell culture media

Substance	Company
Acetic acid	J. T. Baker, Leibzig, Germany
Acrylamide (30 %)	Carl Roth, Karlsruhe, Germany
Agarose	Carl Roth, Karlsruhe, Germany
Ammonium persulfate (APS)	AppliChem, Darmstadt, Germany
Ampicillin	Carl Roth, Karlsruhe, Germany
Anti-Flag M2 agarose beads	Sigma, Deisenhofen, Germany
Bovine serum albumin (BSA)	Sigma, Deisenhofen, Germany
Crystal violet (CV) powder	Carl Roth, Karlsruhe, Germany
Cycloheximide (CHX)	Sigma, Deisenhofen, Germany
Dimethyl sulfoxide (DMSO)	Carl Roth, Karlsruhe, Germany
DMEM medium	PAA, Pasching, Austria
Ethanol	J. T. Baker, Leibzig, Germany
Ethidium bromide	Carl Roth, Karlsruhe, Germany
Ethylenediaminetetraacetic acid (EDTA)	Carl Roth, Karlsruhe, Germany
Fetal bovine serum (FCS)	PAA, Pasching, Austria
Flag peptide	Sigma, Deisenhofen, Germany
Killer-TRAIL	Sigma, Deisenhofen, Germany
Methanol	PAA, Pasching, Austria
Nonfat dried milk powder	J. T. Baker, Leibzig, Germany
Paraformaldehyde	Sigma, Deisenhofen, Germany
Penicillin-Streptomycin (100 x)	Carl Roth, Karlsruhe, Germany
Peptone	PAA, Pasching, Austria
Phosphatase inhibitor II	Carl Roth, Karlsruhe, Germany

Table 2 continued: Chemicals, reagents and cell culture media

Substance	Company
Phosphate buffered saline (PBS)	Sigma-Aldrich, Darmstadt, Germany
Poly(I:C)	Sigma-Aldrich, Darmstadt, Germany
Polymyxin B (PMB)	PAA, Pasching, Austria
Prestained protein marker (broad range)	InvivoGen, Toulouse, France
Protease inhibitor cocktail	Biomol, Hamburg, Germany
Protein G agarose	Roche, Mannheim, Germany
Blue Protein standrds	New England Biolabs, Frankfurt, Germany
RPMI 1640 Medium	Roche, Mannheim, Germany
Silver gel marker (low molecular weight)	PAA, Pasching, Austria
Sodium dodecyl sulfate (SDS)	GE Healthcare, Garching, Dassel, Germany
Sucrose	Carl Roth, Karlsruhe Garching, Germany
Tetramethylethylenediamine (TEMED)	Sigma, Deisenhofen, Germany
Tris	Sigma, Deisenhofen, Germany
Triton X-100	Carl Roth, Karlsruhe, Germany
Trypsin-EDTA solution (10X)	Sigma, Deisenhofen, Germany
Tween-20	PAA, Pasching, Austria
Yeast extract	Carl Roth, Karlsruhe, Germany
β-Mercaptoethanol	Carl Roth, Karlsruhe, Germany

2.2. Antibodies

Table 3: Antibodies

Antibody	Source	Company
Anti-A20/TNFAIP3	Rabbit monoclonal, clone D13H3	Cell Signaling Technology, Beverly, MA, USA
Anti-caspase-3	Rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
Anti-caspase-8	Mouse IgG2b, clone C15	Enzo Life Sciences, Lörrach, Germany
Anti-caspase-9	Rabbit polyclonal,	Cell Signaling Technology, Beverly, MA, USA
Anti-c-IAP1	Rabbit monoclonal, clone D5G9	Cell Signaling Technology, Beverly, MA, USA
Anti-c-IAP2		
Anti-p-c-JUN	Rabbit monoclonal, clone D47G9	Cell Signaling Technology, Beverly, MA, USA
Anti-FADD	Rabbit polyclonal	Santa Cruz Biotechnology, Heidelberg, Germany
Anti-Flag mAb M2	Mouse IgG1 monoclonal	Sigma, Deisenhofen, Germany
Anti-FLIP (NF6)	Mouse IgG1 monoclonal	Enzo Life Sciences, Lörrach, Germany
Anti- Fn14	Rabbit monoclonal	Cell Signaling Technology, Beverly, MA, USA
Anti-IκBα	Mouse monoclonal, clone L35A5	Cell Signaling Technology, Beverly, MA, USA
Anti-mouse IRDye 800	Goat polyclonal	LI-COR Bioscience, Bad Homburg, Germany
Anti-mouse-HRP	Rabbit polyclonal	Dako-Cytomation, Denmark
Anti-PARP	Mouse IgG1, clone 7D3-6	BD Biosciences, Heidelberg, Germany
Anti-p-IκBα	Rabbit polyclonal	Cell Signaling Technology, Beverly, MA, USA
Anti-p-IRF3	Rabbit monoclonal	
Anti-rabbit-HRP	Goat polyclonal	Dako-Cytomation, Glostrup, Denmark
Anti-rabbit-HRP	Goat polyclonal	Cell Signaling Technology, Beverly, MA, USA

Table 3 continued: Antibodies

Anti-TRAF1	Rabbit monoclonal	Cell Signaling Technology, Beverly, MA, USA
Anti-TRAF2	Rabbit monoclonal	Cell Signaling Technology, Beverly, MA, USA
Anti-tubulin	Mouse monoclonal	Dunn Labortechnik, Asbach, Germany
ITEM4 PE	Mouse monoclonal	BioLegend Way, San Diego, USA
IgG2B-PE	Mouse monoclonal	R&D Systems, Wiesbaden, Germany
β-actin	Mouse monoclonal	Sigma-Aldrich, Darmstadt, Germany

2.3. Kits

Table 4: kits

Kit	Company
OptEIA IL8-ELISA	BD Biosciences, Heidelberg, Germany
Pierce ECL Western Blotting Substrate	Fermentas, St. Leon-Rot, Germany
Pierce® Silver Stain	Fermentas, St. Leon-Rot, Germany
Pure Yield Plasmid Midi prep System	Promega, Mannheim, Germany

2.4. Instruments and disposable materials/equipments

Table 5: Instruments and disposable materials/equipments

Instrument or material/equipment	Company
96-well ELISA plates (high binding)	Greiner, Frickenhausen, Germany
Agfa Curix 60 processing maschine	Agfa, Düsseldorf, Germany
Casting chambers for SDS-PAGE	PeqLab, Erlangen, Germany
Cell culture bottles	Greiner, Frickenhausen, Germany
Cell culture petri dishes	Greiner, Frickenhausen, Germany
Cell culture plates	Greiner, Frickenhausen, Germany
Centrifuge Rotana 460R	Hettich, Tuttlingen, Germany
CO_2 incubator Heraeus Cell Safe	Heraeus, Hanau, Germany
Cryotubes	Greiner, Frickenhausen, Germany
Dialysing tubes, Viking, MWCO 15kDa	Carl Roth, Karlsruhe, Germany
Electrophoresis system	BioRad, München, Germany

Table 5 continued: Instruments and disposable materials/equipments

ELISA-reader	Anthos Labtec, Krefeld, Germany
Eppendorf tubes, 1,5 ml und 2 ml	Eppendorf, Hamburg, Germany
Equibio EasyjecT Plus electroporator	PeqLab, Erlangen, Germany
Flow cytometer FACScaliber	BD Biosciences, Heidelberg, Germany
Flow cytometry tubes	Falcon, Heidelberg, Germany
Heat block	PeqLab, Erlangen, Germany
LI-COR Odyssey® Infrared Imager	LI-COR Biosciences, Lincoln, USA
MACS LS columns	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS multistand	Miltenyi Biotec, Bergisch Gladbach, Germany
MACS separator	Miltenyi Biotec, Bergisch Gladbach, Germany
Microcentrifuge 5417C	Eppendorf, Hamburg, Germany
Nitrocellulose membranes, 0,2 μM pore size	Whatman, Dassel, Germany
PCR-Thermocycle Primus	MWG Biotech, Ebersberg, Germany
Pipetus	Hirschmann Laborgeräte, Eberstadt, Germany
Polyallomer tubes	Seton, Los Gatos, CA, USA
Polypropylene tubes	Greiner, Frickenhausen, Germany
Power supply EPS 301	GE Healthcare, Garching, Germany
Sterile filters (0,2μm)	Sarstedt, Nümbrecht, Germany
Sterile plastic Pasteur pipettes	Hartenstein, Würzburg / Versbach, Germany
Ultracentrifuge OPTIMA-L70	Beckman Coulter, Krefeld, Germany
Well plates for cell culture	Greiner, Frickenhausen, Germany
Wet/tank blotting system	PeqLab, Erlangen, Germany
Whatman papers	Hartenstein, Würzburg / Versbach, Germany

2.5. Preparations and buffers

Table 6: Preparations and buffers

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

Table continued: Preparations and buffers

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

2.6. Cells

2.6.1. Eukaryotic cells

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

Table 7: Eukaryotic cells

Cell line	Source	Origin of cancer
HaCaT	Institution's own stock	keratinocyte cell
HaCaT-$FLIP_L$	Stably transfected cell line has been generated in the group of Prof. Wajant	keratinocyte cell
HaCaT-$FLIP_S$	Stably transfected cell line has been generated in the group of Prof. Wajant	keratinocyte cell line
HEK293	*Institution's own stock*	Human embryonic kidney
HeLa	*Institution's own stock*	Human cervical carcinoma
HeLa-RIK3	A kind gift of Prof. Martin Leverkus (University Hospital Aachen)	Human cervical carcinoma
HeLa-RIK3-$FADD_{KO}$	Stably transfected cell line has been generated in the group of Prof. Wajant	Human cervical carcinoma
HeLa-RIK3-$TRADD_{KO}$	Stably transfected cell line has been generated in the group of Prof. Wajant	Human cervical carcinoma
HeLa-RIK3-$RIPK1_{KO}$	Stably transfected cell line has been generated in the group of Prof. Wajant	Human cervical carcinoma
HeLa-RIK3-$caspase8_{KO}$	Stably transfected cell line has been generated in the group of Prof. Wajant	Human cervical carcinoma
HeLa-RIK3-TRADD+RIP_{DKO}	Stably transfected cell line has been generated in the group of Prof. Wajant	Human cervical carcinoma

2.6.2. Prokaryotic cells

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

3. Methods

3.1. Cell culture

HeLa-RIPK3 and HeLa-RIPK3 knockout variants were sustained in RPMI1640 medium containing 10% heat-inactivated fetal bovine serum (FBS). HeLa-RIPK3-FLIPL/S transfectants were generated by sleeping beauty transposon-based expression constructs encoding FLIPL and FLIPS. HaCaT cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. HaCaT cells stably transfected with FLIPL or FLIPS have been described in (Kavuri et al., 2011). All cells were grown at 37 °C and 5% CO2.

All cells were harvested after washing with PBS (1x) and incubating the cells with trypsin-EDTA solution for 10 minutes. Then the cells were centrifuged at 1200 rpm for 4 minutes and were counted under the microscope using hemocytometer before seeding on cell culture plates for further experiments. The rest of the cells were diluted to the ratio of 1:3 till 1:10 and further cultivated in fresh medium with 10 % FCS and were regularly freezed at -80 °C in 1 ml freezing medium (10 % DMSO in FCS) using cryotubes.

3.2. Cloning and production of the Flag-TWEAK expression plasmid

The Flag-TWEAK plasmid was cloned by the help of one of my colleges. Then, I received the DNA and I did Midi preparation.

3.2.1. Isolation of Plasmid DNA (Midi Preparation)

For the isolation of plasmid DNA, a single positively screened clone from the master plate was injected in 100 ml of overnight cultures were prepared in LB medium containing 1 % glucose and 100 µg/ml ampicillin. The next day, a glycerol stock (26 % glycerol) was prepared from an aliquot of the bacterial suspension for long-term storage of the clone, and the remaining cells were harvested by centrifugation for 15 min at 6,000 × g and 4°C. The isolation of plasmid DNA was completed by using Plasmid Midiprep System from Promega following the manufacturers' instructions. The eluted DNA was finally stored at –20°C. The DNA concentration was measured photometrically at OD260 using the spectrophotometer GeneQuant *pro* from Pharmacia.

3.2.2. Protein production

The Flag-TWEAK expression plasmid was transfected in HEK293 with PEI transfection, was performed as follows: A plasmid-PEI mixture was prepared by adding 36 µl of a 1mg/ml water solution of PEI dropwise under vortexing in 2 ml of serum-free RPMI 1640 medium containing 12 µg plasmid DNA. After incubating the plasmid-PEI mixture for 10 min at room temperature, the mixture was added to a 15-cm tissue culture dish with close to confluent HEK293 cells

which had received immediately before 15 ml fresh serum free medium. Next day, the old medium was replaced by 1% P/S and 2% FCS RPMI 1640 medium. After 7 days, the supernatant was collected and centrifuged at 4600 rpm for 10 min to remove the dead cells. The status of protein production was controlled regularly by measuring the protein concentration using Western Blot technique after boiling protein samples for 5 min at 95 °C (see section 3.9). The nitrocellulose membranes were then incubated overnight with anti-Flag mAb M2 and incubated in the next day for one hour with the second antibody anti-mouse IRDye 800 after washing the first antibody. Finally, the nitrocellulose membranes were scanned using LI-COR Odyssey® Infrared Imager to determine the concentration of each protein depending on the intensity of the detected bands of the corresponding proteins and a standard protein of known concentration

3.3. Protein purification

The Flag-TWEAK protein supernatants produced by HEK293 cells were further purified using affinity chromatography on agarose beads of anti-Flag M2. The beads were settled in a column and then washed with autoclaved TBS. Then, the supernatant was applied on the column with adjusted flow rate of at least one drop/30 sec. The column was left at 4 °C until all the flow-through was collected. Afterwards, the beads were washed with TBS and the bound protein molecules were eluted from the beads in 0,5 ml fractions using TBS containing 100 µg/ml of Flag peptide and the flow rate was at least one drop/min. The eluted proteins were dialyzed against PBS overnight at 4 °C and then sterile filtered in the next day and stored at -20 °C for further analysis. The percent of protein recovery after purification was controlled by measuring the protein concentration in all the followings: the supernatant before purification, the flow-through, the elution-fractions, the TBS washing flow-through after purification and beads using Western Blot technique (see section 3.7).

3.4. Silver staining.

The purified Flag-TWEAK was separated by SDS-PAGE (See section 3.7.1) and the gel was stained using Pierce® Silver Stain Kit according to the instructions of the manufacturer.

3.5. FACS analysis

To detect the surface expression of Fn14 receptors on HeLa-RIPK3 and HaCaT cells. $5x10^5$ of cells were transferred to U shape 96-well plate. The plate were centrifuged for 4 min at 1200 rpm and the cells were washed 2 times with ice-cold PBS. Afterwards, the cells were incubated for 1hour at 4 °C with the α-Fn14 antibody (ITEM4) and its corresponding isotype control according the instruction of the manufacturer. After that, the cells were washed 3 times with PBS and resuspended in 300 µl PBS, transferred into FACS tubes, and analysed by flow

cytometry using BD FACS Calibur. Finally, the data were analysed with the help of WinMDI 2.8 software.

3.6. Immunoprecipitation (IP) analysis

For detection of Fn14 complex, 5 × 10^6 of HeLa-RIPK3 and HaCaT cells were seeded for each group. Cells were stimulated with 2 µg/ml of Fc-Flag-TWEAK for 0, 10, 30 minutes at 37°C and 5 % CO_2. The cells were washed 4 times with ice-cold PBS to stop the receptor complex formation. Afterwards, the cells were scratched and collected on ice in 50 ml falcon tubes and the volume was completed to 50 ml with ice cold PBS. The falcon tubes were centrifuged for 3 minutes at 2300 rpm and the supernatant was discarded. Afterwards, the pellets were centrifuged again with 50 ml ice cold PBS. The cell pellets were mixed with1.5 ml IP lysis in the presence of protease inhibitor in 2 ml Eppendorf tubes and left for 20 minutes on ice. Then, the Eppendorf tubes were centrifuged for 5 min at 5000 rpm (4 °C) and the supernatants were transferred to new 2 ml Eppendorf tubes. The supernatants were centrifuged again for 45 min at 14000 rpm (4 °C). 200 µl of the supernatants from each group was stored at -20 °C for further analysis of lysates by Western Blot and the rest were used for the IP experiment. 5 ng/ml of each Fc-Flag-TWEAK was added to the corresponding negative control group. After that, the receptor complexes were precipitated from the supernatants by co-incubation with 40 µl of protein G beads in two ml Eppendorf tubes overnight on shaker at 4 °C. In the next day, the Eppendorf tubes were centrifuged for 30 sec at 5000 rpm (4 °C). The supernatants were then discarded using 1 ml insulin syringes. Then, two ml of IP lysis buffer without protease inhibitor was added on the beads and the Eppendorf tubes were centrifuged again for 30 sec at 5000 rpm (4 °C). The previous step was repeated three times and then the supernatants were removed completely using one ml insulin syringes. In the next step, 60 µl 4× Laemmli buffer and 60 µl IP lysis buffer were added on the beads in Eppendorf tubes and the mixture was further heated at 80 °C for 10 min. Then, the Eppendorf tubes were left on ice for 10 sec and then centrifuged for 5-10 sec. Finally, the supernatants from each group were carefully transferred away from the beads in other new Eppendorf tubes using 1 ml insulin syringe and stored at -20 °C for further analysis of immunoprecipitates by Western Blot.

3.7. Western Blot

3.7.1. SDS-PAGE

For separation of the proteins using SDS-PAGE gels. First, the separating gel was prepared from 0,374 M Tris (pH 8,8) , 0,0035 M SDS, dis. H2O 12 % or 10 % acrylamide and then polymerized using 0,1 % APS and 0,1 % TEMED. Isopropanol was added on the surface of the separating gel immediately after pouring of the gel and before its polymerization to obtain a straight surface. After polymerization, isopropanol was taken off and the stacking gel was

added which was composed of 6 % acrylamide in 0,123 M Tris (pH 6,8), 0,00375 M SDS, 0,1 % APS and 0,1 % TEMED. The sample chambers were inserted in the stacking gel before polymerization. After polymerization, these sample chambers were removed, and the samples were added using micropipette to run electrophoretic separation at 120 V and 400 mA for 95 min in case of small gels and 105 min in case of large gels.

3.7.2. Blotting on nitrocellulose membrane

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

3.7.3. Membrane detection

After the blocking of remaining binding sites on the membrane with 5 % PBST-milk for 1 h on the shaker. After that, the membranes were washed 3 times with TBST or PBST for 30 min and then incubated overnight at 4 °C on the shaker with the required first antibody in PBST or TBST according to the manufacturer. In the next day, the membranes were washed again three times with TBS or PBST for 30 min and afterwards the membranes were incubated with secondary antibody in PBST or TBST-milk for 1h at room temperature on the shaker. Finally, the membranes were washed 3 times with PBST or TBST for 30 min and then detected either using LI-COR Odyssey® Infrared Imager or with ECL-system by incubation of the membrane with the ECL substrate solution for 1 – 2 min and was subsequently exposed to an X-ray film for a few seconds. The film was then automatically developed in an X-ray film processor.

3.8. Cell viability assay

Cells were seeded (2×10^4 of HeLa-RIPK3 cells or 3×10^4 of HaCaT cells per well) in 96-well tissue cultures plates in 100 µl cell culture medium containing 1% P/S and 10 % FCS (RPMI 1640 medium for HeLa-RIPK3 or DMEM medium for HaCaT cell line) and incubated overnight at 37 °C. Next day, cells were challenged overnight in triplicates with the reagents of interest such as 40µg/ml of Poly(I:C) in the presence and absence of 200 ng/ml of Flag-TWEAK or 2.5 µg/ml of CHX. Cell viability was measured by crystal violet staining by removal of the supernatant from the plates and 80 µl/well of the CV staining solution was added on the plates and left for 20 minutes at room temperature. Afterwards, the excess CV staining was removed by washing two times with distilled water. The plates were left to dry at room temperature.

Finally, the plates were measured at 595 nm. To normalize cell viability values, each plate included a triplicate of untreated cells considered as 100 % viable and a triplicate of cells incubated with a cytotoxic mixture (200 ng/ml TNF, 200 ng/ml CD95L 200 ng/ml TRAIL, 25 µg/ml CHX, 1 % (w/v) sodium azide) producing maximal cell death to deliver the value for 0 % viability. All other viability values were normalized according to the averages of these triplicates and analysed by the Graph Pad Prism 5 software (La Jolla, CA, USA).

3.9. IL8 ELISA

Cells were cultivated (2×10^4 of HeLa-RIPK3 cells or 2.5×10^4 of HaCaT cells per well) in 96-well tissue culture plates. On the next day, medium was replaced to reduce the background of constitutive cytokine production, and cells were stimulated overnight with 40µg/ml of Poly(I:C) in the presence and absence of 200 ng/ml Flag-TWEAK or 2.5µl/ml CHX. The supernatants were evaluated for production of IL-8 using the human IL-8 ELISA kit BD Biosciences (Heidelberg, Germany) according to the instructions of the supplier.

3.10. Total cell lysates

Total cell lysates were prepared by collecting $1x10^6$ of cells in ice-cold PBS and centrifuging them at 12000 rpm for 2 minutes at 20 °C. Then, the pellets were lysed in 4× Laemmli buffer with freshly added phosphatase inhibitor mixture II and protease inhibitor. Afterwards, cell lysates were sonficated for 20 sec and then boiled for 5 min at 95 °C. Finally, total cell lysates were centrifuged for 10 min at 14000 rpm and stored at -20 °C for further analysis by Western Blot.

3.11. Statistical analysis

All presented figures in this current work were designed by Microsoft Office Excel 2007, GraphPad Prism 5.0 program, CorelDRAW Graphics Suite X4 software and EndNote X9.

4. Results

4.1. Characterization of Flag-TWEAK

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily (TNFSF) and binds the receptor Fn14 to exert its biological functions (Wajant, 2013). We analyzed the expression of Fn14 on the poly(I:C)-responsive HeLa-RIPK3 and HaCaT cells by flow cytometry (**Figure 6**a). To evaluate the effect of soluble TWEAK on poly(I:C)-induced cell death and inflammatory signaling, the soluble form of TWEAK, containing the TNF homology domain (THD), was expressed as a Flag-tagged protein. Flag-TWEAK was secreted into the surrounding media upon expression in HEK293 cells and was purified by affinity chromatography on anti-Flag agarose (**Figure 6**b).

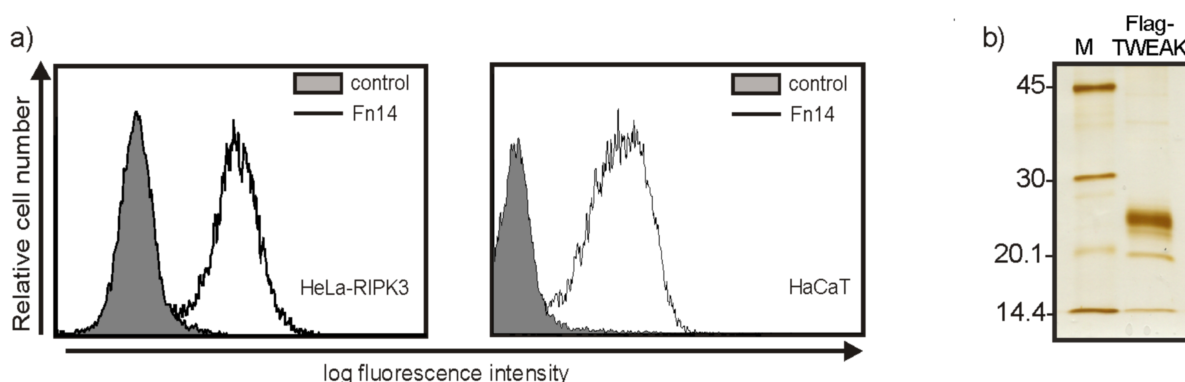


Figure 6: Evaluation of Fn14 expression by Flow cytometry and production and purification of Flag-TWEAK.

a) HeLa-RIPK3 and HaCaT cells were incubated with 1 µg/ml PE-labelled anti-Fn14 antibody (ITEM-4) for 30 minutes at 4 °C (blank area) or, as a control, with a corresponding isotype, PE-labelled IgG2b (filled area). After washing, cell-bound antibodies were quantified identified by flow cytometry. **b)** Affinity purified Flag-TWEAK protein was separated in SDS-PAGE and then detected by silver staining.

4.2. Soluble TWEAK and cycloheximide sensitize HeLa-RIPK3 and HaCaT cells for poly(I:C)-induced cell death

TLR3 is capable of prompting apoptosis by a TRIF/RIP1/FADD/caspase-8-dependent pathway (Maelfait et al., 2008; Galluzzi et al., 2018). We observed that poly(I:C) largely failed to induce cell death in HeLa-RIPK3 and HaCaT cells (**Figure 7**a, b). Treatment with CHX, which down-regulates the expression of short-lived survival proteins. However, sensitized the cells for vigorous killing by poly (I:C) (**Figure 7**a, b). Also, soluble Flag-TWEAK, which depletes the cytosolic pool ofTRAF2-cIAP1/2 complexes, sensitized the HeLa-RIPK3 and HaCaT cells for poly(I:C)-induced cell death (**Figure 7**a, b). Co-treatment with CHX and Flag-TWEAK resulted in enhanced poly(I:C)-induced cell death compared to HeLa-RIPK3 and HaCaT cells treated either with CHX or Flag-TWEAK alone (**Figure 7**c).

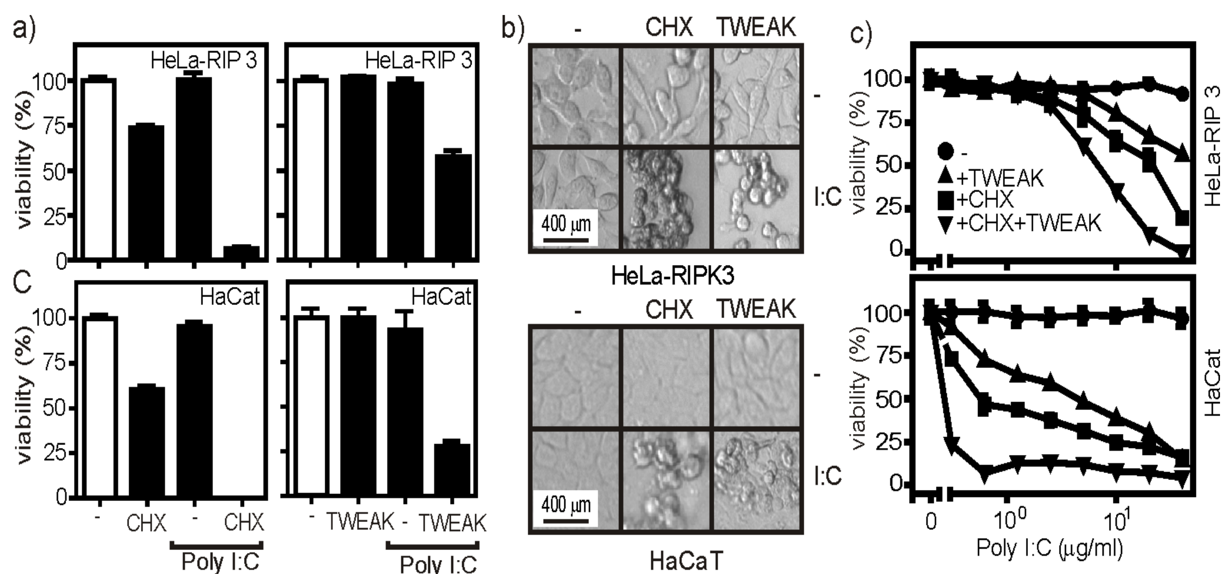


Figure 7: CHX and TWEAK sensitize cells for poly(I:C)-induced cell death.

a) Hela-RIPK3 and HaCaT cells were incubated overnight with the indicated combinations of poly(I:C) (40 µM), CHX (2.5 µg/ml) and Flag-TWEAK (200 ng/ml). Next day, cellular viability was evaluated by crystal violet staining. **b)** Cells were stimulated overnight with CHX (2.5 µg/ml) and Flag-TWEAK (200 ng/ml) in presence and absence of poly(I:C) (40 µM) in comparison to untreated cells. Cells were finally photographed using an EVOS FL digital microscope. **c)** Cells were pre-stimulated with 200 ng/ml of Flag-TWEAK or 2.5 µg/ml CHX or a combination of both in the presence and absence of indicated concentrations of poly(I:C). Finally, cellular viability was assessed by crystal violet staining.

4.3. Poly(I:C) induces inflammatory signaling independent from CHX and TWEAK

Nuclear factor-κB (NF-κB) represents a family of inducible transcription factors, which controls multiple aspects of innate and adaptive immune system and acts as a fundamental mediator of inflammatory responses. NF-κB involves in the expression of different pro-inflammatory genes, such as chemokines and cytokines. The chemokine interleukin (IL)-8 is regulated by transcriptions factor of the nuclear factor-κB (NF-κB) family. Measurement of IL-8 secretion can therefore be used as an indicator of activation of the classical NF-κB pathway. Indeed, several cell lines respond with NF-kB activation and IL-8 secretion after TWEAK stimulation treatment (Chicheportiche et al., 2002). Poly(I:C) induced IL-8 independent from treatment with CHX and Flag-TWEAK. Poly(I:C) also upregulated the survival proteins TRAF1 and p-IRF3 (**Figure 8**a, b).

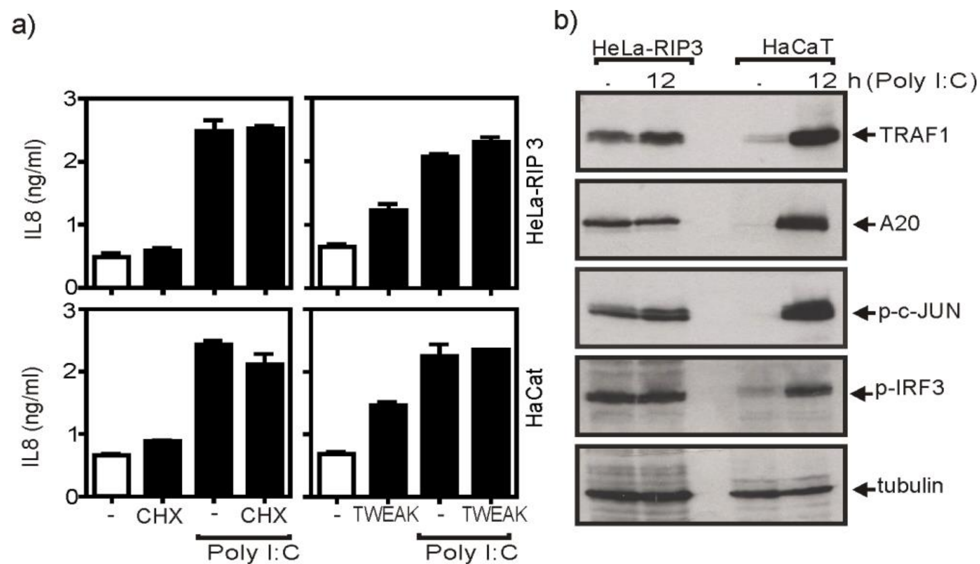


Figure 8: Poly(I:C) stimulates expression of inflammatory proteins.

a) HeLa-RIPK3 and HaCaT cells were incubated overnight in triplicates with poly(I:C) plus the indicated combinations of CHX (2.5 µg/ml) and Flag-TWEAK (200 ng/ml). IL8 were measured by ELISA. b) Cells were stimulated for 12 h with poly(I:C), and total cell lysates were analyzed for the expression of the indicated proteins by western blotting.

4.4. TWEAK primes preferentially for poly(I:C)-induced necroptosis.

It has been reported that TWEAK boosts both TNF-induced necroptosis and apoptosis (Chopra et al., 2015; Grabinger et al., 2017，Karl et al., 2014; Schneider et al., 1999; Wicovsky et al., 2009; Zimmermann et al., 2011). Moreover, TWEAK can also enhance TRAIL death receptor-induced necroptosis (Karl et al., 2014). Therefore, we wondered whether TWEAK affects apoptosis- and/or necroptosis-induction by poly(I:C). A different contribution of apoptosis and necroptosis to poly(I:C)-induced cell death in the presence of CHX- and Flag-TWEAK became evident from inhibitor studies with zVAD-fmk (ZVAD) and necrostatin-1 (nec1). While the pan-caspase inhibitor ZVAD protects cells from death receptor-induced apoptosis, nec1 inhibits the kinase activity of RIPK1, which has a vital role in necroptosis signaling by various inducers (Degterev et al., 2005).

Treatment of CHX-sensitized HeLa-RIPK3 and HaCaT cells with ZVAD alone caused a significant rescue from poly(I:C)-induced killing (**Figure 9**a). In contrast, ZVAD enhanced poly(I:C) induced cell death in Flag-TWEAK-treated HeLa-RIPK3 cells but led to a partial protective effect on poly(I:C)-induced cell death of Flag-TWEAK treated HaCaT cells (**Figure 9**b). On the other side, treatment of the cells with Nec.1 showed an inhibitory effect against poly(I:C)-induced cell death in Flag-TWEAK treated HeLa-RIPK3 cells but Nec.1 showed no protective effect on Flag-TWEAK sensitized HaCaT cells (**Figure 9**b). Treatment with a mixture of ZVAD and nec.1 fully protected against poly (I:C)-induced cell death in both cell types irrespective of the type of sensitizing (**Figure 9**a, b).

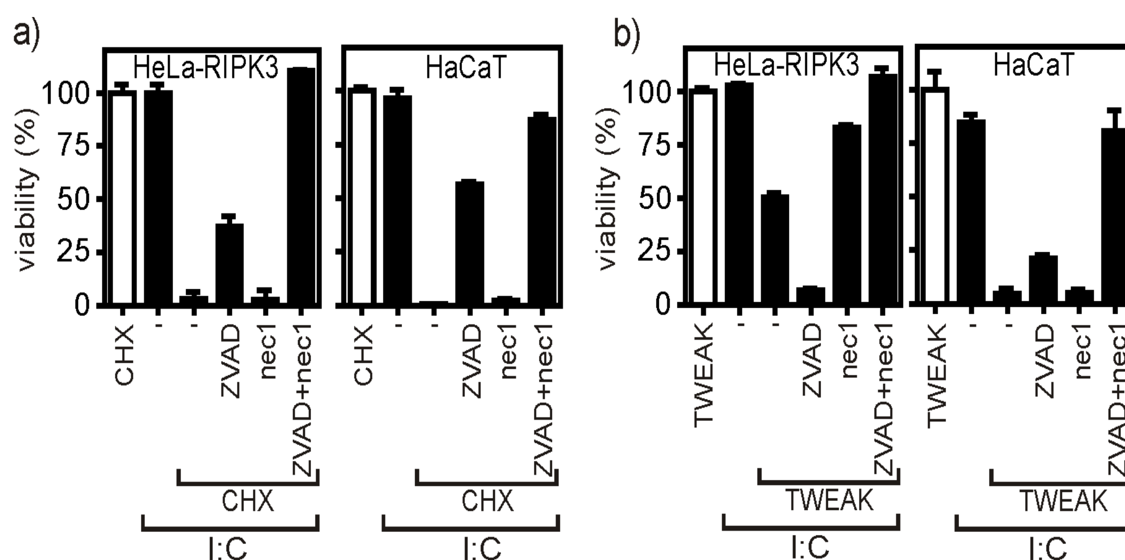


Figure 9: CHX sensitized cells for poly(I:C)-induced apoptosis while TWEAK primes cells preferentially for necroptosis.

HeLa-RIPK3 and HaCaT cells were pre-stimulated for 30 minutes with ZVAD (20 µM), necrostatin-1 (90 µM) and combination of both. Then the cells were challenged overnight with poly(I:C) (40 µg/ml) in the presence and absence of CHX (2.5 µg/ml) **a)** and TWEAK (200 ng/ml) **b)**. Next day, the viability of the cells was evaluated by crystal violet staining.

CHX treatment sensitized better than Flag-TWEAK for poly(I:C)-induced processing of caspases. Flag-TWEAK facilitated poly(I:C)-induced RIPK1 phosphorylation at serine 166 much more efficiently than CHX. Interestingly, CHX antagonized the sensitizing effect of Flag-TWEAK on poly(I:C)-induced RIPK1 phosphorylation, while treatment the cells with a mixture of CHX and Flag-TWEAK was neutral to larger caspase processing to the single treatment (**Figure 10**). The latter result confirms the fact that the apoptotic caspases inhibit the phosphorylation of RIPK1 and RIPK3, which leads to necroptosis by processing both (Humphries et al., 2015).

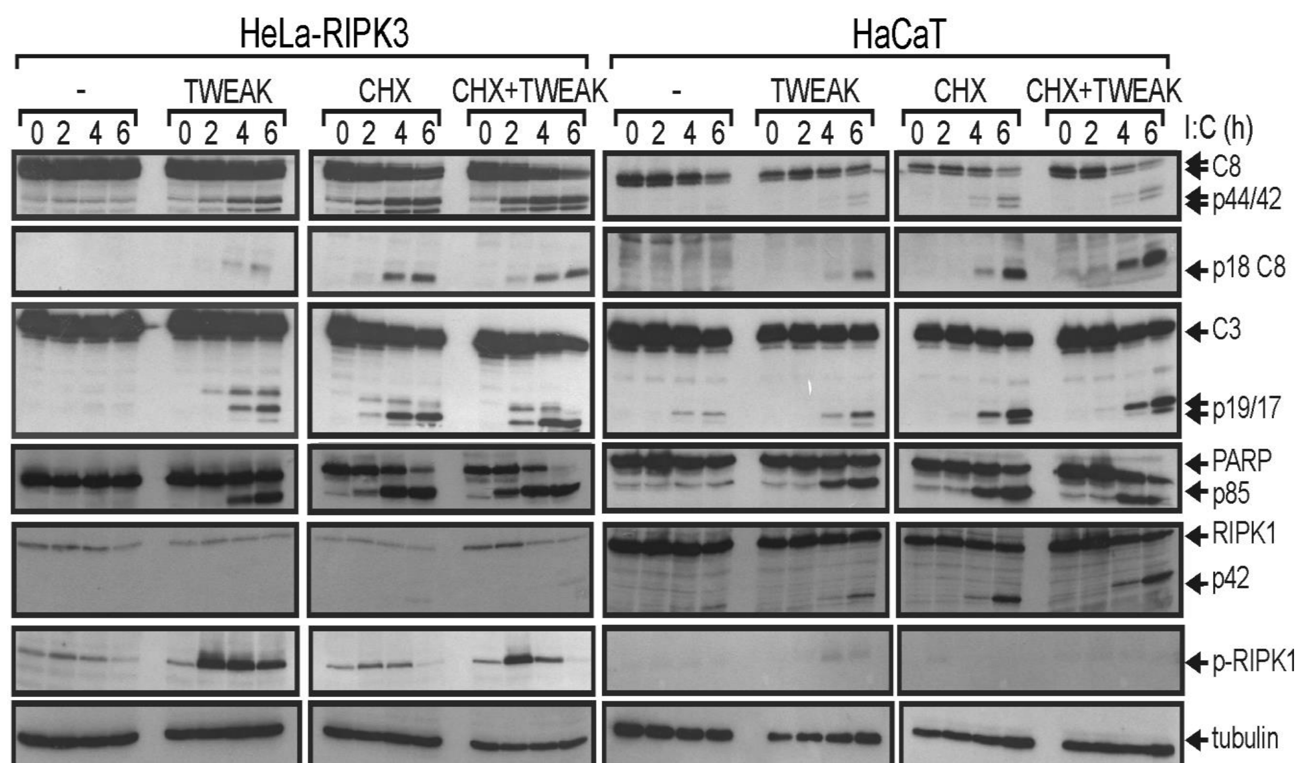


Figure 10: Poly(I:C) induces caspase processing in CHX and TWEAK treated cells but triggers phosphorylation of RIPK1 only in the presence of TWEAK.

Cells were treated with CHX (2.5 µg/ml), Flag-TWEAK (200 ng/ml) or a combination of CHX and TWEAK and were then challenged for the indicated times with poly(I:C) (40 µg/ml). Total cell lysates were analyzed for the presence of the indicated proteins by western blotting.

4.5. Poly(I:C)-induced apoptosis and necroptosis are TNF-independent.

TWEAK stimulates apoptosis by triggering TNF production and subsequent stimulation of TNFR1 in a few cell lines, e.g. Kym1 cells (Schneider et al., 1999; Vince et al., 2008). To address the possible relevance of endogenous TNF production for poly(I:C)/TWEAK-induced cell death, we evaluated the effect of a TNF-blocking antibody. The latter professionally antagonized TNF-induced apoptosis and necroptosis, however, showed no influence on poly(I:C)/TWEAK-induced cell death (**Figure 11**). This rules out a significant contribution of endogenously produced TNF to poly(I:C)/TWEAK-induced cell killing and suggests that the latter is primarily dependent on the ability of Flag-TWEAK to deplete the available cytosolic pool of cell death protective TRAF2-cIAP1/2 complexes (Vince et al., 2008; Wicovsky et al., 2009).

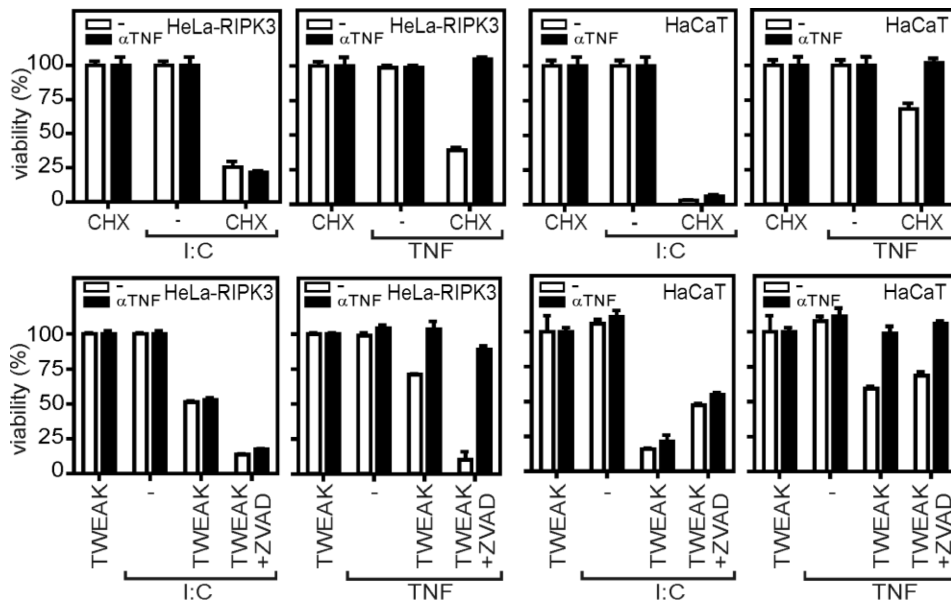
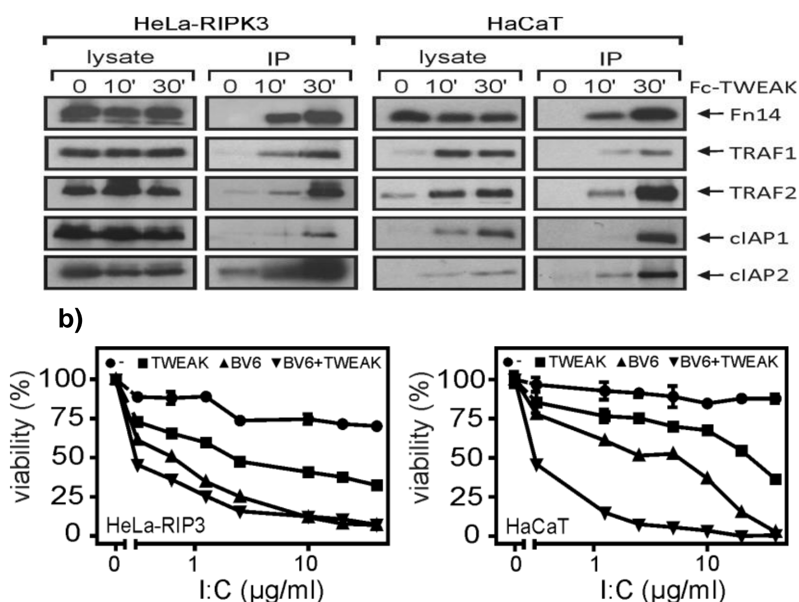


Figure 11: Induction of cell death by poly(I:C) is TNF-independent.

HeLa-RIPK3 and HaCaT cells were challenged overnight with the indicated mixtures of poly(I:C) (40 µg/ml), CHX (2.5 µg/ml), Flag-TWEAK (200 ng/ml), ZVAD (20 µM), TNF (100 ng/ml), anti-TNF and control IgG1 (both 25 µg/ml). Next day, cellular viability was evaluated by crystal violet staining.

By this idea, Flag-TWEAK sensitizes the HeLa-RIPK3 and HaCaT cells for poly(I:C)-induced cell death by recruitment of TRAF2, cIAP1, and cIAP2 to Fn14 (**Figure 12**a). Furthermore, Flag-TWEAK caused efficient recruitment of TRAF1, which forms heterotrimers with TRAF2 and lead to cIAP1 recruitment better than TRAF2 homotrimers (Zheng et al., 2010). In further accordance with this idea, the SMAC mimetic (BV6), which causes the autoubiquitination and degradation of cIAPs, NF-κB activation, and TNF alpha-dependent apoptosis (Varfolomeev et al., 2007) also sensitized HeLa-RIPK3 and HaCaT cells for poly(I:C)-induced cell death (**Figure 12**b).



a) Cells were treated with Fc-Flag-TWEAK (2 µg/ml), a fusion protein of soluble TWEAK with an N-terminal Fc domain. TWEAK-bound Fn14 and Fn14-associated signaling proteins were immunoprecipitated with protein G beads.

b) Hela-RIPK3 and HaCaT cells were stimulated with the indicated concentrations of poly(I:C) in the presence and absence of BV6 (10 µM) and Flag-TWEAK (200 ng/ml). Cell viability was finally measured again using crystal violet staining.

Figure 12: Depletion of TRAF2-cIAP1/2 complexes by TWEAK and BV6 results in enhanced poly(I:C)-induced cell killing.

4.6. Membrane TWEAK upregulates IL8 production more than soluble TWEAK.

The transmembrane form of TWEAK activates the classical NF-κB pathway better than soluble TWEAK (Roos et al., 2010). It has been published that anti-Flag mAb M2 leads to oligomerization of soluble Flag-TWEAK to mimic the activity of membrane TWEAK (Fick et al., 2012; Fick et al., 2012). We wondered whether the type of TWEAK plays a role in TWEAK/poly(I:C)-induced cell death. Consequently, we evaluated the effect of Flag-TWEAK in the presence and absence of the anti-Flag mAb M2. Interestingly, membrane TWEAK-mimiking Flag-TWEAK/M2 complexes elicited strong IL8 production while Flag-TWEAK remained largely inactive in HeLa-RIPK3 cells (**Figure 13**a). However, there was no significant difference between the two Flag-TWEAK prepartions in TWEAK/poly(I:C)-induced cell death (**Figure 13**b).

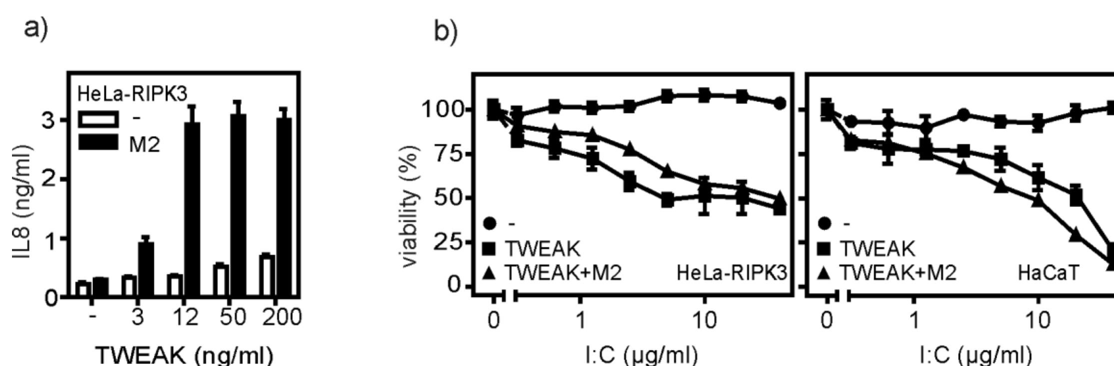


Figure 13: Membrane TWEAK-mimiking Flag-TWEAK/M2 aggregates stimulate IL-8 production more efficiently than soluble TWEAK.

a) HeLa-RIPK3 cells were challenged with the indicated concentrations of Flag-TWEAK in the presence and absence of the anti-Flag mAb M2 (1 µg/ml). Next day, IL8 production was measured by ELISA. **b)** HeLa-RIPK3 cells were stimulated with poly(I:C) and Flag-TWEAK (200 ng/ml) in the presence and absence of M2 (1 µg/ml). Next day, cellular viability was evaluated by using crystal violet staining.

4.7. Evaluation of the role of RIPK1, TRADD, FADD and caspase-8 in poly(I:C)-induced cell death.

4.7.1. RIPK1 is essential for induction of apoptosis and necroptosis by poly(I:C)

TLR3 proapoptotic potential is related to the C-terminal RHIM motif of TRIF that physically interacts with RIPK1 (Kaiser et al., 2005). To address the relationship between RIPK1 protein and the induction of apoptosis and necroptosis by poly (I:C,) we used HeLa-RIPK3-RIPK1$_{KO}$ cells which have been generated using the CRISPR/Cas9 system (**Figure 14**c). Our results showed that the RIPK1 protein is crucial for the induction of apoptosis and necroptosis by poly(I:C) in the presence of CHX, TWEAK, or BV6 (**Figure 14**a). In contrast, TNF/CHX-induced cell death was still evident in the HeLa-RIPK3-RIPK1$_{KO}$ cells. As well, TRAIL was able to induce cell death in the RIPK1-deficient HeLa-RIPK1 cells under all three conditions (**Figure 14**a). There is evidence that RIPK1 has an essential role in the induction of necroptosis by TNF and

TRAIL. Indeed, HeLa-RIPK3-RIPK1$_{KO}$ cells challenged with TNF or TRAIL in the presence of CHX, TWEAK or BV6 were completely rescued by the pan-caspase inhibitor ZVAD indicating pure apoptosis (**Figure 14**b). Moreover, RIPK1 was needed for processing of caspase-9, 8 and 3 by poly(I:C) in the presence of CHX or TWEAK, while the processing and activation of these caspases by TNF and TRAIL was RIPK1-independent (**Figure 14**c).

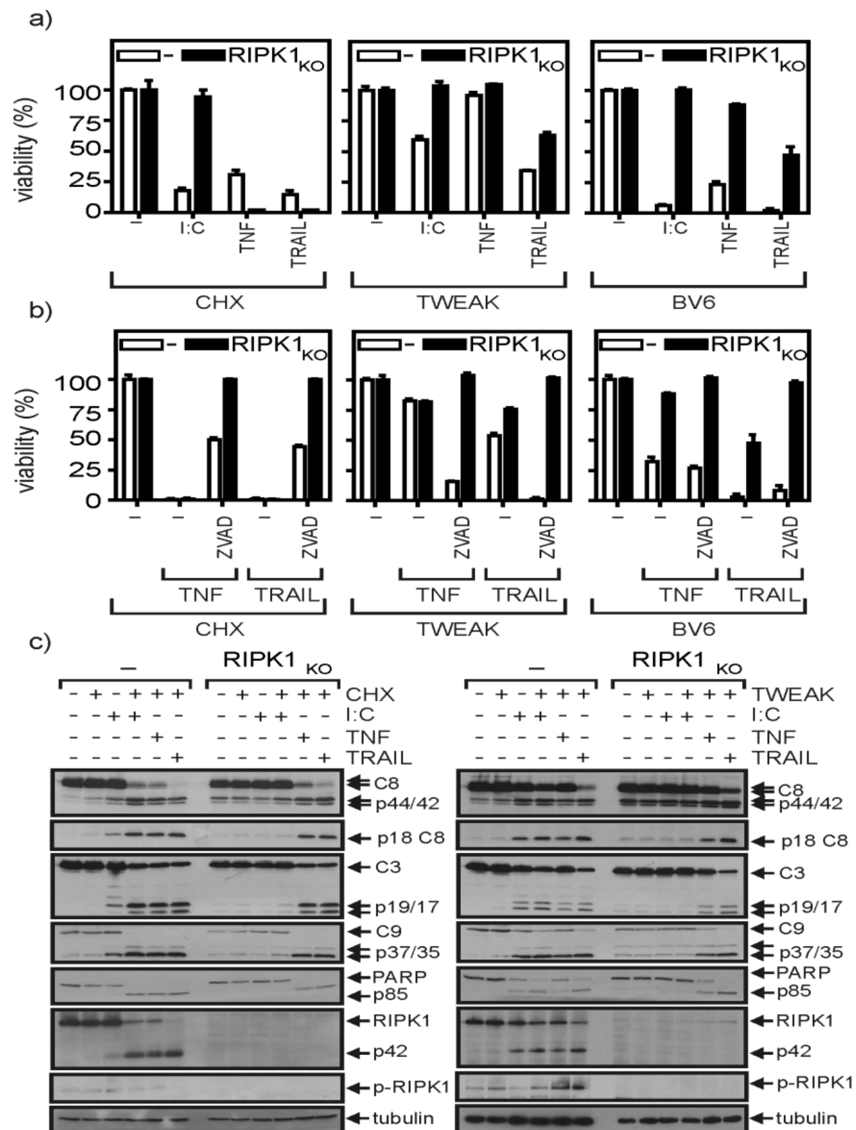


Figure 14: RIPK1 protein plays a crucial role in poly(I:C)-induced cell death.

HeLa-RIPK3 and HeLa-RIPK3-RIPK1$_{KO}$ cells were treated overnight as indicated with poly(I:C) (40 µg/ml), CHX (2.5 µg/ml), Flag-TWEAK (200 ng/ml), BV6 (10 µM), TNF and TRAIL (100 ng/ml) **a)** HeLa-RIPK3 control versus RIPK1 knocked out cells were prestimulated with ZVAD (20 µM) and then stimulated as indicated with CHX (2.5 µg/ml), Flag-TWEAK (200 ng/ml), BV6 (10 µM), TNF and TRAIL (100 ng/ml) **b)**. Finally, the cellular viability was evaluated by crystal violet staining. **c)** HeLa-RIPK3 (-) and HeLa-RIPK3-RIPK1$_{KO}$ cells were challenged with poly(I:C) (40 µg/ml), TNF (100 ng/ml) and TRAIL (100 ng/ml) in the presence of CHX (2.5 µg/ml) or Flag-TWEAK (200 ng/ml). After 6 hours, total cell lysates were prepared and analyzed by Western Blotting.

4.7.2. Caspase-8 and FADD are crucial for poly(I:C)-induced apoptosis.

For better understanding the role of TRADD, FADD and caspase-8 in context of poly(I:C)-induced cell death, we investigated HeLa-RIPK3 variants with knock out of TRADD, FADD and caspase-8 (**Figure 15**).

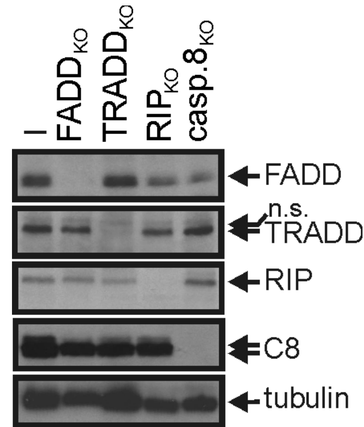


Figure 15: Evaluation of FADD, TRADD, RIPK1 and caspase-8 expression in HeLa-RIPK3 knock out variants.

Total cell lysates were analyzed for expression of FADD, TRADD, RIPK1 and caspase-8 proteins by Western Blotting.

TRADD deficency primed the HeLa-RIPK3 cells for poly(I:C)-induced necroptosis in the presence of CHX. However, absence of TRADD has no significant effect on poly(I:C)/TWEAK-induced apoptosis or necroptosis (**Figure 16**a). Furthermore, lack of caspase-8 or FADD sensitized the cells for poly(I:C)-induced necroptosis in the presence of TWEAK (**Figure 16**a) but rescued the cells from caspase processing and apoptosis induction by poly(I:C)/CHX (**Figure 16**b). Noteworthy, when cells were treated with the RIPK1 kinase inhibitor nec1, they were unable to prevent caspase activation by poly(I:C) in the presence of CHX (**Figure 16**b). This may be indicating that RIPK1 can act as a scaffold protein in poly(I:C)-induced caspase-8 activation/apoptosis independent from its kinase activity.

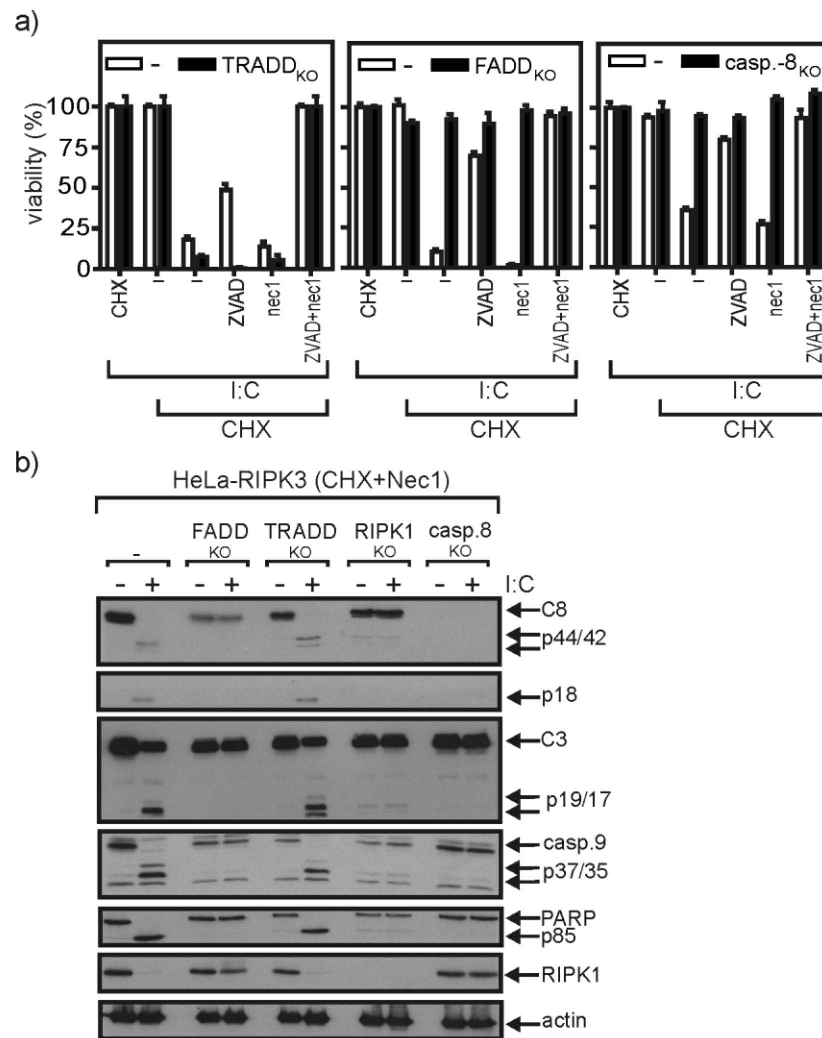


Figure 16: Role of TRADD, FADD and caspase-8 in the poly(I:C)/CHX-induced cell death.

a) HeLa-RIPK3 (-), HeLa-RIPK3-TRADD$_{KO}$, HeLa-RIPK3-FADD$_{KO}$, and HeLa-RIPK3-Casp8$_{KO}$ were pretreated with CHX (2.5 µg/ml), ZVAD (20µM/ml), nec1 (90µM) and a combination of ZVAD and nec1. Cells were stimulated as indicated with poly(I:C) (40 µg/ml). Next day, the viability of the cells was evaluated by crystal violet staining. **b)** HeLa-RIPK3 variants were pretreated with CHX (2.5 µg/ml) and nec1 (90 µM) and stimulated with poly(I:C) (40 µg/ml) for 6 hours. Total cell lysates were analyzed by Western Blotting.

In accordance with the established fact that inhibition of caspases activities induces necroptosis (Tummers and Green, 2017), HeLa-RIPK3 cells pretreated with caspase inhibitor ZVAD and stimulated with poly(I:C) in presence of TWEAK showed vigorous killing and phosphorylation of RIPK1 at serine 166 (**Figure 17**b). Interestingly, FADD and caspase-8 deficiency primed the cells for poly(I:C) induced necroptosis without treating the cells with the caspase inhibitor ZVAD (**Figure 17**a, b).

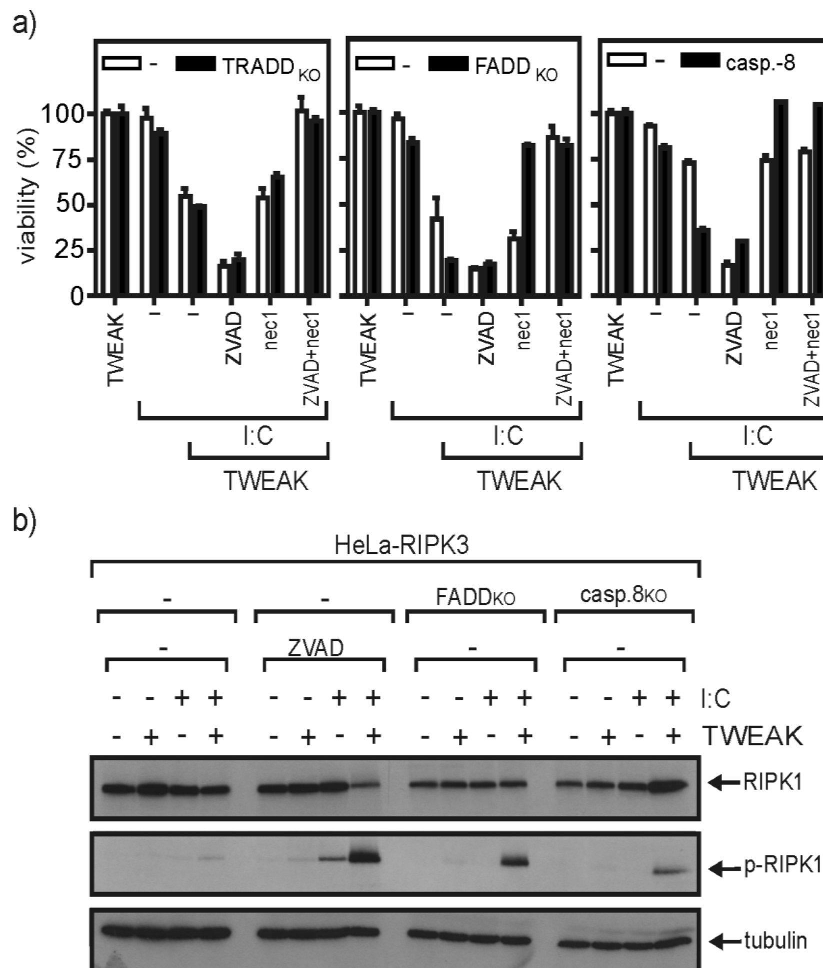


Figure 17: Role of TRADD, FADD and caspase-8 in poly(I:C)/TWEAK-induced cell death.

a) HeLa-RIPK3 control cells versus varaints lacking expression of TRADD, FADD, and caspase-8 were pretreated with Flag-TWEAK (200 ng/ml), (ZVAD 20µM), nec1 (90µM) and a combination of ZVAD and nec1, Then the cells were challenged as indicated with poly(I:C) (40 µg/ml). Next day, the viability of the cells was estimated by crystal violet staining. **b)** HeLa-RIPK3-control was pretreated with Flag-TWEAK (200 ng/ml), and TWEAK in the presence of (ZVAD 20µM), while the cells lacking expression of FADD, and caspase-8 were pretreated only with Flag-TWEAK (200 ng/ml) then the cells were induced with poly(I:C) (40 µg/ml) for 6 hours. Total cell lysates were analyzed by Western Blotting.

4.8. Evaluation of the role of TRADD, FADD, RIPK1 and caspase-8 in poly(I:C)-induced proinflammatory signaling.

4.8.1. Caspase-8 contributes for TRAF1 expression but is unessential for IL8 production.

It has been shown that FADD, TRADD, RIPK1 and caspase-8 play a role not only in the induction of the cell death but also in the initiation of inflammatory signaling. Stimulation of the various HeLa-RIPK3 variants with poly(I:C) showed upregulation of the cytokine IL8, which is regulated by NF-κB signaling and different MAP kinase cascades (**Figure 18a**). Poly(I:C) also induced the expression of TRAF1 which is a target for both canonical and non-canonical NF-κB signaling in all HeLa-RIPK3 variants except caspase-8 knock out cells which showed

regularly a decrease in TRAF1 induction (**Figure 18b**). Moreover, we analyzed the processing of p100 a key event of the non-classical NF-κB pathway, HeLa-RIPK3, and its variants do not affect p100 cleavage. Thus, it seems that the non-canonical NF-κB signaling pathway is not activated in poly(I:C)-stimulated HeLa-RIPK3 cells (**Figure 18b**).

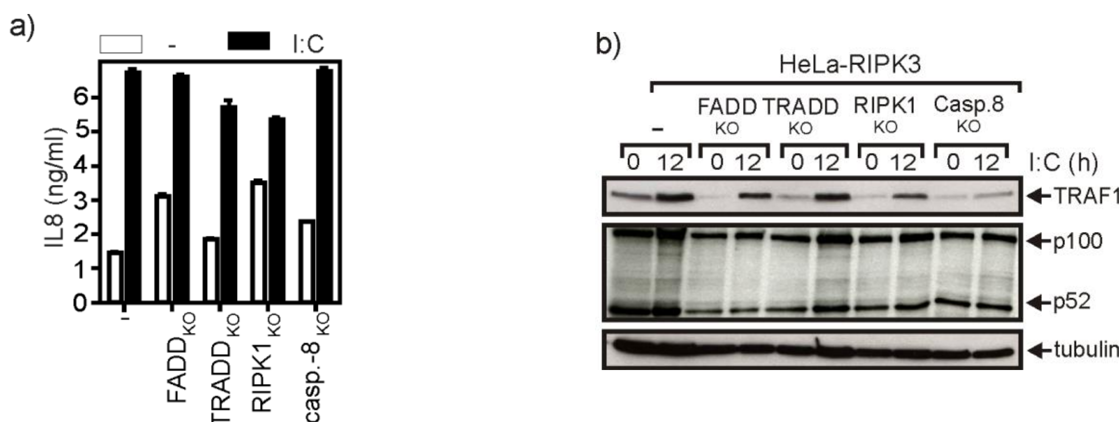


Figure 18: Role of FADD, TRADD, RIPK1, and caspase-8 in poly(I:C)-induced proinflammatory signaling

a) HeLa-RIPK3 control cells versus cells lacking expression of TRADD, FADD, RIPK1, and caspase-8 were challenged overnight with poly(I:C) (40 µg/ml). Finally, IL8 was measured in the supernatant of the stimulated cells by ELISA. **b)** The different variants of HeLa-RIPK3 cells were stimulated with poly(I:C) (40 µg/ml) for 12 hours. Total cell lysates were prepared and analyzed by Western Blotting for expression of TRAF1 and p100 proteins.

4.8.2. TRADD and RIPK1 are essential for IL8 production by TNF but are not crucial by poly(I:C).

As we observed in another study that RIPK1 and TRADD are redundantly essential for proinflammatory signaling by TNF (Fullsack et al., 2019), we analyzed TRADD/RIPK1-double knock out HeLa-RIPK3 for poly(I:C) signaling, too (**Figure 19**a). As observed before, TRADD and RIPK1 double deficiency in HeLa-RIPK3 abrogated IL8 and TRAF1 expression induced by TNF but not in the single knockout variants (**Figure 19**b, c). Concerning gene induction by poly(I:C) the situation was more complicated. Induction of TRAF1 by poly(I:C) was significantly reduced in HeLa-RIPK3-TRADD/RIPK1DKO cells (**Figure 19**c). Moreover, the phosphorylation of IκBα, an early stage in classical NF-κB signaling, was strongly decreased (**Figure 19**c). In contrast to TNF, IL8 production was still upregulated in the HeLa-RIPK3-TRADD-RIPK1 double knocked out cells by poly(I:C) (**Figure 19**b). The finding that poly(I:C) in difference to TNF upregulates IL8 production in HeLa-RIPK3-TRADD/RIPK1DKO cells may reflect the fact that IL8 production can also be enhanced via the IRF-3/ISRE pathway (Matsukura et al., 2006; Wagoner et al., 2007), however, this needs further study and examination.

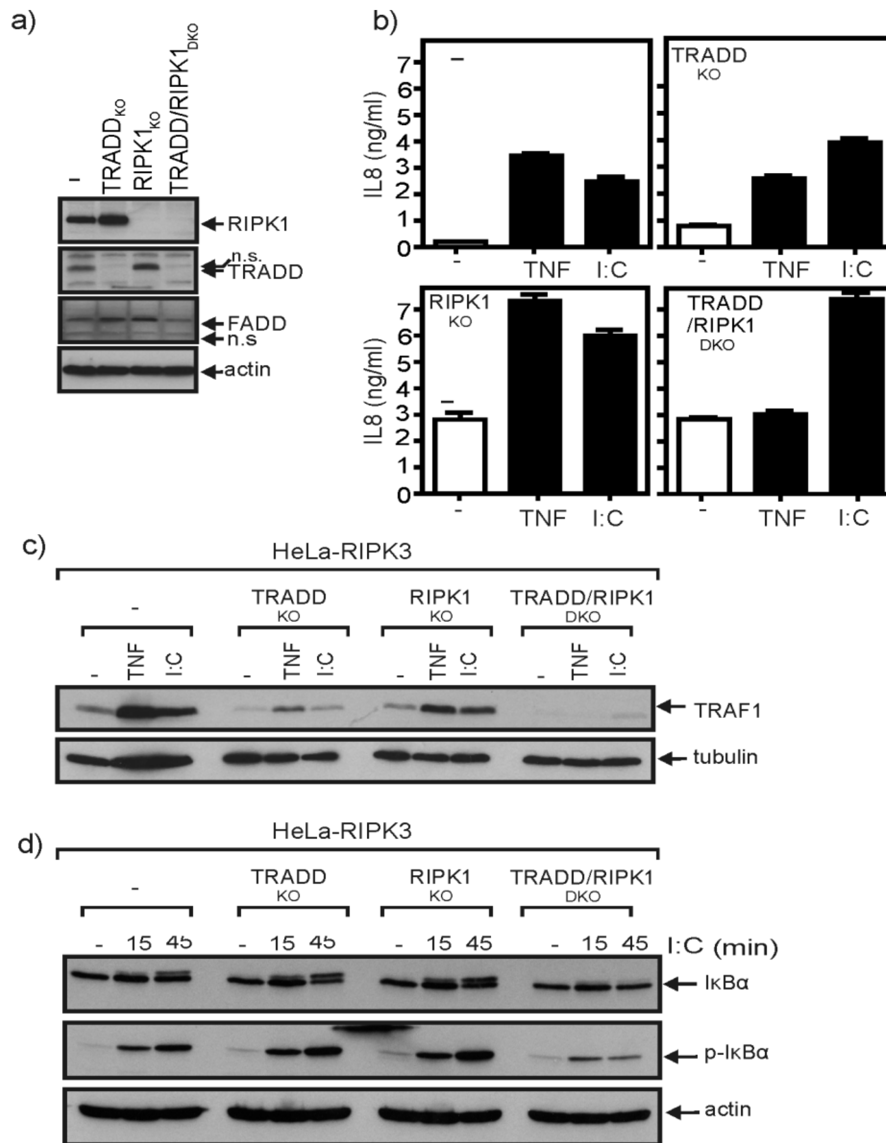


Figure 19: TRADD/RIPK1$_{DKO}$ cells have strongly reduced poly(I:C)-induced proinflammatory signaling.

a) HeLa-RIPK3-control and cells lacking expression of RIPK1$_{KO}$, TRADD$_{KO}$, and TRADD/RIPK1$_{DKO}$ were challenged with poly(I:C) (40 µg/ml) and TNF (100ng/ml) for 12 hours. Finally, IL8 was measured in the supernatant of stimulated cells by ELISA. **b)** Generation of HeLa-RIPK3 deficiency in RIPK1, TRADD, and both by using CRISPR/Cas9.**c)** The different variants of HeLa-RIPK3 cells were treated overnight with poly(I:C) (40 µg/ml) and TNF (100 ng/ml). Total cell lysates were prepared and analyzed by Western Blotting for expression of TRAF1. **d)** The different variants of HeLa-RIPK3 cells were treated as indicated with poly(I:C) (40 µg/ml). Total cell lysates were prepared and analyzed by Western Blotting for expression of IκBα and p- IκBα.

4.9. Evaluation of the role of FLIP proteins in poly(I:C)-induced signaling.

4.9.1. FLIP proteins rescue cells from poly(I:C)-induced cell death.

FLIPproteins regulate not only the classical death receptor-induced extrinsic apoptosis pathway but also the non-conventional pattern recognition receptor-dependent apoptotic pathway (Tsuchiya et al., 2015). To evaluate the role of $FLIP_L$ and $FLIP_S$ in poly(I:C)-induced signaling, the HaCaT and HeLa-RIPK3 cells were stably transfected with $FLIP_L$ and $FLIP_S$ by the Sleeping Beauty transposon system (**Figure 20**a, c). Several studies revealed that $FLIP_L$ can form heterodimers with procaspase-8 (Boatright et al., 2004). Additionally, it has been found that the long isoform of FLIP acts as an inducer but also inhibitor of death receptor-induced apoptosis (Tummers and Green, 2017). For more understanding of the role of FLIP proteins in cell death mediated by poly(I:C), we treated the HaCaT and HeLa-RIPK3 stably expressing $FLIP_L$ and $FLIP_S$ with poly(I:C) in the presence of TWEAK or BV6. Our results clearly showed the ability of $FLIP_L$ and $FLIP_S$ to inhibit cell death-induction by poly(I:C)/TWEAK or poly(I:C)/BV6 (**Figure 20**b, d).

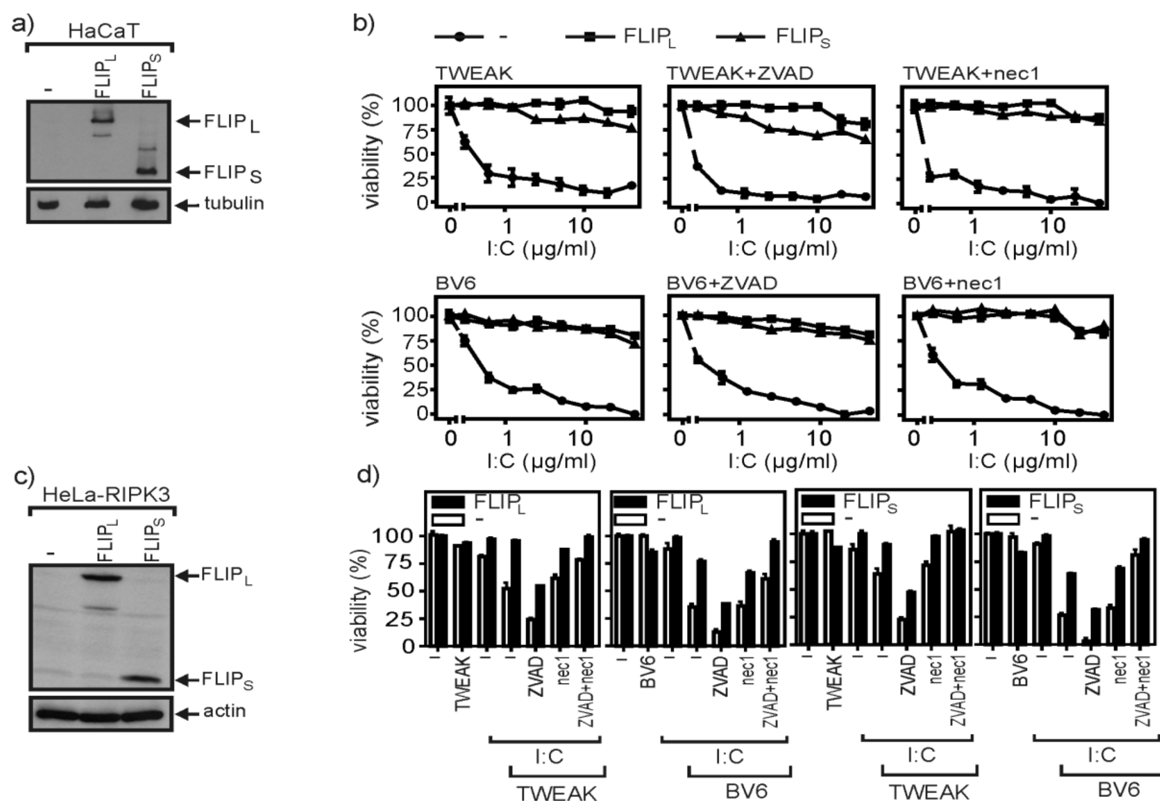


Figure 20: FLIP proteins inhibit induction of cell death by poly(I:C).

a) Stable transfection of HaCaT and HeLa-RIPK3 with FLIPL and FLIPS by using the Sleeping Beauty transposon system. The cell lysates were prepared and analyzed by Western Blotting for expression of FLIPL and FLIPs **a, c)**. The cells were prestimulated with the indicated combination of Flag-TWEAK (200 ng/ml), BV6 (10 µM), ZVAD (20 µM) and nec1 (90 µM) then stimulated overnight with increasing the concentration of poly(I:C) in case of HaCaT cells **b)** and with only one concentration of poly(I:C) (40 µg/ml) in HeLa-RIPK3 **d)**. Next day, viability of cells was evaluated by crystal violet staining.

4.9.2. The short isoform of FLIP protects the cells from poly(I:C)-mediated necroptosis.

As shown previously, the short isoform of FLIP leads to inhibition of apoptosis (Tummers et al., 2017) but it has been claimed that it also enhances necroptosis (Schneider et al., 2017; Shindo et al., 2016). In contrast, our results showed a significant reduction in phosphorylation of RIPK1 protein by FLIPs as well as $FLIP_L$ (**Figure 21**a). Moreover, the two FLIP isoforms have also anti-necroptotic activity when the HaCaT cells pre-stimulated with TWEAK or BV6 in the presence of ZVAD or (ZVAD+CHX) and then enhanced by TNF or TRAIL (**Figure 21**b).

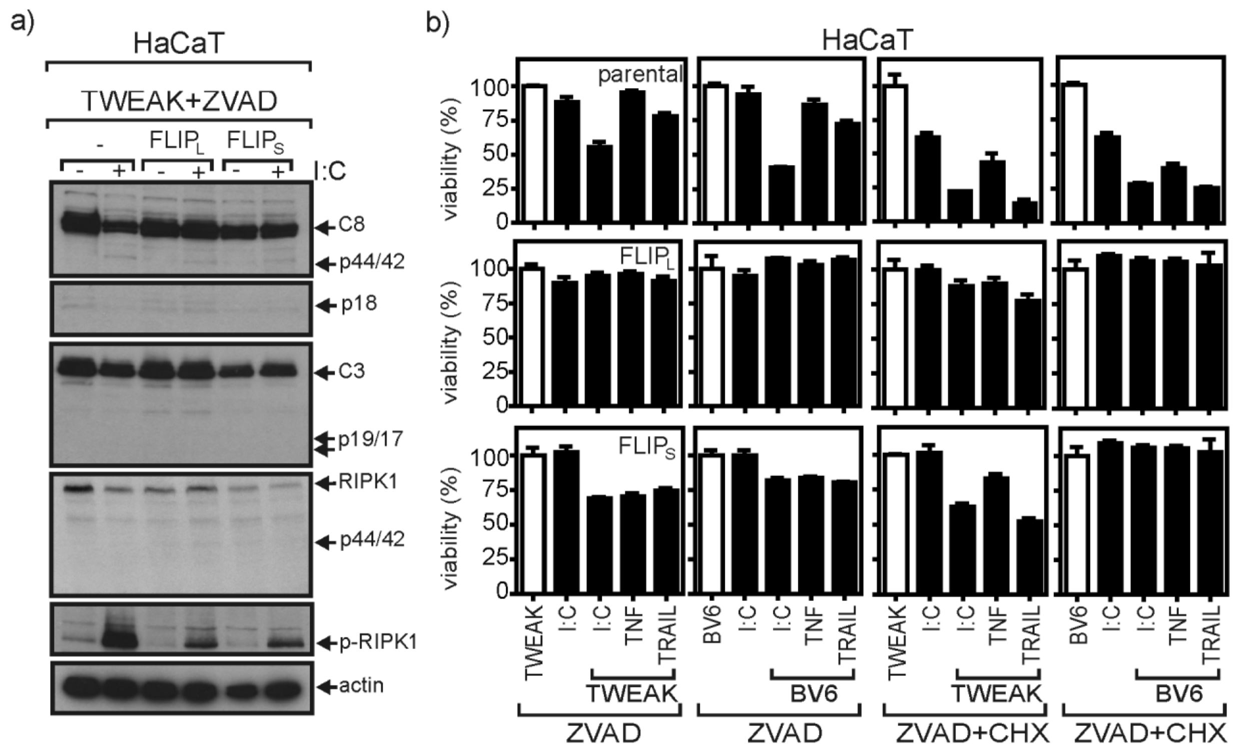


Figure 21: Poly(I:C)-induced necroptosis is markedly reduced by both FLIP isoforms.

The different variants of HaCaT cells were prestimulated with ZVAD (20 µM) then stimulated for 6 hours with poly(I:C) (40 µg/ml). Total cell lysates were prepared and analyzed by Western Blotting. **b)** The cells were challenged overnight with poly(I:C) (40 µg/ml) and TNF (100 ng/ml), TRAIL (100 ng/ml), Flag-TWEAK (200 ng/ml), and BV6 (10 µM) in the presence of ZVAD and CHX. Finally, viability of cells was evaluated by crystal violet stainin

4.9.3. FLIP proteins are dispensable for induction of the proinflammatory signaling by poly(I:C).

FLIP proteins can play a role in the induction of the inflammatory signaling like upregulation of IL-2 production in T cells, activation of ERK and increasing NF-kB activation (Kataoka et al., 2000; Hughes et al., 2016). In contrast, TNF–mediated JNK and ERK stimulation, but not p38 MAPK phosphorylation, are prevented by c-FLIP (Nakajima et al., 2006). We therefore analyzed the role of FLIP proteins in the activation of proinflammatory signaling by poly(I:C). Our results demonstrated $FLIP_L$ and $FLIP_S$ have no significant effect on poly(I:C)-induced proinflammatory signaling neither with respect to IL8 production nor with respect to to TRAF1 and IRF3 expression (**Figure 22**a, b).

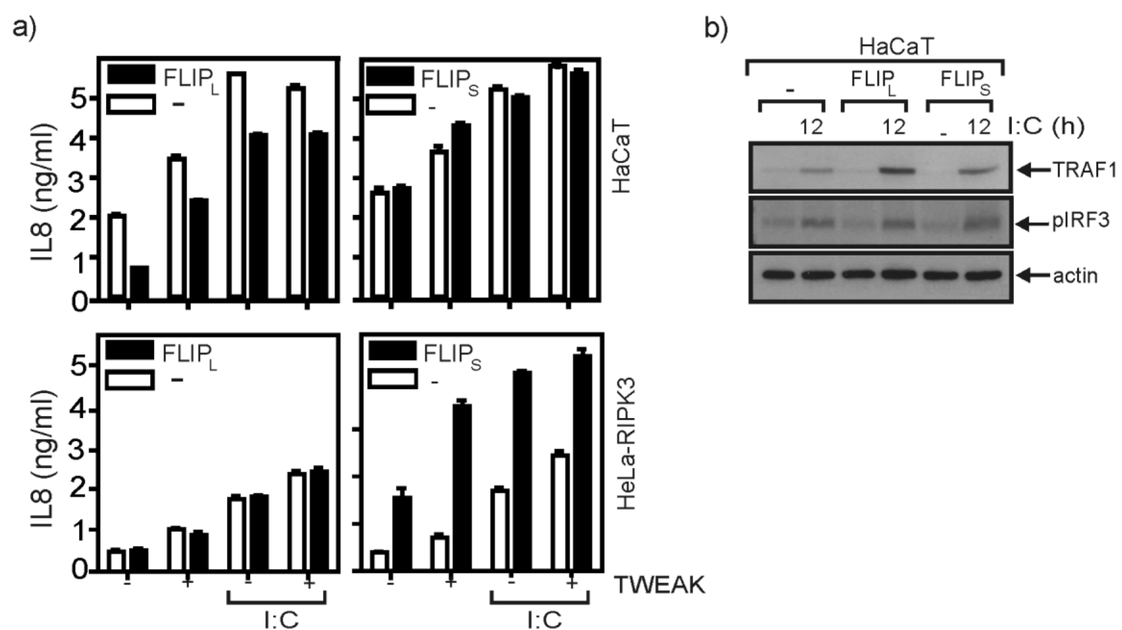


Figure 22: FLIP proteins play no role in poly(I:C)-mediated proinflammatory signaling.

a)The various HaCaT and HeLa-RIPK3 variants were stimulated overnight with poly(I:C) (40 µg/ml) in the presence or absence of Flag-TWEAK (200 ng/ml). Finally, IL8 was analyzed in the supernatant by ELISA. **b)** HaCaT cells were challenged for 12 hours with (40 µg/ml). Total cell lysates were prepared and analyzed by Western Blotting for expression of TRAF1 and pIRF3 proteins.

5. Discussion

5.1. TWEAK and CHX enhances poly(I:C)-induced cell death signalling but has no major effect on its proinflammatory signaling activities

The receptor Fn14 is a member of the TRAF-interacting subgroup of TNFRSF receptors. The TWEAK-Fn14 system triggers cell death induction only in very few cell lines such as Kym-1, OVCAR-4, and SKOV-3 (Wajant, 2013). The capability of TWEAK-Fn14 system to induce apoptotic signaling without having a death domain has been traced back to an indirect mechanism, namely the production of TNF and subsequent stimulation of the prototypic death receptor TNFR1 (Chopra et al., 2015; Grabinger et al., 2017; Karl et al., 2014; Schneider et al., 1999; Wicovsky et al., 2009; Zimmermann et al., 2011). The expression of Fn14 is very high and variable during the embryonal development; however, its expression is very low and restricted to very few cell types in the healthy adult tissue (Wajant, 2013). It has been published that tissue injury strongly induces the expression of Fn14 in different types of non-hematopoietic cells (Wajant, 2013). Tissue injury leads to release of damage- and pathogen-associated molecular patterns (DAMPs/PAMPs) such as double-stranded RNA, LPS, which are released during viral replication, and intracellular molecules produced after cells destruction (e.g., ATP) (Bryant et al., 2015). Both innate immune cells and non-immune cells can detect DAMPs and PAMPs by the help of cytosolic and membranous receptors called pattern recognition receptors (PRRs) (Bryant et al., 2015).

It has been previously shown that double-stranded RNA is sensed not only by cytosolic RIG1-like receptors but also by the transmembrane receptor toll-like receptor-3 (TLR3) (Bryant et al., 2015; Yu and Levine, 2011). Moreover, PRRs trigger not only proinflammatory signaling but may also stimulate cell death signaling under certain conditions (Bryant et al., 2015; Bianchi et al., 2017; Yu and Levine, 2011). Therefore, the TWEAK/Fn14 system and PRRs share some cellular activities and functions under similar circumstances. For that reason, we raised the question of whether TWEAK is also capable of enhancing the poly(I:C)-mediated signaling. We investigated this issue in HeLa-RIPK3- and HaCaT cells, two cell lines that respond well to poly(I:C) (Estornes et al., 2012; Feoktistova et al.,2011; Jiang et al., 2008; McAllister et al., 2013; Weber et al., 2010). Our results showed that poly(I:C) were not able to robustly stimulate cell death alone in HeLa-RIPK3 and HaCaT cells except cells were primed with TWEAK or cycloheximide (**Figure 7**). Moreover, poly(I:C) triggered proinflammatory signaling such as upregulation of IL8 production in the two cell lines independent from TWEAK and CHX (**Figure 8**).

5.2. Poly(I:C) preferentially induces necroptotic cell death in the presence of TWEAK.

Necroptosis and Apoptosis are two distinct types of programmed cell death. Necroptosis is programmed necrosis, as it is controlled by specific proteins including RIPK3 and MLKL. These proteins are responsible for the formation of a necrosome complex which needs RIPK1 to phosphorylate and activate RIPK3. Then, the activated RIPK3 phosphorylates and activates MLKL. Finally, the phosphorylated MLKL binds to N-terminal four-helix bundles domains to form oligomer which translocates to the plasma membrane and leads to cell death by pores formation on the plasma membrane (Pasparakis et al., 2015). On the other side, apoptosis is characterized by processing of apoptotic effector caspases, such as caspase-3/7 and pro-apoptotic proteins, such as BID, which leads to the destruction of mitochondria and apoptosis (Weiss et al., 1998). The death receptors activate the extrinsic pathway of apoptosis by activation of caspase-8 (Fernandes-Alnemri et al., 1996; Muzio et al., 1996). Moreover, it was proved that caspase-8 also plays a vital role in the suppression of necroptosis besides its role in apoptosis induction (Degterev et al., 2005; Kataoka et al., 2000). Recently, It has been indicated that necroptosis is related to many of human diseases accompanied with inflammation and cell death such as ischemic heart and brain injuries, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) (Ofengeim et al., 2015; Quarato et al., 2016). Previous findings indicated that FasL or TNF-α could activate necroptosis when the cells treated by zVAD.fmk, which leads to inhibition of caspase-8 (Shan et al., 2018). Our results showed that blocking of caspase-8 by using ZVAD enables poly(I:C) to trigger necroptosis in the presence of TWEAK, while poly(I:C)/CHX primarily triggered caspase-dependent apoptotic cell death (**Figure 9**). This proposes that the two sensitizers TWEAK and CHX act by different mechanisms protecting cells from killing by poly(I:C). The mechanism by which CHX sensitizes cell for apoptosis is mainly decreasing the expression of the caspase-8 inhibitory short-lived FLIP proteins (Fulda et al., 2000; Kreuz et al., 2001). While TWEAK promotes cell death by two indirect mechanisms:(i) induction of TNF production which binds to TNFR1consequently, leads to cell death, and (ii) depletion of protective TRAF2-cIAP1 and TRAF2-cIAP2 complexes. These complexes reduce the death-receptor-mediated necroptotic activity of RIPK1 by cIAP1/2-mediated K63 ubiquitination (Ikner and Ashkenazi, 2011; Schneider et al., 1999; Vince et al., 2008; Moriwaki and Chan, 2014). Our results revealed that poly(I:C)-induced cell death in the presence of TWEAK or CHX is TNF independent (**Figure 11**). This suggests that the sensitizing effect of TWEAK for poly(I:C)-mediated cell death depends mainly on depletion of protective TRAF2-cIAP1 and TRAF2-cIAP2 complexes (**Figure 12**a). The latter result was confirmed by treatment of cells with poly(I:C) in the presence of the cIAP antagonist BV6 (**Figure 12**b). Previous findings indicated that membrane TWEAK stimulates the canonical NF-κB better than the soluble TWEAK (Rose et al., 2010). We observed the same results regarding IL8 production in HaCaT and HeLa-RIPK3 cells (**Figure 13**a). However, our results showed

no significant variance between the soluble and membrane TWEAK regarding poly(I:C)-induced cell death (**Figure 13**b). This suggests that soluble TWEAK is sufficient to enhance necroptotic signaling what is in good agreement with the idea of TRAF2-cIAP1/2 depletion as the crucial mechanism.

5.3. The mechanistic study of poly(I:C)-induced signaling

TRADD, FADD, RIPK1, and caspase-8 play a crucial role in transduction of apoptotic, necroptotic and proinflammatory signaling mediated by TNFRSF receptors and TLR3 (Estornes et al., 2012; Feoktistova et al., 2011; Meylan et al., 2004; Imtiyaz et al., 2006; Maelfait et al., 2008; Pobezinskaya et al., 2008; Ermolaeva et al., 2008; Chen et al., 2008; Weber et al., 2010; Weber et al., 2010). Investigation of HeLa-RIPK3 variants lacking the expression of RIPK1, TRADD, FADD, and caspase-8 or both TRADD and RIPK1 revealed an essential role of RIPK1, caspase-8, and FADD in poly(I:C)-induced cell death. Despite caspase-8 and FADD turned out to be crucial for poly(I:C)-induced apoptosis (**Figure 16**a, 17a) and caspase activation (**Figure 16**b) They are dispensable for induction of necroptosis by poly(I:C) (**Figure 16**a, 17a, 17b). Furthermore, our results showed significant differences in the mode of how TNF, TRAIL, and poly(I:C) use these proteins for induction of death even in the identical cellular model (HeLa-RIPK3 cells). First, while RIPK1 is only crucial for induction of necroptosis by TNF and TRAIL, in poly(I:C) signaling, RIPK1 is essential for both apoptosis and necroptosis (**Figure 14**a, b). Second, caspase-8 and FAAD are essential for the induction of apoptosis and necroptosis by TRAIL (Fullsack et al., 2019). Indeed, our results revealed that these two proteins are unessential for TNF and poly(I:C)-induced necroptosis (Fullsack et al., 2019 and **Figure 16**a, 17a). Moreover, analysis of HeLa-RIPK3 cells double deficient for the expression of RIPK1 and TRADD showed that these two proteins are unnecessary for poly(I:C)-induced IL8 expression, despite their essential and redundant role in the corresponding response to TRAIL and TNF (**Figure 19**b and Fullsack et al., 2019).

The role of RIPK1, TRADD, FADD and caspase-8 in signaling induced by poly(I:C), TNF and TRAIL could be explained mainly by a simple considering three issues: First, that TRADD and RIPK1 on the one side and caspase-8 and FADD on the other side, work in the opposite order in poly(I:C)- (or (TNF)) and TRAIL-induced signaling. Second, that the RIPK1/TRADD couple elicits proinflammatory and necroptotic signaling while the caspase-8/FADD couple triggers apoptosis. Third, that the RIPK1/TRADD and the caspase-8/FADD couples commonly stimulate each other if suitable receptors have bound one of both (e.g., TNFR1, TLR3, TRAIL death receptors). The TRADD/RIPK1 couple acts in this system upstream of the caspase-8/FADD couple in poly(I:C) and TNF signaling but downstream of the latter in TRAIL signaling. For that reason, the caspase-8/FADD couple is not required for induction of RIPK1-mediated necroptosis and proinflammatory signaling by TNF and poly(I:C) but is essential in the TRAIL

as an adaptor to bind RIPK1/TRADD couple with TRAIL death receptors. Contradictory, the downstream site of the caspase-8/FADD couple in TNF and poly(I:C) signaling describes the importance of RIPK1 and/or TRADD for caspase-8 activation and apoptosis (**Figure 23**). Our results showed that the deficiency of RIPK1 is already enough to inhibit poly(I:C)-induced apoptosis and necroptosis (**Figure 14**), however, both RIPK1 and TRADD are essential for the TNF-induced apoptosis (Fullsack et al., 2019). The later might merely reveal that both molecules actively interact with TNFR1 receptor while the receptor of poly(I:C) TLR3, in a TRIF-dependent manner, favourably recruits TRADD only indirectly via RIPK1 (Ermolaeva et al., 2008). Remarkably, the absence of RIPK1 but not blocking of its kinase activity inhibited induction of caspases activation by poly(I:C) in CHX treated cells (**Figure 14**). Accordingly, it seems that RIPK1 acts independent from its kinase activity as a scaffold protein in caspase-8 activation/apoptosis by poly(I:C). This mode of apoptosis induction has no priority and has been addressed in the context of endoplasmic reticulum stress-induced death and in models of RIPK3-induced apoptosis (Estornes et al., 2012; Estornes et al., 2012; Nogusa et al., 2016). In the framework of TNFR1, however, caspase-8 activation/apoptosis present either RIPK1-independent or dependent under control of its kinase activity (Nogusa et al., 2016). It would be interesting to know in the future whether the differential importance of RIPK1 kinase activity in TNF- and poly(I:C)-induced RIPK1-mediated apoptosis reveals a significant role of TRADD in apoptotic signaling.

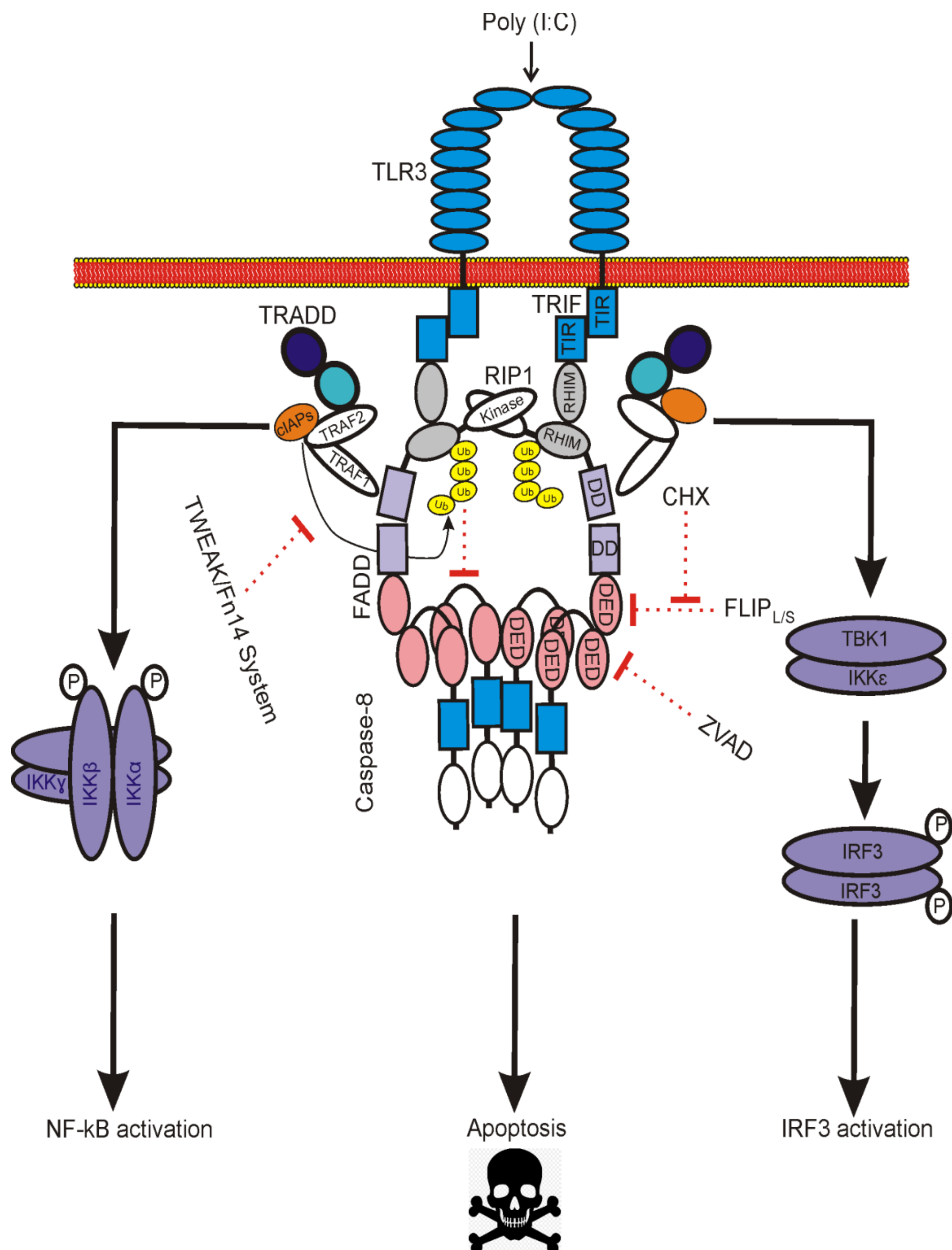


Figure 23: Signal transduction complexes in TLR3-induced survival and cell death.

Upon stimulation with poly(I:C), TLR3 signals through adapter TRIF (TICAM-1) which in turn recruits either RIPK1 with TRAF2 and c-IAP1/2 complex, which is responsible for the induction of NF-kB or RIPK1 with TBK1 and IKKε complex, which mediates the induction of IRF3 signaling. The treatment of cells with CHX causes inhibition of FLIP proteins, the inhibitor of FADD-mediated caspase-8 maturation. While, TWEAK activates the recruitment of TRAF2 and c-IAP1/2complex, which causes the ubiquitination of RIPK1 and inhibits apoptosis. Therefore, CHX and TWEAK sensitize the cells for poly(I:C)-induced apoptosis.

5.4. Effect of $FLIP_L$ and $FLIP_S$ on poly(I:C)-induced signaling.

Cellular FLICE-like inhibitory protein is structurally linked to caspase-8 but is lacking enzymatic activity. There are three different isoforms of c-FLIP: two short isoforms (c-$FLIP_{R/S}$), which only have the prodomain including the two DED domains, and a long isoform (c-$FLIP_L$). The c-terminal part of $FLIP_L$ also contains a caspase domain that is inactive because of the replacement of a cysteine residue in the protease site with tyrosine (Tsuchiya et al., 2015). Our results showed that both $FLIP_L$ and $FLIP_S$ protect HaCat cells from poly(I:C)-induced apoptosis and necroptosis and the response was attenuated in HeLa-RIPK3 cells (**Figure 20**b, d). The antiapoptotic effect of $FLIP_L$ and $FLIP_S$ proteins is expected in the frame of their well-known blocking effect on FADD-mediated caspase-8 maturation. In addition to its role in apoptosis, caspase-8 leads to inhibition of necroptosis by cleavage of RIPK1 and RIPK3, the anti-necroptotic activity of the FLIP proteins suggests that these molecules also have an unclearly defined caspase activity-independent survival role. Another study showed a variance influence of the two FLIP isoforms on the induction of necroptosis by poly(I:C) whereby $FLIP_S$ expression led to an enhancement of necroptosis (Feoktistova et al., 2011). Markedly, the heterodimers of $FLIP_L$ and caspase-8 in variance to the heterodimers of $FLIP_s$ and caspase-8, permit the first of the two maturation steps of caspase-8, causing formation of a complex with limited range of substrates, which still cleaves RIPK1 and RIPK3. Therefore, it seems that the RIPK1/RIPK3 inhibitory potential of $FLIP_L$ is dominant on $FLIP_S$. For that reason, the described effect of ectopic $FLIP_S$ expression for sensitization of necroptosis does not mean an active stimulation of necroptosis, but relatively the competitive inhibition of the superior endogenously expressed necroptosis inhibitor $FLIP_L$. We found that FLIPs reduced significantly the phosphorylation of RIPK1, which plays a crucial role in necroptosis (**Figure 21**a). The opposing effect of $FLIP_S$ on induction of necroptosis by poly(I:C) in our study and to Feoktistova et al., 2011 may also show this complex interaction between caspase-8, $FLIP_{L/S}$, RIPK1 and RIPK3 proteins in the frame of cell death signaling which may cause subtle changes in the expression levels to lead to many variance effects.

6. Summary

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) is a member of the TNF superfamily (TNFSF) and is as such initially expressed as type II class transmembrane glycoprotein from which a soluble ligand form can be released by proteolytic processing. While the expression of TWEAK has been detected at the mRNA level in various cell lines and cell types, its cell surface expression has so far only been documented for dendritic cells, monocytes and interferon-γ stimulated NK cells. The fibroblast growth factor-inducible-14 (Fn14) is a TRAF2-interacting receptor of the TNF receptor superfamily (TNFRSF) and is the only receptor for TWEAK. The expression of Fn14 is strongly induced in a variety of non-hematopoietic cell types after tissue injury. The TWEAK/Fn14 system induces pleiotropic cellular activities such as induction of proinflammatory genes, stimulation of cellular angiogenesis, proliferation, differentiation, migration and in rare cases induction of apoptosis. On the other side, Toll-like receptor3 (TLR3) is one of DNA- and RNA-sensing pattern recognition receptors (PRRs), plays a crucial role in the first line of defense against virus and invading foreign pathogens and cancer cells. Polyinosinic-polycytidylic acid poly(I:C) is a synthetic analog of dsRNA, binds to TLR3 which acts through the adapter TRIF/TICAM1, leading to cytokine secretion, NF-κB activation, IRF3 nuclear translocation, inflammatory response and may also elicit the cell death. TWEAK sensitizes cells for TNFR1-induced apoptosis and necroptosis by limiting the availability of protective TRAF2-cIAP1 and TRAF2-cIAP2 complexes, which interact with the TNFR1-binding proteins TRADD and RIPK1. In accordance with the fact that poly(I:C)-induced signaling also involves these proteins, we found enhanced necroptosis-induction in HaCaT and HeLa-RIPK3 by poly(I:C) in the presence of TWEAK (**Figure 24**). Analysis of a panel of TRADD, FADD, RIPK1 and caspase-8 knockout cells revealed furthermore similarities and differences in the way how these molecules act in cell death signaling by poly(I:C)/TWEAK and TNF and TRAIL. RIPK1 turned out to be essential for poly(I:C)/TWEAK-induced caspase-8-mediated apoptosis but was dispensable for these responses in TNF and TRAIL signaling. Lack of FADD protein abrogated TRAIL- but not TNF- and poly(I:C)-induced necroptosis. Moreover, we observed that both long and short FLIP rescued HaCaT and HeLa-RIPK3 cells from poly(I:C)-induced apoptosis or necroptosis.

To sum up, our results demonstrate that TWEAK, which is produced by interferon stimulated myeloid cells, controls the induction of apoptosis and necroptosis by the TLR3 ligand poly(I:C) and may thus contribute to cancer or anti-viral immunity treatment.

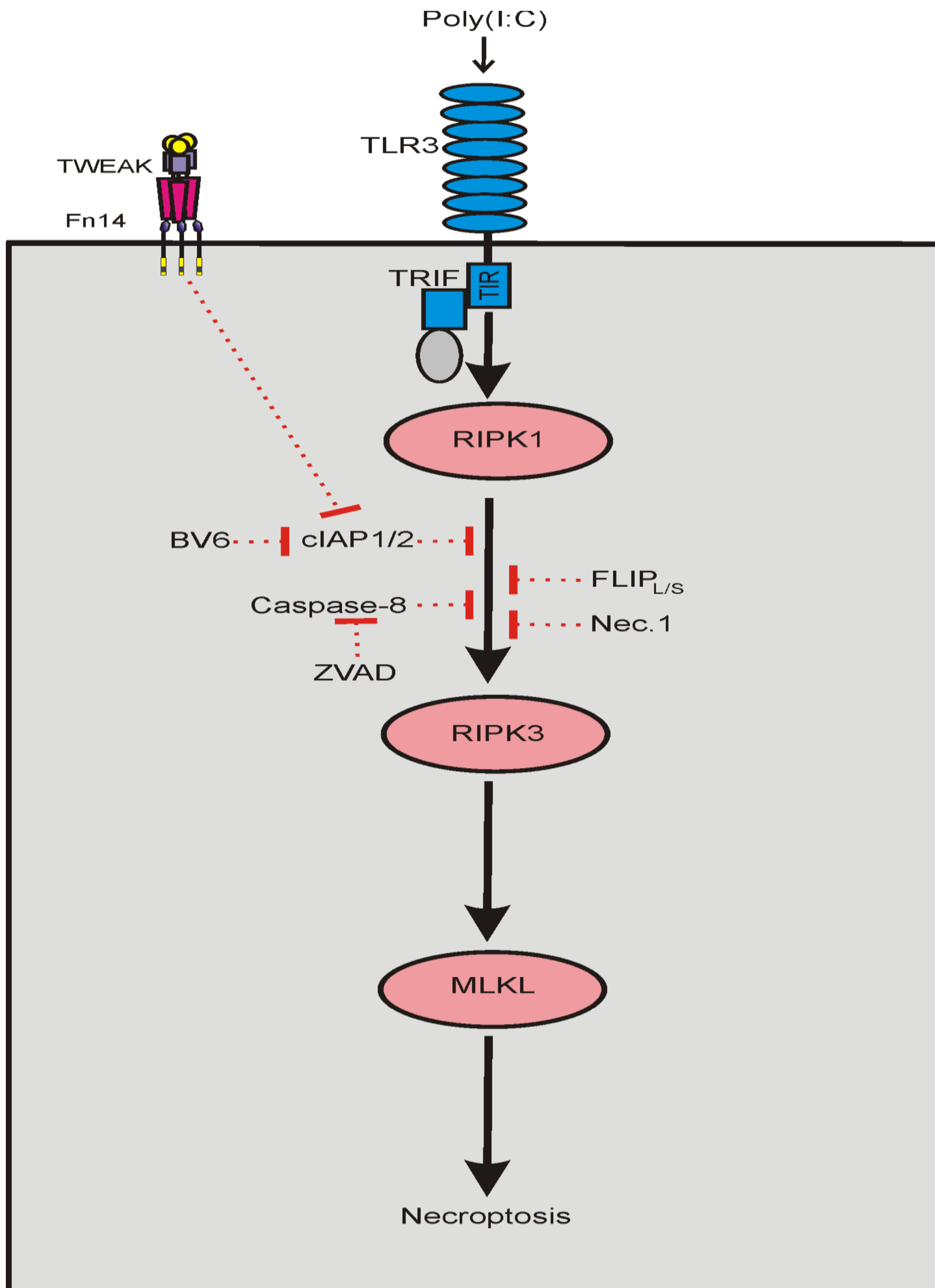


Figure 24: TWEAK sensitize for poly(I:C)-induced necroptosis.

Binding of poly(I:C) to TLR3 causes the recruitment of its adaptor molecule TRIF, which in turn interacts with RIPK1. In the absence of TWEAK, the cIAPs induce the ubiquitination of RIPK1 and the inhibition of necroptosis. On the other side, the presence of TWEAK or BV6 triggers the auto-ubiquitination of cIAPs. Therefore, RIPK1 can induce the phosphorylation of RIPK3 which mediates the phosphorylation and activation of MLKL and necroptosis.

7. Zusammenfassung

Tumor necrosis factor (TNF)-like weak inducer of apoptosis (TWEAK) ist ein Mitglied der TNF-Superfamilie (TNFSF) und wird als solches anfänglich als Transmembranglykoprotein der Klasse II exprimiert, aus dem eine lösliche Ligandenform durch proteolytische Prozessierung freigesetzt werden kann. Während die Expression von TWEAK auf mRNA-Ebene in verschiedenen Zelllinien und Zelltypen nachgewiesen wurde, konnte ihre Zelloberflächenexpression bisher nur für dendritische Zellen, Monozyten und Interferon-γ-stimulierte NK-Zellen dokumentiert werden. Fibroblast growth factor-inducible-14 (Fn14) ist ein TRAF2-wechselwirkender Rezeptor der TNF-Rezeptor-Superfamilie (TNFRSF) und der einzige Rezeptor für TWEAK. Die Expression von Fn14 wird nach Gewebeverletzung in einer Vielzahl von nicht hämatopoetischen Zelltypen stark induziert. Das TWEAK / Fn14-System induziert pleiotrope zelluläre Aktivitäten, die von der proinflammatorischen Geninduktion über die Stimulierung der Angiogenese, Proliferation und Zelldifferenzierung bis hin zur Zellmigration und in seltenen Fällen zur Induktion von Apoptose reichen. Auf der anderen Seite spielt der Toll-like Rezeptor3 (TLR3), einer der DNA- and RNA-sensing pattern recognition receptors (PRRs), eine entscheidende Rolle in der ersten Verteidigungslinie gegen Viren und eindringende fremde Krankheitserreger und Krebszellen. Polyinosin-Polycytidylsäure-Poly (I: C) ist ein synthetisches Analogon von dsRNA, das an TLR3 bindet, das über den Adapter TRIF / TICAM1 wirkt und zu Zytokinsekretion, NF-κB-Aktivierung, IRF3-Kerntranslokation und Entzündungsreaktion führt der Zelltod.

TWEAK sensibilisiert Zellen für TNFR1-induzierte Apoptose und Nekroptose, indem es die Verfügbarkeit von schützenden TRAF2-cIAP1- und TRAF2-cIAP2-Komplexen begrenzt, die mit den TNFR1-bindenden Proteinen TRADD und RIPK1 interagieren. Entsprechend der Tatsache, dass diese Proteine auch von Poly (I: C) induziert werden, fanden wir eine verstärkte Nekroptose-Induktion in HaCaT und HeLa-RIPK3 durch Poly (I: C) in Gegenwart von TWEAK (**Figure 24**). Die Analyse eines Panels von TRADD-, FADD-, RIPK1- und Caspase-8-Knockout-Zellen ergab außerdem Ähnlichkeiten und Unterschiede in der Art und Weise, wie diese Moleküle bei der Zelltodsignalisierung durch Poly (I: C) / TWEAK und TNF und TRAIL wirken. RIPK1 erwies sich als essentiell für die Poly (I: C) / TWEAK-induzierte Caspase-8-vermittelte Apoptose, war jedoch für diese Reaktionen bei TNF- und TRAIL-Signalen entbehrlich. Das Fehlen von FADD-Protein hob TRAIL-, aber nicht TNF- und Poly (I: C) -induzierte Nekroptose auf. Darüber hinaus beobachteten wir, dass sowohl langes als auch kurzes FLIP HaCaT- und HeLa-RIPK3-Zellen vor Poly (I: C) -induzierter Apoptose oder Nekroptose retteten.

Zusammenfassend zeigen unsere Ergebnisse, dass TWEAK, das von Interferon-stimulierten myeloischen Zellen produziert wird, die Induktion von Apoptose und Nekroptose durch den TLR3-Liganden Poly(I: C) steuert und somit zur Krebsbehandlung oder antiviralen Immunität beitragen kann.

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9. Attachments

9.1. Sequence of Flag-TWEAK

aagcttcaaaacatgaacttcggcttcagcctgatcttcctggtgctggtgctgaagggc
K L Q N M N F G F S L I F L V L V L K G
gtgcagtgcgaagtgaagctggtgccccggggatccgattacaaagacgatgacgataaa
V Q C E V K L V P R G S **D Y K D D D D K**
gaattggcgatcgcagcccattatgaagttcatccacgacctggacaggacggagcgcag
E L A I A A H Y E V H P R P G Q D G A Q
gcaggtgtggacgggacagtgagtggctgggaggaagccagaatcaacagctccagccct
A G V D G T V S G W E E A R I N S S S P
ctgcgctacaaccgccagatcggggagtttatagtcacccgggctgggctctactacctg
L R Y N R Q I G E F I V T R A G L Y Y L
tactgtcaggtgcactttgatgaggggaaggctgtctacctgaagctggacttgctggtg
Y C Q V H F D E G K A V Y L K L D L L V
gatggtgtgctggccctgcgctgcctggaggaattctcagccactgcggccagttccctc
D G V L A L R C L E E F S A T A A S S L
gggccccagctccgcctctgccaggtgtctgggctgttggccctgcggccagggtcctcc
G P Q L R L C Q V S G L L A L R P G S S
ctgcggatccgcaccctcccctgggcccatctcaaggctgcccccttcctcacctacttc
L R I R T L P W A H L K A A P F L T Y F
ggactcttccaggttcactgatctaga
G L F Q V H - S R

9.2. Abbreviations

Table 8: Abbreviations

ALRs	absent in melanoma 2 -like receptors
AMD	age-related macular degeneration
AMPs	antimicrobial peptides
ATP	adenosintriphosphat
BAFF	B-cell activating factor
BAFFR	B-cell activating factor receptor
CNS	central nervous system
Caspases	cysteinyl-aspartate-specific proteinases
CLRs	c-type lectin receptors
C_pG DNA	cytosine-phosphate-guanine oligodeoxynucleotides
DAMPs	damage-associated molecular patterns
DCs	dendritic cells
DD	death domain
DISC	death inducing signaling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DRs	death receptors
dsRNA	double-stranded ribonucleic acid

Table 8 continued: Abbreviations

ECL	enhanced chemiluminescence
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EMCV-D	encephalomyocarditis virus strain D
FACS	fluorescence activated cell sorting
FADD	fas-associated death domain
Fas or CD95L	tumor necrosis factor receptor family member
FasL or CD95L	tumor necrosis factor ligand family member
FC- portion	fragment crystallizable portion of IgG
FCS	fetal calf serum
FGF	fibroblast growth factor
FLAG	Flag epitope
Fn14	fibroblast growth factor-inducible 14 kDa protein
FOXO1	forkhead box protein O1
HCl	hydrogen chloride
HEK293	human embryonic kidney cell line
HeLa	human cervical cancer cell line
hMSCs	human mesenchymal stem cells(
HMW	high molecular weight

Table 8 continued: Abbreviations

HRP	horseradish peroxidase
IFN	interferon
IFN-γ	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-8	interleukine-8
IP	immunopreceptation
IRFs	Interferon regulatory factors
IRF3	interferon regulatory factor3
JNK	c-Jun N-terminal kinase
kDa	kilo Dalton
LPS	lipopolysaccharide
LR	linker region
LRRs	leucine-rich repeats
LTα	lymphotoxin alpha
LTβ	lymphotoxin beta

Table 8 continued: Abbreviations

M	molar
mA	milliampere
mAb	monoclonal antibody
MAPK	mitogen activated protein kinase
min	minutes
ml	milliliter
mM	millimolar
mm	millimeter
MMP-1/2/3/9	matrix metalloproteinase 1/2/3/9
mRNA	messenger-ribonucleic acid
MYD88	myeloid differentiation primary response 88
n/a	not applicable
Na2CO3	sodium carbonate
Na2HPO4	disodium phosphate
NaCl	sodiumchloride
NaH2PO4	monosodium phosphate
NaHCO3	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

Table 8 continued: Abbreviations

ng	nanogram
NIK	NFκB inducing kinase
NK cells	natural killer cells
NLRs	nod-like receptors
nM	nanomolar
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
PAMP	pathogen-associated molecular pattern
pDCs	plasmacytoid dendritic cells
Poly(A:U)	polyadenylic–polyuridylic acid
Poly(I:C)	polyinosinic-polycytidylic acid
PRRs	pattern recognition receptors
RIP1	receptor interacting protein 1
RIP3	receptor interacting protein 3
RIPK1	receptor interacting protein kinase1

RIPK3	receptor interacting protein kinase3

Table 8 continued: Abbreviations

RLRs	RIG-I-like receptors
RNA	riboneucliec acid
Src	proto-oncogene tyrosine-protein kinase
ssRNA	single strand ribonucleic acid
TAK1	transforming growth factor-β-activated kinase 1
TBK1	serine/threonine-protein kinase-1
TIR	toll/interleukin-1 receptor
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor1
TLRs	toll-like receptors
TLR1	toll-like receptor1
TLR2	toll-like receptor2
TLR3	toll-like receptor3
TLR4	toll-like receptor4
TLR5	toll-like receptor5
TLR6	toll-like receptor6
TLR7	toll-like receptor7

TLR8	toll-like receptor8
TLR9	toll-like receptor9

Table 8 continued: Abbreviations

TRIF	toll/interleukin-1 receptor-domain-containing adapter-inducing interferon-β
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
TWEAK	tumor necrosis factor (TNF)-like weak inducer of apoptosis
Tyr759	tyrosine759
Tyr858	tyrosine858
WNV	west nile virus

9.3. List of Publications

1- TRAF2 controls death receptor-induced caspase-8 conversion and facilitates proinflammatory signaling. Kreckel J, **Anany MA,** Siegmund D, Wajant H. Frontiers Immunology. 2019 (In revision).

2- Bioactive brominated oxindole alkaloids from the Red sea sponge Callyspongia siphonellaeham S. El-Hawary, **Anany MA** , Ahmed M. Sayed2, Rabab Mohammed3, Hossam M. Hassan3 , Mostafa E. Rateb3,5,6, Tarek A. Mohammed, Elham Amin, Mohamed El-Mesery, Abdullatif Bin Muhsinah, Abdulrhman Alsayari, Harald Wajant and Usama Ramadan Abdelmohsen. Mar Drugs. (Submitted).

3- A Near-Complete Series of Four Atropisomeric Jozimine-A2 Type Naphthylisoquinoline Dimers with Antiplasmodial and Cytotoxic Activities and Related Alkaloids from Ancistrocladus abbreviates. Shaimaa Fayez, Jun Li, Doris Feineis, Laurent Aké Assi, Marcel Kaiser, Reto Brun, **Anany MA**, Harald Wajant, and Gerhard Bringmann. Natural Products. 2019 (Submitted).

4- Soluble TNF-like weak inducer of apoptosis (TWEAK) enhances poly(I:C)-induced RIPK1-mediated necroptosis. **Anany MA,** Kreckel J, Füllsack S, Rosenthal A, Otto C, Siegmund D, Wajant H. Cell Death Dis. 2018 Oct 22;9(11):1084. doi: 10.1038/s41419-018-1137-1.

5- New Cytotoxic Cyclic Peptide from the Marine Sponge-Associated Nocardiopsis sp. UR67. Ibrahim AH, Attia EZ, Hajjar D, **Anany MA,** Desoukey SY, Fouad MA, Kamel MS, Wajant H, Gulder TAM, Abdelmohsen UR. Mar Drugs. 2018 Aug 21;16(9). pii: E290. doi: 10.3390/md16090290.

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