CD95-induced Apoptosis in Health and Disease
Dimers at the DISC
and
The Effect of c-FLIP$_R$ on *L. Monocytogenes* Infection

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I. Introduction

1. Cell Death

Cell death plays an important role during development and in maintaining homeostasis in the adult organisms, especially in the immune system. Several forms of cell death have been described, amongst them the three major forms: apoptosis, necrosis and autophagy-associated cell death [1] (Fig. 1).

During necrosis the cell swells, its content is leaking and may cause inflammation (Fig.1). Newer studies show some forms of necrosis and autophagy to be regulated on the molecular level similar to apoptosis, which used to be seen as solely “programmed” cell death form [2]. Therefore, this kind of cell death was named necroptosis to discriminate between the regulated forms and uncontrolled necrosis. Especially after inhibition of death receptor-induced apoptosis, molecular signaling cascades similar to apoptotic cascades, which lead to necroptosis can occur [3].

Autophagy serves as a system to remove and recycle organelles and intracellular components [4]. The autophagy system helps the cell to eliminate pathogens, even though some pathogens evolved strategies to block it or even used it for survival [5]. Autophagy usually acts as survival mechanism for the cell, nevertheless there are some studies showing it can as well be involved in cell death [6] . Autophagic cell death has been shown in vitro, its role in vivo remains controversial [7].

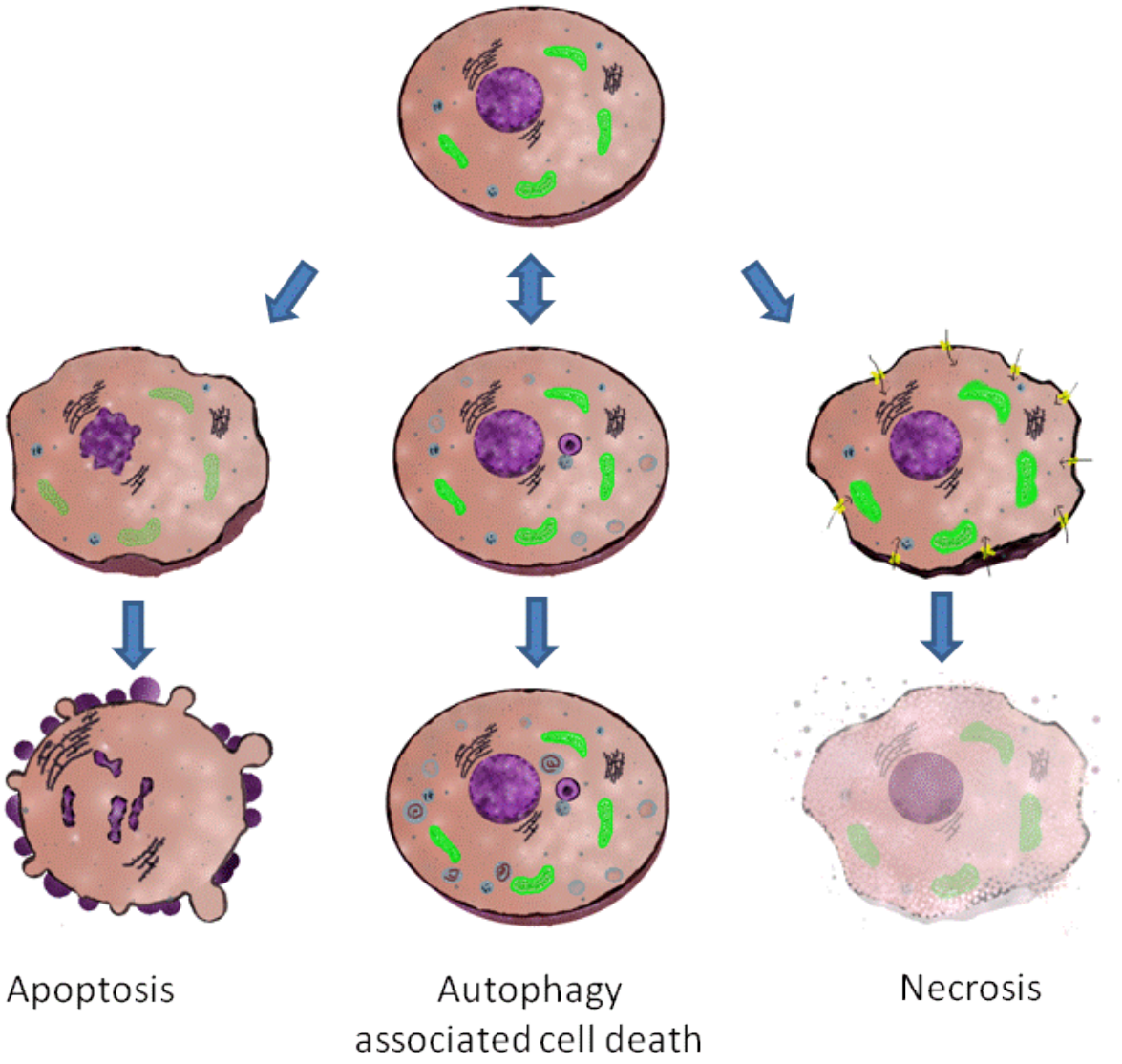

Figure 1. Forms of Cell Death
Cells can die via apoptosis (left), autophagy (middle) or necrosis (right). Apoptotic cells shrink and form apoptotic blebs becoming apoptotic bodies, which are taken up by phagocytes. Auotphagic cells include cell material into autopagosomes, whilst necrotic cells swell and leak content into the surrounding areas, possibly causing inflammation.

Apoptosis, which was first described by Whylie and Kerr [8], is a tightly regulated form of cell death mediated by activation of caspases. Apoptotic cells show condensation of chromatin and the cytoplasm, DNA fragmentation and destruction of pro-survival factors [9]. Apoptotic cells expose posphatidylserine (PS), first bleb and later form apoptotic bodies, which are surrounded by a membrane [10]. These apoptotic bodies are taken up by surrounding phagocytic cells [10], preventing leakage of the cell content into the surroundings and inflammation [11]. Deregulation of apoptosis leads to several diseases. Excess apoptosis can cause tissue destruction in autoimmunity or neurodegenerative diseases. Insufficient apoptosis can lead to tumor growth or survival of autoreactive cells, thereby promoting autoimmunity [12].

2. Molecules Involved in Cell Death

2.1 Death Receptors

So far 19 members of the tumor necrosis factor superfamily (TNF) are known. TNF is a highly pleiotropic molecule that elicits diverse cellular processes, which range from proliferation and differentiation to activation of apoptosis [13, 14]. Their receptors belong to the tumor necrosis factor receptor (TNFR) superfamily, which includes 23 related receptors. Most receptors are type I transmembrane proteins, some are anchored to the plasma membrane by glycophospholipid moieties and some can be secreted [15]. Within the TNFR superfamily some receptors contain death domains (DD) and transduce signals, which may lead to cell death [15]. Therefore, these receptors are called death receptors (DRs) [16]. Next to death receptors, decoy receptors, lacking a DD completely or having a non-functional DD have been described [17]. Soluble decoy receptors have been reported to be capable of competing with death receptors for ligands and thereby modulate DR signaling [18].

In humans six death receptors have been identified: TNFR1, CD95, DR3, DR4, DR5, and DR6 [14]. DRs do not solely signal cell death [15, 19], the outcome of their signaling can as well be proliferation, survival or secretion of cytokines

[15]. Upon binding of their specific ligands DRs recruit DD containing adaptor molecules Fas associated-death domain (FADD) or TNF receptor associated-death domain (TRADD). FADD controls cell death signaling by recruitment of caspase-8, -10 and c-FLIP proteins [15]. TRADD recruits RIP1, TRAF2 and cIAPs and thereby controls non-apoptotic signaling [15]. Nevertheless crosstalk between pro- and anti apoptotic signaling does occur. TNFR1 for example can recruit FADD via TRADD or RIP1 and DR4 and DR4 can activate NF-kB and MAPK via caspase-8 and FADD dependent RIP1 activation [15]. Whilst CD95, DR4 and DR5 signaling primarily lead to cell death, TNFR1 induces pro-inflammatory and immune stimulatory activity via JNK, p38-MAPK and NF-κB. Molecules involved in death receptor signaling are involved in other signaling pathways as well, for example signaling downstream of the T cell receptor (TCR) or pattern recognition receptors (PRRs) like Toll like receptors (TLRs) [15].

Each death receptor has its unique ligand, apart from DR4 and DR5, which both bind TNF-related apoptosis-inducing ligand (TRAIL) [20]. Regarding DR6 the ligand is less clear. For neurons beta-amyloid precursor protein (APP) has been shown as ligand [21, 22]. APP is expressed solely in the nervous system [21], where its role in DR6 signaling is discussed controversial [21]. DR6 is more broadly expressed, e.g. as a marker for several tumors, e.g. solid tumors [23] and sarcoma [24], which makes DR6 an interesting target for tumor treatment.

CD95 is a classical DR belonging to the TNFR superfamily [25]. It is a type I transmembrane receptor, which is activated via external binding of its ligand and induces intracellular formation of a multiprotein complex named death inducing signaling complex (DISC) [26].

TRAIL is a ligand, which occurs like CD95 ligand as trimer [27]. TRAIL signaling and its effect on disease is not as extensively characterized as CD95L signaling. TRAIL signaling is assumed to proceed in a similar fashion as CD95 induced apoptosis [28]. Initially, it was believed that TRAIL specifically kills tumor cells leaving non-transformed cells unharmed [29].

However, recent evidence suggests that other cell types, e.g. hepatocytes, are affected, too, especially after cellular stress induced by triggers like alcohol or HCV infection [30].

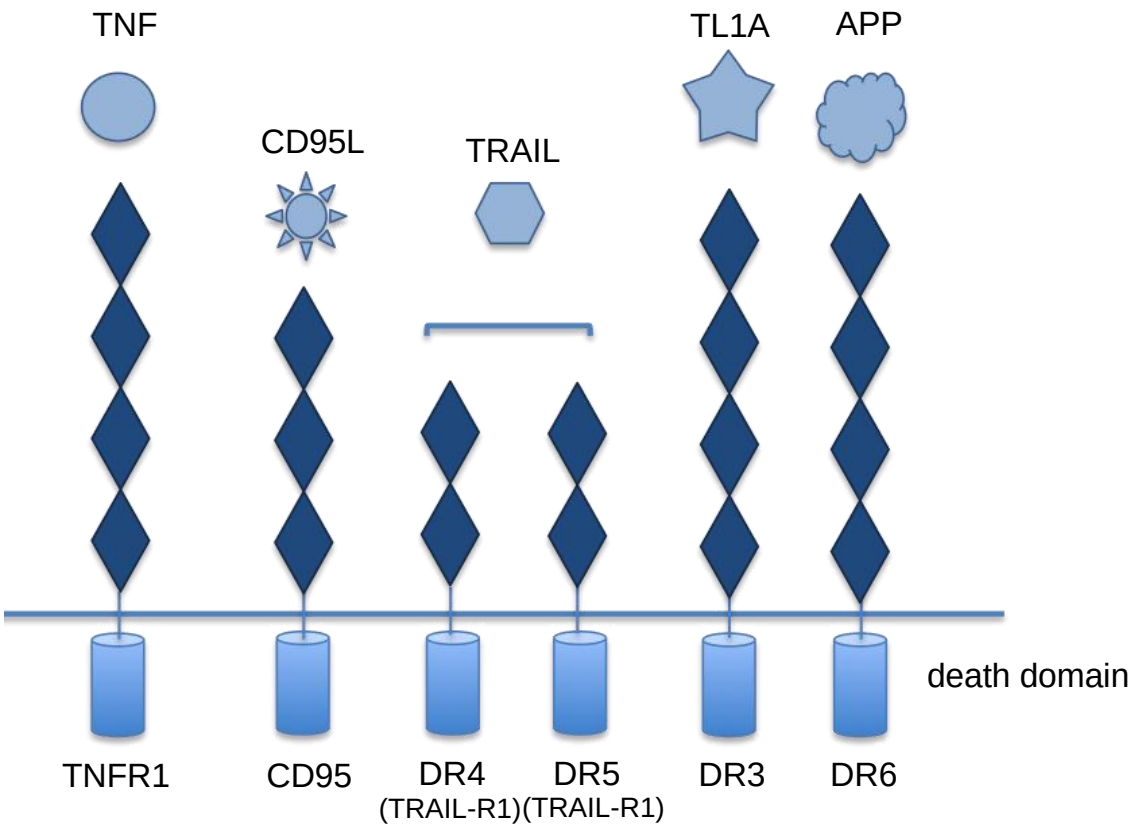

Figure 2. The Death Receptor Superfamily and their Ligands
Each death receptor binds to its unique ligand. The exceptions are DR4 and DR5, which both bind TRAIL. DR6 is known to bind amyloid precursor protein (APP), which does not belong to the TNF superfamily. APP is solely expressed in the nervous system.

2.2 Caspases

The first discovered cysteine-dependent aspartate specific protease (caspase), caspase-1, was found to cleave interleukin-1β and, therefore was named at first interleukin-1β-converting enzyme (ICE) [31, 32]. In humans so far twelve caspases have been described. Depending on the cellular process they initiate or execute they are subdivided into inflammatory or apoptotic caspases. Nevertheless, most apoptotic caspases are known to have functions in survival and proliferation, too [33]. Inflammatory caspases in contrast are capable of inducing cell death as well, e.g. in associated with massive inflammation [34]. Based on their position in signaling cascades, structure of their prodomains and sequence homology apoptotic caspases are grouped into initiator (caspase-2; -8; -9; -10) and effector caspases (-3;-6; 7) [9]. Initiator caspases are activated via dimerization and stabilized by auto-proteolysis [35].

All Caspases are synthesized as preforms called zymogens [9]. Caspase zymogens consist of a C-terminal prodomain and an N-terminal domain containing a large and a short subunit. Apoptotic signaling leads to a cascade where caspases activate further downstream caspases [36]. Initiator caspases activate effector caspases, which are already present as dimers, by proteolytical cleavage [9]. Activated effector caspases cleave directly or indirectly via activation of other proteinases substrates, which lead to the apoptotic phenotype of the cell as described above. Caspases have an active site cysteine (Cys285) and a specificity for substrate cleavage after an Asp residue [34]. The only other protease known to have an Asp preference for cleavage is granzyme B [37]. An Asp residue alone does not make a protein cleavage site though. Several other factors, like surrounding amino acids and resulting bonds, conformation etc. define if the protein is a possible substrate for a certain caspase [34]. The stable, active enzyme is a heterotetramer consisting of two large and two small subunits [38]. Apoptotic executioner caspases cleave preferable at DEV(D^VG) sites, whilst inflammatory caspases prefer WEH(D^VG) sites. Nevertheless caspase substrates do overlap.

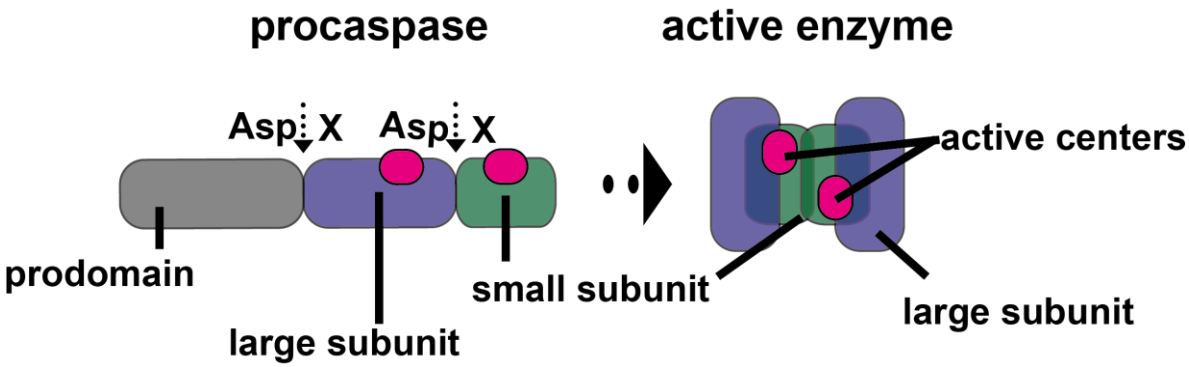

Figure 3. Simplified Overview of Formation of a Stable Active Caspase Enzyme.
After cleavage between the subunits two short and two long subunits form the enzyme. This step is needed for increased stability of caspases.

Caspase-8 is the main initiator caspase within CD95-, DR4- and DR5-induced apoptotic signaling [39, 40]. Caspase-8 is present in the cell as inactive monomer. The current accepted model of caspase-8 activation is the induced proximity model [41]. According to this model caspase-8 is recruited to activated and oligomerized death receptors via FADD [41]. Within the DISC caspase-8 is brought in close proximity with another caspase-8, which would lead to dimerization as described below. During this process a conformational change takes place making the catalytic dyad consisting of His-237 and Cys-285 accessible [42].

Whilst caspases-8's function and its role in apoptotic signaling have been extensively analyzed, the role of caspase-10, the closest homolog of caspase-8, remains unclear [43-47]. Caspase-10 was lost in the rodent line [48], which may explain the research focus on caspase-8. Still caspase-10 seems not to be replaceable in humans, since mutations in its gene lead to autoimmune lymphoproliferative syndrome (ALPS) type II [49]. The term ALPS describes diseases caused by mutations in caspase-10, CD95 or the CD95 ligand. Patients with ALPS show heterogeneous disease syndromes, amongst them chronic lymphoproliferation and autoimmunity [50].

Caspase-8 and caspase-10 share the same gene locus with c-Flip on chromosome 2q33-34 [51] [52]. They have probably arisen by gene duplication. Both enzymes are recruited to the DISC via DED [43]. Both caspases seem to have overlapping as well as distinct substrate specifies [43]. Fischer *et al.* could detect more efficient cleavage of receptor interacting protein (RIP) by caspase-10 than caspase-8 or -3 suggesting that caspase-10 through the cleavage of RIP will also inhibit nuclear factor-kB (NF-κB) signaling [43]. Caspase-10 causes further cleavage of Bid at IEAD/A (caspase-8 at LQTD/G), which is likely to have consequences for downstream signaling. Cleavage sites are conserved within caspase-8 and -10.

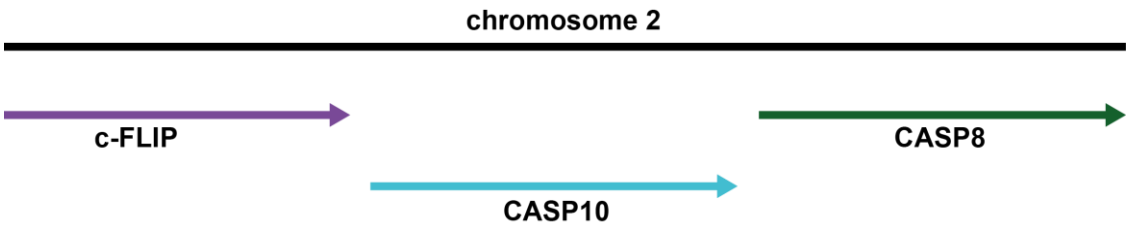

Figure 4. Chromosome 2 Gene Locus of Caspase-10.
Caspase-10 localizes on chromosome 2 in one gene locus with c-FLIP (CFLAR) and caspase-8**.** Likely they have arisen from a common ancestor gene.

Effector caspases have short prodomains and are present in the cell as inactive dimers [34]. They require cleavage by active initiator caspases or granzyme B for activation [53]. The best-characterized effector caspase is caspase-3. It is activated by almost all apoptotic initiator caspases and leads to cleavage of apoptotic substrates resulting in the apoptotic phenotype of the cell. Caspase-3 is involved in CD95-, DR4- and DR5-induced apoptosis signaling [54] and downstream of mitochondria [55]. In the absence of caspase-3 in vivo other caspases, e.g. caspase-7, can compensate [56].

3. Death Receptor-induced Apoptotic Signaling

After binding their ligand, clustering of death receptors is needed for induction of signaling [57]. The activated death receptors recruit adapter molecules, e.g. CD95 recruits FADD, which then leads to recruitment and activation of initiator caspases via autoproteolytical cleavage [58]. How the apoptosis cascade proceeds depends on the apoptosis type of the cell.

Death receptor-induced apoptosis can be subdivided into type I and II apoptosis based on the signaling cascade leading to apoptosis [59]. Most cell types use almost exclusively one of the two apoptosis signaling pathways [59]. Nevertheless, for CD95 it has been shown that expression of the receptor and sensitivity towards CD95-induced apoptosis does not differ between type I and II cells, making the amount of the receptor unlikely as determining factor for the apoptosis type [59]. Tumor cell lines differ in a wide variety of hundreds of genes expressed due to being type I or type II apoptotic cells [60] [61]. During carcinogenesis tumor cells can develop from type II to type I cells [61].

The classic model of signal transduction involves cell-surface receptors that are activated after binding a ligand [62]. The activation of many receptors also triggers endocytosis of ligand receptor complexes [62]. Endocytosis does not only mean termination of signaling; certain pathways require receptor internalization for full activation [62]. Type I cells recruit large amounts of caspase-8 to the DISC, at which caspase-8 is cleaved. Algeciras-Schimnich *et al.* could detect a strong link between actin and CD95 signaling in type I cells where internalization and clustering of CD95 is found [61]. The huge amount of activated caspase-8 activates in turn the effector caspases, caspase-3 and caspase-7.

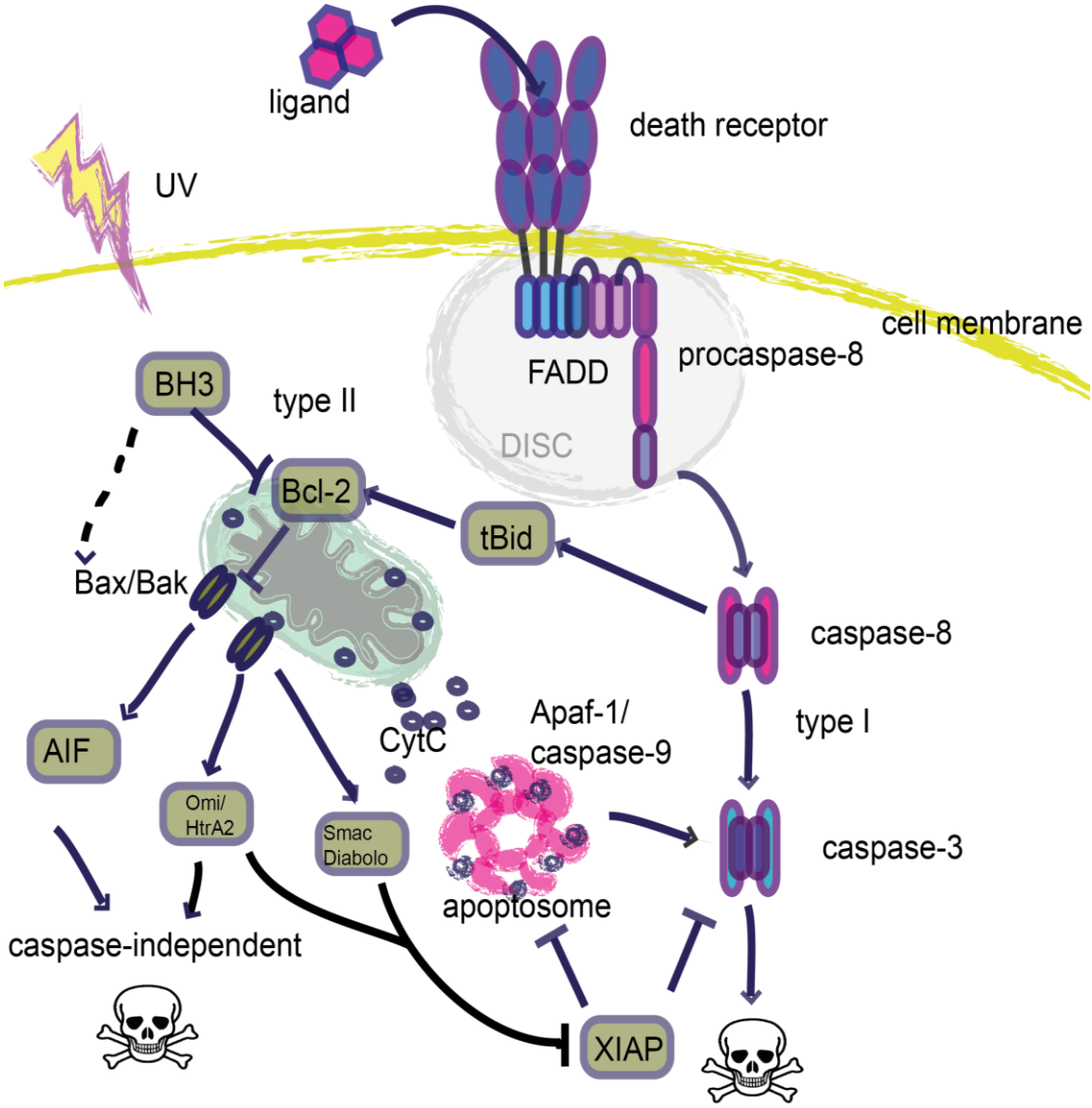

Figure 5. Schematic Overview of Apoptosis Signaling via Death Receptors and External Stimuli, e.g. UV light

Upon ligand binding transmembrane death receptors get activated and built a multiproteincomplex named Death Inducing Signaling Complex (DISC) in the cytoplasm. Caspase-8 gets cleaved and activated at the DISC and activates in turn caspase-3 by cleavage in type I cells. In type II cells caspase-8 cleaves Bid leading to truncated Bid (tBid). tBid mediates amplification of the apoptotic signal via the mitochondrial pathway. The mitochondrial pathway is induced by external stimuli like UV light as well. In this pathway caspase-9 is activated at the apoptosome. Caspase-9 activation results in caspase-3 cleavage as well. Caspase-3 cleaves directly or indirectly via activation of further downstream proteins apoptotic substrates leading to the apoptotic phenotype of the cell.

Type II cells depend on mitochondria for amplification of the apoptotic signal because caspase-3 and caspase-8 are primarily activated downstream of the mitochondria in this apoptosis type [59, 63]. In type II cells low amounts of caspase-8 are cleaved at the DISC and the apoptotic signal is amplified via the mitochondria. This is mediated by cleavage of Bid by activated caspase-8 [64, 65].

Bid belongs to the B-cell lymphoma 2 (Bcl-2) family proteins, which can be divided into three groups. The anti-apoptotic Bcl-2-like and the pro-apoptotic Bcl-2–associated X protein (Bax) like proteins have four Bcl-2-homology domain 3 (BH3) domains, whilst the pro-apoptotic BH3 proteins have one [66]. The ratio of pro- to anti-apoptotic family members is essential for the outcome of signaling, apoptosis or survival. Bid cleavage results in translocation to the mitochondria where it induces via other Bcl-2 proteins the release of mitochondrial factors, which ultimately enhance the apoptotic signal [67] [66].
The pore forming proteins Bax/Bak are essential for permabilization of the mitochondrial membrane and the release of cytochrome c (cytc) and other soluble pro-apoptotic factors from the mitochondria leading to apoptosome formation [68, 69]. Nevertheless the exact mechanisms of pore formation remains unclear [66]. Additionally at late stages of several apoptotic pathways the mitochondrial membrane is cleaved, the mitochondria break down and the pro-apoptotic factors are released, too [67]. Since type II DR mediated apoptosis relies on the function of the mitochondria it is regulated by Bcl-2 family proteins overexpression of Bcl-2 and Bcl-xl in type II cells can block apoptosis [59].

4. FLIP Proteins as Regulators of Apoptosis

Proteolysis is an irreversible process, in case of apoptotic caspases leading to cell death. To prevent unwanted cell death, caspase activation is not only regulated at mitochondrial level, but as well at death receptor level. Several cellular regulatory mechanisms interfering with cell death have evolved. Proteins of the Bcl-2 family [66] and XIAP [70] regulate mitochondrial apoptosis

signaling. Cellular FLICE inhibitory proteins (c-FLIP) proteins act directly at DR level interfering at different steps with caspase-8 [71]. Next to cellular mechanisms some pathogens interfere with apoptosis, as well. Viruses for example express viral Flip proteins (vFLIPs) [72].

c-FLIP proteins also contain DEDs, which allow them to compete with caspase-8 for FADD binding [64] [73], resulting in altered DISC composition and apoptosis inhibition. c-FLIP is expressed in different isoforms, which can have opposing functions [74] [75] and inhibit different steps of caspase-8 activation [71]. For instance, c-FLIP$_L$ contains a caspase-like domain lacking proteolytical activity and acts pro-apoptotic when expressed in low amounts but anti-apoptotic when highly expressed [75]. In addition, humans generate two solely anti-apoptotic acting short isoforms called c-FLIP$_S$ and c-FLIP$_R$ [76] [64], whose expression is regulated by a single nucleotide polymorphism (SNP) [77]. Strikingly c-FLIP$_R$ expression in humans is associated with an increased risk of follicular lymphoma [77], whilst in mice c-FLIP$_R$ is the only short isoform [78].

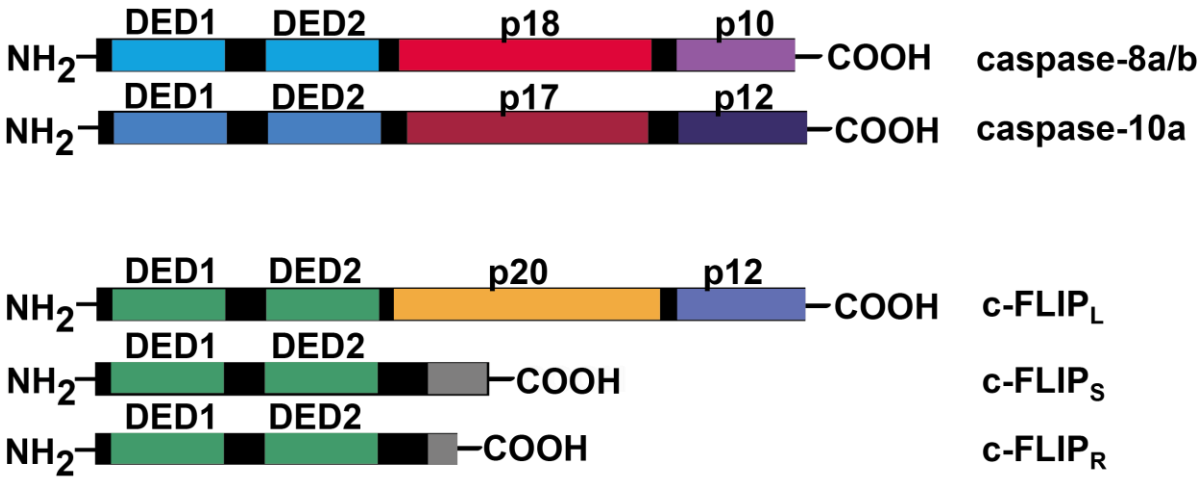

Figure 6. Comparison of Caspase-8, -10 and c-FLIP Isoforms
All proteins contain N-terminal tandem DEDs, which allow them to interact. Caspase-8 and -10 and c-FLIP$_L$ contain C-terminal a small and a long subunit. The main difference between the two caspases and c-FLIP$_L$ is the enzymatic activity. c-FLIP$_L$ lacks an active site and is thereby inactive. The short c-FLIP isoforms, c-FLIP$_S$ and c-FLIP$_R$ contain a very short C-terminal fragment.

5. Dimerization and Activation of Initiator Caspases and their Regulator c-FLIP

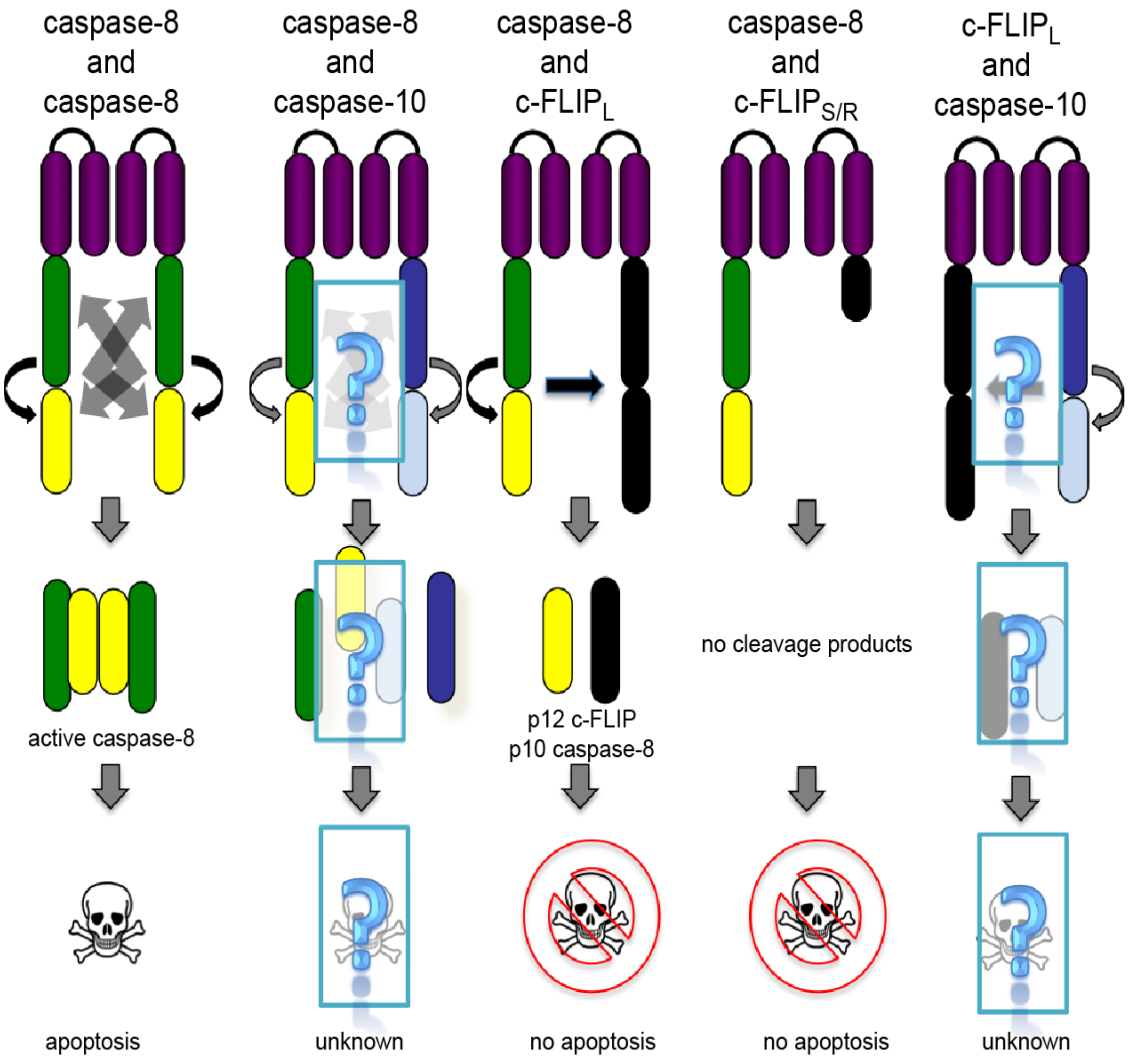

Fig. 7 Dimers Formed at the DISC
Caspase-8 homodimers lead to apoptotic signaling, whilst the short c-FLIP isforms block apoptosis. c-FLIP$_L$ seems to act dose dependent. The role of dimers containing caspase-10 remains unknown. Most experiments have been performed in vitro or with altered recruitment domains. Dimer formation upon apoptotic stimuli has not been shown in living cells so far.

As described above caspases-8, -10 and c-FLIP proteins are recruited to the DISC where they are brought into close proximity. Dimerization seems to be the essential activation process leading to a conformational change, which makes the active site dyad of caspase-8 accessible [42]. In vitro experiments

with kosmotrophic salts showed that caspase-8 forms caspase-8-caspase-8 homodimers as well as dimers with c-FLIP isoforms [79]. Initiator caspases dimerize in vitro in kosmotrophic salts, which leads to enzymatic activity, even in the absence of proteolytical processing. If the salt is removed the proteins dissociate and loose their activity. Cleavable proteins, in contrast, retain the enzymatic activity. Thus, cleavage, which occurs after dimerization, is essential for the stability oft the active caspase [34]. Uncleaved active caspase-8 has a lower enzymatic activity and cleaves different substrates. Dimerization alone seems insufficient to induce apoptosis [35]. In contrast, activation without cleavage seems to be sufficient to prevent defects in T cell proliferation, which are caused by defects or lack of caspase-8 [80].

Different known c-FLIP isoforms act at different steps of caspase-8 activation [71]. The short isoforms directly interfere with DISC binding and the first cleavage step of caspase-8 [71]. They lack a long C-terminal domain and thereby can't stabilize caspase-8 in a conformation to form an active center dyad. Since caspase-8 becomes not activated they inhibit any possible processing steps. Due to its structural similarity with caspase-8 c-$FLIP_L$ is thought to form dimers with caspase-8 and stabilize caspase-8 in an active manner. This makes the first cleavage step possible [71]. Since c-$FLIP_L$ is enzymatically inactive, it is unable to cleave caspase-8 in return. The activated caspase-8 can cleave itself and c-$FLIP_L$ generating a p10 caspase-8 and a p12 c-$FLIP_L$ fragment [71]. Reports in the literature regarding the pro- or anti-apoptotic effect of the cleavage products are controversial. Using a system based on mathematical modeling Fricker and colleagues explain these varying results by multiple factors adding to the outcome of signaling: the amount of c-$FLIP_L$, cell type and strength of receptor signal [81]. Interestingly caspase-8-c$FLIP_L$ heterodimers have been shown to protect cells from necrosis [35, 82]. This means the composition of dimers at the DISC is not solely a yes or no decision on death or survival, but rather a multi-step regulation between various pathways. Strong overexpression of c-$FLIP_L$ prevents apoptosis [59, 83]. Most likely this block occurs due to competing with caspase-8 homodimers for receptor recruitment. If caspase-10 forms dimers with caspase-8 or c-FLIP

or if c-FLIP proteins dimerize with other c-FLIP isoforms or their same isoforms remains is currently unknown.

6. DISC Molecules in Proliferation/ Non-apoptotic Signaling

Several DISC components are found in non-apoptotic pathways as well. For example caspase-8 activity is also required for proliferation. Seemingly this does not require autoproteolytic cleavage processes. Mutants lacking cleavage sites do not interfere with T cell proliferation [84]. In humans a non-functional mutation of caspase-8 results in an immunodeficiency and reduced IL-2 production [85, 86]. Mice lacking caspase-8 die prenatal at day 12.5 [40]. After T cell activation cleavage of known caspase substrates, such as c-FLIP$_L$ or RIP1, occurs but Bid is not cleaved [87]. c-Flip$_L$ has been described to associate with RIP1 and TRAF2 for NF-κB activation [88]. Cancer cells in general, regardless of their CD95 apoptosis sensitivity, depend on constitutive activity of CD95, stimulated by CD95L for optimal growth [84].

7. Apoptosis in the Immune System

The immune system is an organ system that is regulated by dramatic alterations in clonal populations. This is controlled by various mechanisms: clonal activation, expansion due to high rates of cell division after contact with an antigen and termination by cell death. Apoptosis, a highly regulated form of cell death, plays an important role in deletion of autoreactive and excess cells in the immune system [12].

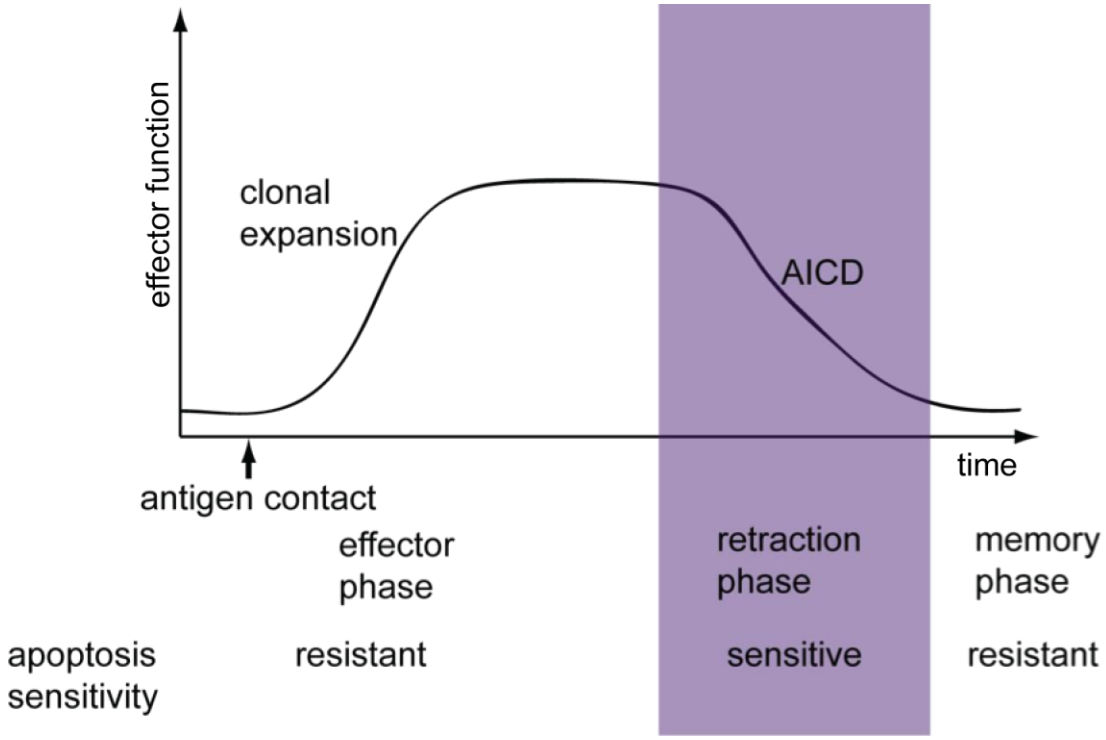

Figure 8. T cell Activation During an Immune Response
After antigen contact T cells enter the expansion/effector phase where they proliferate. During this phase they are resistant towards death receptor induced apoptosis. At the end of the immune response excess T cells become sensitive towards apoptosis and most cells are removed. Finally memory cells survive, which are resistant to apoptosis.

The role of c-FLIP$_S$ in the human immune response has been extensively analyzed. T cells are protected from DR-mediated cell death during the initial phase of an immune response. Thus, c-FLIP$_S$ is highly induced in freshly activated human T cells and contributes to resistance to CD95-induced apoptosis in the early phase of an immune response [78, 89, 90]. The function of the other short isoform, c-FLIP$_R$, however, remains unknown. This is very interesting since c-FLIP$_R$ is the solely short murine c-FLIP splice variant due to the gene structure in mice. In humans in contrast c-FLIP$_R$ is associated with a higher risk of developing follicular lymphoma [77].

8. *Listeria Monocytogenes* as Model for an Immune Response

Listeria monocytogenes (L. monocytogenes) is a gram positive bacterium, which causes listeriosis [91]. *L. monocytogenes* can thrive in a variety of environments like soil, water or food products. This can, especially in immune compromised people, pregnant women and newborns, cause listeriosis [91]. *L. monocytogenes* enters the body via endothelial cells, but it is able to cross the fetal barrier as well and infect the fetus [91]. In addition, *L. monocytogenes* is able to penetrate the blood brain barrier and cause infection of meninges and brain [91].

L. monocytogenes enters cells via two surface receptors, Internalin A (InlA; and Internalin B (InlB) [92], which both bind host cell proteins [91]. InlB binds to the c-Met receptor, whose endogenous ligand is the hepatocyte growth factor (HGF). c-Met is usually expressed by cells of epithelial origin. Binding of InlB to c-Met leads to rearrangement of the actin cytoskeleton and internalization of bacterium via clatherin-mediated endocytosis [91]. InlA binds the host protein E-cadherin [93], which is a transmembrane protein required for the formation of adherence junctions. After *L. monocytogenes* has entered the host cell forming a vacuole, it lyses the phagosome via two phospholipases (PlcA and PlcB) and Listeriolysin O (LLO) and enters the host cytoplasm [91, 94]. LLO is a cholesterol dependent cytolysin which is pH sensing and unique for Listeria [95]. LLO shows highest activity in the acidic phagosome [95]. In the cytoplasm *L. monocytogenes* can polymerize actin of the host cell and propel itself through the cell to a neighboring cell [96].

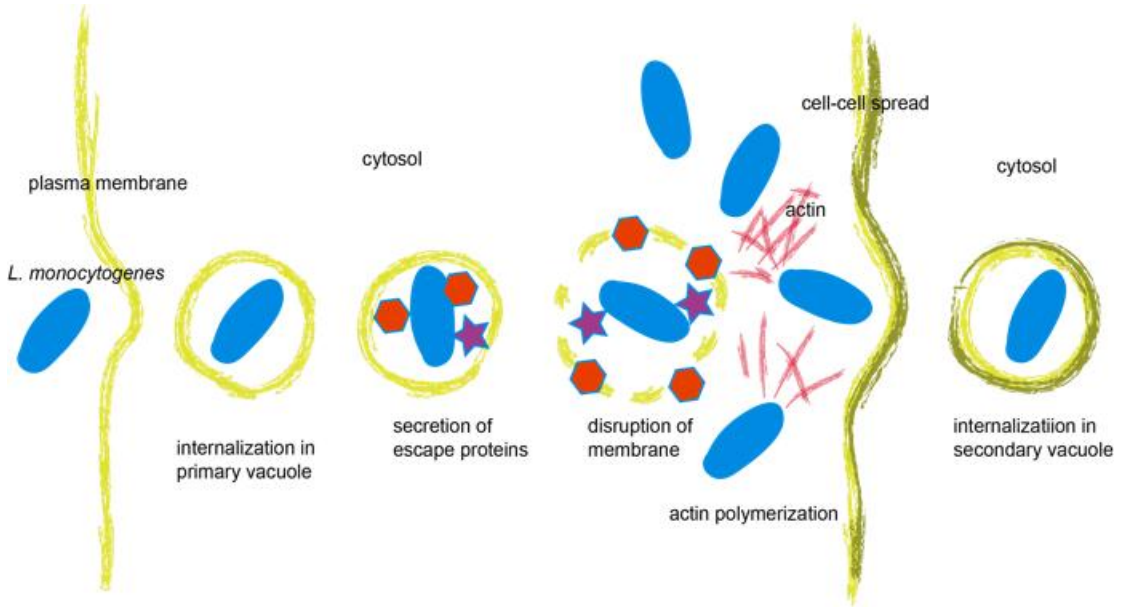

Figure 9. Intracellular Life Cycle of *L. monocytogenes*
The bacterium enters the cell forming a primary vacuole, secretes escape proteins, which disrupt the membrane, this enables the bacteria to escape to the cytoplasm. In the cytoplasm it polymerizes cellular actin for motility. Listeria can spread to the next cell, where it is enclosed in a double membrane vacuole called secondary vacuole. Here it starts again to secrete virulence factors to escape into the cytosol.

In contrast to humans mice are resistant to oral *L. monocytogenes* infection. This is due to murine E-cadherin differing in a single amino acid, which prevents binding of InlA [97]. Intravenous infection of mice is a dose-dependent model, which is widely used to study T cell activation. Infection with a sub-lethal dose induces in mice a strong immune response with bacterial clearance [98].

Minutes after injection Listeria can be detected in the liver and spleen where they are taken up by resident macrophages [98]. A strong T cell response is mounted to eliminate bacteria and memory cells are generated [98]. Protective immunity is only generated if the injected Listeria are alive and contain LLO [99]. The first phase of *L. monocytes* infection is controlled by innate immunity keeping growth in check and preventing spread into a systemic, lethal infection. Macrophages are very essential for Listeria infection since the bacteria replicate within them [100] and they are essential for mediating clearance [101].

Listeria induces IFNγ and type I Interferons, which are usually important for anti-viral responses and can induce apoptosis [98]. Recognition of bacteria by toll like receptors (TLRs) leads to activation of dendritic cells (DCs), which are a bridge between the innate and adaptive immune system [102]. Later on the adaptive immune system eliminates the pathogen and memory cells are formed. In Balb/c mice the adaptive response is mediated be $CD8^+$ cells, whilst Black6 mice react to MHCII epitopes leading to a $CD4^+$ immune response [98].

9. Listeria monocytogenes and cell death

Apoptosis is a component of several infections. Infected cells may be killed via apoptosis to prevent spread of the infection, but some microorganisms use apoptosis to their advantage, *L. monocytogenes* being such [101]. After *L. monocytogenes* infection two phases of cell death occur, a fast granzyme B induced one and slower one which is not really well understood. During the first phase LLO, which is a member of the cholesterol-dependent cytolysin/ membrane attack complex/ perforin superfamily of toxins [101]. LLO-induced apoptosis depends on the activation state of the T cells. Rapidly dividing lymphocytes are susceptible, whilst resting lymphocytes are resistant [103]. This process proceeds via granzyme B-induced activation of caspase-3, -6 and 9 and therefore, this phase can be abolished by granzyme B depletion [104]. The following slower apoptotic phase seems to be independent of granzyme B. Death receptor mediated apoptosis is known to alter infection with *L. monocytogenes*, e.g. TRAIL-deficient mice show a reduction in apoptosis and a lower bacterial burden [105]. Nevertheless, different factors contributing to cell death after *L. monocytogenes* infection are discussed controversial. Moreover, the role of *L. monocytogenes*-induced cell death in macrophages and dendritic cells remains controversial. They are the first carriers of *L. monocytogenes* after infection [101].

II. Aims

Deregulated death receptor-induced apoptosis is known to play an essential role in the development of severe diseases, e.g. autoimmunity and immune deficiency in patients with nonfunctional caspase-8 mutations [12, 85]. Therefore, a better understanding of signaling downstream of death receptors is needed. Caspase-8 is assumed to form caspase-8-caspase-8 homodimers and heterodimers with c-FLIP proteins. Nevertheless, this has never been shown in living cells with unaltered recruitment domains. The exact localization, where dimerization takes place is unknown, too. Composition and localization of caspase-8 dimers will be analyzed to gain a better understanding of these processes. This will be done using bimolecular fluorescence complementation (BiFC), which allows in vivo visualization of dimer formation. This technique has been used prior for visualization of caspase-2 dimerization [106]. Here, dimer formation between caspase-8, -10 and c-FLIP proteins and the localization will be analyzed using BiFC.

c-FLIP proteins are known to regulate death receptor signaling at the DISC level, e.g. during an immune response. Humans express two short c-FLIP isoforms, c-FLIP$_R$ and c-FLIP$_S$, but solely c-FLIP$_R$ is expressed in the murine system [77]. While human c-FLIP$_S$ has been extensively analyzed, the role of murine c-FLIP$_R$ remains unknown. c-FLIP$_S$ is known to regulate lymphocyte apoptosis during an immune response [89, 90]. Here murine c-FLIP$_R$'s functions during an infection as counterpart of human c-FLIP$_S$ will be analyzed. Therefore, vavFLIP$_R$ mice, which express c-FLIP$_R$ in cells of hematopoietic origin, will be infected with *Listeria monocytogenes*. Cell numbers, apoptosis, bacterial burden and histology will be analyzed. This will show if c-FLIP$_R$ alters the immune response. Taken together this project aims at clarifying the direct interactions of signaling molecules in the DISC and the physiological function of c-FLIP$_R$ in health and disease. This should lead to a better understanding of the role of death receptor-induced apoptosis and possible ways to interfere with it in diseases, e.g. infections or autoimmunity.

III. Material and Methods

1.1 Chemicals

If not stated otherwise chemicals were obtained from Sigma Aldrich (Munich, Germany), Roth (Karlsruhe, Germany) or Merck (Darmstadt, Germany).

1.2 Cell Culture

1.2 Cell Culture Materials and Devices

Cell culture flasks, 10 cm dishes, 6- and 12-well plates were obtained from NUNC (Thermo Fisher Scientific, Rochester, USA). 15ml and 50ml plastic tubes used were from Greiner bio-one (Frickenhausen, Germany). 5ml, 10ml and 25ml sterile pipettes were obtained from Thermo Scientific (Rochester, USA). Sterile pipet tips were used from Starlab (Ahrensburg, Germany).

1.3 Cell Culture Conditions

All cells used were cultured at 37 °C, 5 % CO_2 and 95 % humidity in a HERAcell a 240i incubator (Thermo Scientific, Vantaa, Finland). Cells were spun down in 5810R centrifuge at 1500rpm and RT (Eppendorf, Hamburg, Germany) and handled under a sterile Guard III clean bench (Baker Company; Sanford, FL, USA).

2. Cell Biological Analyses

2.1 Caspase-8-deficient A3 Jurkat Cells

Due to high endogenous caspase-8 expression caspase-8-deficient Jurkat cells generated in the lab of John Blenis were used [39]. The cells were cultured in RPMI1640 with 10% fetal calf serum. The parental cell line (A3) was used as control. A3 cells were cultured similar as caspase-8-deficient A3 cells.

2.2 SH-SY5Y Cells

SH-SY5Y cells are human neuroblastoma cells. The cell line used was a kind gift of Dr. Marcus Rehm (Dublin, Ireland). Caspase-8 is silenced in this cell line via methylation and can be induced by demethylation [107, 108]. SH-SY5Y cells were cultured in DMEM containing 10% fetal calf serum.

2.3 HeLa Cells

HeLa cells are a cell line derived from a human patient with cervical cancer [109]. HeLa cells were grown adherent in DMEM containing 10% fetal calf serum.

2.4 HT1080 Cells

HT1080 cells are a human fibrosarcoma cell line [110]. They were grown in DMEM containing 10% fetal calf serum.

2.5 293 and 293T cells

293 cells were derived from a human embryonic kidney culture and were transformed with adenovirus 5 DNA [111]. 293T cells contain additionally the SV40 large T-antigen, which allows episomal replication of transfected plasmids [111].

antibody	clone	supplier
CD95 2R2	2R2	kind gift of Dr. K. Schulze-Osthoff, Tuebingen, Germany
TRAIL-R1	HS101	Enzo Life Sciences, Loerrach, Germany
TRAIL-R2	DJR1	PE-conjugated, eBioscience San Diego, CA
TNFR1	H398	kind gift of Dr. H. Wajant, Wuerzburg, Germany

Table 1. Antibodies Used for Staining of Cell Surface Markers on Cell Lines from Human Origin

2.6 Transfection of Cells

Chemical Transfection

Adherent cells were transfected using JetPei (Polyplus Transfection-SA, Illkirch, France). SH-SY5Y cells were treated with a mixture of 2 µl Jetpei: 1 µg

DNA diluted with 150 mM NaCl solution (Polyplus Transfection-SA, Illkirch, France). 293 cells, HT1080 and HeLa cells were treated similar, but using 1µl Jetpei : 1 µg DNA. Transfection media was left on cells overnight, than media was changed and cells used for experiments.

Electroporation

Cell were grown at a density between $2x10^5$/ ml and $3x10^5$/ml. $20x10^6$ cells were taken for electroporation, spun down (1500 rpm; RT) and washed once with PBS with Ca^{2+} and Mg^{2+} (BioChrom, Berlin, Germany), resuspended in 350µl PBS and transferred to a 4mm gap cuvette (BTX Harvard Apparatus, Holliston, MA USA). 30 µg DNA were added, mixed by slightly shaking and pulsed with 230 V/950 µF on a gene Pulser (BioRad, Hercules, CA, USA) with capacitance on high. Particles formed were removed with a sterile pasteur pipette and cells transferred to a cell culture flask containing a 1:1 mixture of old and new cell culture media).

2.7 Stimulation of Cells with TRAIL and CD95

Cells were either stimulated with TRAIL (TEC-375, R&D; 1µg/ml) or CD95 ligand supernatant 1 ng/ml (produced in 293T cells) or anti-CD95 antibody (2R2; 0.5 µg/ml) for 10minutes up to two hours as indicated.

2.8 Flow Cytometry

PI Uptake Assay (Nicoletti Assay)

To analyze the amount of dead cells a Nicoletti assay was performed [112]. During a Nicoletti assay cells are stained with propidium iodide (PI). PI can't cross the membrane, therefore a hypotonic buffer is used to lyse the cell membrane. This way apoptotic cells can be measured as sub G1 peak by flow

cytometry. Cells were harvested, spun down and incubated for 2 hours in Nicoletti buffer (0.1% v/v Trition X 100; 0.1% Trisodium citrate; ddH_2O). Then cells were analyzed by flow cytometry using a FACScalibur (BD). In case of adherent cells supernatant was collected, cells washed with PBS and trypsinized, than treated like adherent cells described above.

3. Bimolecular Fluorescence Complementation Analyses as a Tool to Study Caspase Dimerization

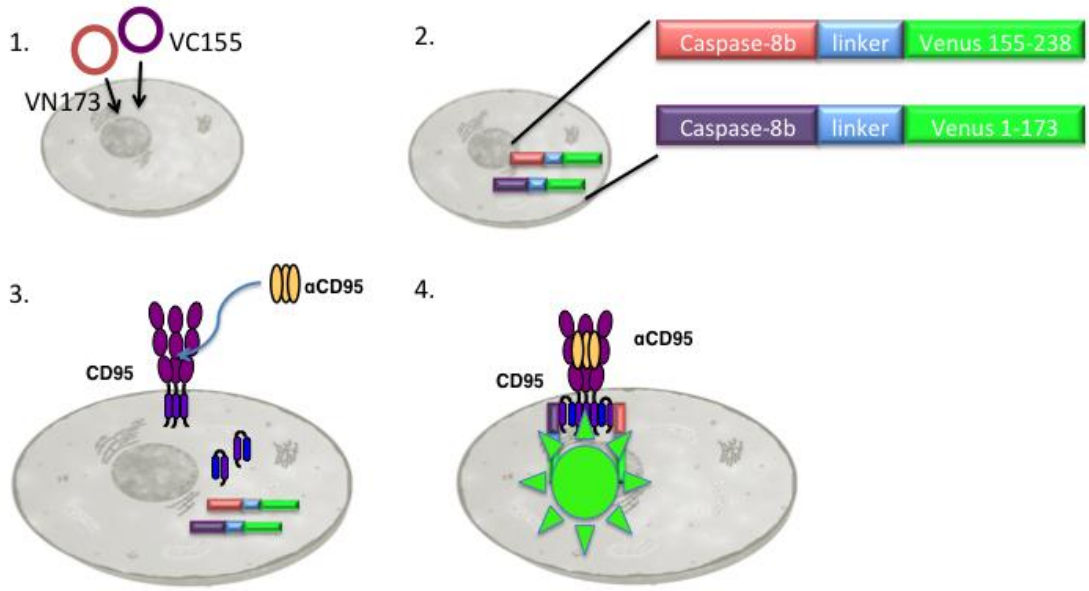

Figure 10. Schematic Overview of BiFC Analyses

1. Cells are transfected with BiFC plasmids. 2. The plasmids code for the protein of interest coupled to a fluorescent fragment. 3. If a death receptor is stimulated by its ligand, e.g. CD95 ligand, the Death Inducing Signaling Complex (DISC) is formed. If both proteins of interest are recruited and dimerize at the DISC, the fluorescent fragments are brought into close proximity resulting in formation of the intact fluorescent molecule, which than can be detected via confocal microscopy or FACS analyses.

Caspases are assumed to form homodimers and heterodimers with other caspases and regulatory molecules like c-FLIP proteins. Most techniques like conventional antibody staining do not allow visualization of homodimer formation. During BiFC the two proteins of interest are each labeled with a

fluorescent fragment, but not the complete fluorescent protein [113]. The fragments alone cannot be excited. If the proteins tagged with the fragments interact the fluorescent fragments are brought into close proximity and reunite to form the fluorescent molecule [113]. The fluorescent signal can be detected via confocal microscopy in localization studies or quantified by flowcytometry. Bouchier-Hayes and colleagues used this approach to show real time formation of caspase-2 homodimers [106].

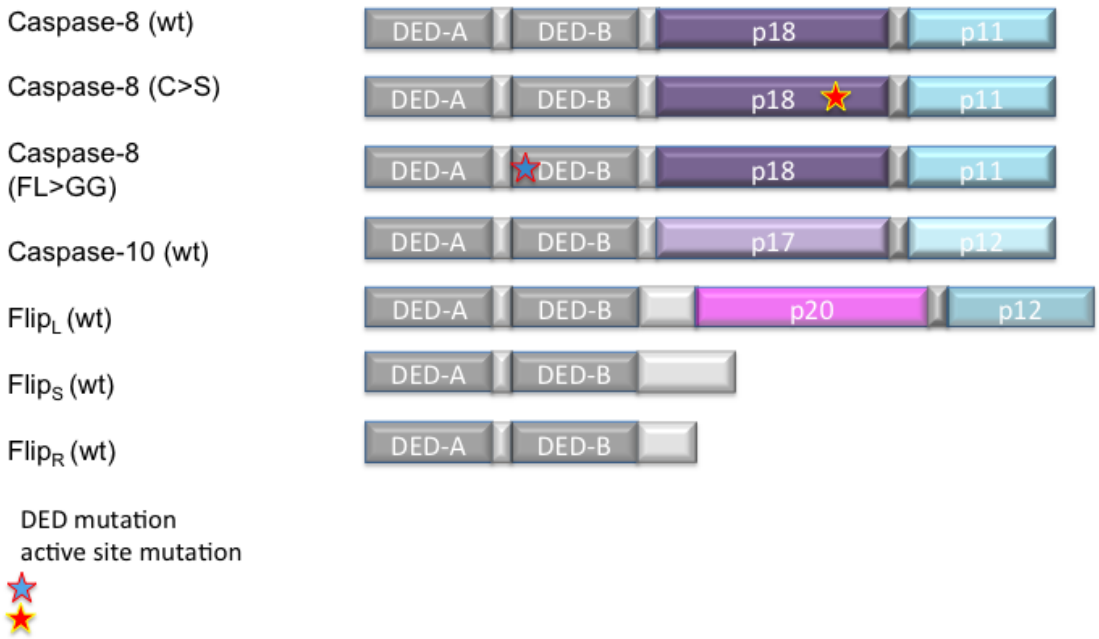

Figure 11. BiFC Constructs
Overview of BiFC constructs used to analyze interactions between c-Flip molecules and caspases. Caspase-8 and -10 are mutated in the active site cysteine (C>S) to prevent toxicity for the cells. Additionally constructs of c-Flip, caspase-8 and -10 with a mutated hydrophobic patch, which prevents DISC recruitment, are used as negative controls. These constructs are brought into BiFC vectors via PCR cloning.

4. Confocal Microscopy

Cells were grown untransfected or after transfection on poly-L-lysine coated coverslips, stimulated or left untreated, than fixed using 2% formaldehyde. If

using mitotracker or lysotracker cells were incubated before for 20 min in lyso- or mitotracker containing cell culture media. After fixation slides were washed with PBS and stained with DAPI (20 µM; 20 min; RT). If antibody stainings were done cells were treated with saponin containing blocking buffer (1% BSA, 0.1% Saponin, 0.02% sodium azide in PBS), incubated with primary antibody overnight in a damp, dark chamber (4°C) washed 3 times and incubated with secondary antibodies (1:1000) for 2 hours at RT. Cover slips were put with Dako (Glostrup, Denmark) Mounting Medium onto slides and after drying for 2 hours borders were closed with nail polish. Samples were analyzed using a Leica TCS SP5 confocal microscope (Leica Company, Wetzlar, Germany) and Leica LAS AF software for acquiring (Leica Company, Wetzlar, Germany). Samples were analyzed using Volocity 3D Image Analysis software (PerkinElmer, Waltham, MA, USA).

5. Mouse Strains

vavFLIP$_R$ Mice

vavFLIP$_R$ mice express the c-FLIP$_R$ transgene under the control of the vav Promoter (see figure 12), which leads to expression in all cells of hematopoietic origin. The transgenic construct consists of three fragments of the vav promotor region surrounding the murine c-FLIP$_R$ cDNA attached to a SV40 polyadenylation sequence. The transgenic vector has been described by Ogilvy, *et al.* [114].

6. Mouse Infection Experiments

L. monocytogenes (EGD wildtype) were grown in brain heart infusion medium (BHI) and mice were infected intravenously via the tail vein with $5x10^3$ colony forming units (CFUs). Mice were sacrificed on day 5 past infection. Liver and spleen were separated into three parts and used for histology, CFU coun and FACS analyses. One third of the spleen and liver was weighed and homogenized in PBS containing 0.2% NP40. $1x10^{-2}$ to $1x10^{-5}$ dilutions were

plated onto BHI agar plates. Colonies were counted 24 hours after plating and CFU/g were calculated.

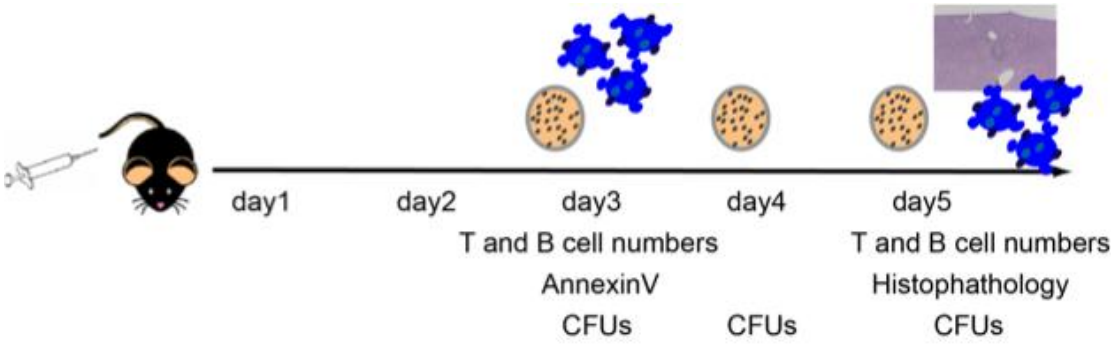

Figure 12. Analyses of vavFLIP$_R$ Mice after *L. monocytogenes* Infection
Mice were injected intravenously with *L. monocytogenes*. Mice were sacrificed on day 3, 4 and 5. Colony forming units were analyzed on day 3, 4 and 5. On day 3 apoptosis was measured using AnnexinV and 7AAD. On day 5 T and B cell numbers were analyzed and spleen and liver used for histopathology.

6. Protein Biochemistry

6.1 Cell Lyses

$1x10^6$ cells were lysed in TPNE Buffer (300mM NaCl; 1% v/v TritonX100, 2 mM EDTA in PBS (pH 7.4)) 100 x Protease Inhibitors (100µg/ml apopeptin; 100 µg/ml leupeptin; 100 µg/ml chymostatin; 100µg/ml pepstatinA) and Na_3VO_4 (1mM) for 15 min at 4°C. Then lysates were centrifuged (15 min, 4° C; 1400 rpmi) and the supernatant was used for further experiments. The pellet was discarded.

6.2 Determination of Protein Content

The protein concentration of lysates was determined using a bicinchoninic acid (BCA) protein assay (Pierce – Thermo Fisher Scientific). This assay is based

on two reactions. First peptide bonds reduce Cu^{2+} to Cu^{+}. This process is proportional to the amount of protein in the sample. In the second step two bicinochoninic acid chelate with each Cu^{+}, which results in an at 562nm wavelength light absorbing product. The absorption was measured using a TECAN infinite 200M (Männedorf, Switzerland) plate reader.

6.3 SDS PAGE

After determination of protein concentration via BCA assay the lysate was mixed with 5x Laemmli buffer (10% w/v SDS, 24% v/v β-mercaptoehtanol, 50% v/v glycerol, 0.25 mg/ml bromphenolblue) to a final 1 x concentration and incubated at 95°C for 3 min. 20 µg of protein lysate was separated in a 12% polyacrylamid gel in 1x running buffer using a Biorad tetra cell. Proteins were run in the upper gel at 80 V and separated on the lower gel at 160 V.

6.4 Western blot

After SDS separation proteins were transferred to a nitrocellulose membrane (GE healthcare, Chalfont St Giles, GB) using a Biorad Mini Tetra Cell blotter (100 V; 60min) in 1 x transfer buffer (25mM Tris pH 8.0; 192 mM glycerol; 20% v/v methanol). After transfer the membrane was incubated in blocking buffer (5% non-fat dry milk; PBS; 0,2% Tween) agitating for one hour followed by an overnight incubation with an primary antibody in blocking buffer overnight. Before incubation in secondary antibody (in wash buffer; 2 hours) unbound protein was removed by washing the Blot three times (0.05% v/v Tween; PBS). Afterwards it was washed again three times. Detection was done using ECL (SuperSignal West Dura substrate, Pierce, Thermo Scientific).

antibody	Species reactivity	clone	supplier
caspase-3	hu	CPP32	R&D Systems
caspase-8	hu	12F5	Biocheck
caspase-10	hu	4C1	MBL
FADD	hu; mu	IF1	Biomol
c-FLIP	hu	Dave-2	Enzo
c-FLIP	hu	NF6	Alexis
CD95	hu	C-20	Santa Cruz
Flag		M2	Sigma Aldrich
HA		12CA5	Roche
RIP	Hu ms dog rt ch	38	BD
β-actin	Hu ms dog rt ch	AC-74	Sigma-Aldrich

Table 5 Primary Antibodies Used for Western Blot Analyses

reactivity	origin	clone/notation	supplier
Mouse IgG	goat	Sc-2055	Santa Cruz
Mouse IgG1	goat	1070-09	Southern Biotech
Mouse IgG2a	goat	1080-05	Southern Biotech
Mouse IgG2b	goat	1090-05	Southern Biotech
Goat	donkey	Sc-2056	Santa Cruz
Rabbit	goat	5030-05	Southern Biotech
Rat	goat	Sc-2065	Santa Cruz

Table 6. Secondary Antibodies (horseradish peroxidase conjugated)

6.5 DISC Immunoprecipitation

$2x10^7$ caspase-8-deficient Jurkat cells were spun down (1500 rpm; RT), washed with PBS and suspended in 5 ml CD95L supernatant (1ng/ml) for 30 minutes, then washed twice with PBS and pelleted. Pellets were lysed in 1ml DISC lysis buffer (15min; 4°C), centrifuged and supernatant was used for immunoprecipitation. To exclude unspecific binding the supernatant was first incubated with uncoupled sepharose beads, than with anti-FLAG agarose (Sigma-Aldrich, Saint Louis, MO, USA) overnight. Beads were washed three times with DISC buffer, then boiled at 96°C for 3min with 5xRSB Buffer (see Western blot) and loaded onto a 12% SDS gel (see WB).

7. Molecular Biology

7.1 PCR Cloning

enzyme	manufacturer	BSA added	incubation temperature	Cat No	Recognition Site
BamH I HF	NEB		37°C	R3136S	5'…GvGATCC…3' 3'…CCTAG∧G…5'
EcoR I HF	NEB		37°C	R3101S	5'…GvAATTC…3' 3'…CTTAA∧G…5'
Hind III	NEB		37°C	R0104S	5'…AvAGCTT…3' 3'…TTCGA∧A…5'
Kpn I	NEB	x	37°C	R0142S	5'…GGTACvC…3' 3'…C∧CATGG…5'
Not I HF	NEB	x	37°C	R3189S	5'…GCvGGCCGC…3' 3'…CGCCGG∧CG…5'
Sal I HF	NEB		37°C	R3138S	5'…GvTCGAC…3' 3'…CAGCT∧G…5'

Xba I	NEB	x	37°C	R0145S	5'…TvCTAGA…3' 3'…AGATCʌT…5'
Xho I	NEB	x	37°C	R0146S	5'…CvTCGAG…3' 3'…GAGCTʌC…5'

Table 2. Restriction Enzymes
Restriction enzymes, conditions and recognition sites used for PCR cloning of caspase-8, caspase-10 and c-FLIP proteins into BiFC vectors (all enzymes were purchased at New England Biolabs, Ipswich, MA, USA)

Constructs for BiFC experiments were generated using PCR cloning. BiFC vectors and control vectors were obtained from Addgene (Cambridge, MA, USA). PCR primers with restriction sites were designed using Serial Cloner Software. As templates vectors coding for the protein of interest were used. Caspase-10a was a kind gift of Ute Fischer (Heinrich Heine Universität, Düsseldorf, Germany), caspase-8b from Frank Kischkel (TherapySelect, Heidelberg, Germany) and c-FLIP from Frida Ewald (HZI, Braunschweig, Germany). Inserts were generated via PCR with PhusionFlash polymerase (New England Biolabs, Ipswich, MA, USA). The final BiFC vectors (VN173; VC155) and PCR products were restricted generating complementary ends. PCR products were ligated into DNA vectors using T4 Ligase (NEB; Ipswich, MA, USA) for 10min at RT.

7.2 Plasmid Amplification

Bacteria from glycerol stocks or transformed bacteria were plated on agarose plates containing lysogeny broth (LB) media (1% NaCl; 1% bacto trypton/pepton; 0.5 % yeast extract, pH 7.0; 50 µg/ml kanamycin or 100 µg/ml ampicillin) and incubated at 37°C overnight. Single colonies were picked and grown in 2 ml liquid LB media. DNA was isolated from competent bacteria using Zyppy Plasmid Mini Kit (Zymo Research, Irvine, CA, USA) and in case

of new constructs sequenced at MWG Operon Eurofins (Ebersberg, Germany) and sequences were analyzed using Geneious software (Auckland, New Zealand). For amplifying bacteria containing constructs were grown in 300ml LB medium containing ampicillin or kanamycin (as described above) and endotoxin free plasmids were isolated using Nucleobond Xtra Maxi Plus EF (Macherey-Nagel, Düren, Germany) according to the manufacturers instructions.

Name	Sequence
BiFC C8 155 (fwd)	aag cat GTC GAC gga gAT GGA CTT CAG CAG AAA TCTT
BiFC C8 155 (rev)	cga agt gGG TAC Cga tAT CAG AAG GGA AGA CAA GTTT
BiFC C8 173 (fwd)	aag cat GCG GCC GCg gag ATG GAC TTC AGC AGA AAT CTT
BiFC C8 VN173 (rev)	cga agt gGT CGA Cga tAT CAG AAG GGA AGA CAA GTT T
BiFC C10 155 (fwd)	tag gaa ttc cta caA TGA AAT CTC AAG GTC AAC A
BiFC C10 155 (rev)	tag cct cga gta tcT GAA AGT GCA TCC AGG GGC AC
BiFC C10 173 (fwd)	cta cga att cta gcg agA TGA AAT CTC AAG GTC AAC A
BiFC C10 173 (rev)	acg tgt cga cta tTG AAA GTG CAT CCA GGG GCA C
BiFC_FLIP$_L$ 155 (fwd)	tac gca tgt cga cta tgt ctg ctg aag tca tcc a
BiFC FLIP$_L$ 155 (rev)	ctc att agg tac ctg tgt agg aga gga taa gt
BiFC FLIP$_L$ 173 (fwd)	gcg gcc gca ATG TCT GCT GAA GTC ATC CA
BiFC FLIP$_L$ 173 (rev)	cga atg tct aga TGT GTA GGA GAG GAT AAG TT
BiFC FLIP$_R$ 155 (fwd)	gta cga att ccg tcg ATG TCT GCT GAA GTC ATC CA
BiFC FLIP$_R$ 173 (rev)	tgc agg tac cct aTG CTG GGA TTC CAT ATG TT

Table 3. PCR Cloning Primer

PCR Primers used for PCR cloning of genes coding for Proteins of interest into BiFC vectors (vectors and genes as indicated in primer names).

7.3 PCR Mutagenesis

DEDs and active sites were mutated by quikchange site directed mutagenesis (Agilent Technologies; Loveland; CO). Within the DEDs the hydrophobic patch was mutated (FL>GG), which is known to prevent receptor recruitment. In the active center the cysteine was mutated into an serine, resulting in loss of enzymatical activity. This kit uses a *Pfu*Ultra high-fidelity DNA polymerase and two complementary mutagenesis primer containing the desired mutation. Plasmids were amplified via PCR followed by digestion of the parental DNA by DpnI. After digestion plasmids were transformed into competent bacteria (see below). Additionally some constructs were mutated using Phusion HF polymerase (Thermo Scientific, Vantaa, Finland) instead. After PCR mutagenesis samples were treated as described above.

Mutation	Mutagenese Primer
Casp8M1f	CA GAA TTG AGG TCT TTT AAG GGC CTT TTG CAA GAG GAA ATC TCC
Casp8M1rev	GGA GAT TTC CTC TTG CAA AAG GCC CTT AAA AGA CCT CAA TTC TG
Casp8M2fwd	G AGG TCT TTT AAG GGT GGC TTG CAA GAG GAA ATC TC
Casp8M2rev	GA GAT TTC CTC TTG CAA GCC ACC CTT AAA AGA CCT C

Table. 4 PCR Primers Used for Mutagenesis

8. Statistical Analysis

Statistical analyses were performed by non-parametric Mann-Whitney *U*-test using GraphPad Prism software (Graph-Pad-Software, La Jolla, CA, USA). Data are presented as the mean with standard error of the mean (S.E.M.) and standard deviation (S.D.) used as error bars.

IV. Results

1. Generation of vavFLIP$_R$ Mice

c-FLIP$_R$ is the solely short c-FLIP isoform expressed in mice [77]. Transgenic mice were generated to analyze if c-FLIP$_R$ functions as murine counterpart of the human short isoform c-FLIP$_S$. vavFLIP$_R$ mice express c-FLIP$_R$ under the vav promoter that directs expression to the hematopoietic lineage [114]; Fig 12.A). The transgene is present in vavFLIP$_R$ mice as shown via genomic PCR (Fig 12.B). On protein level c-FLIP$_R$ is expressed in vavFLIP$_R$ mice in cells within the thymus and spleen (Fig. 12.C).

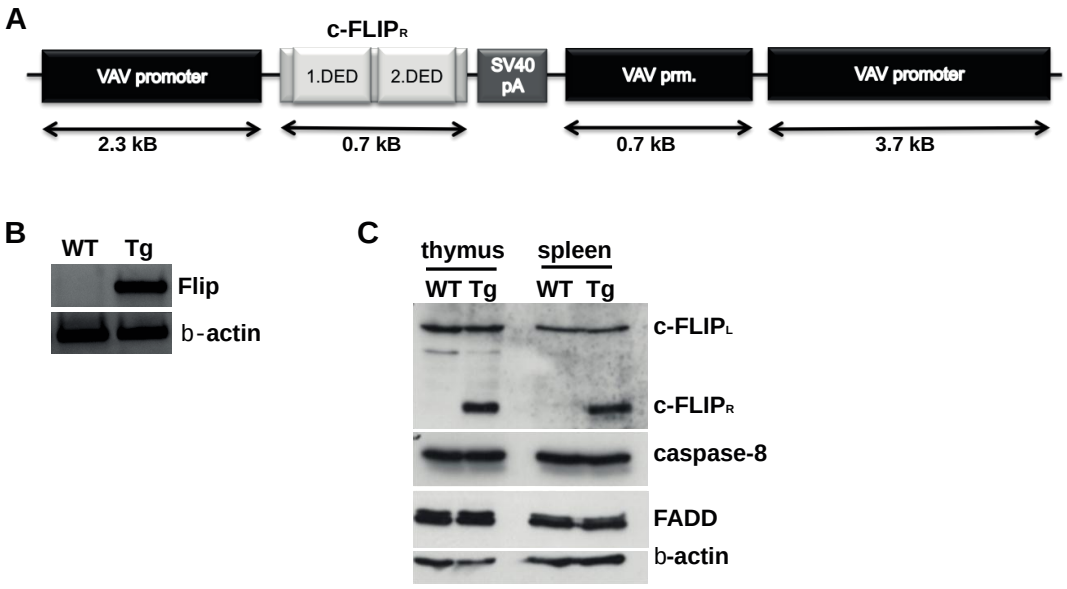

Figure 12. (A) vavFLIP$_R$ Transgenic Mice The transgenic construct consists of three fragments of the vav promoter region surrounding the murine c-FLIP$_R$ cDNA attached to a SV40 polyadenylation sequence. The transgenic vector has been described by [114]. **(B)** Expression of the transgene was analyzed by genotyping with primers specific for c-FLIP (forward primer) and SV40 polyA (reverse primer) **(C)** Expression of the transgene on protein level in lysates from spleen and thymus of vavFLIP$_R$ mice and wildtype littermates. Detection of c-FLIP, caspase-8 and FADD analyzed by Western blot and β-actin as loading control.

2. c-FLIP$_R$ Overexpression Does not Alter Thymic T Cell Development

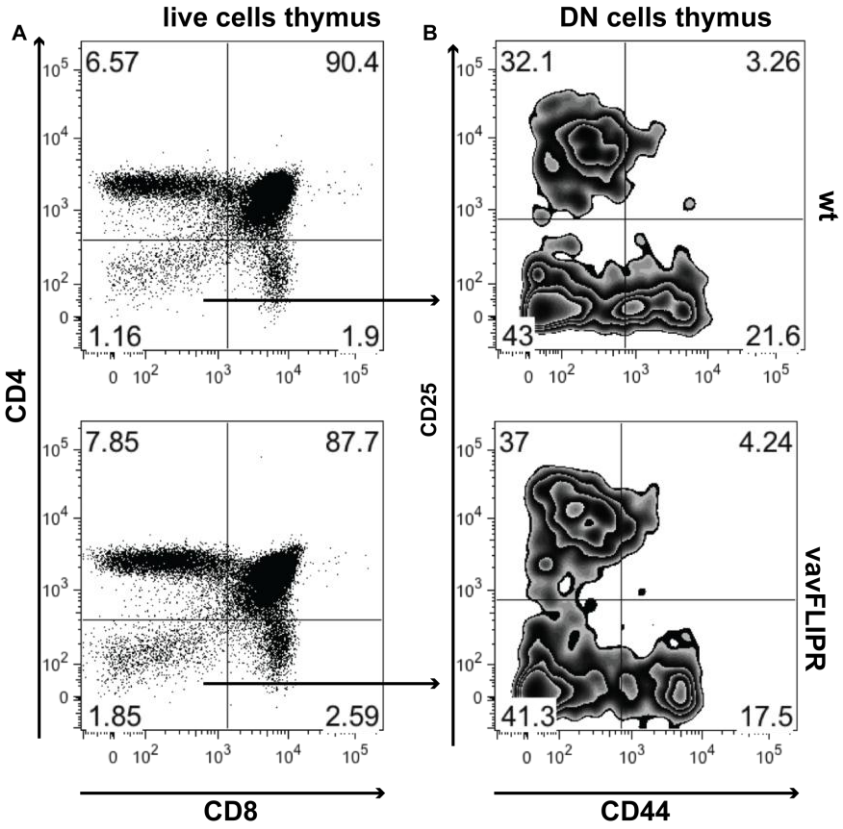

Figure 13. Thymic CD4 to CD8 Ratio and CD25$^+$ and CD44$^+$ cells Representative dot blots for Thymic cellularity in 7-week-old wildtype (WT; n=6; upper panel) and vavFLIP$_R$ mice (lower panel; n=6). **(A)** Comparison of CD4$^+$ single positive, CD4$^+$CD8$^+$ double positive, CD8$^+$ single positive and CD4$^-$CD8$^-$ double negative cells. **(B)** Analyses of CD25$^+$ and CD44$^+$ ratio amongst double negative cells.

Inhibition of CD95 signaling is often associated with abnormal lymphocyte numbers or development. Therefore, lymphocytes from the thymus of 7 weeks old vavFLIP$_R$ mice (n=6) and WT littermates (n=6) were analyzed. T cell developmental status in the thymus was determined by CD4$^+$ and CD8$^+$ expression. The ratio of single positive CD4$^+$ and CD8$^+$ did not differ between wildtype and vavFLIP$_R$ animals (Fig 13.A). Overexpression of the apoptosis regulator Bcl-2 is known to interfere with CD4$^-$CD8$^-$ double negative progenitor cell survival and proliferation [115]. To determine whether the anti-apoptotic c-FLIP$_R$ causes a similar effect double negative cells were further analyzed staining for CD25 and CD44 (Fig 13.B). No differences were detected between

vavFLIP$_R$ and WT animals (Tab.5). Therefore an effect of c-FLIP$_R$ on T cell development in the thymus is unlikely.

3. B and T cell Ratios In the Spleen Are Not Altered in vavFLIP$_R$ Mice

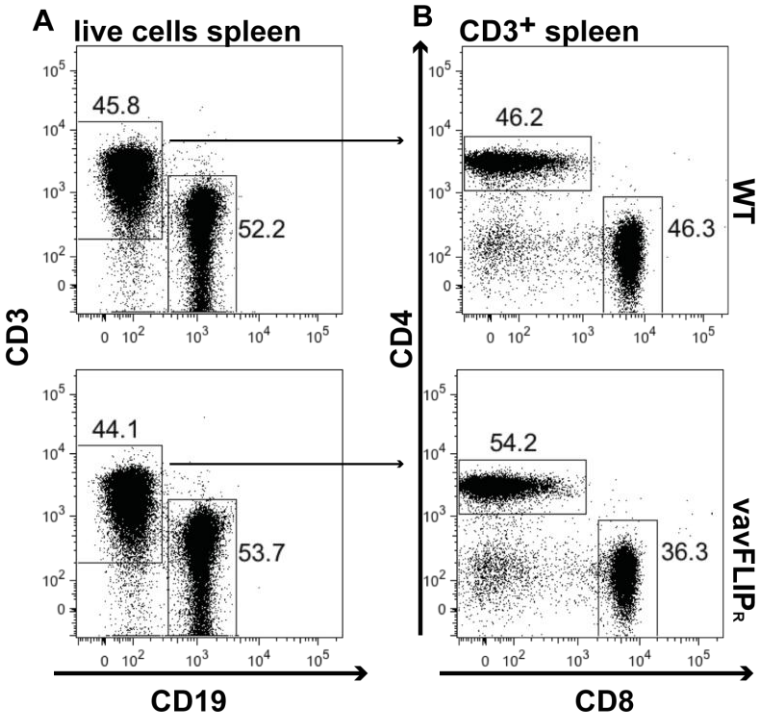

Figure 14. T to B Cell Ratio and CD4 to CD8 Ratio in the Spleen
(A) Exemplary CD3 to CD19 ratios amongst live cells in the spleen and **(B)** CD4 to CD8 ratio within the CD3$^+$ spleen cells of wildtype (upper panel) and vavFLIP$_R$ (lower panel) mice.

Upregulation of the human short isoform c-FLIP$_S$ *in vitro* and its transgenic overexpression in mice *in vivo* lead to a reduction in lymphocyte apoptosis [116, 117]. To analyze if c-FLIP$_R$ interferes with T cell death and survival *in vivo* T cell numbers were analyzed in vavFLIP$_R$ mice and compared to wildtype littermates. The ratio of B to T cells in spleens from 7-week-old-mice did not differ between wildtype and vavFLIP$_R$ mice (Fig.14; Tab.5). T cells were further subdivided into CD4$^+$ and CD8$^+$ cells to see if subpopulations differ. There was

no difference detected regarding the $CD4^+$ to $CD8^+$ ratio between wildtype and $vavFLIP_R$ animals. Even though percentages may vary between individual animals (Fig 14), average percentages do not differ between wildtype and transgenic animals (Tab. 5).

4. B and T Cell Ratios in the Peripheral Lymph Nodes are Not Altered in $vavFLIP_R$ Mice

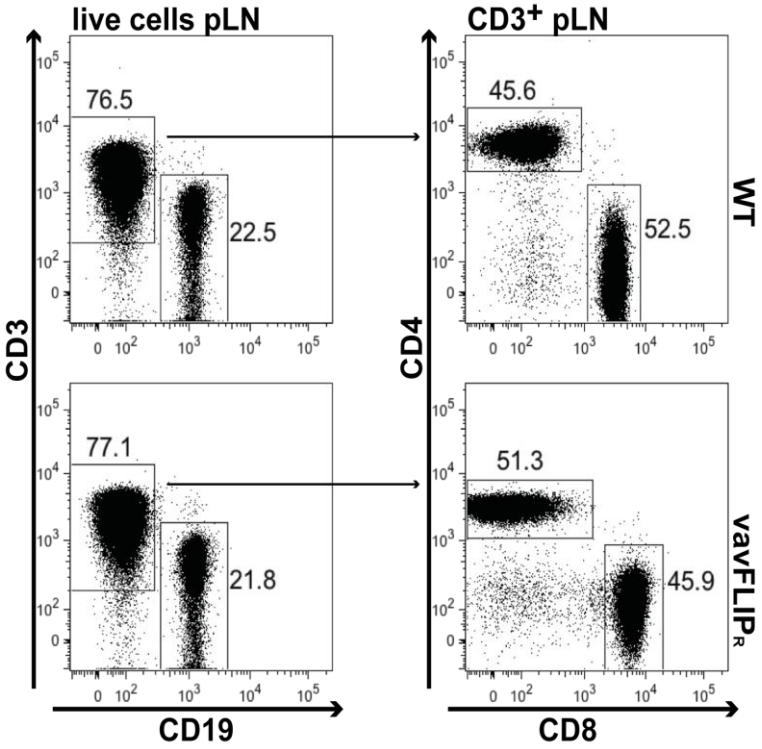

Figure 15. T to B Cell Ratio and CD4 to CD8 Ratio in Peripheral Lymph Nodes (A) CD3 to CD19 ratio amongst live cells in peripheral lymph nodes of wildtype (upper panel) and $vavFLIP_R$ mice (lower panel). **(B)** CD4 to CD8 ratios amongst $CD3^+$ cells in peripheral lymph nodes.

B and T cell ratios in peripheral lymph nodes were analyzed and within the $CD3^+$ cells the $CD4^+$ to $CD8^+$ ratio was determined. There were no differences between the $vavFLIP_R$ mice and their WT littermates regarding percentages or absolute cell numbers (Fig.15; Tab.5). Similar to spleen lymphocyte numbers

peripheral lymphocytes numbers in young mice seem to be unaffected by c-$FLIP_R$ expression.

5. Numbers of Double Negative (DN; $CD3^+CD4^-CD8^-$) Cells are not Enhanced in $vavFLIP_R$ Mice

Mutations in CD95 signaling have been described to lead to autoimmunity in the human autoimmune lymphoproliferative disease (ALPS) and the murine lpr and gld mouse models. These conditions lead to an accumulation of DN cells. Numbers of DN negative cells analyzed in $vavFLIP_R$ mice and wildtype littermates did not differ (Fig. 16) in peripheral lymph nodes (Fig. 16). Numbers of $B220^+$ cells within the DN cells were not elevated in $vavFLIP_R$ mice (Fig. 16).

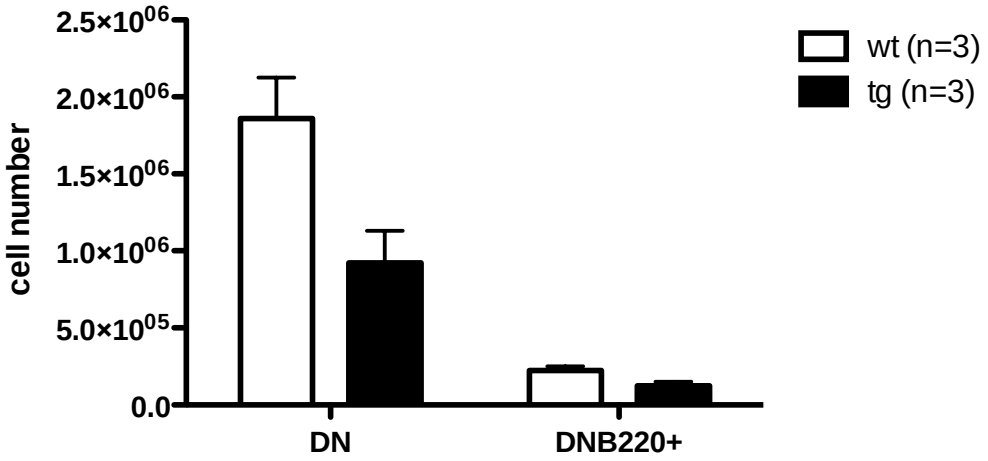

Figure 16. Double Negative (DN; $CD3^+CD4^-CD8^-$) Cells in $vavFLIP_R$ Transgenic Mice (tg) Analyses of absolute cell numbers of double negative (DN; $CD3^+CD4^-CD8^-$) cells and DN $B220^+$ cells in $vavFLIP_R$ mice (tg) and wildtype littermates (wt).

6. Altered B cell Developmental Stages in vavFLIP$_R$ Mice

To further analyze B cell subsets B cells from bone marrow of three-month-old mice (wt n=2; tg n=2) were stained for subpopulations. Regarding the total amount of B220$^+$ cells no difference could be detected between vavFLIP$_R$ mice and wildtype littermates. B cells were further subdivided into Hardy fractions separating them gating B220 against CD43 to identify the fractions D-F (pre B cells, immature B cells and recirculating B cells and A-C (earliest developmental phases; Fig. 17). Analyses of the two main groups (A-C and D-F) did not show differences between vavFLIP$_R$ mice and their wildtype littermates. Interestingly within the fraction D-F a further subdivision showed in the wildtype more cells in fraction F (recirculating B cells) and less in fraction D (pre B cells) than in the vavFLIP$_R$ mice. However, the analyzed animal number is not sufficient enough to conclude a statistical significance.

	percentages of all aquired cells						absolute numbers
	CD3+	CD4+	CD8+	DN	DP	CD19+	1.00E+07
wt thy (n=6)	n.d	5.7±1.5	1.4±0.3	3.4±3.3	67.7±9	n.d.	10.0±4.0
tg thy (n=6)	n.d	5.2±0.6	1.4±0.5	4.4±4.3	68.8±7.9	n.d.	8.0±4.4
wt pLN(n=6)	48.0±5.3	25.6±2.9	19.4±0.5	n.d.	n.d	10.2±1.6	1.8±3.6
tg pLN (n=6)	48.6±5.3	23.3±2.3	19.1±2.4	n.d.	n.d	10.8±1.1	1.4±0.5
wt spleen (n=6)	21.8±6.3	9.2±2.5	7.0±1.5	n.d.	n.d.	21.2±6.4	6.7±2.0
tg spleen (n=6)	22.9±6.6	10.2±2.1	7.4±1.0	n.d.	n.d.	20.6±6.1	6.1.0±1.6

Table 5. **Percentages and Absolute Cell Numbers in Young vavFLIP$_R$ Mice and WT Littermates** DN = double negative (CD4$^-$CD5$^-$); DP = double positive (CD4$^+$CD8$^+$); thy = thymus; pLN = peripheral lymph nodes

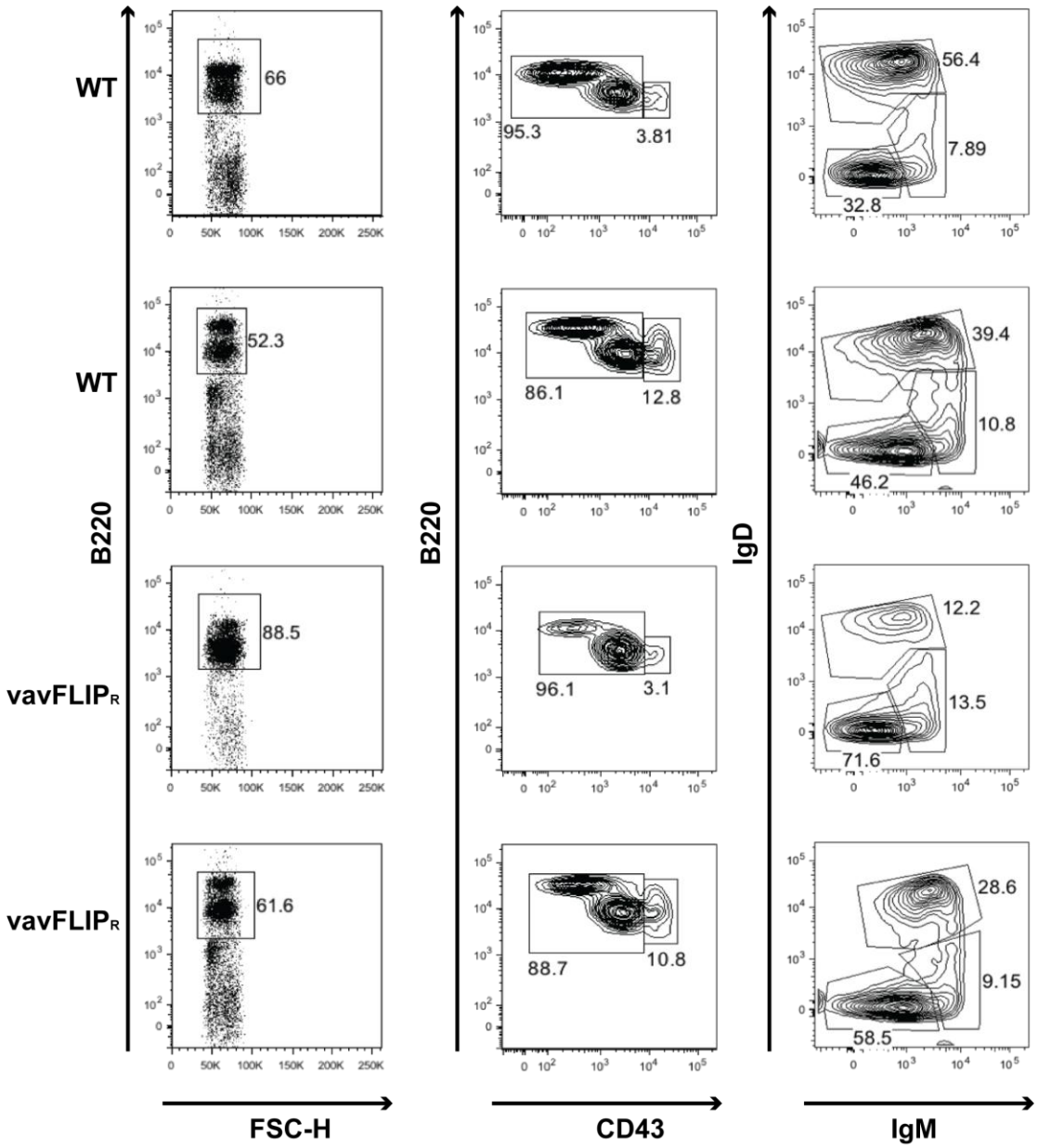

Figure 17. B Cell Subpopulations/ Hardy Fractions
B cell subpopulations in the bone marrow of vavFLIP$_R$ mice (n=2) and wildtype littermates (n=2). Cells were gated on lymphocytes and doublets being excluded. First B220$^+$ cells were analyzed, than subdivided into Hardy fractions D-F and A-C (second panel). The D-F fraction was further subdivided into pre B cells (fraction F).

7. c-FLIP$_R$ Overexpression Does Not Effect Regulatory T Cell Numbers in Peripheral Lymph Nodes or Spleen

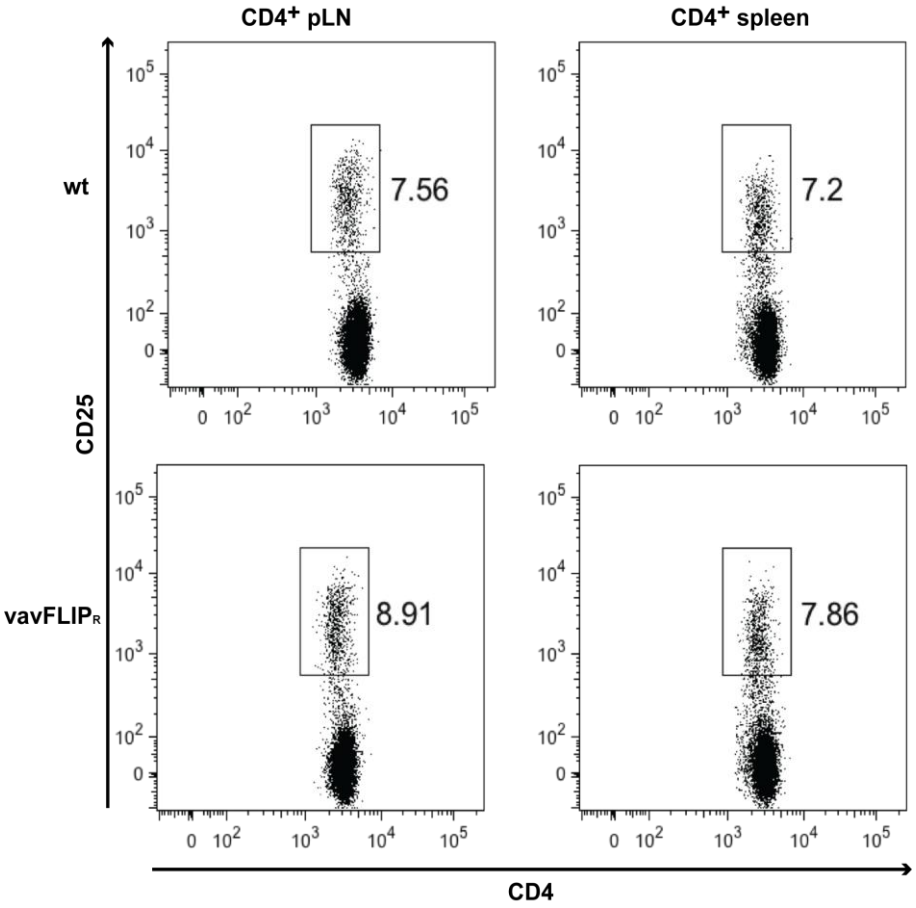

Figure 18. Treg Frequencies in vavFLIP$_R$ Mice and WT Littermates
Treg frequencies amongst CD4$^+$ cells in vavFLIP$_R$ mice and WT littermates in peripheral lymph nodes (right) and spleen (left).

Defects in CD95 signaling are often associated with autoimmunity and deregulation of regulatory T cells (Tregs) as well. To analyze if c-FLIP$_R$ overexpression interferes with Treg frequencies CD4$^+$CD25$^+$ cells were analyzed in peripheral lymph nodes and spleen of vavFLIP$_R$ and wildtype animals. No differences were detected. Therefore, it can be concluded, that c-FLIP$_R$ has no effect on Treg frequencies in young mice (Fig. 18).

8. c-FLIP$_R$ Interferes with T Cell Numbers after *L. Monocytogenes* Infection

L. monocytogenes infection is known to evoke an immune response and induce massive, unspecific T cell apoptosis [101]. To determine if c-FLIP$_R$ overexpression interferes with these processes frequencies of CD4$^+$ and CD8$^+$ cells in vavFLIP$_R$ mice were analyzed via flow cytometry and compared to cells from infected and uninfected wildtype animals. This showed lower T cell numbers in infected vavFLIP$_R$ mice and WT littermates compared to uninfected wildtype animals as expected. Infected vavFLIP$_R$ mice have higher cell numbers compared to infected wildtype animals (Fig.19). Therefore it can be concluded, that c-FLIP$_R$ overexpression modulates cell numbers after *L. monocytogenes* infection.

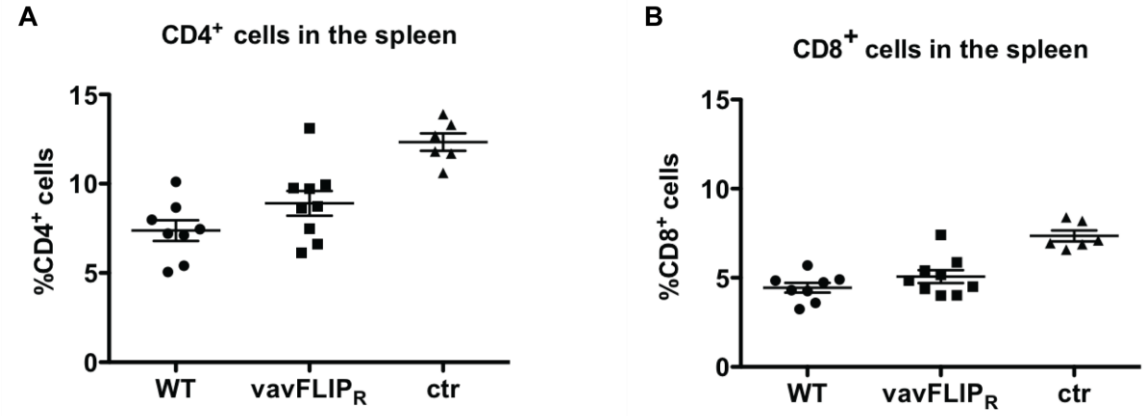

Figure 19. Percentages of CD4$^+$ and CD8$^+$ T Cells after *L. monocytogenes* Infection
Effect of *L. monocytogenes* infection on percentages of CD4$^+$ and CD8$^+$ cells in vavFLIP$_R$ mice, wildtype littermates (WT) and uninfected wildtype animals (ctr).

9. Percentages of B Cells Do Not Differ between *L. monocytogenes* Infected vavFLIP$_R$ Mice and Wildtype Littermates

Even though there was no general difference between B and T cell ratios in young mice, B cell developmental stages were slightly altered. Therefore, B cell numbers in vavFLIP$_R$ mice after *L. monocytogenes* infection were analyzed. There was no significant difference regarding percentages of B cells in vavFLIP$_R$ or infected wildtype animals. *L. monocytogenes* infection does not significantly alter B cell apoptosis and c-FLIP$_R$ overexpression seems to have no effect on this process (Fig. 20).

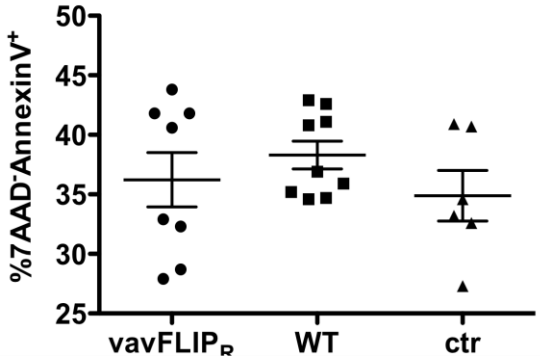

Figure 20. Percentages of apoptotic (7AAD$^-$Annexin$^+$) B cells after *L. monocytogenes* Infection Effect of *L. monocytogenes* infection (5x10^3) on percentages of B cells in the spleen of vavFLIP$_R$ mice (n=8), wildtype littermates (WT; n=9) and uninfected wildtype animals (ctr; n=6).

10. Altered Apoptosis of CD8$^+$ Cells in vavFLIP$_R$ Mice upon *L. Monocytogenes* Infection

Cell numbers were altered in vavFLIP$_R$ mice at day three post-infection, which is most likely due to apoptotic cell death. To analyze the effect of vavFLIP$_R$ on apoptosis after *L. monocytogenes* infection an AnnexinV 7AAD staining was performed. Within the CD4$^+$ T cell compartment no difference regarding the number of apoptotic cells could be detected. In contrast, apoptosis of CD8$^+$ T

cells was increased in infected when compared to non-infected animals. Less apoptosis of $CD8^+$ cells was detected in $vavFLIP_R$ mice compared to WT mice (Fig. 21). It can be concluded, that $c\text{-}FLIP_R$ does interfere with apoptosis of $CD8^+$ cells after *L. monocytogenes* infection.

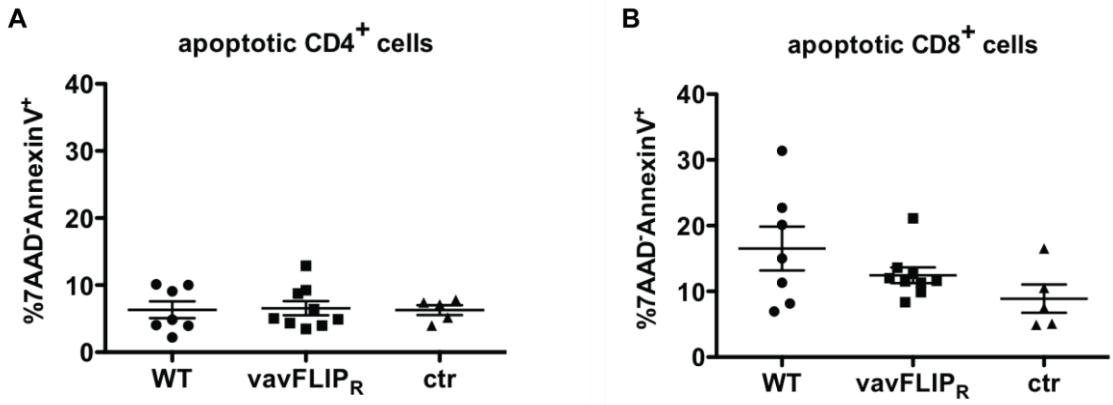

Figure 21. Apoptosis of $CD4^+$ and $CD8^+$ Cells after *L. Monocytogenes* Infection 3-month-old $vavFLIP_R$ mice and wildtype littermates were infected with L. monocytogenes ($5x10^3$). Apoptotic $CD4^+$ and CD8+ cells were stained with AnnexinV and analyzed by flowcytometry. (A) Percentages of apoptotic $CD4^+$ cells and (B) of $CD8^+$ cells.

11. $vavFLIP_R$ Mice Show a Reduced Bacterial Burden after *L. Monocytogenes* Infection

To understand if the bacterial load in $vavFLIP_R$ mice is altered after *L. monocytogenes* infection colony-forming units (CFUs) were determined in liver and spleen of $vavFLIP_R$ mice and wildtype littermates. During *L. monocytogenes* infection bacteria accumulate in spleen and liver and cause massive cell death [101]. CFUs in the organs indicate capability of mice to clear the infection. CFUs were measured on day 3 and 4 past infection. Strikingly $vavFLIP_R$ mice always showed a tendency for better bacterial clearance compared to wildtype littermates (Fig. 22).

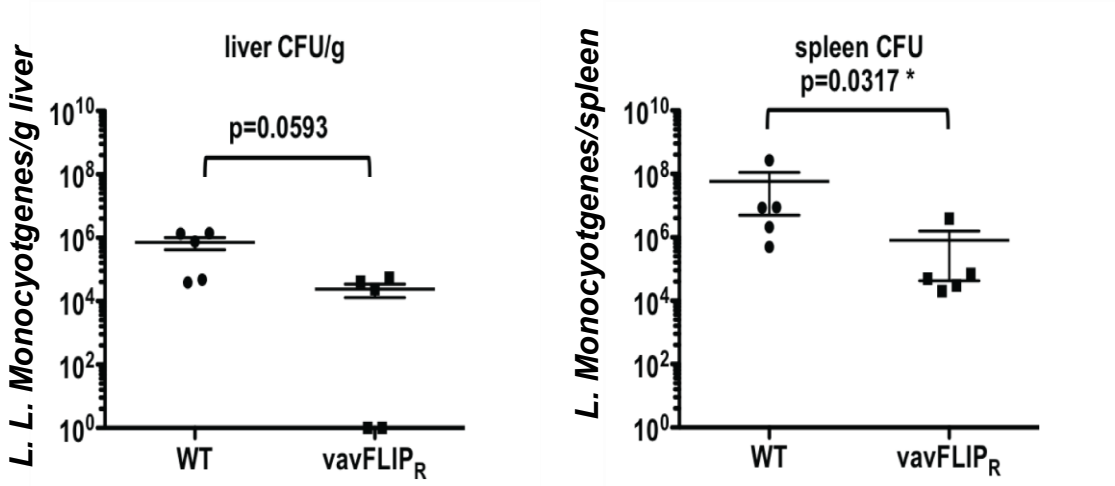

Figure 22. Bacterial Burden of vavFLIP$_R$ Mice after *L. Monocytogenes* Infection

Colony Forming Units at day 3 past *L. monocytogenes* infection. Livers and spleens from infected vavFLIP$_R$ mice and wildtype littermates were meshed and lysed with NP40 containing lyses buffer and plated onto brain heart infusion media agar plates. After 24 hours colonies were counted and CFUs calculated.

12. Reduced Hepatocyte Necrosis After *L. Monocytogenes* Infection in vavFLIP$_R$ mice

Since vavFLIP$_R$ mice show a lower bacterial burden the histology of liver and spleen was analyzed. All liver samples analyzed showed multifocal and randomly distributed necrotic foci with infiltration of predominantly neutrophils with fewer and scattered macrophages and neutrophils (Fig. 23). Strikingly infected vavFLIP$_R$ mice (0.39% SD +/-0.173) show a severely lower number of necrotic foci in the liver compared to infected wildtype animals (6.15% SD +/-4.82).

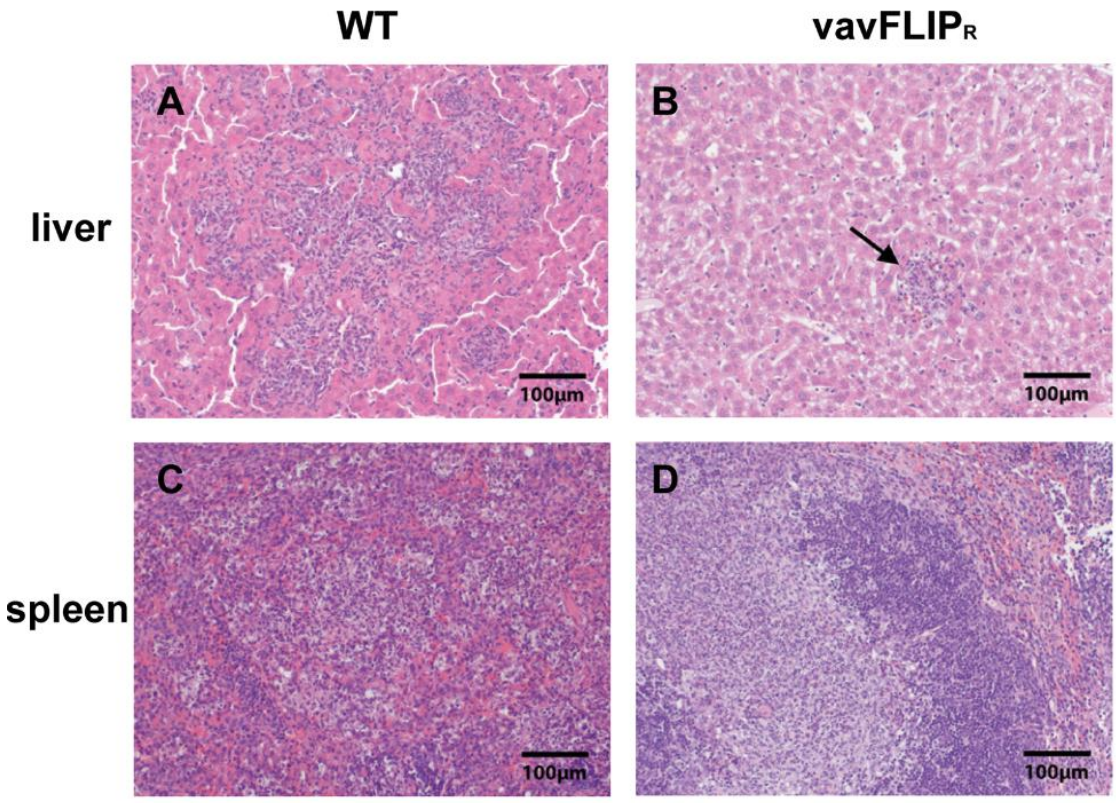

Figure 23. Histological Analyses of Liver and Spleen Tissue after *L. Monocytogenes* Infection. Mice were infected with *L. monocytogenes* and an H&E staining was performed. Livers of wt (A) and vavFLIP$_R$ mice (B) were analyzed for necrotic foci.

13. BiFC Constructs Coding for Caspase-8 are Expressed in 293T Cells in Lower Amounts than Endogenous Caspase-8

vavFLIP$_R$ mice seem to be protected against *L. monocytogenes* infection due to c-FLIP$_R$ overexpression. This effect is probably partly due to different dimers being formed at death receptors. This highlights the importance of dimer formation at the DISC. Nevertheless how this protection is generated remains unclear since the exact interactions of DISC molecules are not known. In previously described analyses of DISC interactions altered recruitment domains were used or experiments were performed *in vitro* with kosmotrophic salts [34, 79, 118]. To gain a better understanding of interactions at the DISC Bimolecular Fluorescence complementation (BiFC) was applied. This method allows visualization of dimer formation in living cells (see material and methods). Constructs for DISC molecules coding for the molecule of interest, a tag and the Venus fragments were generated.

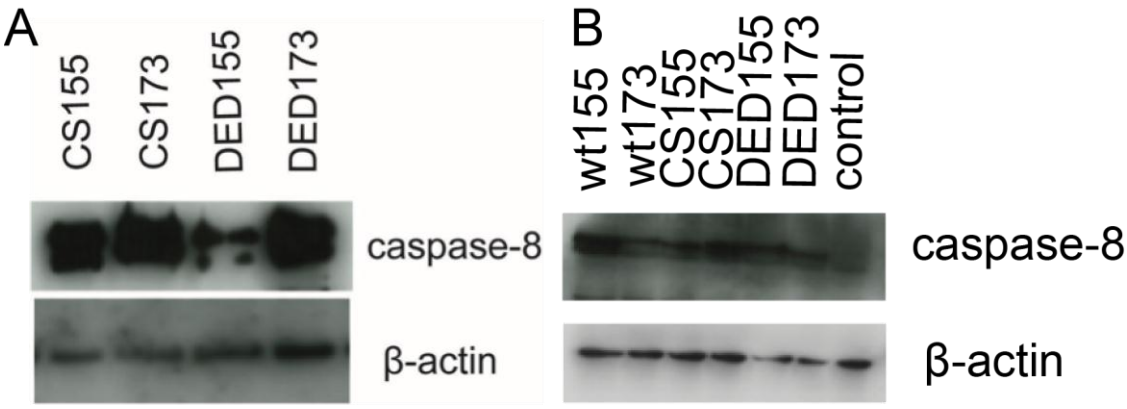

Figure 24. Analyses of expression of BiFC constructs in 293T cells (A) and SH-SY5Y cells (B) Wildtype caspase-8 (wt), active center mutated caspase-8 (C>S) and a DED mutant were expressed in both BiFC constructs (indicated as 155 containing the c-terminal Venus fragment and 173 containing the N-terminal Venus fragment). Caspase-8 expression was analyzed via Western Blot and detected using an anti-caspase-8 antibody (12F5).

14. Death Receptor Expression on SH-SY5Y and Jurkat Cells

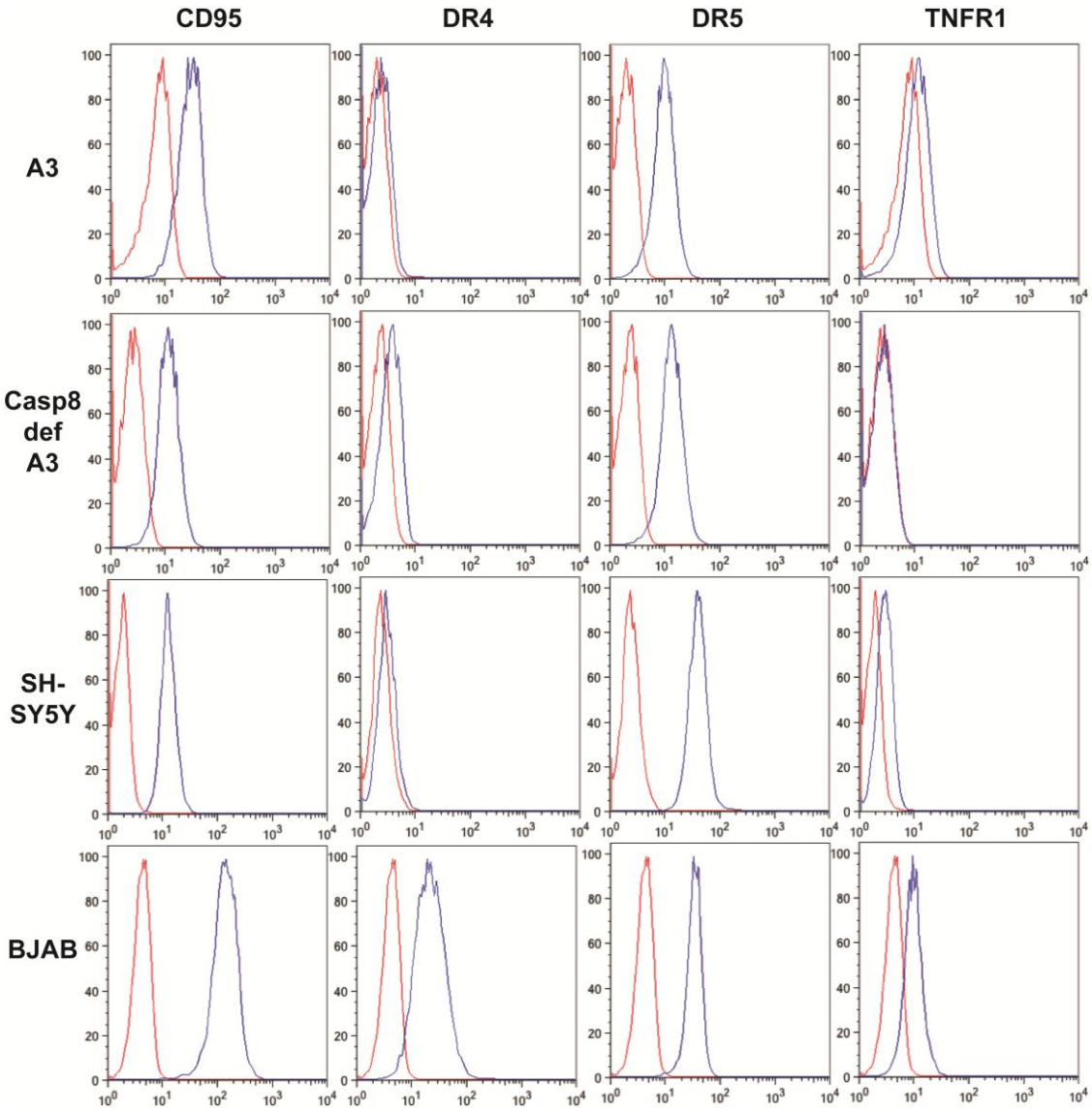

Figure 25. (A) Receptor Expression on A3, caspase-8-deficient Jurkat cells (I9-2**), SH-SY5Y and BJAB cells**
The death receptors CD95, DR4, DR5 and TNFR1 were analyzed by flow cytometry. Histograms for each death receptors are shown for all cell lines used later for analyses.

Expression of constructs was analyzed by Western blot in 293T cells. Since BiFC constructs contain additionally a Venus fragment they differ in size

compared to endogenous expressed caspase-8 (Fig. 24). Therefore endogenous caspase-8 and the BiFC tagged versions can be distinguished based on their molecular weight. Interestingly the constructs showed lower expression levels than endogenous caspase-8. Caspase-8 is expressed endogenously at very high levels in 293T cells (Fig. 24).

To prevent endogenous caspase-8 to interfere with dimer formation the following experiments were performed with cell lines lacking endogenous caspase-8 on the protein level. Caspase-8-deficient A3 cells, in which caspase-8 was knocked out by chemical mutagenesis [39] and SH-SY5Y neuroblastoma cells, in which caspase-8 is epigenetically silenced, were used. Since DISC formation is triggered by stimulation of death receptors [16], presence of such was analyzed. All cells used express CD95 (Fig. 26), therefore further DISC analyzes in these cell lines was carried out using CD95 ligand or anti-CD95 (clone 2R2). Next to CD95 all cells analyzed express high amounts of DR5.

15. Jurkat I9-2 Cells are Resistant Towards CD95-mediated Apoptosis

All analyzed cells express CD95 (Fig. 25) and caspases for apoptosis signaling, apart from the caspase-8-deficient A3 clones and SH-SY5Y, which are caspase-8 knockout cells ([39]; Fig. 27). Susceptibility towards CD95-induced apoptosis of Jurkat clones was analyzed stimulating them with CD95L To determine the amount of death cells a PI uptake assay was performed. As expected caspase-8-deficient Jurkat cells (I9-2) are, in contrast to their parental A3 and the E6.1 Jurkat-clone, not susceptible to apoptosis (Fig. 26) and thereby an excellent tool for reconstitution with caspase-8 containing BiFC constructs.

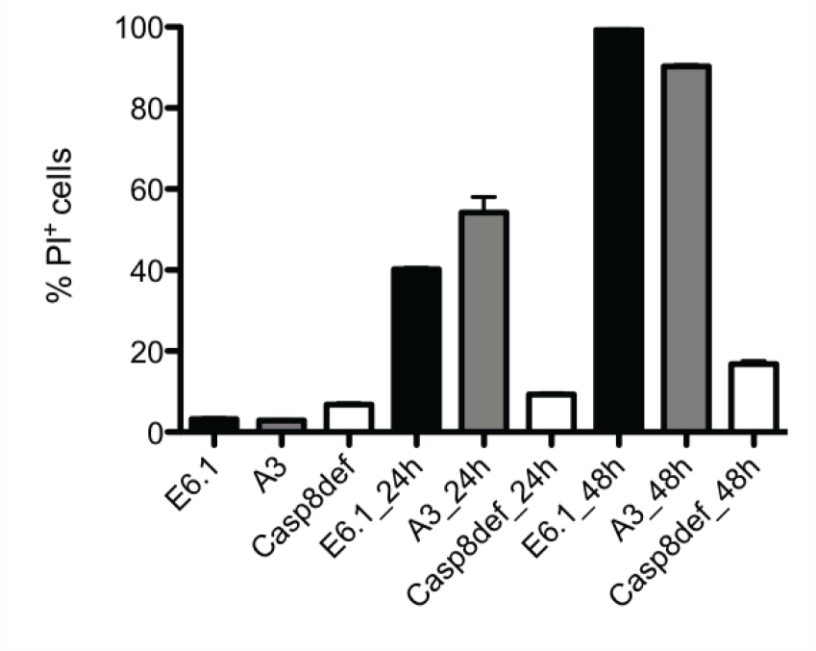

Figure 26. Susceptibility Towards CD95-induced Apoptosis
Different Jurkat clones (E6.1 and A3) and caspase-8-deficient Jurkat cells (I9-2) were analyzed for apoptosis susceptibility using a PI uptake assay modified after Nicoletti. Cells were stimulated with CD95 Ligand (200 ng/ml) for 24 or 48 hours or left untreated (n=4).

16. Expression of Proteins Involved in DR-Mediated Apoptosis

Cells were analyzed by Western blot to investigate if lack of caspase-8 expression interfered with expression of other proteins involved in death receptor-induced signaling. As expected A3 cells express caspase-8, caspase-3 and FADD, which are essential for CD95- or TRAIL-induced apoptosis (Fig. 27). The caspase-8-deficient I9.2 Jurkat clone and SH-SY5Y cells express normal amounts of FADD, caspase-3, but no caspase-8 (Fig. 27). Therefore, I9.2 and SH-SY5Y cells are suitable for BiFC analyses of caspase-8.

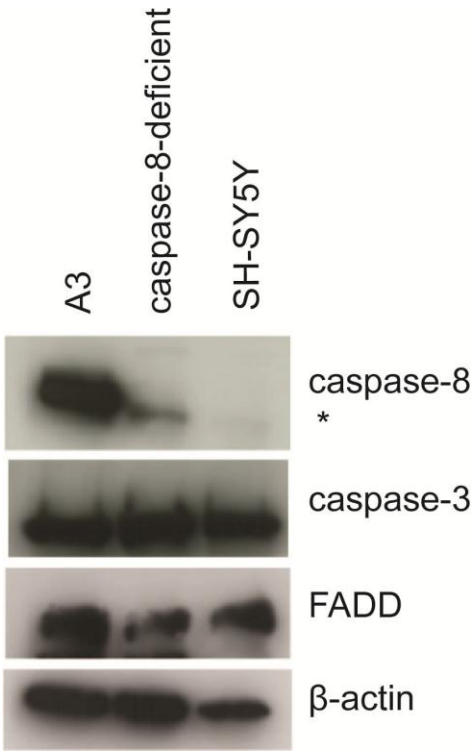

Figure 27. Expression of Apoptosis Proteins
Expression of apoptotic proteins in SH-SY5Y, A3 and caspase-8 deficient Jurkat cells (I9-2). Western Blot Analyses of caspase-8, caspase-3, FADD and β-actin in cell lines used for BiFC analyses.

17. Caspase-8 BiFC Constructs are Recruited to the CD95 Receptor

To further analyze functionality of caspase-8 proteins transcribed from BiFC constructs, they were transfected into caspase-8-deficient Jurkat cells and a DISC immunoprecipitation (DISC IP) was performed (Fig. 28). Upon DISC stimulation FADD is always recruited to the DISC, in the active center mutants even without CD95 ligand treatment weak amounts of FADD are detectable. Caspase-8 is always present at the DISC if cells are transfected with active center mutants or DED mutants. This is surprising because the DED mutations should abolish DISC binding [119]. The controls used, non-BiFC caspase-8 transfected A3 cells and untransfected A3 cells, show weaker recruitment of caspase-8 (Fig 28.; longer exposure time). Taken together BiFC constructs, even with mutated DEDs, are recruited to the DISC.

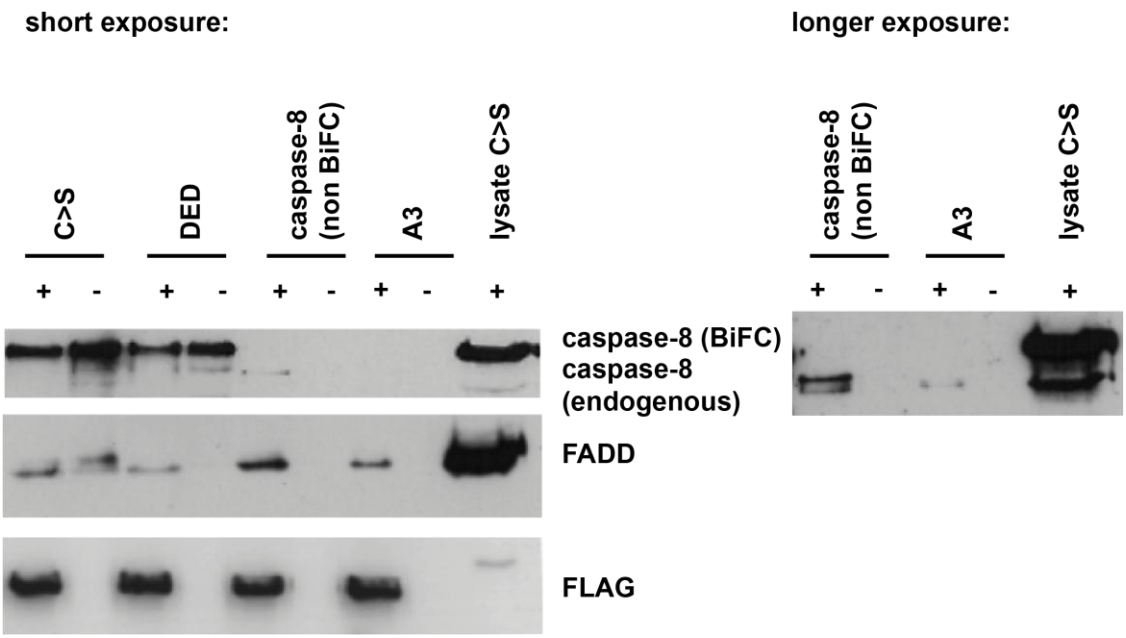

Figure 28. DISC Recruitment of Proteins Transcribed from BiFC Constructs Analyses of recruitment of Venus tagged caspase-8 to the DISC. Cells were transfected with active center mutants (C>S), DED mutants (DED) and a non- BiFC caspase-8 construct. As control A3 cells, which endogenously express caspase-8 were used. DISC proteins were pulled with Flag-tagged CD95 ligand supernatant after 30min incubation (+= incubated with ligand; - control incubated with cell culture medium).

18. Dimerization is Abolished upon Mutation of the DEDs

To further analyze if the observed dimer formation is rather an artifact of overexpression of fluorescent fragments or indeed is based on DISC recruitment/interaction of DISC proteins interaction between DED mutants were analyzed. DED mutations have been described to abolish DISC recruitment [119], but in the IP caspase-8 was detected at the DISC even with mutated DEDs (Fig. 28). Nevertheless dimer formation is abolished upon mutations of caspase-8s DED (Fig. 29). Therefore, dimer formation seems to be caspase-8 specific and not caused by fluorescent fragments.

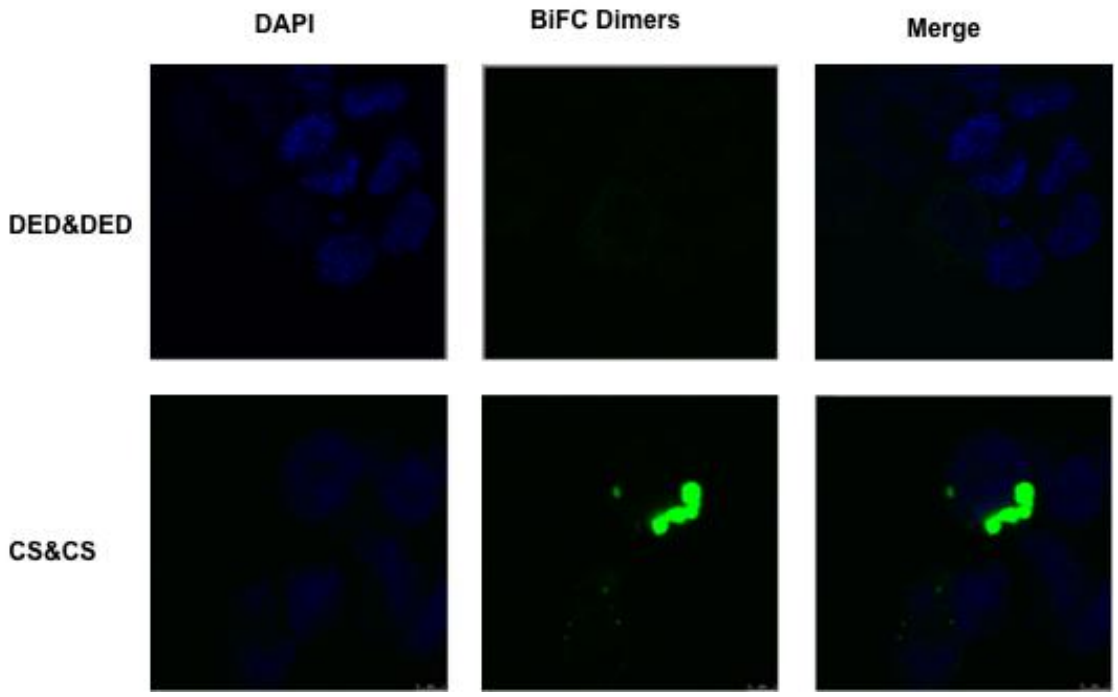

Fig 29. DED Mutations abolish DISC Binding
SH-S5Y5 cells were transfected with BiFC constructs coding for caspase-8 with mutated DEDs (upper panel) and active center (C>S; lower panel). Cells were fixed and treated with DAPI. The green signal shows caspase-8 dimers, red Mitochondria and blue nuclei stained with DAPI.

19. Dimerized Versus Single Caspase-8

Overexpression leads to dimer formation and green fluorescence can be detected using confocal microscopy. Using BiFC, dimerized molecules can be shown, but single caspase-8 molecules are not visualized. To visualize all transfected caspase-8 molecules, cells were transfected with active center mutant BiFC constructs and counterstained with an anti-caspase-8 antibody (12F5; Fig. 30). More single caspase-8 molecules could be detected close to the cell surface, whilst within the cells more dimerized caspase-8 was present (Fig. 30). Addition of CD95 ligand did not alter ratios of single to dimerized caspase-8 or localization of caspase-8.

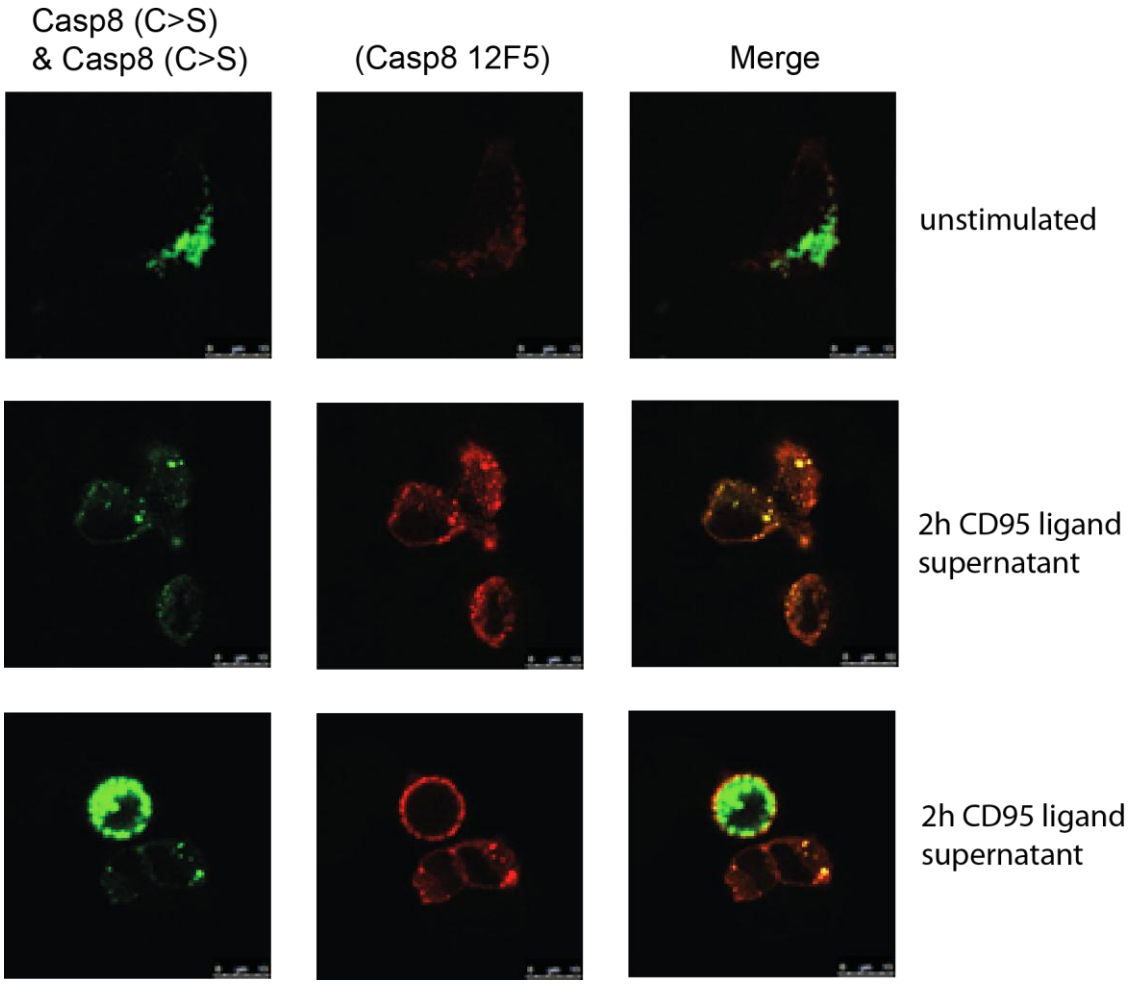

Figure 30. Dimerized Versus Single Caspase-8
Confocal microscope images of SH-SY5Y cells overexpressing mutated active center (C>S) caspase-8 constructs and being stained with an anti-caspase-8 antibody (12F5).

20. Caspase-8 Dimers do Not Colocalize with the Golgi Apparatus

The exact localization of caspase-8 dimer formation is unknown. Cell organelles were stained to see if colocalization with these organelles could be detected. To analyze if caspase-8 dimers colocalize with the Golgi a cotransfection with a Golgi marker (RFP Golgi) was performed. No colocalization between Golgi and caspase-8 dimers could be detected. Therefore, it seems unlikely that caspase-8 homodimers localize at the Golgi apparatus (Fig. 31).

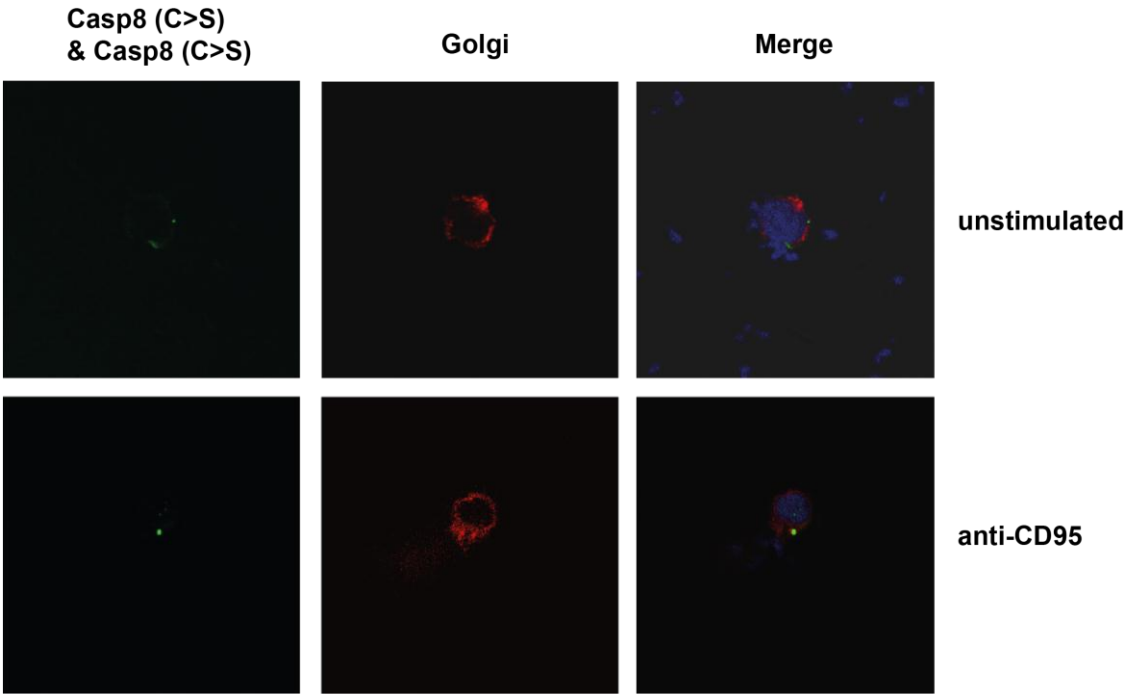

Figure 31. Localization of Caspase-8 Dimers: No Colocalization with Golgi Apparatus SH-SY5Y cells were transfected with active centre mutated (C>S) caspase-8 constructs and additionally a plasmid coding for an Golgi marker in RFP. Cells were analyzed unstimulated and 20min after anti-CD95 treatment (2R2). Cells were fixed and analyzed by confocal microscopy. 18. Dimerization of Caspase-8 with Caspase-10 and c-FLIP proteins

21. Colocalization of Caspase-8 Dimers with Mitochondria

Caspase-8 has been described to localize to the mitochondria [120], but it remains unclear if these are dimers or single caspase-8 molecules. Therefore, colocalization of Mitochondria with BiFC dimers was analyzed. Mitochondria were stained in caspase-8 transfected cells using Mitotracker red. The smaller dot like structures seen do not colocalize with mitochondria, but more vast dimer structures can be seen at the mitochondria (Fig. 32). Cells, where colocalization was detected, had an apoptotic seeming phenotype.

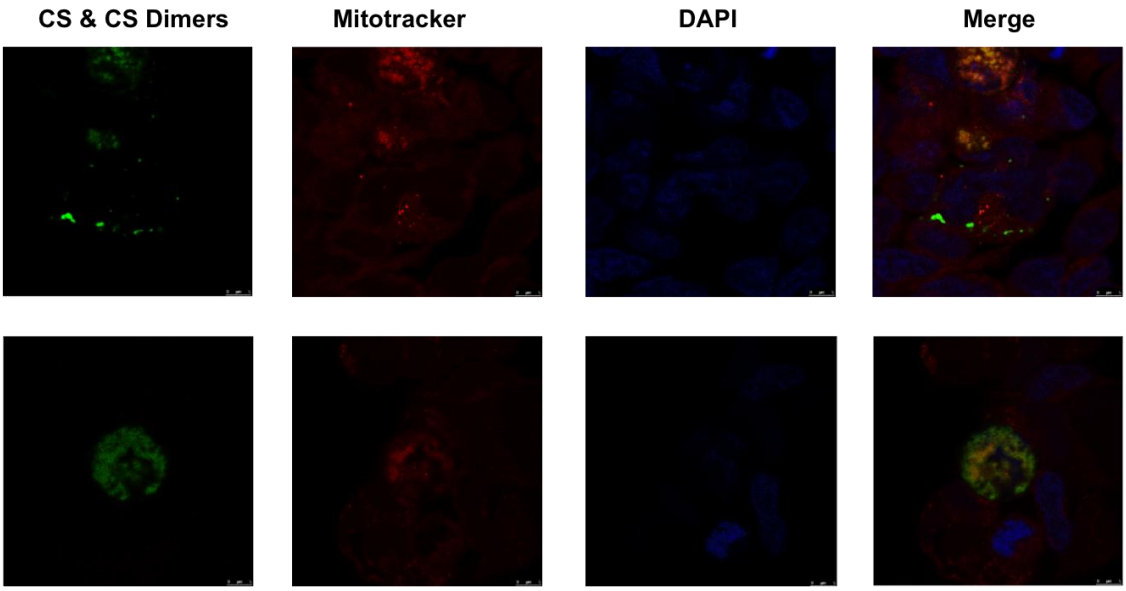

Figure 32. Colocalization of Caspase-8 Dimers with Mitochondria
SH-S5Y5 cells were transfected with BiFC constructs coding for caspase-8 with mutated active center (C>S). Cells were treated with Mitotracker and afterwards fixed and stained for caspase-8 (12F5). The green signal shows caspase-8 dimers, red Mitochondria and blue nuclei stained with DAPI.

22. Dimers Containing c-FLIP and Caspase-10

Caspase-8 is assumed to dimerize with c-FLIP proteins and caspase-10, but this has never been shown in living cells with unaltered recruitment domains. The role of caspase-10 remains enigmatic, therefore dimers containing c-FLIP and caspase-8 were analyzed. Transfection with BiFC constructs for these proteins showed Caspase-8 seems to form heterodimers as well with c-FLIP and caspase-10 (Fig. 33 B; Fig 34). Caspase-10 does as well form caspase-10-caspase-10 homodimers (Fig. 33 A).

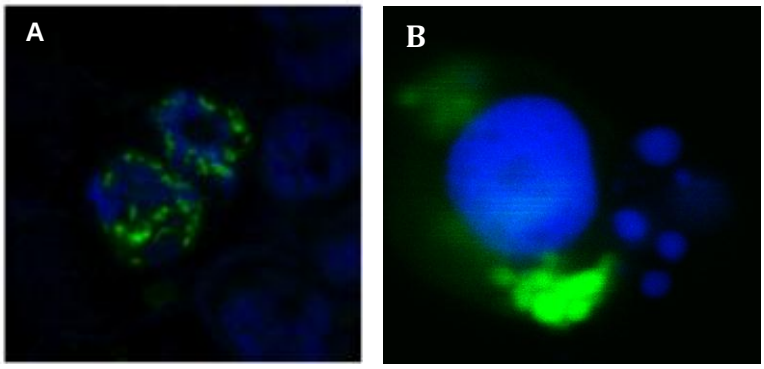

Fig. 33 Hetero and Homodimer Formation of caspase-10 (A) Expression of caspase-10/a and -10/a in HeLa cells. Caspase-10/a dimers are shown in green, nuclei in blue. **(B)** Overexpression of caspase-10/a and caspase-8/b in Jurkat (Caspase-8 deficient A3 I9.1) cells. Caspase-10/a dimers are shown in green, nuclei in blue.

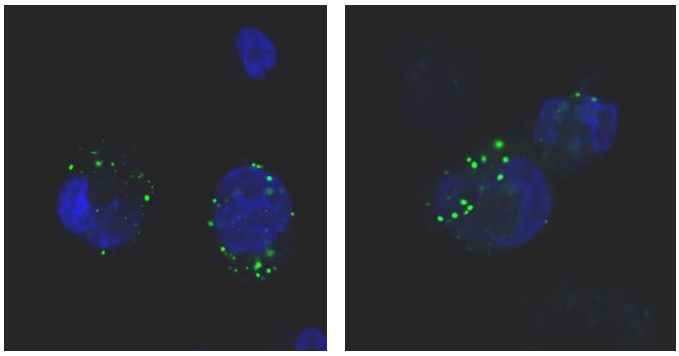

Fig. 34 Caspase-8-c-FLIP$_L$ dimer formation
Expression of caspase-8/b and c-FLIP$_L$ in HT1080 cells. Caspase-8/b and c-FLIP heterodimers are shown in green and nuclei are stained with DAPI (blue).

V Discussion

Interactions of DISC Proteins

Deregulation of death receptor signaling is often associated with severe diseases. Too much apoptosis may lead to neurodegenerative diseases or destruction of tissue, whilst too little may lead to cancer or survival of autoreactive cells [12]. One form of apoptosis signaling is death receptor-induced cell death, where a protein complex named death inducing signaling complex (DISC) is formed inside the cell at the activated receptor. Most likely the exact protein composition of the DISC decides about the outcome of signaling [121]. To gain further understanding of DISC proteins and their interactions the role of c-FLIP$_R$ during Listeria monocytogenes infection in mice and dimerization of DISC proteins in living cells were analyzed in this study.

c-FLIP$_R$ Overexpression Alters B Cell Maturation, but Does Not Interfere with T and B Cell Numbers in General

c-FLIP$_R$ is the solely expressed c-FLIP isoform in the murine system [77], but still its function remains unknown. To analyze the function of murine c-FLIP$_R$ we generated a mouse model overexpressing c-FLIP$_R$ under the *vav*-promotor. The *vav*-promoter has been described to induce transgenic expression in all cells of hematopoietic origin [77, 114]. This makes analyses of the effect of c-Flip$_R$ on the immune system possible. As Western blot analyses show, the vavFLIP$_R$ mice express the transgene. In wildtype littermates the expression is below detection level under steady state conditions. This is similar to c-FLIP$_S$ in humans, which is induced upon TCR stimulation [90]. vavFLIP$_R$ mice are viable and show no obvious defects. In that respect they resemble c-FLIP$_S$ transgenic mice reported in the literature [116, 117]. In contrast, complete c-FLIP knockout in mice is lethal [122]. Zhang and colleagues described c-FLIP knockout mice to be rescued by expression of human c-FLIP$_S$ via BAC transgene [123], showing short isoforms to have an essential impact on embryonic development. Nevertheless it remains questionable if this reflects

the natural occurring situation since without stimulation higher amounts of c-FLIP$_L$ than c-FLIP$_R$ are expressed. Possibly high levels of c-FLIP$_S$ are in the absence of c-FLIP$_L$ capable of counteracting apoptosis and thereby substituting c-FLIP$_L$ regarding apoptosis inhibition. Altered CD95-induced signaling has been described to lead to accumulation of double negative (DN; CD3$^+$CD4$^-$CD8$^-$) cells, e.g. DN cells have been described to accumulate in human ALPS patients and mice with lpr or gld phenotype [124]. Generation and function of DN T cells are being discussed rather controversial. One theory states DN survive thymic development due to lack of depletion via FAS-induced apoptosis [125] other attribute regulatory effects to these cells [126]. Oberst and colleagues conclude that a caspase-8-c-FLIP$_L$ complex inhibits RIPK3 dependent necrosis during development [127]. Additionally, the presence of c-FLIP prevents caspase-8-dependent apoptosis, too. Endogenous c-FLIP$_L$, FADD and caspase-8 are present in the vavFLIP$_R$ mice in amounts comparable to wildtype littermates and therefore do not affect the phenotype of vavFLIP$_R$ mice. Taken together c-FLIP$_R$ overexpression does not interfere with cell numbers in general.

vavFLIP$_R$ Mice Show Normal T Cell Development

Being phenotypically normal vavFLIP$_R$ mice were analyzed further with respect to T cell development. During thymic T cell development cell death is an essential process to remove potentially harmful cells, only 5% of the developing cells survive [128]. Whilst FADD-binding receptors seem to be non-essential for negative selection [128], c-FLIP proteins have been described to protect thymocytes during the last maturation steps to the single positive stage [129]. To analyze if c-FLIP$_R$ overexpression impacts T cell development thymic T cells from vavFLIP$_R$ mice were compared to wildtype mice. vavFLIP$_R$ mice show no differences in thymic T cell developmental stages though, numbers of double positive (CD4$^+$CD8$^+$) single positive (CD4$^+$ or CD8$^+$) or double negative stages CD4$^-$CD8$^-$) are similar to wildtype littermates. This is in agreement with transgenic mice overexpressing the human short variant c-FLIP$_S$, which show no alterations in T cell development [116, 117]. Taken together overexpression of short c-FLIP isoforms does not interfere with thymic T cell development. c-

FLIP knockout mice in contrast have been described to phenotypically resemble caspase-8 and FADD knockout which are embryonically lethal [130]. T cell specific c-FLIP knockouts are viable and show a severe lack of T cell developmental stages [122, 129]. Zhang and He describe a partial developmental block during transition to the SP stage [129]. They assume SP cells detected at later time points originate from homeostatic proliferation and not from thymic development [129]. Interestingly reconstitution with c-FLIP$_S$ is sufficient to rescue T cell development and prevent spontaneous apoptosis of in T cells [123]. This may be explained by redundancy between the long and short isoforms in their function during development.

T Cell Homeostasis is Unaffected by c-FLIP$_R$ Overexpression

Next to apoptosis-signaling c-FLIP proteins and other DISC-associated proteins play an essential role in modulating cell survival and proliferation [121, 131]. This underscores the importance of c-FLIP as double-sided regulator of cell homeostasis. The short forms of c-FLIP proteins are assumed to act only anti-apoptotic [71, 76], therefore overexpression of c-FLIP$_R$ is likely to shift the live/death balance in a cell towards survival and proliferation. Looking at T cell numbers there is no increase in vavFLIP$_R$ mice, which show similar T to B cell ratios as wildtype mice. Within the T cell subgroup no altered CD3$^+$, CD4$^+$ and CD8$^+$ ratios are detectable in vavFLIP$_R$ mice. This is in agreement with the normal thymic T cell development and phenotype described above. Consistently c-FLIP$_S$ overexpressing mice described by Oehme *et al.,* and Hinshaw Makepeace and colleagues show no alterations of cell numbers in vivo [116, 117]. Both groups describe a defect in NF-κB activation in vitro, which they suggest to be counteracted in *vivo* by other mechanisms, which keep up immune homeostasis [117]. In the human system c-FLIP$_S$ in lymphocytes is known to be upregulated upon TCR stimulation [89, 90], whilst the long isoform is expressed constantly in humans and mice. The mice analyzed were kept under specific pathogen free (SPF) conditions and thereby without pathogen contact. Therefore, c-FLIP$_R$ in mice and c-FLIP$_{S/R}$ in humans may not be relevant for T cell homeostasis as long as c-FLIP$_L$ is present in

endogenous occurring amounts. This is as well supported by c-FLIP knockouts being rescued by BAC transgenic expression of one of the isoforms. Transgenic overexpression of the long isoforms in contrast has been described to cause a lupus-like syndrome at least on a BALB/c background [132]. vavFLIP$_R$ mice express the transgene heterozygous on a C57BL/6 background, which renders them less lupus-prone and may dampen effects. Additionally the long isoforms is known to act pro- and anti-apoptotic, being able to support the first cleavage steps of caspase-8 [71]. c-FLIP$_R$'s structure renders it unable to stabilize caspase-8 during cleavage. This difference in structure results probably in different functions and may explain why overexpression of the long and short isoforms leads to different phenotypes. Some viruses are known to express viral Flip (vFLIP) proteins, which prevent death receptor induced apoptosis [72]. Overexpression of the viral MC159 variant in mice leads to an autoimmune phenotype resembling CD95 mutations described below [133]. Even though viral c-FLIP proteins resemble in some structural details the short c-FLIP isoforms, they prevent apoptosis in a divergent way from c-FLIP$_S$ [119]. Taken together the phenotype of vavFLIP$_R$ mice resembles most transgenic c-FLIP$_S$ mice and differs from mice overexpressing the long isoform or vFLIPs.

Since short c-FLIP variants inhibit caspase-8 activation at the DISC level comparison with known mutations regarding CD95, CD95 ligand and DISC proteins are of interest. Mutations in CD95 lead in humans to ALPS, in mice to a gld/lpr phenotype. In both cases lymphoproliferation and accumulation of $CD3^+B220^+CD4^-CD8^-$ have been described. No such drastic effect can be observed in vavFLIP$_R$ mice. This shows differences between inhibition at death receptor level or at caspase-8/c-FLIP level. This may be explained by comparison with the human c-FLIP$_S$ isoform. Inhibition of the human c-FLIP$_S$ isoform after CHX treatment does render cells only in part apoptosis sensitive, since resistance is regulated at multiple levels, e.g. Bcl-x$_L$ [90]. In contrast humans with mutations rendering caspase-8 non-functional show an immunodeficiency, but no accumulation of double negative cells or lymphoproliferation has been described. The situation in humans may not be completely comparable to mice since humans have caspase-10 as additional

initiator caspase [47]. Mutations in caspase-10 have been described to lead to ALPS type II [134]. Caspase-10s function remains enigmatic and its ability to substitute for caspase-8 is discussed controversial [43, 45, 135]. This makes comparison between mice and humans regarding caspase-8 and its regulation complicated.

The necessity of c-FLIP, FADD and caspase-8 for cell proliferation can be seen in the knockout situation. Whilst overexpression of short isoforms does not interfere with T cell homeostasis conditional knockouts have been described to alter T cell proliferation. For example Chau and colleagues describe c-FLIP deficient $rag^{-/-}$ chimera [131]. These mice show reduced T cell counts in thymus and periphery resembling similar models for FADD and caspase-8 [131, 136]. Knockout of FADD abolishes c-FLIP and caspase-8 binding to the DISC. FADD knockout mice show dramatic alterations in T cell numbers and development [136]. RAG chimera's reconstituted with dominant negative FADD show during the first days normal T cell developmental stages, but by the time they reach 5 weeks of age almost completely lack cells in the double positive stage [137]. c-FLIP, FADD and caspase-8 are assumed to have various non-apoptotic functions as well, which may lead to different effects [138]. Since caspase-8 and c-$FLIP_L$ are both present in vavFLIP$_R$ mice at normal endogenous levels, their anti-apoptotic functions are most likely not affected in vivo in vavFLIP$_R$ mice. Interestingly a RIPK3 and caspase-8 double knockout is able to rescue mice from embryonic lethality [139]. RIPK3 and caspase-8 double knockout mice do not show double negative cells at young age, but later on [139]. Further analyses of aged vavFLIP$_R$ mice may be interesting to see if c-FLIP$_R$ overexpression does interfere with T cell homeostasis and lead to autoimmunity at later stages.

vavFLIP$_R$ Mice Show Unaltered T cell Activation Status

Unaltered T cell development and unaltered T cell numbers as described above lead to the assumption of steady-state T cell homeostasis being similar in vavFLIP$_R$ mice and wildtype littermates. This is further supported by lack of

obvious differences regarding T cell activation in vavFLIP$_R$ mice and wildtype littermates. In the human system c-FLIP$_S$ is upregulated in response to TCR stimulation [90]. Nevertheless c-FLIP$_R$ overexpression does not vice versa lead to activation of T cells in vavFLIP$_R$ mice.

Treg Numbers are Not Altered by c-FLIP$_R$ Overexpression

CD95-induced signaling is known to regulate Treg homeostasis [140]. Tregs from healthy mice are sensitive to CD95 ligand, even though they express less stimulation-induced CD95 ligand themselves [140]. Weiss and colleagues showed it is possible to deplete in vivo Tregs using CD95 [140]. Therefore, the effect of c-FLIP$_R$ overexpression on Tregs was analyzed. Nevertheless, this seems not the case since vavFLIP$_R$ mice show normal Treg numbers like c-FLIP$_S$ transgenic mice described by Oehme and Hinshaw-Makepeace and colleagues [116, 117].

c-FLIP$_R$ Interferes with B Cell Fractions

Surprisingly further analyses of the B cell compartment did show a difference in certain Hardy fractions in vavFLIP$_R$ mice compared to wildtype littermates. The number of naïve B cells was higher in vavFLIP$_R$ mice compared to wildtype animals whilst the number of developed B cells was lower. This is in agreement with c-FLIP being essential for B cell homeostasis, c-Flip knockout mice showing less peripheral B cells [141]. c-FLIP has been described to be essential for the germinal center reaction in a model proposed by van Eijk and colleagues [142]. During clonal expansion and receptor diversification B cells are protected against apoptosis. Germinal center B cells behave like type I apoptotic cells and depend on CD40 ligation to upregulate c-FLIP$_L$, which leads to apoptosis protection [142]. Loss of stimulation is followed by downregulation and increased apoptosis sensitivity. Thereby hyperreactive B cells as well as non-responsive B cells could be eliminated via c-FLIP$_L$ downregulation. Increased c-FLIP$_R$ expression in the vavFLIP$_R$ mice may interfere with this process and block death receptor signaling. This blockage

would prevent c-FLIP$_L$ as well as caspase-8 binding. Nevertheless one would expect more mature B cells, compared to wildtype littermates, and autoimmunity if c-FLIP$_R$ protects cells from apoptotic death. On the other hand c-FLIP$_L$ and caspase-8 have been implicated as well in proliferation and to counteract RIP1 at the DISC [88, 143]. Interference with these processes may lead to a decreased number of mature B cells. Data from B cell specific c-FLIP knockout mice are controversial. Zhang and colleagues describe reduced numbers of mature B cells, but no effect on the bone marrow during development [144]. This would make further analyses of B cell development in vavFLIP$_R$ mice and analyses of autoimmunity during aging highly interesting and supports a role for c-FLIP in B cell maturation, which would as well cause altered B cell numbers in vavFLIP$_R$ mice. A later publication by two of the authors attributes these effects rather to cre lox mediated inactivation of CD19 and describes no significant developmental differences due to B cell specific c-FLIP knockout [145]. Further analyses of the effects of c-FLIP isoforms on B cells and their development are needed. Comparison of vavFLIP$_R$ mice to knockout mice could reveal at which developmental or activation stages c-FLIP regulates B cells. Taken together lymphocyte numbers in young vavFLIP$_R$ mice are close to normal, showing differences regarding B cell developmental stages.

vavFLIP$_R$ Mice Show Better Bacterial Clearance After *L. Monocyotgenes* Infection

Though the function of murine c-FLIP$_R$ has not been analyzed before, its structure and expression makes it a likely functional counterpart of the human short isoform c-FLIP$_S$. c-FLIPs has, like murine c-FLIP$_R$, a short half-life [119]. c-Flip$_S$ is known to be upregulated in human T cells in response to TCR stimulation [89, 90]. This may explain why during the steady state only mild alterations could be detected between wildtype and vavFLIP$_R$. Additionally viral Flip proteins, which resemble the short isoforms in structure, are known to modify immune responses [146]. vavFLIP$_R$ characterized here have been kept under specific pathogen free (SPF) conditions, which means mice have not encountered many pathogens. To investigate if alterations in vavFLIP$_R$ mice

can be detected during an immune response vavFLIP$_R$ mice and littermates were infected with *L. monocytogenes*, which causes lymphocyte apoptosis [101]. How this *L. monocytogenes*-induced cell death proceeds remains unclear. Death-signaling systems like TRAIL have been discussed to contribute to this massive cell death [105]. Similar to CD95 TRAIL receptor stimulation induces formation of a DISC, which contains c-FLIP proteins [28]. Knockout of c-FLIP has previously been shown to interfere with *L. monocytogenes* infection [122] making an involvement of DR-mediated apoptosis likely. We challenged vavFLIP$_R$ mice with *L. monocytogenes* to see if c-FLIP$_R$ interferes with the immune response and cell death triggered by *L. monocytogenes* and found vavFLIP$_R$ mice being better protected against *L. monocytogenes* than wildtype littermates.

In detail, vavFLIP$_R$ mice showed a reduced bacterial burden in liver and spleen, the effect being stronger in the spleen. In agreement with this, c-FLIP knockout mice show the opposite phenotype: no CD8$^+$ effector function in response to *L. monocytogenes* infection [122]. The lower bacterial load may be explained by c-FLIP$_R$ mediated protection of T cells from unspecific apoptosis occurring after *L. monocytogenes* infection and thereby making survival of more effector cells possible.

Less Necrosis in vavFLIP$_R$ Mice After *L. monocytogenes* Infection

To further investigate the above-described differences in bacterial load between vavFLIP$_R$ mice and wildtype littermates, spleens and livers from *L. monocytogenes* infected vavFLIP$_R$ mice and wildtype littermates were histologically analyzed. c-FLIP proteins are known to modulate apoptosis and necrosis [88, 121, 147]. Several inducers of death and various forms of cell death are discussed in the context of *L. monocytogenes* infection [101]. In accordance with lower bacterial burden vavFLIP$_R$ mice show in response to *L. monocytogenes* infection less necrotic foci. This reduction in necrotic foci is more pronounced in the liver, compared to CFUs differing stronger in the spleen. Different routes of infection and cell types involved in the two organs may explain this. In the liver the first peak of cell death occurs 12 hours after

infection and is caused by death of infected macrophages [148]. Within the liver resident Kupffer cells take up 80% of the bacteria and the majority of the bacteria is killed [148]. The exact mechanisms of this killing are not clear, but most likely neutrophils are involved in this process [149]. The remaining bacteria infect hepatocytes, which die later during the infection [150]. 20% of the inoculum is taken up by the spleen, which lacks a early inactivation mechanisms [148]. Microabscesses are formed in the spleen within 24 hours past infection [148]. These differences during the infection process are a possible explanation for differences seen between the two organs. Regardless of the involvement of different immune cells and infection routes c-FLIP$_R$ leads to better protection against *L. monocytogenes* infection in liver and spleen.

Less T Cell Apoptosis in *L. Monocytogenes* Infected vavFLIP$_R$ Mice

Within the necrotic foci massive, unspecific T cell apoptosis has been described. To gain a better understanding if c-FLIP$_R$ overexpression in hematopoietic cells does interfere with *L. monocytogenes* infection-induced T and B cell apoptosis cells from spleen and lymph nodes were analyzed. There were no differences regarding B cells, but less T cells from vavFLIP$_R$ mice were apoptotic. This is in accordance with TRAIL knockout mice described in the literature showing less apoptosis [105] and supports the theory of DR involvement in cell death after *L. monocytogenes* infection. Protection against the early phase of *L. monocytogenes* infection as seen in vavFLIP$_R$ mice and TRAIL null mice has been reported as well for SCID mice that lack T and B cells [151]. Comparing the different phenotypes *L. monocytogenes*-induced T cell death seems to be beneficial for the bacteria.

Obvious differences regarding cell death, bacterial burden and liver necrosis show that signaling pathways regulated by c-FLIP proteins are involved in *L. monocytogenes*-induced cell death in mice. Listeria secrete LLO, which is known to induce cell death [103], but the exact pathways it triggers are not known. The first directly occurring cell death of infected cells is most likely to be granzyme based [104]. c-FLIP$_R$ interferes with cell death at death receptor level, while granzymes activate effector caspases directly [152] or via Bid

cleavage [153]. Kataoka and colleagues showed that perforin/granzyme B-induced apoptosis is unaffected by c-FLIP [154]. The cause of the second, slower phase of cell death after *L. monocytogenes* infection is less well understood. Here a contribution of death receptors and thereby an impact of c-$FLIP_R$ overexpression seems to be very likely. The exact cause of spontaneous liver apoptosis after *L. monocytogenes* remains unknown, but three signaling events have been proposed. The first is signaling pathway is recognition of bacteria within lysosomes via diverse receptors, e.g. Toll like receptors, which signal via MyD88 [155].

In *L. monocytogenes* infection one mode of recognition of the bacteria by the innate immune system strongly involves IRF3 (Interferon regulatory factor 3) [156], which eventually leads to TNFα expression. TNFR1 recruits FADD via the adaptor TRADD and c-FLIP is described as part of an TNFα signaling complex, modulating the balance between NF-kB activation and cell death [88]. Therefore overexpression of c-$FLIP_R$ may interfere with this aspect of signaling after *L. monocytogenes* infection. A second option of apoptosis induction after *L. monocytogenes* infection is type I interferon signaling [98]. Whilst type one interferon signaling is needed for clearance of many viral pathogens they seem rather detrimental to the host during *L. monocytogenes* infection [98]. In the literature c-FLIP has been reported to interfere with interferon-induced cell death. Caspase-8-deficient mice have been described to show impaired degradation of IRF3 resulting in high cytokine signaling [157]. This makes as well an impact of c-$FLIP_R$ on this pathway possible. In case of viral infections IRF3 signaling has been described to dampen the immune response, but in case of *L. monocytogenes* infection the effect seems to be beneficial. Interferon signaling has been described to be involved in immune regulation and macrophages apoptosis after *L. monocytogenes* infection, too [158]. In vavFLIP$_R$ mice all cells of hematopoietic origin express c-$FLIP_R$, including macrophages and dendritic cells, which develop from monocytes. Therefore, interference with macrophage cell death or DC survival may be one factor contributing to less necrotic foci in the livers of vavFLIP$_R$ mice.

Taken together c-$FLIP_R$ overexpression mediates a better protection against

the early phase of *L. monocytogenes* infection. This supports strongly the theory of death receptor involvement in cell death after *L. monocytogenes* infection. Since *L. monocytogenes* is the leading cause of food-borne infections this may be interesting for further therapeutic approaches. Therefore, analyses are required to investigate if clearance is complete and if a memory response is generated in vavFLIP$_R$ mice to mediate protection in the long run. c-FLIP$_R$'s capability of modifying an immune response argues for functional homology with human c-FLIP$_S$.

Bimolecular Fluorescence Complementation (BiFC) to Analyze Dimerization Processes at the DISC

Signaling at DISC level and regulation of DISC proteins is a very complex procedure. As discussed before overexpression of c-FLIP$_R$ alters the immune response to *L. monocytogenes*. Another example are knockouts of DISC proteins, that show a lethal phenotype, which can be rescued by knocking out a second protein component, e.g. caspase-8 and RIPK3 [139]. Alterations in these pathways can cause severe diseases like cancer, neurodegeneration and autoimmunity [12]. Autoimmune lymphoproliferative syndrome (ALPS) being probably the most striking example for deregulated death receptor signaling. ALPS patients show lymphoproliferation and autoimmunity [159]. A better understanding of interaction of DISC proteins on a molecular level is needed to gain insights into these conditions. Here dimerization processes involving caspase-8, c-FLIP and caspase-10 were analyzed using bimolecular fluorescence technique (BiFC). This approach allows visualization of hetero- and homodimers as well [160]. Each protein analyzed is tagged with a fragment of a fluorescent protein. Only if the proteins interact the fluorescent fragments are brought into close proximity and the complete fluorescent protein is formed. The fluorescence can be detected using FACS or confocal laser scanning microscopy. The current model of caspase-8 activation is the induced-proximity model. This model assumes caspase-8 dimerizes with another caspase-8 molecule and is fully activated by subsequent self-

processing [41, 161, 162]. In addition, caspase-8 is assumed to dimerize with c-FLIP proteins, dimerization with the long isoform c-$FLIP_L$ leading to an intermediate cleavage product [71]. The molecules, recruited after death receptor stimulation, decide about whether the outcome of signaling is cell death, survival or proliferation [81, 163]. The actual process of caspase-8-caspase-8 interaction is not detectable with antibody staining, since it is impossible to distinguish between two molecules bound by the same antibodies. *Ex vivo* dimerization was analyzed with kosmotrophic salts and *in vivo* with altered recruitment domains, which make dimerization inducible [79]. Nevertheless altered prodomains may affect dimerization dynamics since the DEDs are crucial for DISC recruitment [119]. Here BiFC constructs of caspase-8,-10 and the c-Flip isoforms c-$FLIP_R$ and c-$FLIP_L$ were generated and used for visualization of dimers. This technique has been shown by Bouchier-Hayes and colleagues to work efficiently for visualization of caspase-2 dimerization [106].

BiFC as Tool to Analyze Dimerization of DISC Proteins

Analyses of caspase-8 expressed from BiFC constructs in 293T cells showed expression to be weaker than endogenous caspase-8 expression. This makes dimerization of the constructs with endogenous caspase-8 more likely and thereby diminishes the fluorescent signal. Therefore, cell lines were chosen, which do not express caspase-8 on the protein level. SH-SY5Y, a neuroblastoma cell line, in which caspase-8 is silenced [108], and an I9.2 Jurkat cell, in which caspase-8 is mutated by the frameshifting agent ICR191 and therefore not expressed [39], were used for further experiments. Another criterion, by which both cell lines were chosen, is expression of death receptors. Caspase-8-deficient Jurkat cells and their parental cell line A3 express high amounts of CD95, which makes them suitable for DISC analyses. SH-SY5Y express TRAIL receptors and have been shown to undergo apoptosis after demethylation of caspase-8 [108].

To exclude fluorescence signals, which were detected being caused by unspecific interactions additionally DED mutants were used. Constructs with mutated DEDs do not show dot-like or larger structures. They show only background fluorescence. In contrast analyses of caspase-8 overexpression revealed caspase-8-caspase-8 homodimers in 293T cells, SH-SY5Y, caspase-8-deficient Jurkats and A3 cells. Two different structures were formed by dimers, dot-like structures, which spread over the whole cells and larger structures, which localized closer to the nucleus.

This shows the fluorescent signals detected do not result from unspecific interactions of the fluorescent fragments. Additionally the fact that expression in 293T cells did show low expression of BiFC caspase-8 compared to endogenous caspase-8 argues against the fluorescent signal being caused by simple overexpression. Constructs are expressed in SH-SY5Y cells, but only on a moderate level. Since SH-SY5Y cells and caspase-8-deficient A3 cells lack caspase-8 on protein level the overall amount should be very low. Stimulation of cells with TRAIL, CD95 ligand or anti-CD95 (2R2) did not show any increase in dimer formation. Therefore dimerization of BiFC constructs is not inducible.

Caspase-8-Caspase-8 Dimers Are Formed in Living Cells

Nevertheless the fact that caspase-8-caspase-8 dimers are formed in large quantities shows the capability of caspase-8 molecules to form homodimers in living cell. Previous models made before were based on *in vitro* experiments with kosmotrophic salts [35, 118]. The data generated here using living cells shows, that caspase-8 forms indeed homodimers. Pop and colleagues and Oberst and colleagues showed that caspase-8 forms caspase-8-caspase-8 homodimers *in vitro* [35, 118]. Initiator caspases, even if containing mutated cleavage sites, dimerize in vitro in kosmotrophic salts. After removal of salt the proteins dissociate and loose their activity due to conformational changes showing that dimerization leads in part to activation [35, 118]. Cleavable

proteins, in contrast, retain the enzymatic activity. This means that cleavage, which occurs after dimerization, is essential for the stability of active caspases [118]. For apoptosis signaling coordinated dimerization and cleavage of caspase-8 is essential, whilst the uncleaved form of caspase-8 is able to signal for NF-κB activation [84]. The cell culture cells used here for the BiFC analyses are tumor cell lines, which proliferate strongly. Proliferation signaling caused by caspase-8 is not as well understood as apoptotic signaling.

Rather large structures detected are in accordance with recent publications, which show chains of DED containing proteins instead of simple caspase-8 dimers being formed at the DISC [82, 164]. The described chain formation may explain the rather large structures resulting from caspase-8 and cFLIP$_L$ dimers as well. The caspase-8-caspase-8-homodimers visualized prove, that caspase-8 with unaltered recruitment domains dimerizes in living cells. If these dimers result from cell proliferation or simple overexpression needs further investigation.

Caspase-8 and c-FLIP form Heterodimers in Living Cells

Even though the role of c-FLIP within the DISC has been analyzed extensively, direct dimerization of c-FLIP proteins had never been shown before. The BiFC experiments done here show Caspase-8 forms in living cells heterodimers with c-FLIP proteins, most prominent with c-FLIP$_L$.

Different known c-FLIP isoforms act at different steps of DISC formation [71]. c-FLIP$_L$ has been described to be cleaved at the DISC, whilst the short isoforms directly block any cleavage processes at the receptor [64, 71]. All three isoforms require DISC recruitment for their action. The chain model showed lower amounts of c-FLIP$_L$ present within the DISC chains [164, 165]. Here c-FLIP$_L$ was highly overexpressed. Therefore, the amount of c-FLIP$_L$ was above the endogenous occurring levels. This may be the cause of huge dot-like structures, which could be detected. This supports the hypothesis of c-FLIP$_L$ being in large amounts able to interfere with caspase-8 dimerization and

chain formation at the DISC. This was as well supported by c-FLIP$_L$ being able to form large quantities of homodimers as well. In contrast heterodimers of caspase-8 and c-FLIP$_R$ or caspase-10 always lead to smaller dots and no vast structures. One possible explanation for this difference may be c-FLIP$_R$ and caspase-10 being not capable of supporting further chain formation at the DISC and thereby terminating the chains. Another explanation may be, that they block the receptor and terminate recruitment of other DISC proteins. This is in accordance with the assumed role of the short c-FLIP isoforms c-FLIP$_R$ and c-FLIP$_S$ as apoptosis inhibitors. Differences in dimerization behavior between c-FLIP$_R$ and c-FLIP$_L$ are as well in agreement with the data from vavFLIP$_R$ mice described above leading to a different phenotype than c-FLIP$_L$ overexpression described by Zhang and colleagues [122].

Caspase-10-Caspase-8 Heterodimers Are Formed in Living Cells

The role of caspase-10 in the human system has been discussed very controversial. Some reports argue that it can, at least partially replace caspase-8, other doubt its function in death receptor-induced apoptosis [43-45, 47, 135]. Human patients with mutations of caspase-8 rendering it non-functional are able to survive whilst mice die upon caspase-8 knockout since mice do not have a caspase-10 gene. The smaller dots observed in the current study may argue for a weaker mode of action and explain why caspase-10 cannot substitute caspase-8 completely. This is as well in accordance with the experiments by Schleich and colleagues showing weak amounts of caspase-10 within the DISC chains [164]. The moderate levels of dimer formation by caspase-10 and -8 and different substrate specificities described in the literature may explain why caspase-8 deficient patients are viable, but show immunodeficiencies [85]. Nevertheless, this study shows clearly caspase-8 and -10 are capable to dimerize in living cells.

Localization of Dimers

Localization is an important factor influencing the outcome of signaling processes. Lee and colleagues could show that during CD95-induced signaling internalization of the receptor has an important impact on the outcome [166]. In the absence of CD95-internalisation proliferation and NF-κB activation are induced instead of apoptosis [166]. Due to the difficulties of visualizing homodimer formation reports on the activation site are quite controversial. The BiFC technology allows direct visualization of dimers and thereby makes localization analyses of these processes possible [160].

Colocalization with Mitochondria

Caspase-8-caspase-8 homodimers show two structures. Whilst the dot-like structure does not colocalize with mitochondria, the larger structures do. This is very interesting because Stegh and colleagues reported that caspase-8 localizes at the mitochondria in the human breast cancer cell line MCF7 [120]. One possible explanation may be that the small dots are the first initiation platform of apoptotic signaling. This is in accordance with small dots being on cells, which are morphological normal cells, whilst the cells containing larger structures are often rounded up suggesting an apoptotic phenotype. In the model proposed by Stegh and colleagues dimerization occurs at the receptor and than dimers are activated at the mitochondrial membrane [120]. The BiFC constructs used for confocal microscopy contained an active center mutation. Therefore cleavage, which is supposed to take place at the DISC, does not occur. The large structures, which colocalize with the mitochondria, may be accumulating caspase-8 dimers.

Crosstalk Between Apoptosis and Autophagy

Recently a new complex named intracellular death-inducing signaling complex (iDISC) containing FADD, caspase-8, which associates with components of the autophagy system has been described [167]. After stimulation with the cancer drug SKI-I, a caspase-8-ATG5 complex is formed, which colocalizes with LC3

and p62 at autophagosomal membranes [167]. SH-SY5Y cells expressing BiFC constructs were analyzed for colocalization of dimers with LC3 to see if a similar complex is formed. There was no detectable colocalization of c-FLIP, caspase-8 or caspase-10 dimers with LC3 with or without TRAIL or CD95 stimulation. This may be explained by crosstalk having an impact on LC3, but no direct interaction between apoptotic components and LC3 [167].

No Colocalization with Endosomes or Golgi

As mentioned above Schütze and colleagues describe a receptor internalization processes [62]. This would make localization in endosomes likely. Staining for Rab5 and EEA1 did not show any colocalization with endosomes. Neither could co-localization with lysosomes or Golgi be detected with or without DR stimulation. No colocalization of caspase-8 with Golgi has been described in the literature; therefore the lack of interaction is to be expected.

Taken together, the BiFC constructs allow visualization of dimers in living cells. This showed strong homo- and heterodimer formation between c-FLIP$_L$ and caspase-8 and weaker dimer formation with c-FLIP$_R$ and caspase-10. Colocalization analyses showed vast dimer structures co-localizing with mitochondria, whilst smaller structures localize to a different, yet unknown compartment. This makes the smaller structures most likely locations, where signaling is initiated and dimerization occurs, whilst the larger structures could be the place where caspase-8 would be cleaved.

VI. Abbrevations

B cell	B lymphocyte
BiFC	Bimolecular fluorescence complementation
c-FLIP	celluar FLICE inhibitory protein
C>S	cysteine to serine mutation; used here as abbreviation for active center mutation
CD95	cluster of differentiation 95; fas
CD95L	CD95 ligand
CHX	cyclohexamide
DD	death domain
DED	death effector domain
DISC	death inducing signaling complex
DR4	death receptor 4 (TRAIL receptor 1)
DR5	death receptor 5 (TRAIL receptor 2)
FACS	fluorescence activated cell sorting
FADD	fas-associated protein with death domain
FLICE	FADD-like interleukin-1 beta-converting enzyme
FLIP	FLICE inhibitory protein
HGF	hepatocyte growth factor
H&E	hematoxylin and eosin
InlA	internalin A
InlB	internalin B
IP	immunoprecipitation
IRF3	interferon regulatory factor 3
LLO	lysteriolysin O
L. monocytonges	Listeria monocytogenes
Met	HGF-SF receptor
NF-κB	nuclear factor kappa beta
RT	room temperature
T cell	T lymphocyte
tg	transgene

TNFα	tumor necrosis factor-alpha
TNFR1	tumour-necrosis factor receptor-1
TRAIL	TNF-related apoptosis-inducing ligand
Tregs	regulatory T cells
vavFLIP$_R$ mice	transgenic mice expressing c-Flip under the vav promoter
vFLIP	viral FLIP
WT	wildtype

VII. Literature

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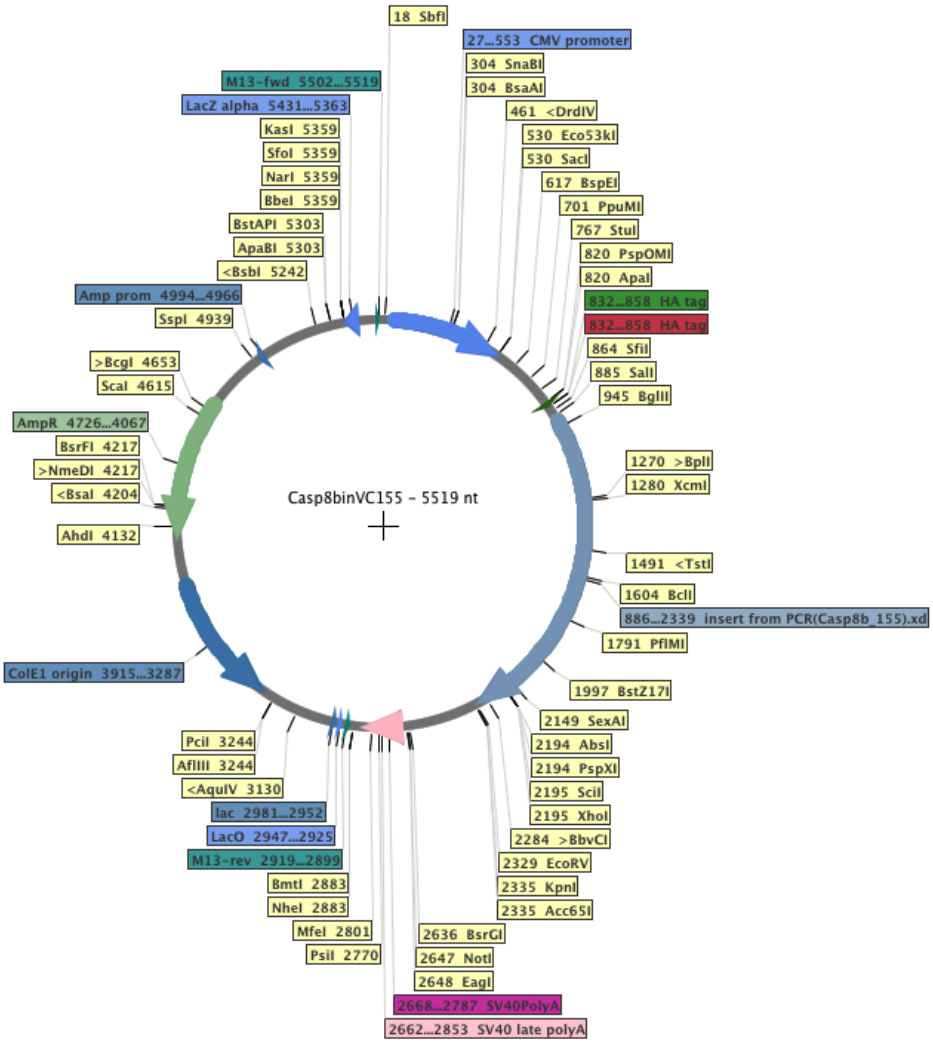
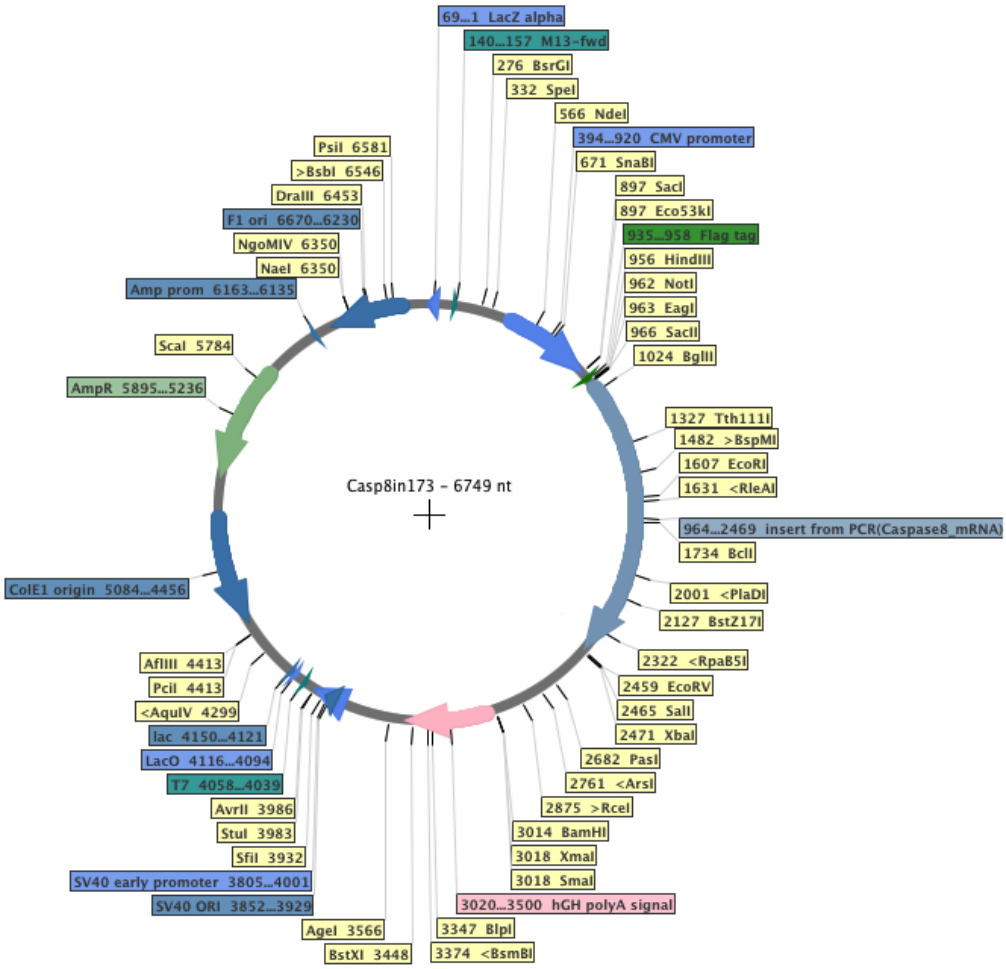
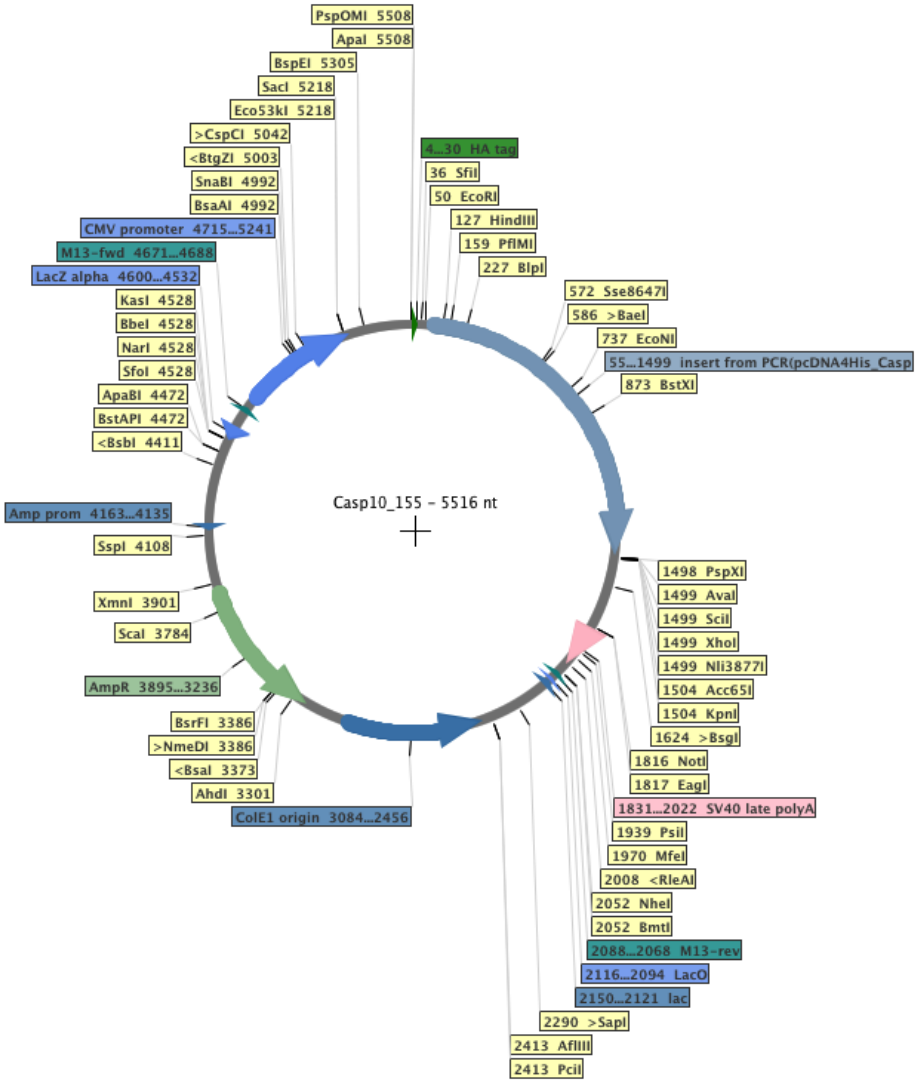
VIII. Plasmid Maps



Caspase-8 in VC155



Caspase-8 in VN173



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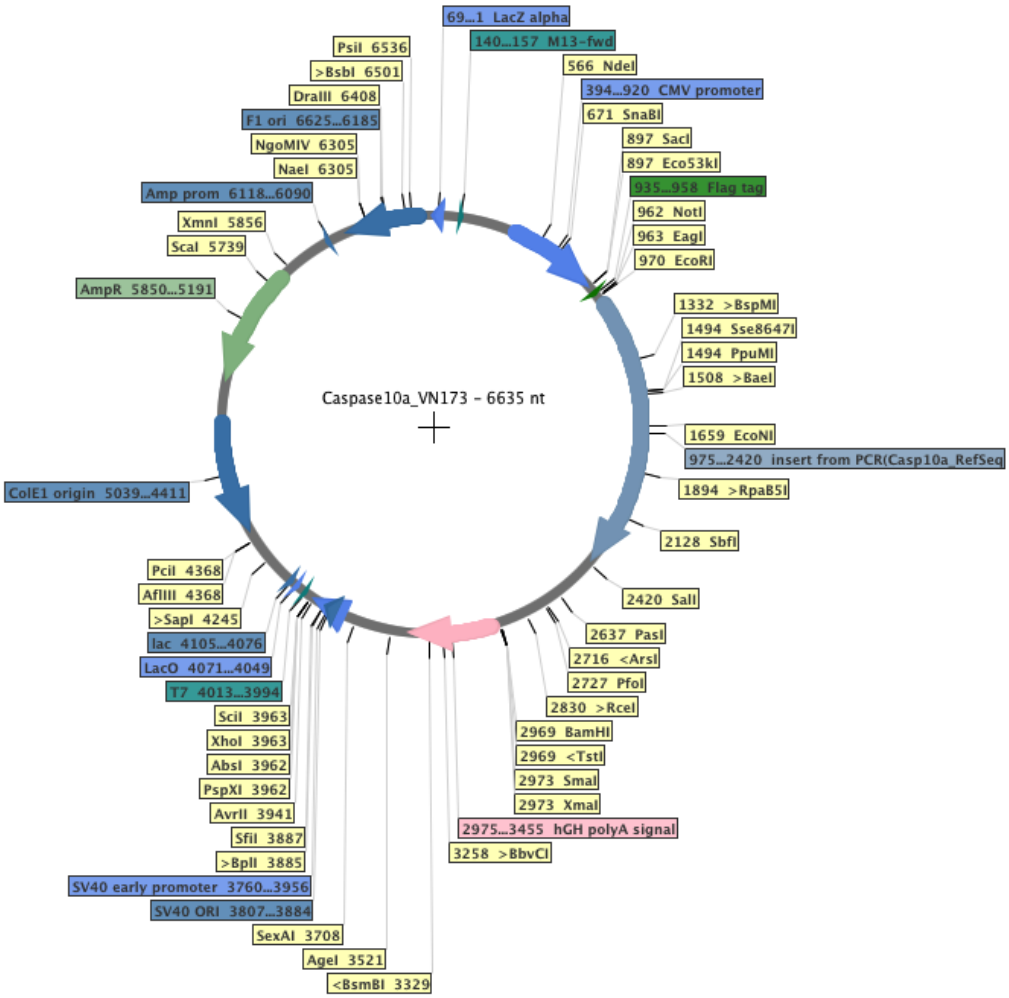

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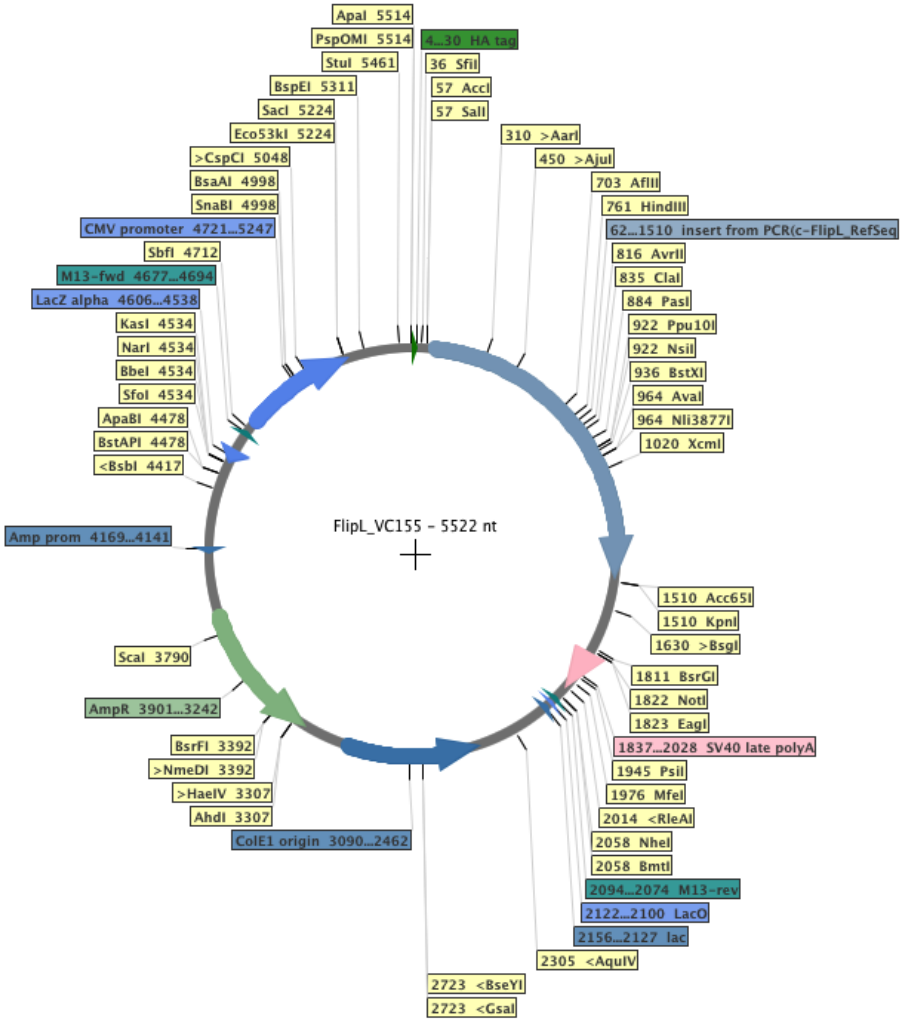
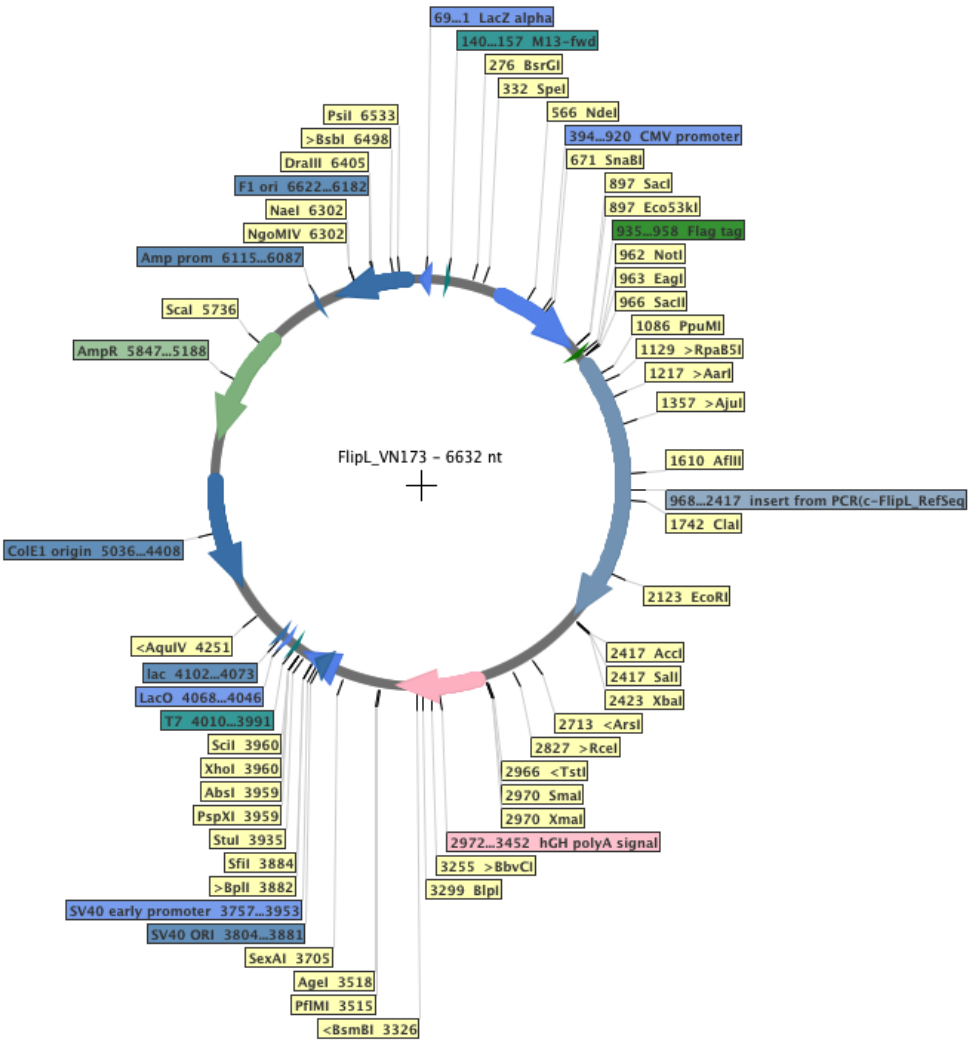


c-FLIP$_L$ in VC155



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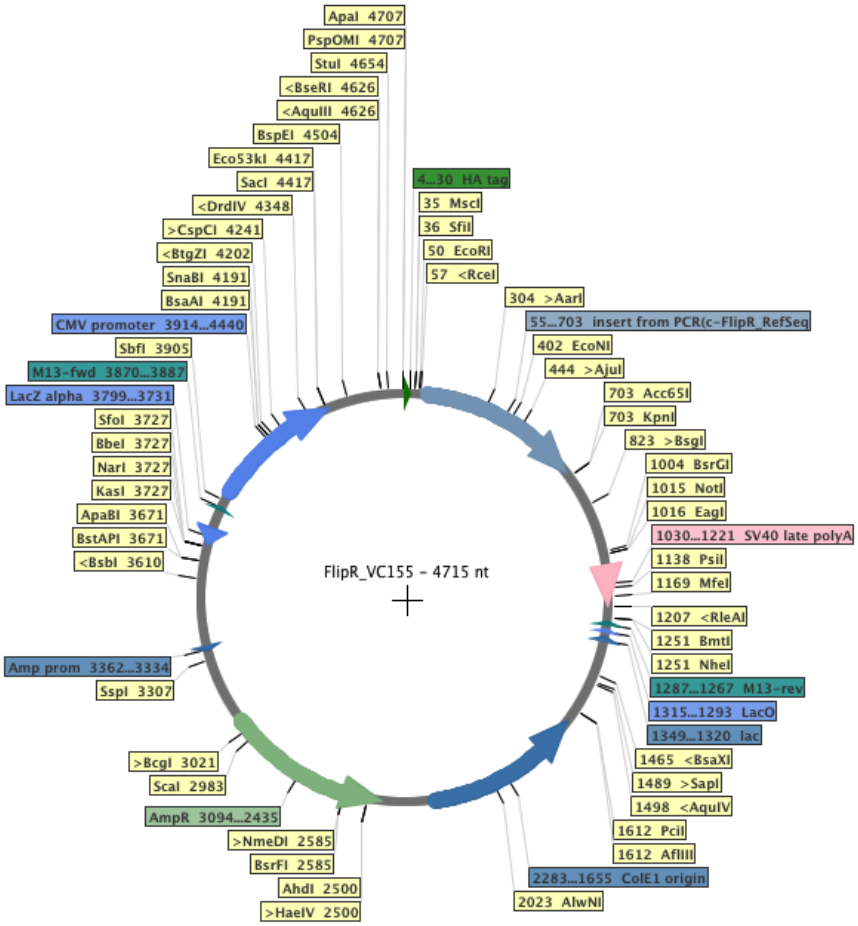

c-FLIP$_R$ in VC155

IX.

Dipl.-Biol. Tanja Telieps

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PERSONAL DATA:

Date / Place of birth: Oct 19th, 1979 / Goslar / Nationality: German/ gender: female

ACADEMIC PREPARATION:

University

2009- 2013	Otto-von-Guericke University of Magdeburg (Magedburg, Germany) and Helmholtz Centre for Infection Research (Brunswick, Germany)
2003-2009	Heinrich-Heine-University (Düsseldorf, Germany)
2007-2009	Member of research stuff – Children's Hospital Düsseldorf/ Medical Microbiology and Hospital Hygiene
2006-2007	Diploma Thesis: CD95-induced Apoptosis in Juvenile Idiopathic Arthritis (JIA); Children's Hospital/Medical Microbiology
2002	Intermediate diploma (Vordiplom)
2000-2003	Johannes Gutenberg University (Mainz; Germany)
1999-2000	University of Vienna (Vienna; Austria)

Students Exchange

1996-1997	East Valley High School Spokane (USA; ½ year)
2002-2003	Erasmus Exchange Scholarship; University Oulu (Finland)

(Higher) Secondary

1999 Abitur (Higher secondary school final exam) with main focus: Biology and English (Robert Koch Schule Clausthal-Zellerfeld; Germany)

LANGUAGES:

- Fluently English, German (oral / written)
- Some Finnish
  French (3-years school program)

RESEARCH INTERNSHIPS

- 2000 Thyssen Krupp VDM (Altena; Germany) Material Testing (6 weeks)
- 2004 University of Turku (Turku; Finland) Immunogentics of Type 1 Diabetes (2 months)

SCHOLARSHIPS:

- 2002-2003 Erasmus Exchange Scholarship; University Oulu
- 2004 Scholarship by the University of Turku (Finland) for internship (2 months)
- 2009-2011 Jürgen Manchot Scholarship
- 2011 ECDO Travel Grant to visit the annual European Cell Death Meeting
- 2012 (july - december) LOM scholarship Otto-von-Guericke University Magdeburg

AWARDS:

- 2006 Preis im Forschungsmeeting der 16. Jahrestagung für Kinder- und Jugendrheumatologie (GKJR) für den Beitrag „Defekte Caspase-8 Aktivierung innerhalb der CD95-vermittelten Apoptose bei Juveniler Idiopathischer Arthritis“ (award for the research meeting contribution: defective caspase-8 activation in CD95 induced apoptosis in juvenile idiopathic arthritis)

PUBLICATIONS

The role of c-FLIP splice variants in urothelial tumours. Ewald F, Ueffing N, Brockmann L, Hader C, **Telieps T**, Schuster M, Schulz WA, Schmitz I.Cell Death Dis. 2011 Dec 22;2:e245. doi: 10.1038/cddis.2011.131.

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Impaired CD95-mediated apoptosis in autoimmunity and occurrence of a p22 Caspase-8 cleavage product in JIA. Feyen O*, **Telieps T***, Schmitz I, Niehues T. Klin Padiatr. 2008 Nov-Dec;220(6):358-64. (*shared first author)

Concepts on the pathogenesis of juvenile idiopathic arthritis, Niehues T, Feyen O, **Telieps T**., Z Rheumatol. 2008 Mar;67(2):111-6, 118-20.

In revision at the European Journal of Immunology: c-FLIP$_R$ modulates cell death induction upon T cell activation and infection **Tanja Telieps***, Frida Ewald,*, Marcus Gereke, Michaela Annemann, Yvonne Rauter, Marc Schuster, Nana Ueffing, Dorthe von Smolinski, Achim D. Gruber , Dunja Bruder and Ingo Schmitz (*co first authors)

X. Declaration of Originality

Tanja Telieps
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Hiermit erkläre ich, dass ich die von mir eingereichte Dissertation zum Thema

CD95-induced Apoptosis in Health and Disease

Dimers at the DISC
and
The Effect of c-FLIP$_R$ on *L. Monocytogenes* Infection

selbständig verfasst, nicht schon als Dissertation verwendet habe und die benutzen Hilfsmittel und Quellen vollständig angegeben wurden.

Weiterhin erkläre ich, dass ich weder diese noch eine andere Arbeit zu Erlangung des akademischen Grades doctor rerum naturalium (Dr. rer. nat.) an anderen Einrichtungen eingereicht habe.

Magdeburg, den 19.03.2012 _____

Tanja Telieps