The Role of c-FLIP in the Regulation of Apoptosis, Necroptosis and Autophagy in T Lymphocytes

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

To maintain homeostasis, T lymphocytes die through caspase-dependent apoptosis. However, blockage of caspase activity in T lymphocytes does not increase cell survival. The loss of caspase 8 activity leads to programmed necrosis (necroptosis) upon T cell receptor (TCR) stimulation in T lymphocytes. Necroptosis is correlated with excessive macroautophagy, an intracellular catabolic process characterized by the sequestration of cytoplasmic compartments through double-membrane vacuoles. Meanwhile, the proper induction of macroautophagy is required for T lymphocyte survival and function. Cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) promotes survival in T lymphocytes. c-FLIP suppresses death receptor-induced apoptosis by modulating caspase 8 activation. Whether this modulation plays a role in the regulation of necroptosis has yet to be studied. Additionally, overexpression of c-FLIP reduces autophagy induction and promotes cell survival in cell lines. It remains unclear whether c-FLIP protects primary T lymphocytes by regulating the threshold at which autophagy occurs. In this study, c-FLIP isoform-specific conditional deletion models were used to study the role of c-FLIP in necroptosis and autophagy in primary T lymphocytes.

Our results showed that the long isoform of c-FLIP (c-FLIP$_L$) regulates necroptosis by inhibiting receptor interacting protein 1 (RIP-1). Upon TCR stimulation,

c-FLIP$_L$-deficient T cells underwent RIP-1-dependent necroptosis. Interestingly, though previous studies have generally described necroptosis in the absence of caspase 8 activity and apoptosis, pro-apoptotic caspase 8 activity and the rate of apoptosis were also increased in c-FLIP$_L$-deficient T lymphocytes. Moreover, c-FLIP$_L$-deficient T cells exhibited enhanced autophagy, which served a cytoprotective function.

Apoptosis can be induced by either death receptors on the plasma membrane (extrinsic pathway), or the damage of the genome and/or cellular organelles (intrinsic pathway). Previous studies in c-FLIP-deficient T lymphocytes suggested that c-FLIP promotes cell survival in the absence of death receptor signals. Independent of death receptor signaling, mitochondria sense apoptotic stimuli and mediate the activation of caspases. Whether c-FLIP regulates mitochondrion-dependent apoptotic signaling remains unknown. Here, by deleting the *c-Flip* gene in mature T lymphocytes, we showed a role for c-FLIP in the intrinsic apoptosis pathway. In naïve T cells stimulated with the apoptosis inducer, c-FLIP suppressed cytochrome c release from mitochondria. Bim-deletion rescued the enhanced apoptosis in c-FLIP-deficient T cells, while inhibition of caspase 8 did not. Different from activated T cells, there were no signs of necroptosis in c-FLIP-deficient naïve T cells. Together, our findings indicate that c-FLIP is a key regulator of apoptosis, necroptosis and autophagy in T lymphocytes.

Dedication

To my parents, for raising me to become the person I am today.

Contents

List of Tables

List of Figures

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1. Introduction

T lymphocytes face multiple checkpoints for life-or-death during their life span. Lymphoid precursor cells migrate from bone marrow to thymus, where they rearrange at the TCR loci and undergo positive selection and negative selection. Only T cell clones that are capable of responding to foreign antigens, but not self-reactive, survive and develop into mature $CD4^+$ and $CD8^+$ T lymphocytes (Palmer 2003). In the periphery, the resting T cells require interleukin-7 (IL-7) (Marrack et al. 2000) and tonic 'tickling' – the low-affinity engagement of TCR and self-peptide presented on the major histocompatibility complex (MHC) – for the maintenance (Rubtsov and Rudensky 2007). Once encountering specific antigens presented by antigen-presenting cells (APCs) with the appropriate co-stimulatory signals, T lymphocytes robustly proliferate and differentiate into populations of effector cells, including T helper cells (T_H cells), inducible regulatory T cells (iT_{reg}) and cytotoxic T lymphocytes (CTLs) (Johansson et al. 2008). The majority of the effector T cells are eliminated during the contraction phase, leaving only a small fraction of them to develop into the memory T cells - cells that are specialized to rapidly respond to the same antigen. The size of the peripheral T lymphocyte pool is critical for a functional adaptive immune system; as a diverse T-cell repertoire is needed for protection against pathogens, while the body can only host a limited number of cells in the blood and lymphoid organs (Jameson 2002). Therefore, cell death is necessary for the proper development and function of T lymphocytes.

Different types of cell death are observed in T lymphocytes. Much attention has been given to apoptosis, a programmed cell-death (PCD). Apoptosis is shown to be essential for the development, homeostasis and function of T lymphocytes (Zhang et al. 2005; Dunkle and He 2011). Another form of programmed cell death, necroptosis (programmed necrosis) is recently suggested to occur in activated T cells (Walsh and Edinger 2010). Additionally, several immunological studies revealed that the survival and proper function of T lymphocytes require autophagy, a conserved intracellular catabolic mechanism (Pua and He 2009a). These three processes, as well as their complex interplay, are essential for the proper T cell response in adaptive immunity.

1.1 Apoptosis in T lymphocytes

1.1.1 The mechanism of apoptosis

Biologists are so busy deciphering the myth of life that death has not been recognized as a regulated process until 1970s, when Kerr *et al*. characterized apoptosis: an active, programmed form of cell death with distinct morphological features, including nuclear condensation, cytoplasm shrinkage (pyknosis), cellular membrane blebbing, membrane-bound apoptotic body formation and subsequent engulfment by neighboring phagocytes (Kerr et al. 1972). During apoptosis induction, chromosomal DNA is initially degraded at the internucleosomal linker site to produce 50-200 kb DNA fragments. This process, termed apoptotic DNA fragmentation, is one of the biochemical hallmarks of apoptosis (Danial and Korsmeyer 2004). Apoptotic cells also redistribute

phosphatidylserine (PS) residues to the cell surface as an "eat me" signal (Fadok et al. 1998). However, under certain conditions PS exposure can be reversible, for instance in granulocytes and monocytes after the incubation of hypotonic 0.2% NaCl or NH_4Cl lysing solution used to remove red blood cells (Yang et al. 2002). Cells in the late stage of apoptosis *in vitro* eventually lose the plasma membrane integrity in the absence of co-cultured phagocytes. Therefore, the evaluation of apoptosis can be performed by combining the PS staining on the cell surface and the incorporation of vital dyes that are excluded in intact cells (i.e. 7-AAD).

1.1.1.1 Caspases

The execution of apoptosis requires the members of the cysteine aspartyl-specific proteases family, abbreviated as "caspases". Not all the members of caspase family are involved in apoptosis. For example, the first identified caspase, caspase 1 (also known as interleukin 1 converting enzyme, ICE), mediates pro-inflammatory pyroptosis in innate immunity (Bergsbaken et al. 2009). The caspases with pro-apoptotic activities in mammalian cells are divided into two groups based on their function: the initiator caspases (caspase 2, 8, 9, 10) and the effector caspases (caspase 3, 6, 7). All caspases are synthesized as catalytic inactive form (referred as zymogens). The proteolytic cleavages at the specific Asp residues lead to the release of a large (~20kD) and a small (~10kD) subunits, which then forms the active enzyme together (Cohen 1997). The initiator caspases undergo auto-proteolysis, but the intra-chain cleavage dose not greatly increase

their catalytic activity and may not be required for their activation (Stennicke et al. 1999). The full activation of initiator caspases generally requires the formation of a multi-component complex serving as the activating platform. For example, caspase 8 activation requires the formation of death–inducing signaling complex (DISC) and caspase 9 activation occurs at the multiple-component apoptosome (Krammer et al. 2007; Riedl and Salvesen 2007). Activated initiator caspases subsequently cleaves the effector caspases at the Asp residues to generate the active form of effector caspases (Riedl and Shi 2004). Upon activation, effector caspases amplify the magnitude of death signals by self-cleavage, and conduct a broad scaled proteolysis on "cell-death substrates".

The destruction of "cell-death substrates" by active effector caspases leads to the apoptotic morphology and eventually cell death. For instance, inhibitor of the caspase-activated DNase (ICAD) is cleaved by caspase 3 and 7 to allow the transportation of endonuclease to the nucleus for DNA fragmentation (Wolf et al. 1999). At the same time, DNA-dependent protein kinase (DNA-PK), an enzyme for double-strand-break repair, is inactivated as the catalytic subunit is cleaved by caspase 3 (Itoh and Horio 2001). Active caspase 3 also cleaves poly(ADP-ribose) polymerase (PARP) to cease DNA repair, as well as to spare the NAD^+ and ATP normally consumed by active PARP (Simbulan-Rosenthal et al. 1998; Boulares et al. 1999). Lamin A and C are cleaved by caspase 3 and 6, leading to chromatin condensation and nuclear shrinkage (Ruchaud et al. 2002;

Krammer et al. 2007). During apoptosis, messenger RNA splicing is blocked, as caspase 3 cleaves U1-70, the protein component of U1 small nuclear ribonucleoprotein particles for the processing of precursor mRNAs (Slee et al. 2001). The cleavage of cytoskeletal proteins, such as actin, gelsolin, fodrin and RAS homologue (RHO)-associated coiled-coil-containing protein kinase 1 (ROCK-1), results in plasma blebbing and the generation of apoptotic bodies (Cohen 1997; Mashima et al. 1999; Krammer et al. 2007). Past the point of no return, now the cell successfully commits suicide.

Based on the original trigger of caspase activation, two signaling cascades are termed: the intrinsic apoptotic pathway, which congregates at the outer membrane of mitochondria; and the extrinsic apoptotic pathway, which senses extracellular death signals. Though initiated and regulated by different factors, both pathways eventually lead to the cleavage and activation of effector caspases.

1.1.1.2 The intrinsic pathway

The intrinsic pathway is triggered by intracellular stimuli, such as DNA damage, endoplasmic reticulum (ER) stress, or growth factor deprivation. Mitochondrial outer membrane permeabilization (MOMP) is the crucial process of intrinsic pathway (Bender and Martinou 2013). MOMP is primarily regulated by the members of Bcl-2 family. The founder of this family, B-cell lymphoma-2 (Bcl-2) was first cloned at the chromosomal breakpoint in the t(14;18) translocation-bearing follicular B-cell lymphoma (Tsujimoto et al. 1984). Bcl-2 contains four conserved α-helical segments, named Bcl-2 homologous

domains (BH domains). Other Bcl-2 family members were identified by the sequence homology to BH domains (Gross et al. 1999). Based on the functional and structural differences, Bcl-2 family is further divided into three subfamilies: anti-apoptotic BH1-4 subfamily, pro-apoptotic BH1-3 subfamily and pro-apoptotic BH3-only subfamily (Figure 1A).

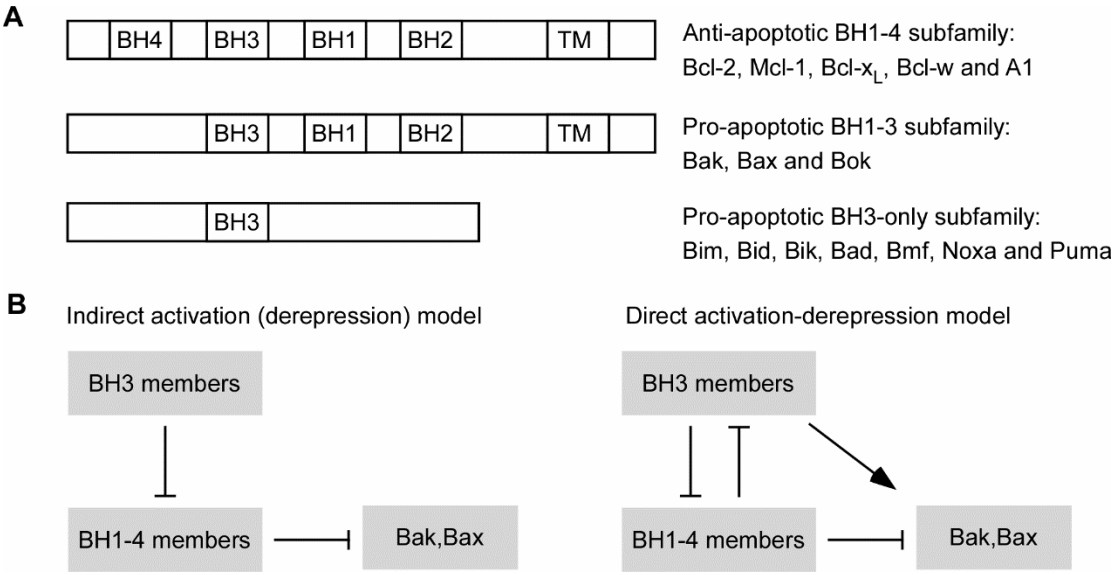

Figure 1: Bcl-2 family members

Figure 1. (A) Schematic structures of Bcl-2 family members. TM: transmembrane; Bcl-x_L: B-cell lymphoma-extra large; Mcl-1: myeloid cell leukemia-1; A1: Bcl-2-related protein A1; Bax: pro-apoptotic Bcl-2-associated X protein; Bak; Bcl-2-antagonist/killer-1; Bok: Bcl-2 related ovarian killer; Bim: Bcl-2-interacting mediator of cell death; Bid: BH3-interacting domain death agonist; Bik: Bcl-2-interacting killer; Bad: Bcl-2 antagonist of cell death; Bmf: Bcl-2-modifying factor; Noxa: Phorbol-12-myristate-13-acetate-induced protein 1; Puma: p53 upregulated modulator of apoptosis. (B) Two major models for the role of BH3-only members during apoptosis induction. The indirect activation model proposes that anti-apoptotic BH1-4 proteins constantly suppress Bax and Bak; and the competitive interactions of BH3-only proteins with anti-apoptotic BH1-4 proteins release Bax and Bak from the inhibition. Bax and Bak are auto-activated once they are unconstrained. The direct activation-depression model suggests that activation of Bax and Bak requires the aids of some BH3-only proteins. Anti-apoptotic BH1-4 proteins promote cell survival by inhibiting both Bax/Bak and pro-apoptotic BH3-only proteins.

BH1-3 subfamily members contain BH1, BH2 and BH3 domains and a transmembrane domain. Three proteins are in this group: Bax, Bak and Bok. Bax and Bak are broadly expressed in tissues and organs (Oltvai et al. 1993; Kiefer et al. 1995), while the expression of Bok is restricted to the reproductive system, including ovary, testis and uterus (Hsu et al. 1997). Bax and Bak are functionally redundant for apoptosis to occur during the embryonic development in mice (Lindsten et al. 2000). $Bak^{-/-}Bax^{-/-}$ fibroblasts show resistance to MOMP after multiple intrinsic apoptotic stimuli, including tBid transfection, apoptosis inducing drugs and ultraviolet (UV) radiation (Wei et al. 2001). During apoptosis, Bak and Bak are believed to undergo conformational change and oligomerize to facilitate cytochrome c release. Recent protein structure study has revealed that the BH3 domain of Bak is transiently exposed upon apoptotic stimuli and subsequently interacts with the hydrophobic groove of another activated Bak to form a symmetric dimmer (Dewson et al. 2008; Dewson and Kluck 2009). The same mechanism applies to the dimerization of Bax (Czabotar et al. 2013). The Bak or Bax dimmers likely associate with each other via other regions away from BH3:groove, forming a high-ordered oligomer (Dewson and Kluck 2009). The role of Bok in apoptosis is yet unclear. Though overexpression of Bok promotes apoptosis, Bok may not be a functional analogue to Bak and Bax, as enforced expression of Bok cannot restore apoptosis induction in the apoptosis resistant $Bak^{-/-}Bax^{-/-}$ fibroblasts (Echeverry et al. 2013).

Bax- and Bak-mediated MOMP allows the release of several pro-apoptotic proteins from the mitochondrial intermembrane space (IMS) to cytosol. Identified proteins that are released during MOMP are cytochrome c, second mitochondria-derived activation of caspase/direct IAP-binding protein with low pI (Smac/Diablo) and high temperature requirement protein A2 (Htra2/Omi) (Tait and Green 2010). In the cytosol, cytochrome c binds to apoptotic protease activating factor-1 (Apaf-1). This interaction leads to the structural alteration and oligomerization of Apaf-1, forming the apoptosome: a 1.4 MD seven-member caspase activating complex (Riedl and Salvesen 2007). The apoptosome recruits pro-caspase 9 through its caspase-recruitment domain (CARD) and serves as a reaction platform for caspase 9 activation. The active caspase 9 then proteolytically cleaves and activates effector caspases, such as caspase 3, 6 and 7. X-linked inhibitor of apoptosis protein (XIAP) inhibits the activation of caspase 3, 7 and 9 (Deveraux et al. 1999; Suzuki et al. 2001). Smac/Diablo and Htra2/Omi released from mitochondria block the activity of XIAP to promote the execution of apoptosis (Tait and Green 2010).

Anti-apoptotic BH1-4 subfamily contains Bcl-2, Bcl-w, Bcl-x_L, Mcl-1 and A1, which contain all four BH domains and a C-terminal transmembrane domain. The anti-apoptotic members generally inhibit Bax and Bak activation (Youle and Strasser 2008). In healthy cells, Bak locates on the outer membrane of mitochondria. Mcl-1 and Bcl-x_L constitutively interact with Bak via the BH3 domain, thus suppressing Bak activation

(Cuconati et al. 2003; Willis et al. 2005). Bax, on the other side, resides in the cytosol compartment as a monomer in the absence of death signals (Hsu and Youle 1998). In response to cellular stress, Bax is "semi-activated" and translocates to mitochondria, where its further oligomerization is suppressed by Bcl-2 (Korsmeyer et al. 1993; Ruffolo and Shore 2003). Therefore, the formation of Bak/Bax oligomer on the outer membrane of mitochondria requires the downregulation of anti-apoptotic BH1-4 proteins. Additionally, members of the anti-apoptotic BH1-4 subfamily protect cell from death beyond restraining Bax and Bak. Bcl-2 associates with both Apaf-1 and caspase 9, and Bcl-x_L inhibits Apaf-1-dependent activation of caspase 9 (Hu et al. 1998). Bcl-2 even provides protection independent of apoptosome, as overexpression of Bcl-2 promotes cell survival in *Apaf-1*$^{-/-}$ and *caspase-9*$^{-/-}$ cells (Ekert et al. 2004; Marsden et al. 2006).

BH3-only subfamily includes Bid, Bim, Bad, Bik, Bmf, Noxa and Puma. The BH3-only members act as sentinels instead of executioners in apoptosis induction. Most BH3 members inhibit anti-apoptotic Bcl-2 family members to promote apoptosis (Figure 1B). For example, Noxa interacts with Mcl-1, displays Bak and lifts the inhibition on Bak for apoptosis-inducing dimerization (Willis et al. 2005). Whether BH3-only proteins directly assist Bax/Bak during MOMP is debatable (Youle 2007). Chemically stabilized BH3 α-helices of Bim and Bid bind to Bax and trigger its activation (Walensky et al. 2006; Youle 2007). However, some studies showed that Bim, Bid and Puma are dispensable for

Bax/Bak-mediated apoptosis (Willis et al. 2007). The two models of BH3-only members promoting apoptosis are illustrated in Figure 1B.

1.1.1.3 The extrinsic pathway

The extrinsic pathway is initiated by the cell surface death receptors upon the ligation of their ligands. Death receptors (DRs) belong to the tumor necrosis factor receptor (TNFR) superfamily and contain a death domain (DD). DD locates in the cytoplasmic part of the DRs and mediates the recruitment of the adaptor protein Fas-associated via death domain protein (FADD) and/or TNFR type 1-associated death domain protein (TRADD) following trimetric ligand engagement (Locksley et al. 2001). Among the 8 homologous DRs identified in the TNFR superfamily, so far 6 of them are shown to be capable of inducing apoptosis. These DRs includes Fas (CD95), TNFR1, death receptor 3 (DR3), death receptor 6 (DR6), TNF-related apoptosis inducing ligand receptor 1 (TRAILR1/DR4), and TRAILR2 (DR5) (Tartaglia et al. 1991; Suda et al. 1993; Chinnaiyan et al. 1996; Schneider et al. 1997; Pan et al. 1998). It needs to be noted that DR signaling does not always lead to apoptosis. For example, apoptosis is prohibited once the engagement of TNFR1 to TNF-α successfully triggers NF-κB signaling and subsequent upregulation of anti-apoptotic proteins, such as c-FLIP (Wang et al. 1998; Micheau et al. 2001; Micheau and Tschopp 2003).

Stimulation of Fas initiates the formation of a membrane-bound receptor complex referred to as the death-inducing signaling complex (DISC) (Lavrik and

Krammer 2012). Upon Fas-FasL interaction, the cytoplasmic tail of Fas recruits cytosolic adaptor protein FADD (Chinnaiyan et al. 1995). FADD contains a C-terminal DD, which mediates its association with Fas, and a N-terminal death effector domain (DED), through which FADD recruits DED containing pro-caspase 8, pro-caspase 10 and c-FLIP (Kim 2002). The catalytic cleavage of pro-caspase 8 in DISC generates the two subunits of active caspase 8: p10 and p18 (Budd et al. 2006; Lavrik and Krammer 2012). Recently, a cytosolic Fas signaling complex is identified, which is termed complex II. Complex II does not contain surface receptor Fas but the rest components of DISC, and mediates the activation of caspase 8 as well (Lavrik et al. 2008). TRAILR1 and TRAILR2 engagements also lead to the formation of DISC, to which adaptor protein TRADD is recruited along with FADD (Schneider et al. 1997; Jin et al. 2004). c-FLIP directly regulates pro-caspase 8 and pro-caspase 10 activation at the DISC. The role of c-FLIP in death receptor-induced apoptosis will be introduced in details in 1.5.1

TNF-α/TNFR1 ligation-mediated apoptosis involves two sequential signaling complexes. The engagement of TNFR1 leads to trimerization of the receptors and recruitment of adaptor protein TRADD. Additionally, DD-containing Serine/threonine kinase receptor interacting protein 1 (RIP-1), TNF receptor-associated factor 2 (TRAF2) and cellular inhibitor of apoptosis protein-1 (c-IAP-1), c-IAP-2 are recruited. Together they form the plasma membrane associated complex I and activate NF-κB/JNK signaling (Wertz and Dixit 2010). TNFR1 is internalized after TNF-α ligation (Schutze et al. 1999).

In cytosol, pro-caspase 8 and pro-caspase 10 are recruited to TRADD and RIP-1, forming the apoptosis-inducing complex, termed complex II. NF-κB upregulates the expression of anti-apoptotic protein c-FLIP$_L$, which suppresses the formation of complex II. Therefore, whether TNFR1 induces NF-κB response or apoptosis depends on the cellular level of anti-apoptotic proteins (Micheau and Tschopp 2003).

1.1.1.4 The crosstalk of intrinsic pathway and extrinsic pathway

A long debate revolves around the question whether death receptor-induced apoptosis requires the Bcl-2 family member-controlled MOMP. One scenario is that DISC can activate the initiator caspases (caspase 8 and 10), while apoptosis cannot be executed because the cellular XIAP blocks the caspase 3 activation and maturation (Sprick and Walczak 2004). This augment is supported by the fact that the activation of caspase 8 does not always result in apoptosis, especially in the hematopoietic system (Galluzzi et al. 2008). The BH3-only protein Bid is shown to be the link of death receptor ligation-induced (extrinsic) and mitochondrion-dependent (intrinsic) apoptotic pathways. Bid is a specific substrate of active caspase 8 upon Fas-FasL ligation. The product after the cleavage, truncated Bid (tBid) translocates to the outer membrane of mitochondria, where it induces the release of cytochrome c (Li et al. 1998; Luo et al. 1998) and Smac/Diablo (Li et al. 2002). Bcl-2 and Bcl-x$_L$ regulates the function of Bid, as well as the release of Smac/Diablo from IMS (Li et al. 1998; Luo et al. 1998; Sun et al. 2002b). Though cytosolic Bid is shown to interact with Bax, caspase 8-cleaved tBid

preferentially utilizes Bak instead of Bax for triggering MOMP (Cartron et al. 2003). The crosstalk of extrinsic and intrinsic apoptotic pathways is illustrated in Figure 2.

Though death receptor signaling can lead to cytochrome c release and apoptosome formation, apoptosome is not always required for the execution of DR-induced apoptosis. For example, thymocytes derived from *Apaf-1*$^{-/-}$ and *caspase 9*$^{-/-}$ mice are not resistant to Fas-induced apoptosis (Hakem et al. 1998; Yoshida et al. 1998). Caspase 8/Bid also mediates the release of Smac/Diablo, which antagonizes the XIAP to remove its inhibition on caspase 3 and 7 (Chai et al. 2000; Du et al. 2000; Srinivasula et al. 2000; Verhagen et al. 2000). Therefore, Bid is probably essential for death receptor-mediated apoptosis in cells with high level of anti-apoptotic protein XIAP. Based on the requirement of Bid during Fas-induced apoptosis, cells are divided into two groups: type I cells that can be killed by Fas signal in the absence of Bid, and type II cells that requires Bid for Fas-mediated apoptosis (Kaufmann et al. 2012).

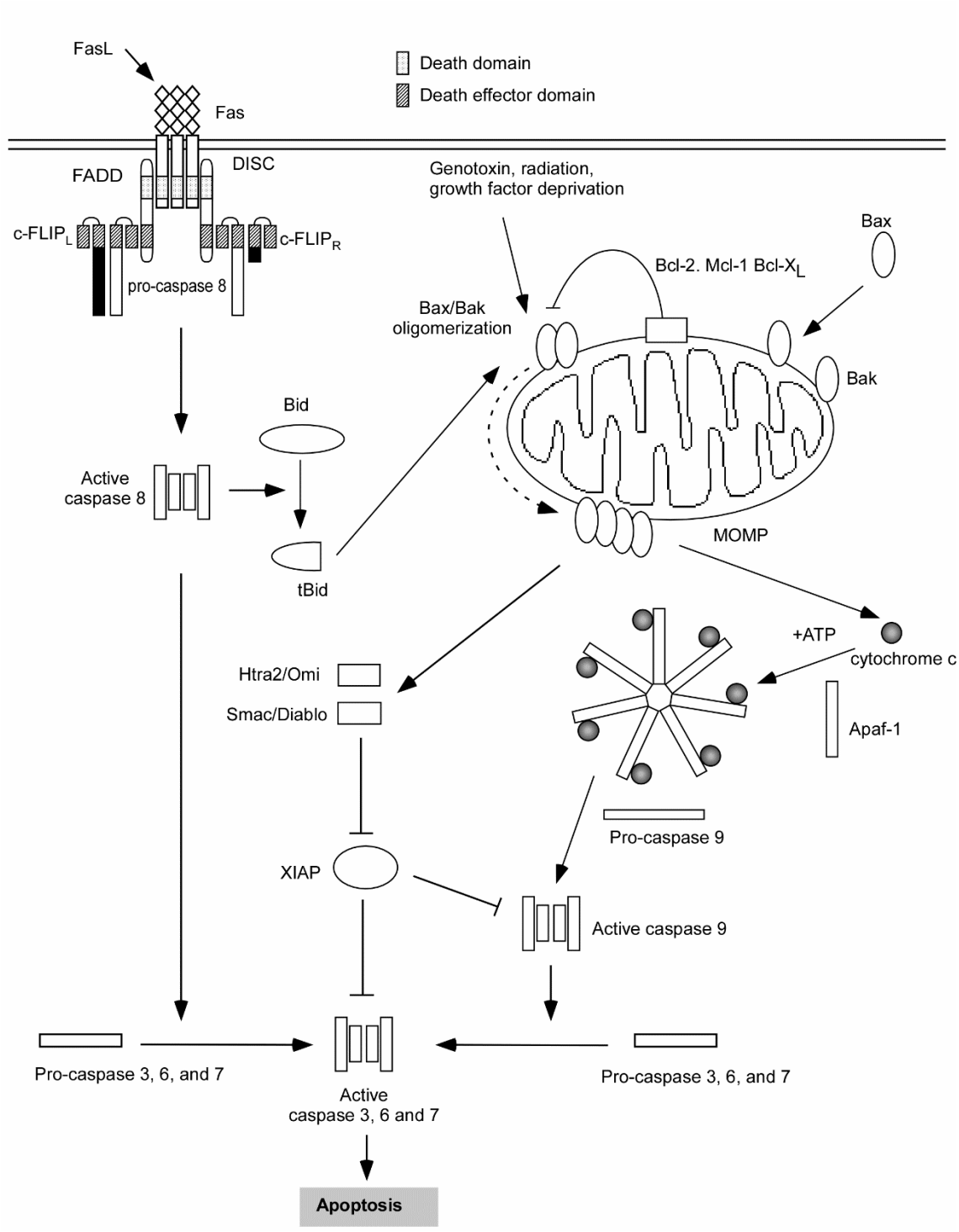

Figure 2: The interplay of intrinsic and extrinsic apoptotic pathways.

Figure 2. The intrinsic pathway is initiated by cellular stress or damage to the genome. Upon death stimuli, cellular Bax Translocates to the mitochondria, where Bax and Bak oligomerize to induce MOMP. Cytochrome c, Smac/Diablo and Htra2/Omi are then released from the intermembrane space of mitochondria. Cytochrome c binds to cytosolic Apaf-1 and changes the structure of Apaf-1 with the aids of dATP. Consequently, the seven-member apoptosome is formed, which leads to the activation of caspase 9. The extrinsic pathway is triggered by death receptors on the plasma membrane. FasL-Fas interaction triggers the formation of death-inducing signaling complex (DISC), which serves as the docking site for the self-cleavage and activation of caspase 8. TRAIL-DR4/DR5 ligation initiates similar death signaling, though TRADD is also recruited to DISC (not shown in this figure). The TNF-α/TNFR1 association may also lead to apoptosis, which involves the formation of the intracellular complex II (illustrated in Figure 3). Active caspase 8 cleaves pro-apoptotic protein Bid. Truncated Bid relocates to mitochondria and promotes MOMP. Both active caspase 8 and caspase 9 cleave the effector caspases: caspase 3, 6 and 7. Active caspase 3, 6 and 7 execute the morphological and biochemical alterations for apoptosis. The activation of caspase 9 and caspase 3/7 are inhibited by XIAP. Smac/Diablo released from mitochondria blocks the activity of XIAP to promote apoptosis (To simply the illustration, caspase 10 and c-FLIP$_S$, which are expressed in human but not in mice, are not shown in this figure).

1.1.2 Apoptosis in T cell homeostasis

1.1.2.1 Apoptosis in thymocyte development

Death is not only a high cost but also a necessity of living, especially in the immune system. Apoptosis is required for the proper thymocyte development. Reconstituted mice with *Bak*$^{-/-}$*Bax*$^{-/-}$ hematopoietic cells show disrupted development and selection of thymocytes. *Bak*$^{-/-}$*Bax*$^{-/-}$ thymocytes are resistant to both death-by-neglect and antigen receptor-induced apoptosis (Rathmell et al. 2002). Similarly, Bim-deficient mice showed autoimmunity caused by defective negative selection in thymocytes (Bouillet et al. 1999).

In the development battlefield of the thymus, thymocytes actively upregulate and utilize different anti-apoptotic Bcl-2 family members to pass the "checkpoint" at different developmental stages. For example, Bcl-2-deficiency leads to the blockage in the progression from double negative (DN) to double positive (DP) stage in mice older than approximately three weeks (Nakayama et al. 1993; Veis et al. 1993). The reason for the age-related requirement of Bcl-2 in thymocyte development may be attributed to the shift from fetal liver-derived to bone marrow-derived progenitors and their different dependence on Bcl-2 (Matsuzaki et al. 1997). Bcl-x_L promotes the survival of double DP thymocytes, though loss of *Bcl-x* gene does not lead to defective thymocyte development, but only reduced cellularity in thymus (Zhang and He 2005a). Mcl-1 is required for thymocytes development and survival at both DP and single positive (SP)

stage (Opferman et al. 2003; Dzhagalov et al. 2008). Genetic study revealed that Bcl-x_L and Mc-1 function redundantly in the development of DP thymocytes. Deletion of either *Mcl-1* or *Bcl-x* at the late DN to DP stage (through CD4-Cre) marginally impairs the cellularity of DP thymocytes. However, *Bcl-x$^{f/f}$ Mcl-1$^{f/f}$* CD4-Cre$^+$ mice display a severe reduction in DP compartment (Dzhagalov et al. 2008). On the other side, overexpression of Bcl-2 fails to rescue the thymocyte developmental defect in the T-cell specific Mcl-1-deficient mice (Dunkle et al. 2010), which suggests Mcl-1 and Bcl-2 function in different pathways in the regulation of thymocyte development.

1.1.2.2 Apoptosis in T cell maintenance

Naïve T cells are long-lived resting cells that generally do not proliferate. The quiescence of naïve T cells is regulated by the transcription factor, lung Kruppel-like factor (LKLF), which is induced during the maturation of SP thymocytes (Kuo et al. 1997). Though naïve T cells do not undergo clonal expansion, the maintenance of peripheral T cells requires the transient, low affinity engagement of TCR with self-ligands presented on MHC. In the absence of MHC-II or MHC-I molecule, periphery CD4$^+$ or CD8$^+$ T lymphocytes fail to survive, respectively (Kirberg et al. 1997; Nesic and Vukmanovic 1998). Cytokine IL-7 is also required for the survival of naïve T cells (Fry and Mackall 2001). It is generally believed that Bcl-2 family proteins are the major downstream effector molecules regulated by IL-7 (Zhang et al. 2005). IL-7 signaling promotes cell survival by upregulating the expression of anti-apoptotic protein Bcl-2

and Mcl-1 (Kim et al. 1998; Opferman et al. 2003), as well as downregulating the expression of Bax and Bad (Kim et al. 1998; Khaled et al. 2002; Li et al. 2004). Bim is also the sensor of the IL-7 signal. Deletion of Bim partially rescues the death phenotype driven by IL-7 deprivation in peripheral T cells. IL-7 withdrawal does not alter the transcription or protein level of Bim, but alters the association of Bim with Mcl-1 (Li et al. 2010). Additionally, stress-induced apoptosis is also mediated by Bcl-2 family members in T lymphocytes. Puma and Bim contributes significantly to apoptosis induction in response to γ-radiation and glucocorticoid in T cells (Erlacher et al. 2005).

Different anti-apoptotic Bcl-2 family members function differently in T cells. Bcl-2 and Mcl-1 are required for the survival of mature T lymphocytes (Nakayama et al. 1993; Veis et al. 1993; Opferman et al. 2003; Dunkle et al. 2011). Mcl-1 protects cell survival by antagonizing Bak in the presence of IL-7 in resting T lymphocytes. During IL-7 deprivation, Bcl-2 and Mcl-1inhibit the BH3-only protein Bim to promote cell survival (Dunkle et al. 2011). Bcl-x_L, however, is dispensable for the survival of primary T cells (Zhang and He 2005a).

1.1.2.3 Apoptosis in activated T lymphocytes: AICD and ACAD

While naïve T cells are generally resistant to apoptosis stimuli except for IL-7 deprivation, activated T cells are prone to apoptosis. The different threshold of sensitivity to apoptosis induction is required for a functional adaptive immune system. After clonal expansion, the majority of activated T cells need to be eliminated to

maintain T cell homeostasis, as well as to avoid excessive inflammation and autoimmunity. Defective apoptosis in activated T cells can be very problematic. For example, the *Bak*$^{-/-}$*Bax*$^{-/-}$ T cells in periphery show reduced apoptotic rate upon endogenous superantigen-induced elimination, which leads to accumulated memory T cells in the spleen and splenomegaly (Rathmell et al. 2002). Activated T cells that are restimulated in the absence of appropriated co-stimulatory signals promptly die through apoptosis, a process known as activation-induced cell death (AICD). *In vitro*, AICD can be mimicked by restimulating TCR-activated T cells by anti-CD3 or PMA/ionomycin in the presence of IL-2 (Krammer et al. 2007). AICD is majorly mediated by death receptor ligation. A death receptor-independent, autonomous apoptosis also occurs in activated T cells. This type of cell death is caused by the lack of appropriate survival signals and termed activated cell autonomous death (ACAD) (Krammer et al. 2007).

AICD usually involves the signals from death receptors, including Fas, TNFR1, TRAILR1 and TRAILR2 (Krammer et al. 2007). Fas plays a critical role in maintaining lymphocyte homeostasis via AICD (Strasser et al. 2009). Human carrying dysfunctional Fas develop a lymphoproliferative autoimmune disease. Similar phenotype is observed in *lpr*/*lpr* mice, a mutant in which functional Fas is not expressed (Watanabe-Fukunaga et al. 1992; Rieux-Laucat et al. 1995). Restimulation of already activated T cells leads to the upregulation of FasL and Fas-dependent apoptosis (Green et al. 2003). Meanwhile, IL-2 signaling downregulates the cellular level of c-FLIP protein, the cellular inhibitor of

death receptor signaling, to sensitize activated T cells to Fas-mediated apoptosis (Algeciras-Schimnich et al. 1999). BH3-only protein Bim is also involved in the execution of AICD. T cells upregulate the expression of Bim after polyclonal or specific TCR stimulation, and the blockage of Bim expression rescues T cells from AICD (Sandalova et al. 2004). *In vivo* study showed that Fas and Bim mediated the $CD8^+$ T cell contraction concurrently. *Bcl2l11*$^{-/-}$*lpr/lpr* mice showed 100 fold more antigen-specific memory T cells than wild type mice after lymphocytic choriomeningitis virus (LCMV) infection (Weant et al. 2008). Mice lacking both Bim and Fas developed systemic lupus erythematosus (SLE)-like phenotype by 16 weeks of age, which is much earlier than the onset of autoimmunity in Bim-deficient or *lpr/lpr* mice. The autoimmunity in *Bcl2l11*$^{-/-}$*lpr/lpr* mice is mediated not only by the apoptosis-resistant T lymphocytes, but also overactive APCs (Hutcheson et al. 2008).

ACAD is mediated by the pro-apoptotic Bcl-2 family members. Though activated T cells do not alter the protein level of Bim, Bim is the major mediator of apoptosis in activated T cells in the absence of Fas or TNFR1 (Hildeman et al. 2002). Bim is prohibited by Bcl-2 and Bcl-x_L in healthy cells, while in activated T cells Bim is preferentially associated with Bcl-x_L, but not Bcl-2 (Zhu et al. 2004). The role of IL-2 is complicated in the survival of activated T cells. While IL-2 signaling contributes to AICD by downregulating anti-apoptotic protein c-FLIP, IL-2 deprivation leads to the upregulation of Puma and ACAD (You et al. 2006).

Altogether, apoptosis plays an essential role in the T cell development and homeostasis. Interestingly, caspase 8- and FADD-deficient T cells, in which the extrinsic apoptotic pathway is impaired, do not show prolong survival or accumulate in the periphery. Instead, they are eliminated promptly upon TCR stimulation (Osborn et al. 2010; Ch'en et al. 2011). A type of caspase-independent programmed cell death is observed in those cells, which will be introduced in details in the following section.

1. 2 Necroptosis in the immune system

Since the identification of apoptosis, "necrosis" has been considered as a passive way of cell death, an accidental consequence of "intolerable" micro-environmental stress. Morphologically, necrosis is characterized by the increased cell volume (oncosis), swelling of the organelles, lack of modifications of the nucleus and the ultimately disruption of the plasma membrane prior to phagocytosis (Kroemer et al. 2009; Vandenabeele et al. 2010). Recently, data emerged to show that necrosis can be associated with a sequence of controlled intracellular events including increased production of reactive oxygen species (ROS), ATP depletion, peri-nuclear clustering, and activation of specific proteases, such as calpains and cathepsins (Golstein and Kroemer 2007). In 2000, Holler et al. described a type of RIP-1-dependent necrotic cell death in human and murine T lymphocytes. Normally, FasL-Fas ligation induces caspase-dependent apoptosis. However, pan-caspase inhibitor z-VAD-fmk treatment on T cells triggers severe cell death. The dying cells showed none of the typical apoptotic

signatures, such as chromatin condensation or oligonucleosomal DNA fragmentation, but necrotic morphological features, such as early plasma membrane damage and shortened time frame between phosphatidylserine exposure on the surface and membrane rupture. This caspase-independent cell death requires RIP-1, which is recruited to the DISC (Holler et al. 2000). Similar phenotypes are detected in other experimental systems, usually when caspase activities are demolished. The term "necroptosis" is proposed then to describe this regulated, serine/threonine kinase RIP-1-dependent necrosis to distinguish it from fortuitous necrosis caused by non-physiological stress, such as base/acid treatment and freeze-thaw circles (Galluzzi and Kroemer 2008; Kroemer et al. 2009).

1.2.1 The mechanism of necroptosis

1.2.1.1 Initiation of necroptosis: plasma membrane bound and cytosolic receptors

Necroptosis can be triggered by the ligation of death receptor to their ligands on plasma membrane. In wild type cells, death receptor ligation almost always triggers the cascades that mediate apoptosis (though apoptosis may not be executed in the end). However, when caspase activities are suppressed, either through addition of caspase inhibitors or genetic deletion of certain molecules in the caspase-activating pathway, ligation of death receptors causes the regulated cell death featuring a necrotic morphology. Several death receptors can trigger necroptosis, including Fas, TNFR-1, TNFR-2, TRAILR-1, and TRAILR-2 (Vercammen et al. 1997; Holler et al. 2000; Los et al.

2002; Chan et al. 2003; Vanlangenakker et al. 2011). Though TNFR2 does not contain a cytoplasmic death domain, it mediates necroptosis through degrading TRAF2 to enhance the death signal of TNFR1 (Chan and Lenardo 2000). Transcription inhibitors, such as actinomycin D (ActD) also sensitized cells to TNF-α-triggered necroptosis (Vercammen et al. 1997; Los et al. 2002). Transcriptional inhibition may promote necroptosis by blocking the synthesis of protective molecules, which will be introduced in details in 1.2.1.3.

Pathogen recognition receptors (PRRs) can also initiate necroptosis. Similar death receptor-induced necroptosis, necroptosis induced by PPRs requires a second "inhibitory signal", which switches the cells from "default" survival/apoptosis to necrotic cell death. Caspase inhibitor z-VAD-fmk sensitizes mouse fibroblasts and macrophage to RIP-3-dependent necrosis when Toll-like receptor 3 (TLR3) is activated by double strand RNA (dsRNA) or poly (I:C) (Li and Beg 2000; Kalai et al. 2002; He et al. 2011). TLR3 activation also leads to necroptosis in cells lacking caspase 8, FADD or functional c-IAPs (Kalai et al. 2002; Feoktistova et al. 2011). TLR4 ligand lipopolysaccharide (LPS) induces RIP-1/RIP-3-dependent necroptosis in z-VAD-fmk-treated macrophages, especially when NF-κB activation is blocked (Ma et al. 2005; He et al. 2011). Cellular ribonucleic acid sensor retinoic acid-inducible gene 1 (RIG-I) is a potential necroptosis initiator. Caspase 8-mediated cleavage of the RIP-1 protein is

mediated by RIG-I signaling (Rajput et al. 2011). In the absence of caspase activity, the inhibition on RIP-1 may be lost and necroptosis may be provoked.

In addition, some types of cells are very susceptible to necroptosis. Pan-caspase inhibitor z-VAD-fmk treatment induces necroptosis in L929 cells in the absence of death receptor or PRR ligation (Yu et al. 2004). Certain viruses are capable of encoding caspase inhibitor to induce necroptosis and inhibit apoptosis in infected cells, such as CrmA encoded by cowpox and B13R encoded by vaccinia virus (Ray and Pickup 1996; Chan et al. 2003; Oberst et al. 2011).

1.2.1.2 The necrosome: RIP-1 and RIP-3

Serine/threonine kinase receptor interacting protein 1 (RIP-1/RIPK1) was first identified in the yeast two-hybrid screening as a binding protein for the intracellular domain of Fas (Stanger et al. 1995). RIP-1 contains a death domain, a RIP-1 homotypic interaction motif (RHIM) and a protein kinase domain. Through its death domain, RIP-1 is recruited to death receptor signaling complex. RIP-1 is dispensable for apoptosis induction (Hsu et al. 1996; Ting et al. 1996; Dohrman et al. 2005a), but required for the pro-survival NF-κB signaling induced by TNFR1 (Ting et al. 1996; Meylan et al. 2004) *Rip-1*$^{-/-}$ newborn mice died 1 to 3 days post-birth, due to extensive apoptosis in lymphocytes and adipocytes (Kelliher et al. 1998). However, emerging data suggested that RIP-1 is also a pro-death factor, as the kinase activity of RIP-1 mediates necroptosis (Degterev et al. 2008). The pro-survival NF-κB inducing activity and the pro-necroptotic

kinase activity of RIP-1 are likely to be independent, as attenuated NF-κB activation has no impact on TNF-α-induced necroptosis in L929 cells (Vanlangenakker et al. 2011).

The posttranslational modification on RIP-1 is the decision-maker of its function. RIP-1 can be phosphorylated and ubiquitinated in response to TNF-α. The kinase activity of RIP-1 is not the requisite factor of its ubiquitination (Lee et al. 2004). In TNFR1 complex I, c-IAP is stabilized by TRAF2 and mediates the ubiquitination of RIP-1 (Bertrand et al. 2008; Csomos et al. 2009). Poly-ubiquitination at Lsy377 transforms RIP-1 to the docking site for NF-κB essential modulator (NEMO), the regulator subunit of the IκB kinase complex (Ea et al. 2006). Ubiquitinated RIP-1 also activates transforming growth factor-β-activated kinase 1 (TAK1) (Lee et al. 2004; Hacker and Karin 2006). The regulatory molecule of TAK1, TAK-binding protein 2 (TAB2) associates with ubiquitinated RIP-1 upon TNF-α stimulation (Kanayama et al. 2004). This recruitment leads to the formation of NF-κB activating TAK1-TAB2-TAB3 complex following TNF-α ligation (Wertz and Dixit 2010). The Lys377 is the critical ubiquitination site for RIP-1. Mutation of this residue to Arg abolishes TNF-α-dependent RIP-1 ubiquitination and NF-κB signaling (Li et al. 2006).

The Lys 63-linked polyubiquitin moieties on the Lys377 of RIP-1 prohibit the association of RIP-1 to caspase 8 (O'Donnell et al. 2007). Cylindromatosis (CYLD) and A20 mediate the deubiquitination of RIP-1 (Harhaj and Dixit 2012), allowing the deubiquitinated RIP-1 to recruit pro-caspase 8 (Rajput et al. 2011). Deubiquitinated RIP-1

may recruit TRADD, FADD and pro-caspase 8 to form the death-inducing complex II, in which RIP-1 either undergoes proteolytic cleavage by caspase 8 or recruits RIP-3 to initiate necroptotic signaling. CYLD is shown to be a requisite factor for necroptosis induction (Hitomi et al. 2008; O'Donnell et al. 2011). The role of A20 in the regulation of necroptosis remains unclear. One study showed that A20 suppresses cell death in TNF-α treated L929 cells (Vanlangenakker et al. 2011). As A20 is also a negative regulator of apoptosis, whether A20 regulate necroptosis in this experimental setting has yet to be tested.

The kinase activity of RIP-1 is dispensable for NF-κB activation, but essential for its pro-necrosis activity (Holler et al. 2000; Lee et al. 2004; Degterev et al. 2005). RIP-1 undergoes autophosphorylation *in vitro* when it is overexpressed and immunoprecipitated (Ting et al. 1996). The detected autophosphorylation sites of RIP-1 all locate in its N-terminal kinase domain, which indicates a regulatory role of phosphorylation in RIP-1 kinase activity (Degterev et al. 2008). Besides auto-phosphorylation, RIP-1 can also be phosphorylated by RIP-3 (Cho et al. 2009). Three RIP-1 kinase inhibitors have been identified: necrostatin-1 (Nec-1), necrostatin-3 and necrostatin-5. Necrostatin-1 allosterically inhibits the kinase activity of RIP-1 *in vitro*. Though necrostatin-3 and necrostatin-5 are structurally quite different from necrtostatin-1, they are as potent inhibitors for immunoprecipitated RIP-1 as necrostatin-1. Necrostatin-3 directly inhibits RIP-1, while necrostatin-5 is likely to indirectly suppress

the kinase activity of RIP-1 through interacting with other components associated with RIP-1 in the cell (Degterev et al. 2005; Teng et al. 2005; Degterev et al. 2008).

Some studies showed that RIP-1 is a negative regulator for apoptosis. Knockdown of RIP-1 in TNF-α-treated L929 cells switches the TRADD-independent necroptosis to TRADD-dependent apoptosis. This effect is RIP-1 specific, as knockdown of RIP-3 only protects L929 cells from TNF-α-induced necroptosis and does not lead to apoptosis (Vanlangenakker et al. 2011). Similarly, knockdown of RIP-1 sensitizes TRAIL-resistant cell lines to apoptosis. Caspase 8-dependent cleavage of RIP-1 is observed in TRAIL-sensitive but not TRAIL-resistant pancreatic cancer cells (Wang et al. 2007). However, it has yet to be examined whether the cleavage of RIP-1 influences apoptosis induction or it is merely a secondary effect of highly active caspase 8 upon TRAIL ligation. The relationship of RIP-1 and apoptosis is further complicated by the discovery of "Ripoptosome", which requires the kinase activity of RIP-1 for its formation and provokes either apoptosis or necroptosis in the cells (Feoktistova et al. 2011; Tenev et al. 2011).

The dual role of RIP-1 in both pro-survival and pro-death signaling suggests that RIP-1 associate with and is regulated by distinct molecules in different signaling pathways. Genome RNA interference screening revealed that RIP-3 is required for TNF-α-induced necroptosis in the presence of c-IAP antagonist or caspase inhibitor z-VAD-fmk (He et al. 2009; Zhang et al. 2009). RIP-3 interacts with RIP-1 through its C-terminal

RHIM and phosphorylates RIP-1 (Sun et al. 2002a). Phosphorylated RIP-1 in turn phosphorylates RIP-3. The assembly of RIP-1-RIP-3 complex stabilizes the binding of RIP-1 to TNF-α signaling complex II and mediates necroptosis (Cho et al. 2009). The acetylation of Lys 530 on RIP-1 inhibits the RIP-1/RIP-3 interaction and consequent necroptosis. NAD-dependent deacetylase sirtuin-2 (SIRT2) constitutively binds to RIP-3 and deacetylates RIP-1 to permit TNF-α-induced necroptosis (Narayan et al. 2012).

The interaction between RIP-1 and RIP-3 shifts the fate of the cell from apoptosis/survival to necroptosis. This necrosis-inducing complex may also contain FADD, caspase 8, c-FLIP and TRADD. As the assembly of RIP-3 containing complex is the critical step for necroptosis induction, it is termed "necrosome" to be distinct from other complexes that function in death receptor-mediated apoptosis or NF-κB signaling (Declercq et al. 2009). The induction of necrosome by death receptors and T cell receptor (introduced in 1.2.3) is illustrated in Figure 3.

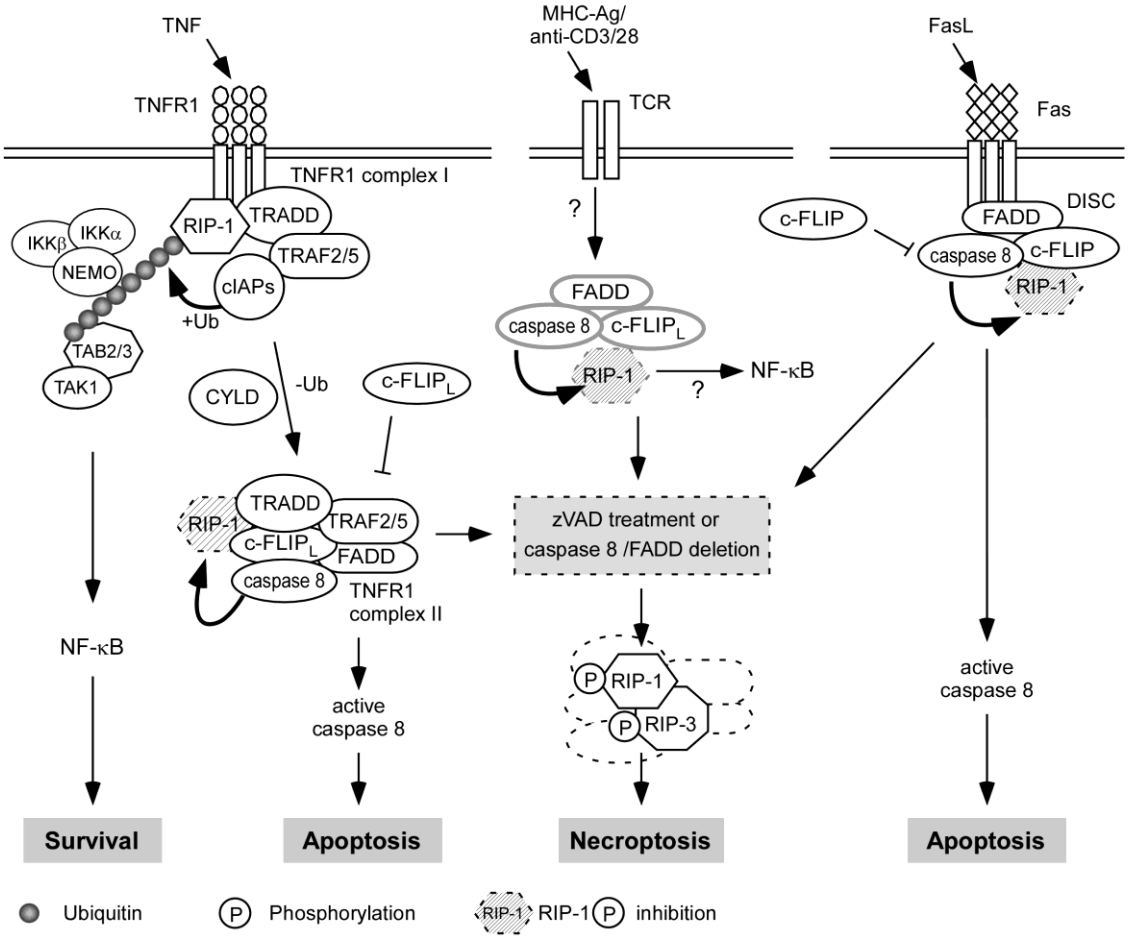

Figure 3: Necroptosis induction by TNFR1, Fas and TCR

Figure 3. TNFR1, Fas or TCR stimulation can induce necroptosis. TNFR1 interaction with TNF-α can lead to one of the three outcomes. The plasma membrane associated complex I leads to RIP-1 ubiquitination and NF-κB activation. A death-inducing complex II will be formed if c-FLIP$_L$ and c-IAPs are insufficient to prohibit its formation. Complex II leads to the inhibition of RIP-1 ubiquitination and activation of caspase 8. When caspase 8 activity is impaired, the repression on the phosphorylation of RIP-1 is withdrawn. Consequently, RIP-3 is recruited to the death-inducing complex and phosphorylated RIP-1. Phosphorylated RIP-1 and RIP-3 form the stable necrosome and induce necroptosis. Fas ligation leads to the formation of DISC, which subsequently activates caspase 8. Active caspase 8 triggers downstream apoptotic signaling and apoptosis. When caspase 8 activity is absent, necroptosis can be induced by necrosome. In T lymphocytes, caspase 8 is required for the inhibition of RIP-1-dependent necroptosis and activation of NF-κB signaling upon TCR stimulation. All the isoforms of c-FLIP proteins inhibit caspase 8 activity, and c-FLIP$_L$ regulates the apoptosis/necroptosis-inducing complex II formation.

RIP-3 can mediate necrosis in the absence of RIP-1. RIP-3-mediated necrosis is found in TNF-α treated L929 cells when both caspase 8 and RIP-1 were knocked down (Vanlangenakker et al. 2011). Similarly, MCMV infection leads to RIP-1-independent necrosis, which requires the expression of RIP-3 and DNA-dependent activator of interferon regulator factors (DAI, also known as ZBP1 or DLM-1) (Upton et al. 2010; Upton et al. 2012).

1.2.1.3 The regulation of necroptosis

Though necroptosis is an active cellular process, the induction of necroptosis generally requires the loss of inhibition to RIP-1 and/or RIP-3. Several regulators participate in this process. The most well studied negative regulator of necroptosis is caspase 8. Necroptosis can be induced upon the loss of caspase 8 activity, either through gene ablation or addition of pharmacological inhibitors (Vandenabeele et al. 2010). Caspase 8-deficient mice are embryonic lethal between E10.5 and E11.5. This defect is attributed to unregulated necroptosis, as crossing to *Rip-3*$^{-/-}$ background completely rescues the defective development of *caspase-8*$^{-/-}$ embryos. *Rip-3*$^{-/-}$*caspase-8*$^{-/-}$ mice are viable and can grow into fertile adults (Kaiser et al. 2011; Oberst et al. 2011).

Previous studies suggested that caspase 8 directly cleaves RIP-1 and RIP-3. Full length pro-caspase 8, instead of active p18/p10 dimer, is the functional anti-necroptotic molecule (Leverrier et al. 2011; Oberst et al. 2011). In HeLa cells treated with TNF-α and cycloheximide (CHX), caspase 8 cleaves RIP-1 at Asp 324. The cleavage product, 42 kD

RIPc, enhances TRAFF and FADD association and apoptosis induction. The same cleavage of RIP-1 is also detected in anti-Fas treated Jurkat cells and TRAIL treated HeLa cells, though it shows no influence on FADD recruitment (Lin et al. 1999). In murine cells, caspase 8 cleaves RIP-1 to generate a 38kD fragment upon ribonucleic acid sensor RIG-I activation. The truncated form of RIP-1 functions as a dominant-negative inhibitor in RIG-I-induced activation of transcription factor IRF3 (Rajput et al. 2011). However, it has yet to be studied whether the spliced fragment of RIP-1 has an inhibitory role in necroptosis induction. RIP-3 is cleaved by caspase 8 at Asp328 in response to possible necroptosis-inducing stimuli, including TRAIL, TNF-α/CHX and etoposide (Feng et al. 2007). De-ubiquitining enzyme CYLD, a molecule required for necroptosis induction, is another identified substrate of caspase 8. Caspase 8 proteolytically cleaves CYLD at Asp215 to suppress CYLD activity in TNF-α treated cells (O'Donnell et al. 2011).

The anti-necroptotic role of c-IAPs was revealed using Smac mimetics. A number of small-molecular Smac mimetics were originally designed to inhibit XIAP, but later shown to function as c-IAP antagonist by inducing auto-ubiquitination and proteasome-mediated degradation of c-IAPs (Gaither et al. 2007; Petersen et al. 2007; Varfolomeev et al. 2007; Vince et al. 2007; Bertrand et al. 2008). Upon TNF-α stimulation, c-IAPs serve as the direct E3 ligase to catalyze poly-ubiquitination of RIP-1. Loss of c-IAP leads to the reduced ubiquitination of RIP-1. Deubiquitinated RIP-1 consequently disassociates from TAK1 and recruits caspase 8 and FADD to form the death-inducing complex II (Petersen

et al. 2007; Bertrand et al. 2008). This deubiquitination may be mediated by enzyme CYLD (Wang et al. 2008). c-IAP antagonists were also shown to sensitize cells to Fas-induced apoptosis and necroptosis (Geserick et al. 2009). Since Fas usually only induces apoptosis in un-manipulated cells, it is likely that c-IAPs restrain RIP-1 kinase activity so necroptosis cannot be triggered. Smac mimetic BV6 primes RIP-1-dependent necroptosis in apoptosis resistant FADD- and caspase 8-deficient Jurkat T cells after TNF-α treatment (Laukens et al. 2011). After TLR3 activation or genotoxin treatment, c-IAPs antagonists trigger the formation of a ~2MD cytosolic RIP-1-containing death-inducing complex, termed "Ripoptosome", which leads to necroptosis when RIP-1 kinase activity is not sufficiently inhibited by c-FLIP$_L$ and caspase 8 (Feoktistova et al. 2011; Tenev et al. 2011).

Contradictory results were presented about the role of adaptor protein FADD in the regulation of necroptosis. The early studies showed that FADD is required for necroptosis (Holler et al. 2000; Lin et al. 2004). However, Jurkat T cells and primary T lymphocytes that lack functional FADD (FADD-deficient or expressing dominant negative FADD) are susceptible to RIP-1-dependent necroptosis (Degterev et al. 2005; Bell et al. 2008; Osborn et al. 2010; Lu et al. 2011). FADD-deficient keratinocytes exhibit RIP-3-dependent necroptosis *in vivo* (Bonnet et al. 2011). *Fadd*$^{-/-}$ embryos showed increased level of RIP-1 and massive necrosis, which is rescued by crossing to *Rip-1*$^{-/-}$ background (Zhang et al. 2011). Other RIP-1 associated proteins may also regulate its

pro-necroptotic activity by competitive binding. For example, NEMO binds to ubiquitinated RIP-1, thus restraining RIP-1 from necroptosis-inducing pathway (O'Donnell et al. 2012).

1.2.1.4 Execution of necroptosis

Multiple distinct cellular events were reported to contribute to the morphological features of necrosis. Whether these events are RIP-1/RIP-3-dependent remains to be identified (Vandenabeele et al. 2010). It is also unclear whether these necrosis-inducing pathways act independently or cooperate with each other to amplify the death signals. Among them, ROS generation by mitochondria is most frequently associated with RIP-1/RIP-3-dependent necroptosis. ROS contributes to the execution of necroptosis in different setting of necroptosis induction, including treatment of viral caspase inhibitor B13R (Los et al. 2002), TLR3/TLR4 simulation in the presence of z-VAD-fmk (Kalai et al. 2002; He et al. 2011), and death receptor ligation in the presence of z-VAD-fmk (Chen et al. 2009; He et al. 2011). ROS generated by NAPH oxidase (NOX) mediates the execution of RIP-1-dependent necrosis in Jurkat T cells co-cultured with amoeba *Naegleria fowleri* (Song et al. 2011). ROS scavenger antioxidant butylated hydroxyanisole (BHA), antioxidant acetylcysteine (NAC) and mitochondrial respiratory chain uncoupler (rotenone) prevent the Fas/zVAD-induced necroptosis (Chen et al. 2009). Interestingly, BHA treatment shifts the cell death pathway from necroptosis to apoptosis in L929 cells

after TLR3 stimulation in the presence of z-VAD-fmk (Kalai et al. 2002), suggesting in certain condition necroptosis is the "first-come" mechanism of death.

RIP-3 induces the generation of ROS during necroptosis. Activated RIP-3 recruits and phosphorylates mixed lineage kinase domain-like protein (MLKL) and mitochondrial protein phosphatase PGAM5, two positive regulators of ROS generation (Wang et al. 2012; Zhao et al. 2012). The short isoform of PGAM5 activates the mitochondrial fission factor Drp1 by de-phosphorylating Drp1 at Ser637 site. Activated Drp1 induces mitochondrial fragmentation, an early step for necrosis execution (Wang et al. 2012). RIP-3 also directly associates with several metabolic enzymes: glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL) and glutamate dehydrogenase 1 (GLUD1). The activation of RYGL permits the degradation of glycogen as an energy source. GLUL and GLUD1 function together in the catabolism of glutamine or glutamate. The activation of GLUL, GLUD1 and PYGL contributes to the TNF/zVAD-induced accumulation of ROS and necroptosis (Zhang et al. 2009).

Loss of mitochondrial transmembrane potential is associated with RIP-1-dependent necroptosis (Ma et al. 2005; Chen et al. 2009). The mechanism of RIP-1 or RIP-3 stimulating mitochondrial permeability transition (MPT) needs to be identified. BH3-only Bcl-2 family member Bmf was hypothesized as a potential regulator in death receptor-induced necroptosis by genome wide RNA interference screening (Hitomi et al. 2008) The current hypothesis is that Bmf antagonizes anti-apoptotic Bcl-2 and Bcl-x_L, two

inhibitors of the permeability transition pore complex (PTPC), to execute MPT (Golstein and Kroemer 2007; Galluzzi and Kroemer 2008).

During apoptosis, the consumption of ATP is promptly ceased, as active caspases shut off the ATP-consuming cellular processes, such as translation, DNA repair and proteasome degradation (Vandenabeele et al. 2010). A dramatic drop of intracellular ATP concentration, on the other side, is associated with necrosis (Leist et al. 1997). Poly(ADP-ribose) polymerase-1 (PARP-1)-mediated ATP exhaustion contributes to the TNF/zVAD-induced necroptosis (Los et al. 2002). Nuclear enzyme PARP-1 directly executes necrosis through "PARP1-AIF loop". DNA damages activates PARP-1 for DNA repair, a process that eventually depletes the cellular storage of ATP and NAD. The NAD depletion and/or poly(ADP-ribose) accumulation induce the relocation of apoptosis-inducing factor (AIF) from the mitochondrial intermembrane space to the nuclear compartment. Consequently, AIF executes large scale DNA fragmentation, which further causes PARP-1 activation and ATP depletion (Vandenabeele et al. 2010). Interestingly, when DNA damage is achieved by alkylating agents in mouse embryonic fibroblasts (MEFs), PARP-1 hyper-activation-induced necrosis requires the expression of RIP-1 and TRAF2, suggesting PARP-1 functions as an upstream regulator of RIP-1, at least in certain conditions (Xu et al. 2006).

1.2.2 Necroptosis in immune response

Apoptosis is considered to be a type of "quiet death", as the apoptotic bodies are promptly engulfed and ingested by surrounding phagocytes. In contrary, cells die through necrosis do not display "eat me" signals on their surface long enough before the plasma membrane rupture. Some of the endogenous components released from necrotic cells are damage-associated molecular patterns (DAMPs), which induce inflammation and promote immune response (Kono and Rock 2008). Admission of z-VAD-fmk in experimental mice stimulated through TLR3 or TLR4 results in increased serum concentration of several pro-inflammatory cytokines, such as IL-6, TNF-α, MCP-1 and IFN-γ. This pro-inflammatory effect of z-VAD-fmk treatment requires the expression of RIP-3 and TRIF. Therefore, it is likely caused by increased necroptosis in macrophages (He et al. 2011). However, because systemic necrosis can be quite pathogenic, whether necroptosis induction is a potential therapeutic methodology remains debatable.

Necroptosis is essential for the clearance of the viruses that induce resistance to apoptosis in the host cells. Vaccinia virus (VV) encodes caspase inhibitor B13R, which is 92% identical to cowpox virus caspase inhibitor CrmA and sensitizes cells to necrotic cell death (Los et al. 2002). The infection of VV induces necroptosis in Jurkat cells and mouse fibroblasts (Li and Beg 2000; Chan et al. 2003). *In vivo* studies showed that RIP-1/RIP-3-containing necrosome is specifically induced in the liver after VV infection (Cho et al. 2009). Necroptosis is essential for the host defense against VV, as necroptosis-resistant

mice (*Rip-3*$^{-/-}$ or *TNFSF1B*$^{-/-}$) showed attenuated inflammation and defective clearance of the virus after VV infection (Chan et al. 2003; Cho et al. 2009).

The pro-inflammatory feature of necroptosis can be detrimental for the host during infection. RIP-3-dependent necroptosis mediates the establishment of TNF-induced systemic inflammatory response syndrome (SIRS). Deletion of RIP-3 or pretreatment with necrostatin-1 protects host against lethal SIRS induced by TNF-α injection, while deletion of effector caspases (caspase 3 and caspase 7) or inflammatory inducer caspase 1 shows no impact. Moreover, *Rip-3*$^{-/-}$ mice show lower level of circulating DAMP than wild type controls do (Duprez et al. 2011). These results suggest that RIP-1 and RIP-3 are potential therapeutic targets for the treatment of systemic inflammation.

Conversely, some studies suggested that programmed necrotic cells could be anti-inflammatory. For example, Jurkat T cells display phosphatidylserine (PS) on their surface when necrosis is induced by ATP depletion. Those necrotic Jurkat cells are preferentially engulfed by macrophages when mixed with apoptotic Jurkat T cells (induced by staurosporine). More importantly, those necrotic cells are capable of suppressing the cytokine production of macrophages, similar to apoptotic Jurkat T cells (Hirt and Leist 2003). Necroptotic L929 cells induced by TNF-α are shown to be anti-inflammatory when co-cultured with macrophages. Macrophages engulf the cellular particles of necrotic cells after they loss the plasma membrane integrity, while apoptotic

bodies are internalized as a whole. Nevertheless, neither apoptotic nor necrotic L929 cells induce pro-inflammatory cytokine production in this experimental setting (Brouckaert et al. 2004). Whether those studies truly reflect the innate immune response *in vivo* is debatable. The cell type of the apoptotic/necrotic cells may also determine whether the cell death is pro-inflammatory.

Some pathogens evolved to manipulate necroptosis to escape the attack from immune system. Murine cytomegalovirus (MCMV) infection induces RIP-3-dependent, but RIP-1-independent necrosis. One strain of MCMV, viral M45, encodes the inhibitor of RIP-3, which attenuates inflammation to avoid consequent virus clearance (Upton et al. 2010). On the other side, some pathogens actively induce necroptosis for efficient invasion. *Naegleria fowleri*, the free-living amoeba that causes primary amoebic meningoencephalitis in human, damages host tissues through lytic necrosis. Co-incubation of *N. fowleri* and Jurkat T cells results in RIP-1-dependent necroptosis in Jurkat T cells (Song et al. 2011).

1.2.3 Necroptosis in T lymphocytes

T lymphocyte was one of the first type of cells in which necroptosis was identified. In 2000, Holler et al. reported that Fas ligation induces RIP-1-dependent cell death when caspase activities are ablated by pan-caspase inhibitor z-VAD-fmk (Holler et al. 2000). Later, necroptosis were identified by several genetically modified T cell models. Caspase 8-deficient T cells fail to undergo clonal expansion due to excessive

TCR activation-induced cell death (Salmena et al. 2003), which is mediated RIP-3-dependent necroptosis (Ch'en et al. 2011). Adapter protein FADD is also involved in the suppression of necroptosis in T lymphocytes. T cells that lack FADD or express dominant-negative FADD (*tFADDdd*) showed RIP-1 kinase activity-dependent cell death upon TCR stimulation (Bell et al. 2008; Osborn et al. 2010).

The mechanism of TCR activation-induced necroptosis in T lymphocytes is not well studied. It is likely to utilize the similar pathways as death receptor and TLR-induced necroptosis (Figure 3). The catalytic activity of pro-caspase 8 suppresses necroptosis (Leverrier et al. 2011), while RIP-1 and RIP-3 are required for necroptosis induction in activated T cells (Ch'en et al. 2011a; Lu et al. 2011). RIP-1 phosphorylation is shown in necroptotic FADD-deficient T lymphocytes by western blot. However, neither phosphorylation of RIP-3 or RIP-1/RIP-3 complex is detected in this system (Osborn et al. 2010). It is unclear whether these results are caused by the limitation of methodology and experimental setting, or necroptosis in T lymphocytes does not require the kinase activity of RIP-3. Nevertheless, the expression of RIP-3 is the requisite factor for necroptosis in T cells, as crossing to *Rip-3*$^{-/-}$ mice rescues the defective proliferation and necroptosis phenotype in *tFADDdd* mice (Lu et al. 2011).

The significance of TCR activation-induced necroptosis for T cell homeostasis is unclear. One possible explanation is that activated T cells resistant to death receptor mediated apoptosis need to be removed to protect the host from sever autoimmunity.

The phenotype of T cell-specific deletion of caspase 8 (*tCasp8*$^{-/-}$) supports this theory to a degree. Though caspase 8-deficient T cells are resistant to Fas-induced apoptosis, *tCasp8*$^{-/-}$ mice do not accumulate double-negative T cells in the blood and lymphoid organs, or develop the SLE-like autoimmune phenotype as *lpr*/*lpr* mice (Salmena and Hakem 2005). It is probably because activated caspase 8-deficient T cells are eliminated by necroptosis. *Rip-3*$^{-/-}$*tcasp8*$^{-/-}$ and *Rip-3*$^{-/-}$*tFADDdd* mice show lymphoproliferative syndrome reminiscent of *lpr*/*lpr* mice (Ch'en et al. 2011a; Lu et al. 2011), as activated peripheral T cells cannot be cleared. The reporters did not address whether SLE-like autoimmunity was developed in those mice, which will be interesting to examine in the future study. On the other side, the protective effect of necroptosis seems to be insufficient with age, as *tcasp8*$^{-/-}$ mice eventually develop lethal age-related lymphoproliferative disease (Salmena and Hakem 2005).

1.3 Macroautophagy in T lymphocytes

This section is adapted from "Macroautophagy in T lymphocyte development ad function". Ming-Xiao He et al., Frontiers in Immunology, 2012;3:22. doi: 10.3389/ fimmu.2012.00022.

Autophagy is a cellular process characterized by the sequestration of cytoplasmic compartments through double-membrane vacuoles, termed autophagosomes. Based on the mechanism by which cargo is delivered for degradation, autophagy is subdivided into three types: macroautophagy, microautophagy and chaperone-mediated autophagy. Microautophagy is characterized by the direct sequestration of cytosolic

component into lysosomes by invagination or arm-like protrusion of lysosomal membrane. The physiological role of microautophagy in mammalian cells is yet unrevealed, as the approaches to detect this process are limited (Mijaljica et al. 2011). Chaperone-mediated autophagy (CMA) is a highly regulated process, in which cytosolic proteins are sorted one-by-one by the heat shock cognate protein 70 (Hsc70) into lysosomes (Kaushik and Cuervo 2012). Among the three types of autophagy, macroautophagy is the most studied in mammalian cells. Macroautophagy has been shown to be essential for providing nutrients during starvation, clearing long-lived proteins as well as unwanted organelles, and fighting intracellular infections in multiple types of mammalian cells (Mortimore and Poso 1987; Levine et al. 2011).

1.3.1 Mechanism of macroautophagy

Macroautophagy (thereafter referred as autophagy) starts with the generation of double-membrane structures termed isolation membranes or phagophores. The phagophore sequestrates intracellular components to form the intracellular double-membrane autophagosomes. Consequentially, autophagosomes fuse with lysosomes and the inside content is digested (Klionsky and Emr 2000). The biogenesis of autophagosomes requires a group of evolutionarily conserved genes, referred to as autophagy-related genes (*Atgs*) (Longatti and Tooze 2009). The initiation of the phagophore requires the class III phosphatidylinositol 3-kinase (PtdINs3K) complex, which further activates two ubiquitin-like conjugation systems: The Atg12-Atg5

conjugation and LC3-processing (Ohsumi and Mizushima 2004; Pua and He 2009b). For Atg12-Atg5 conjugation, Atg12 is first linked to Atg7 (E1), then to Atg10 (E2). Atg10 is subsequently replaced by Atg5 (E3) to form the Atg12-Atg5 conjugate. LC3 processing starts with the association between the inactive form of LC3, LC3-I, with activated Atg7 (E1). Atg3 (E2) then exchanges Atg7, and catalyzes the addition of a hydrophobic phosphatidylethanolamine (PE) tail to LC3. These reactions transform the inactive LC3-I is to the active LC3-II. Atg12-Atg5 conjugates and LC3-II insert into the intracellular membrane, leading to the formation of the isolation membrane/phagophore. While both systems are required for autophagy, the Atg12-Atg5 conjugate assembles on phagophore prior to the insertion of LC3, and contributes to LC3 processing (Hailey et al. 2010; Levine et al. 2011).

In the immune system, autophagy balances the threshold of immune responses to protect against infection, autoimmunity and inflammatory diseases (Levine et al. 2011). The primary role of autophagy in cells depends on the cell type and environment. To study the role of autophagy in primary T lymphocytes, several specific knockout or transgenic models have been developed during the past few years, and it has been revealed that autophagy plays a crucial role in T cell homeostasis and proper T cell response. The phenotypes of different Atg-deficient T lymphocytes showed a great deal of similarity, with certain differences, which can be explained as a result of different deletion efficiency, accumulating effects, or the molecular functions independent of

autophagy. From here I will review the current findings concerning how autophagy regulates the survival and function of T cells, and compare different model to show the role of autophagy, not each individual Atgs, in T lymphocytes.

1.3.2 Autophagy induction in T lymphocytes

Although relatively low, autophagy can be detected in all subsets of thymocytes and freshly isolated naïve T cells (Lu et al. 2006; Pua et al. 2007; Stephenson et al. 2009). These results were fairly surprising, since T cells contain limited cytoplasm. The existence of autophagy in T lymphocytes suggests that autophagy may play a regulatory role in T cells. Aging T lymphocytes, on the other hand, shows an accumulation of autophagic vacuoles. In a study of long term *in vitro* human lymphocyte culture, the percentage of cells with autophagosomes increased during culture, which was associated with the increase in lysosomal mass and accumulation of lipofusion events. (Gerland et al. 2004).

T lymphocytes display TCR activation-induced autophagy (Pua et al. 2007; Pua et al. 2009; Hubbard et al. 2010; Jia and He 2011; Jia et al. 2011; Kovacs et al. 2011). $CD4^+$ T cells upregulate Beclin-1 (Atg6) and LC3 (Atg8) upon TCR stimulation (Arsov et al. 2008). Ultrastructural studies revealed an increase in the number and a decrease in the size of autophagosomes after TCR stimulation. Interestingly, while mitochondria are frequently contained in the autophagosomes of resting T cells, the autophagic cargo switches to almost exclusively cytosolic material in activated T cells (Hubbard et al.

2010). Mitochondria undergo morphological changes during autophagy induction to escape autophagic degradation and maintain energy production in MEFs (Gomes et al. 2011). It is worth to investigate whether T lymphocytes apply similar strategies or utilizes unique pathways to regulate the mitochondrial content.

TCR-induced autophagy requires the key autophagy machinery, as deleting Atg5, Atg7, or Atg3 abolishes autophagosomal induction (Pua et al. 2007; Pua et al. 2009; Hubbard et al. 2010; Jia and He 2011). JNK1/JNK2 is required for TCR-induced autophagy in $CD4^+$ T cells (Lu et al. 2006). Beclin-1, a component of PtdINs3K complex, was shown to be crucial for autophagy initiation (Yue et al. 2003). Nevertheless, overexpression of Beclin-1 in T lymphocytes by a bacteria artificial chromosome (BAC) transgene did not change the basal level of autophagy in multiple organs including thymus and spleen (Arsov et al. 2008). Contradictory results were presented regarding the role of class III PI3K, Vps34, in the autophagy induction in T lymphocytes. One study, in which the ATP binding region of Vps34 was deleted to generate a truncated protein, suggested that Vps34 was dispensable for autophagy induction in mature T lymphocytes (McLeod et al. 2011). However, complete deletion of Vps34 impairs autophagy induction (Willinger and Flavell 2012). It is possible that the kinase activity of Vps34 is not required for autophagy as least in mature T lymphocytes. The autophagy machinery in T lymphocytes is illustrated in Figure 5.

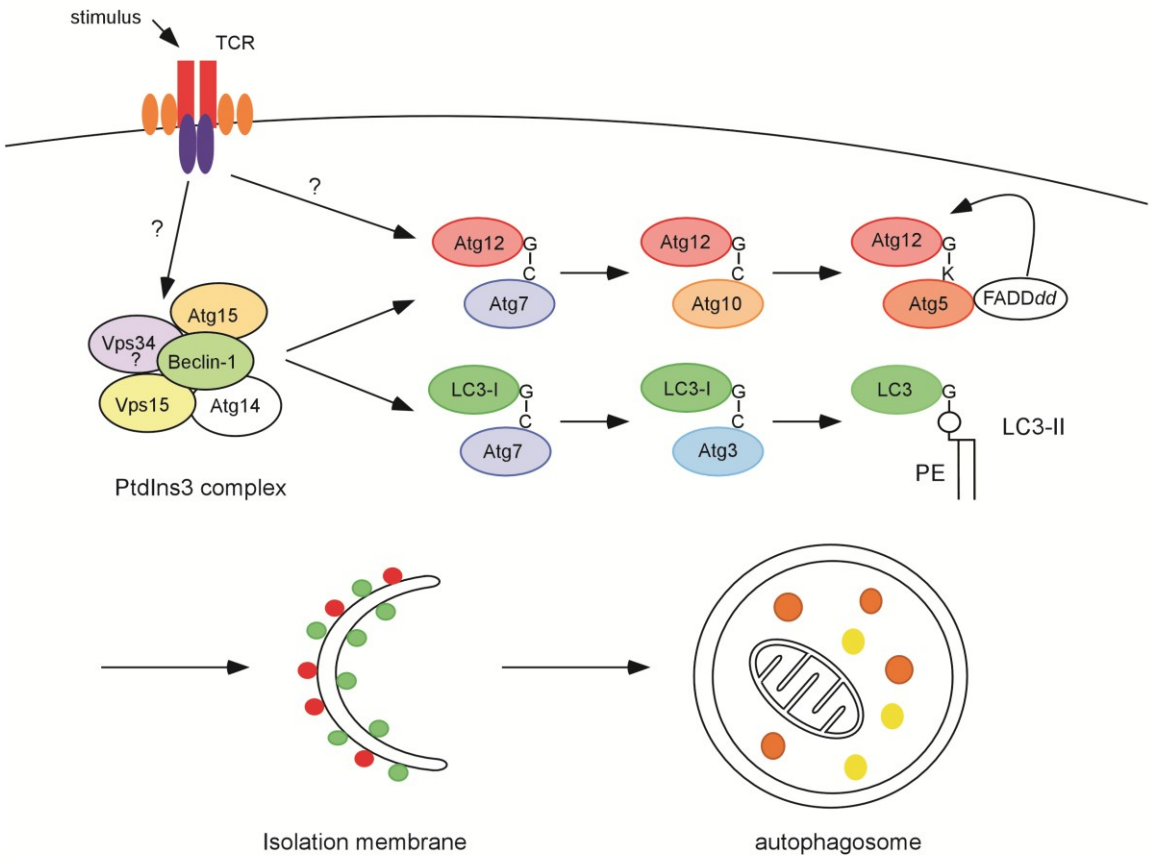

Figure 4: Macroautophagy induction in T lymphocytes

Figure 4. TCR engagement is a strong inducer for autophagy in primary T cells. Autophagy is induced by the PtdIns3k complex in many types of cells, but the role of Beclin-1/Vps34 in autophagy induction in T lymphocytes is controversial. The two autophagosome processing pathways: Atg12-Atg5 conjugation and LC3 processing are essential for autophagy induction in T lymphocytes. Furthermore, FADD, the death adaptor protein in the extrinsic death pathway, may play a key role in mediating a crosstalk between the apoptosis and autophagy pathways.

TCR-induced autophagy is compromised in aged $CD4^+$ T cells (Mattoo et al. 2009). The mechanism by which autophagy is defective during aging is unclear. Some evidence suggests that the Rel family member, p65, may be involved. The nuclear translocation of p65 upon TCR signaling is impaired in aged $CD4^+$ T cells (Mattoo et al. 2009), and p65 has been shown to activate autophagy by upregulating the transcription of Beclin-1 in multiple cell lines (Copetti et al. 2009). It is yet unknown whether defective autophagy induction leads to other defects in aged T lymphocytes, such as increased mitochondrial damage, reduction in glycolysis, or enhanced apoptosis upon primary TCR stimulation.

1.3.3 Autophagy in T lymphocyte homeostasis: different genetic models, similar defects

Several tissue specific knockout models have been developed during the past few years to study the role of autophagy in T lymphocytes. The deficiency in autophagy-related genes leads to a blockage in autophagic flux, as well as impaired T cell homeostasis. In *Atg5*$^{-/-}$ fetal liver chimeric mice, the thymocytes undergo full maturation, but the thymic cellularity is reduced by half. The number of peripheral T lymphocytes is also decreased, which is likely attributed to both the loss of thymocytes and increased cell death rate (Pua et al. 2007). The *Atg5*$^{f/f}$ Lck-Cre^+ mice generated later showed an almost identical phenotype to the *Atg5*$^{-/-}$ chimera (Stephenson et al. 2009). The debate remains as to whether the enhanced cell death is caused by loss of autophagy, but not specific functions of Atg5. Atg5 is certainly involved in apoptosis

pathway. Overexpression of Atg5 increases the apoptotic rate in HeLa cells, a phenotype dependent on FADD. Antisense cDNA knockdown of FADD protects HeLa cells from Atg5-induced apoptosis, whereas autophagy is still upregulated in Atg5 overexpressing cells (Pyo et al. 2005). Atg5 also sensitize cell to apoptosis in a FADD-independent pathway. The truncated form of Atg5 antagonizes Bcl-x_L to enhance MOMP and cytochrome c release (Yousefi et al. 2006). Therefore, Atg5 may regulate apoptosis in extrinsic and intrinsic apoptosis pathways.

To address whether autophagy indeed regulates T cell homeostasis, several other tissue specific knockout models have been analyzed. Mice with tissue specific deletion of Atg3 in T lymphocytes by Lck-Cre system (deletion occurs during double negative stage in thymus) show an almost identical phenotype as the *Atg5*$^{-/-}$ chimera, characterized by normal frequencies of DN, DP, and SP thymocytes in the thymus, but a reduced cellularity in both the thymus and peripheral T cell pools (Jia and He 2011). On the other side, the thymocyte cellularity in *Atg7*$^{f/f}$ Lck-Cre^{+} mice is barely decreased (Stephenson et al. 2009), with only a minor reduction in SP cells. No thymocyte defect was reported in the *BECN*$^{f/f}$ CD4-Cre^{+}, *Vps34*$^{f/f}$ CD4-Cre^{+} models (Kovacs et al. 2011; Willinger and Flavell 2012). Despite the difference inside the thymus, T lymphocytes in all these models (*Atg5*$^{-/-}$ chimera, *Atg5*$^{f/f}$ Lck-Cre^{+}, *Atg7*$^{f/f}$ Lck-Cre^{+}, *Atg3*$^{f/f}$ Lck-Cre^{+}, *BECN*$^{f/f}$ CD4-Cre^{+} and *Vps34*$^{f/f}$ CD4-Cre^{+}) show decreased cellularity in the periphery and an enhanced apoptosis rate (Pua et al. 2007; Pua et al. 2009; Stephenson et al. 2009; Jia and He 2011;

Kovacs et al. 2011). There are several explanations for the difference of the thymocyte phenotypes in these autophagy-deficient T cell models. First of all, different T cell-specific deletion strategies were applied in these studies. The deletion of Beclin-1 and Vps34 was driven by CD4-Cre, which is expressed at DP stage. Therefore, the deletion in the CD4-Cre models may occur much later than that in the Lck-Cre models. Autophagy may be required at DN-DP stage for thymocyte development, but dispensable beyond DP stage. The second possibility is that the defect in thymocytes is accumulated and requires a relative long time of autophagy inhibition. As a result, the CD4-Cre models, in which deletion occurs at a later developmental stage, showed milder defect than the Lck-Cre models. Additionally, low deletion efficiency in CD4-Cre model may cause the differences, as enough protein may be left for autophagy induction. Finally, some other effectors may replace PtdIns3k complex for autophagy induction in thymocytes, so Beclin-1 and Vps34 are dispensable for autophagy induction in thymocytes.

Ortiz's group utilized a *Rag1*$^{-/-}$ (recombination activating gene-1 null) blastocyte complementation to study the role of Beclin-1 in hematopoietic cells. Beclin-1 deficient (*BECN*$^{-/-}$) blastocytes were transferred to a *Rag-1*$^{-/-}$ host in this study. The data showed that Beclin-1 is required for the maintenance of early thymocyte progenitors. Controversially, survival and TCR-induced proliferation remains normal in *BECN*$^{-/-}$ T lymphocytes developed in this system. Moreover, autophagy can be detected in those cells, though decreased compared to controls (Arsov et al. 2011). These results indicate

that a Beclin-1-independent autophagy-inducing pathway may occur in T lymphocytes. The mechanism of autophagy induction in the absence of Beclin-1 needs to be determined.

1.3.4 Autophagy in T cell function

1.3.4.1 Autophagy and intracellular organelle homeostasis

Autophagy plays a role in controlling mitochondria during T cell development (Pua et al. 2009; Stephenson et al. 2009). Transcriptional profiling of Atg5-deficient T lymphocytes shows a remarkable enrichment in mitochondrion-associated genes (Stephenson et al. 2009). The mitochondrial content in T lymphocytes gradually decreases during the transition from thymocytes to peripheral T lymphocytes. The level of mitochondria is the highest in DP thymocytes, followed by SP thymocytes, and mature T lymphocytes contain the lowest volume (Pua et al. 2009). This contraction of mitochondria requires autophagy, as least partially, as mitochondrial content is higher in *Atg5*$^{-/-}$, *Atg7*$^{-/-}$ and *Atg3*$^{-/-}$ T lymphocytes than that in wild-type T lymphocytes (Pua et al. 2009; Stephenson et al. 2009; Jia et al. 2011). Conversely, it was reported that cells from *BECN*$^{f/f}$CD4-Cre^{+} mice showed no change in mitochondrial content (Kovacs et al. 2011). One possible explanation is that genomic deletion only starts in DP thymocytes in this model, and persistent levels of Beclin-1 protein in cells is enough to mediate the autophagic degradation of mitochondria. Still, we cannot rule out the possibility that controlling mitochondrial content is achieved independent of Beclin-1. Nevertheless,

current data suggests that autophagy protects T lymphocytes during the migration from thymus to the periphery by eliminating excessive mitochondria. Mitochondria are believed to be the major reservoir of toxic ROS in cells (Hildeman et al. 2003). The oxidative tension is much higher in the blood than in the thymus (Braun et al. 2001; Sitkovsky and Lukashev 2005). Therefore, maintaining the same level of mitochondria in peripheral T cells as in thymocytes can cause intolerable levels of ROS.

Endoplasmic reticulum (ER) is well regulated by autophagy. An increase in ER volume can be observed in autophagy-deficient T lymphocytes (Jia et al. 2011). Different from mitochondria, ER volume is downregulated in the transition of DN to DP thymocytes, and maintained to a similar level afterwards. The accumulation of ER, as well as mitochondria, requires a long period of time to establish (up to 18 days in *vitro*) after autophagy is abolished by inducible-deletion in mature T lymphocytes. Interestingly, lymphocytes did not show elevated cell death until 24 days after inducible deletion of Atg3. These data suggest that the elevated cell death in autophagy-deficient T lymphocytes is possibly the outcome of cellular organelles' abnormality.

1.3.4.2 Autophagy in T cell activation

Different groups have identified that autophagy-related genes are required for T cell proliferation upon TCR stimulation. T lymphocytes lacking Atg5, Atg7, Atg3, or Beclin-1 all show impaired TCR stimulation-induced proliferation (Pua et al. 2007; Pua et al. 2009; Stephenson et al. 2009; Hubbard et al. 2010; Jia et al. 2011; Kovacs et al. 2011).

Severe TCR stimulation-induced cell death is observed in Beclin-1-deficient lymphocytes (Kovacs et al. 2011). The survival defect may contribute to impaired proliferation in autophagy-deficient T lymphocytes. Meanwhile, autophagy is required for intact TCR signal by maintaining intracellular organelle homeostasis. *Atg7*$^{-/-}$ T cells displayed impaired calcium influx upon TCR stimulation, as well as increased calcium efflux from ER to cytosol. The excessive ER in those cells leads to abnormal redistribution of calcium into the ER upon TCR stimulation (Jia et al. 2011). Besides the calcium signal, other TCR signal components such as PLCγ-1 (the upstream signal of calcium efflux), p38, ERK, and NF-κB activation all remain unchanged in Atg7-deficient T lymphocytes (Jia et al. 2011).

Autophagy is involved in the metabolism of activated T lymphocytes. Upon TCR activation, T lymphocytes undergo a metabolic switch and produce more ATP to ensure sufficient energy for protein synthesis, cytokine secretion, and cellular division (Fox et al. 2005). Without autophagy, the ATP production in activated T lymphocytes is reduced to resting levels. The lack of ATP, at least partially, contributes to impaired transcription and production of IFN-γ and IL-2 in those cells, as methyl pyruvate, a cell-permeable intermediate of glucose, incompletely rescues both ATP generation and cytokine production (Hubbard et al. 2010). However, another study showed of IL-2 secretion is increased in autophagy-deficient T lymphocytes after TCR activation (Jia et al. 2011). This controversy may be the consequence of the autocrine nature of IL-2: IL-2 is

consistently produced and utilized by activated T lymphocytes (Cantrell et al. 1988). Therefore, the IL-2 detected in culture medium at a single time point may or may not reflect the capacity of IL-2 production. To settle the debate, careful study in the kinetics of the transcription and secretion of cytokines is needed.

Autophagy is differentially regulated in T helper subsets. T lymphocytes cultured in T_H2 polarizing condition *in vitro* contain more autophagosomes than those cultured in T_H1 polarizing conditions (Lu et al. 2006). T_H17 cells are relatively more resistant to cell death in the absence of Beclin-1, compared to T_H0, T_H1, and T_H2 cells (Kovacs et al. 2011). T_H17 cells, along with T_H1 cells, mediate the pathogenesis of experimental autoimmune encephalomyelitis (EAE) (El-behi et al. 2010). Though T_H17 cells manage to survive when autophagy is blocked, mice with Beclin-1-deficient T lymphocytes still showed resistance to EAE development. The number of MOG specific T_H1 and T_H17 cells are reduced in the periphery and undetectable in the central nerve system (CNS) in the T cell specific Beclin-1-deficient mice (Kovacs et al. 2011). The protective role of Beclin-1-deficient T lymphocytes in EAE induction may be the result of defective antigen-induced proliferation.

1.3.5 Autophagy in HIV infection

Autophagy is involved in the progressive decline in the number of $CD4^+$ T cells during human immunodeficiency virus infection/acquired immunodeficiency syndrome (HIV/AIDS). HIV infection induces cell death in both infected and uninfected

"bystander" $CD4^+$ T cells (Laurent-Crawford et al. 1993). HIV-1 envelope glycoproteins (Env), composed of gp41 and gp120 subunits, is expressed on infected human lymphocytes. Via CXCR4 and CCR5, Env triggers autophagy in uninfected "bystander" $CD4^+$ T cells, which eventually leads to apoptosis in those cells (Espert et al. 2006; Espert et al. 2009). The gp41 fusion to target membranes is required for Env-mediated autophagy (Denizot et al. 2008). Interestingly, autophagy is repressed in infected $CD4^+$ T cells (Zhou and Spector 2008). The effect of killing surrendering T cells through surface protein Env seems to be T cell specific, as Env expressed on macrophages with CXCR4 and CCR5 fails to induce autophagy in uninfected $CD4^+$ T cells in a co-culture experiment (Espert et al. 2009). From the perspective of virus, downregulation of autophagy in host cells may be beneficial, as autophagy potently attacks virus through xenophagic degradation and facilitates antigen presentation (Kim et al. 2010). At the same time, provoking excessive autophagy in uninfected surrounding lymphocytes promotes cell death, further weakening the host defense by reducing potential adaptive immunity against the virus.

In conclusion, autophagy is required for the homeostasis and function of T lymphocytes. Deletion of different autophagy-related genes results in similar phenotypes in T lymphocytes, indicating the defects are caused by a loss of autophagy instead of other functions of those proteins. T lymphocytes utilize autophagy to maintain intracellular organelle homeostasis, intact TCR signaling, and metabolic switch

upon TCR activation. Identifying new players in the regulation of autophagy in T lymphocytes will broaden our understanding in the role of autophagy in T cell homeostasis.

1.4 The interplay of apoptosis, necroptosis and autophagy in T lymphocytes

1.4.1 The interplay of autophagy and apoptosis in T lymphocytes

As mentioned before, the loss of autophagy-related genes generally leads to increased cell death (Pua et al. 2007; Pua et al. 2009; Stephenson et al. 2009; Jia et al. 2011). The crosstalk between autophagy and apoptosis has been observed in various eukaryotic cells (Thorburn 2008), but how autophagy regulates apoptosis in T lymphocytes remains unclear. Atg7-deficient T lymphocytes upregulate the expression of Bcl-2, but the expression levels of other pro-apoptotic or anti-apoptotic proteins are unchanged in the absence of Atg7 (Pua et al. 2009). Beclin-1-deficient T lymphocytes show highly increased protein levels, but not mRNA levels of pro-apoptotic pro-caspase 8, pro-caspase 3, and Bim, which suggests a block in the post-translational regulation of these proteins (Kovacs et al. 2011). Caspase 8 is detected in p62/ubiquitin-containing aggregates in *BECN*$^{-/-}$ T cells (Kovacs et al. 2011). It is reasonable to hypothesize that p62 targets caspase 8 for autophagic degradation. However, that hypothesis is condemned by the fact that pro-caspase 8 level is comparable between wild-type and p62-deficient T cells. Further studies are required to identify whether Beclin-1 controls pro-apoptotic protein levels through Bcl-2 binding, autophagy induction, or other pathways.

Though autophagy is required for survival, excessive autophagy seems to be destructive for T lymphocytes. Autophagy promotes, rather than protects, growth factor withdrawal-induced cell death in a T_H2 cell line (Lu et al. 2006). In long-term human $CD8^+$ T cell cultures, cells with higher numbers of autophagosomes died out first (Gerland et al. 2004). However, it is still unclear whether the high number of autophagosomes reflects an increase in flux or a blockage of autophagosome–lysosome fusion. Whether those cells undergo enhanced autophagy remains to be verified. When exposed to IFN-γ, *Irgm 1*$^{-/-}$ (Interferon-γ inducible, immunity-related GTPase$^{-/-}$) T cells showed greatly enhanced death in an autophagy-dependent manner (Feng et al. 2008). Ginsenoside Re suppresses autophagy by inhibiting Irgm 1 and IFN-γ production in human T lymphocytes, which leads to enhanced proliferation and reduced cell death (Son et al. 2010).

1.4.2 The interplay of autophagy and necroptosis in T lymphocytes

Necroptosis and enhanced autophagy were observed in the same population of cells when caspase 8 activity was blocked (Yu et al. 2004; Degterev et al. 2005; Lu et al. 2006; Wu et al. 2011). Whether autophagy contributes to necroptosis in T lymphocytes is debatable. FADD is required for death receptor-mediated caspase 8 activation (Zhang et al. 1998). Similar to caspase 8-deficient T cells, T cells lacking FADD die through TCR activation-induced necroptosis. TCR-induced necroptosis in *Fadd*$^{-/-}$ and *caspase-8*$^{-/-}$ T cells is independent of autophagy (Osborn et al. 2010; Ch'en et al. 2011). However,

hyperactive autophagy seems to be involved in the RIP-1-dependent necroptosis in T cells expressing a dominant-negative FADD (*FADDdd*). Inhibition of autophagy with 3-methyladenine (3-MA), dominant-negative Vps34 or silencing of Atg7 rescued the cell death phenotype in *FADDdd* T cells (Bell et al. 2008). Autophagy-dependent cell death in *FADDdd* T cells may be attributed to the specific cellular function of the dominant-negative FADD instead of the loss of FADD function. FADD and caspase 8 associate with Atg12-Atg5 conjugates, the essential molecule complex for autophagy induction (Bell et al. 2008). Therefore, the mutated FADD may modulate the autophagy machinery.

Studies in other type of cells also provided controversial results in the relationship between necroptosis and autophagy. While some studies show that autophagy contributes to the induction of necroptosis, other studies show opposite results (Yu et al. 2004; Bell et al. 2008; Osborn et al. 2010; Ch'en et al. 2011b; Chen et al. 2011). Necroptosis is usually associated with increased ROS production and dysfunctional mitochondria, so the enhanced autophagy may be a desperate survival attempt by the cell to clean damaged organelles. In some studies, attenuation of autophagy by siRNA knockdown benefited the cells, while thoroughly abolishing autophagy may create additional stress, thus killing instead of protecting the cells. Careful analyses of cell death pathways in necroptotic T cells, especially with modest inhibition of autophagy, will provide us valuable information to understand the

relationship between autophagy and necroptosis. Altogether, autophagy is shown to play a dual role in T lymphocytes: autophagy is critical for cell survival and T cell response, while too many autophagic vacuoles may create intolerable stress.

1.5 The role of c-FLIP in T lymphocytes

1.5.1 c-FLIP proteins

Cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP), also known as CASP8 and FADD-like apoptosis regulator (CFLAR), is a homologue and negative regulator of caspase 8. c-FLIP was cloned by several groups back to 1997, when it was given several names including CLARP, FLAME, I-FLICE, MRIT, Usurpin, Casper, and CASH (Goltsev et al. 1997; Han et al. 1997; Hu et al. 1997; Irmler et al. 1997; Shu et al. 1997; Srinivasula et al. 1997; Rasper et al. 1998). *c-Flip* gene locates upstream of caspase 10 and caspase 8 gene in human genome and only 40kb away from caspase 8 gene in mouse genome. The genomic location and structure suggest a gene-duplication event during the evolution. Multiple splice variants of *c-Flip* gene have been reported. In mammalian cells, three isoforms of c-FLIP protein have been identified: 24 kD c-FLIP$_R$, 26kD c-FLIP$_S$ and 55kD c-FLIP$_L$ (Irmler et al. 1997; Rasper et al. 1998; Golks et al. 2005). Only c-FLIP$_R$ and c-FLIP$_L$ are expressed in murine cells. Like caspase 8, all c-FLIP proteins contain two DED domains. c-FLIP$_L$ also shows distinct homology to the protease region of caspase 8 at the C-terminal (Goltsev et al. 1997). However, it lacks the conserved caspase active residues, including the cysteine residue within the Gln-Ala-Cys-X-Gly motif and the histidine

residue within the His-Gly motif (Cohen 1997). Interestingly, retro-mutation of these residues back to their functional counterparts (in caspase 8) is not sufficient to restore the proteolytic activity, which suggests other inhibitory elements are within c-FLIP protein (Rasper et al. 1998). c-FLIP$_R$ and c-FLIP$_S$ contain short C-terminus and lack the catalytic sites for caspases (Golks et al. 2005; Poukkula et al. 2005). Therefore, they are resistant to cysteine protease cleavage (Figure 5A).

All three isoforms of c-FLIP are degraded through the ubiquitin-proteasome pathway. However, the half-lives of c-FLIP$_S$ and c-FLIP$_R$ are shorter than that of c-FLIP$_L$ (Poukkula et al. 2005; Kaunisto et al. 2009). The ubiquitination and rapid turnover of c-FLIP$_S$ is dependent on its specific 19 amino acid C-terminal tail, in which Lys192 and Lys195 are ubiquitinated (Poukkula et al. 2005). c-FLIP$_R$ contains Lys195 but not Lys192, and it is regulated in a similar pattern as c-FLIP$_S$ (Golks et al. 2005). The *in vivo* phosphorylation of the conserved S193 is critical for the ubiquitination of all three isoforms. While the ubiquitination leads to fast degradation of c-FLIP$_S$ and c-FLIP$_R$, it surprisingly does not affect the stability of c-FLIP$_L$ (Kaunisto et al. 2009). Cellular ROS stimulates ubiquitination on c-FLIP$_L$ at Lys167, which consequently leads to the degradation of c-FLIP$_L$ by the proteasome. Phosphorylation at the Thr166 residue is also required for ROS-induced ubiquitination and degradation of c-FLIP$_L$ (Wilkie-Grantham et al. 2013).

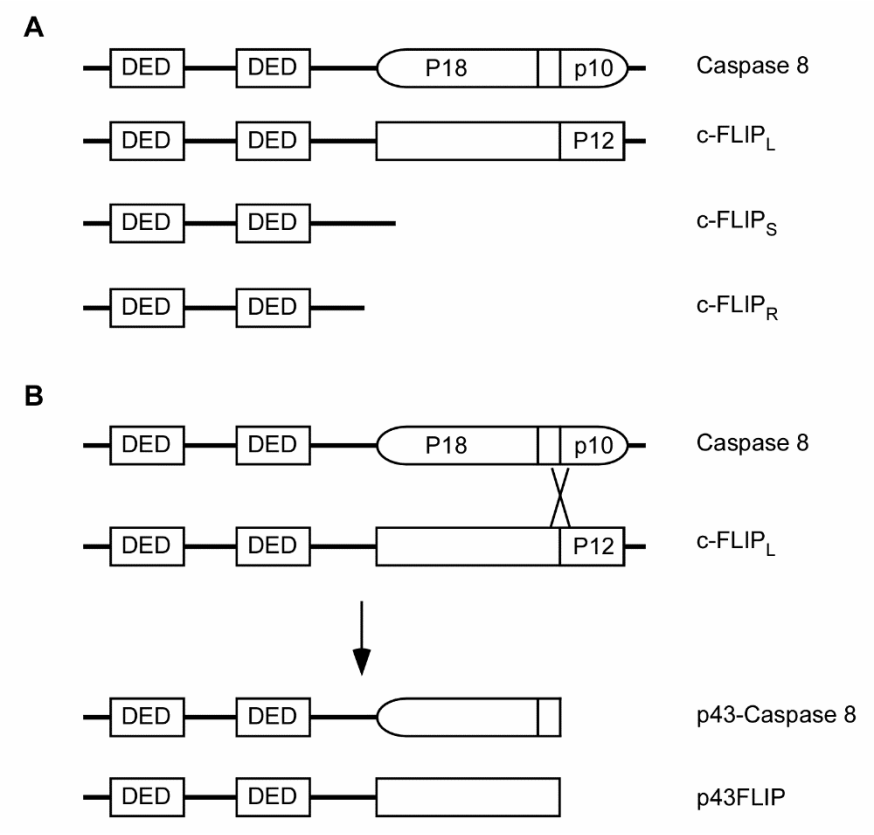

Figure 5: The structure of c-FLIP proteins.

Figure 5. (A) Schematic structure of c-FLIP$_L$, c-FLIP$_S$ and c-FLIP$_R$. All c-FLIP proteins and caspase 8 contain two DEDs on the N-terminus. c-FLIP$_L$ contains a caspases-like domain in the C-terminal region, which shares homology with caspases 8. (B) Processing of c-FLIP$_L$ by caspase 8. c-FLIP$_L$-caspase 8 interaction leads to incomplete cleavage of caspase 8 and c-FLIP$_L$.

1.5.2 c-FLIP in the regulation of apoptosis

c-FLIP is originally identified as an anti-apoptotic protein. Down-regulation of c-FLIP in cultured leukemic T cells is correlated with the increased sensitivity to Fas-induced apoptosis (Peter et al. 1997). c-FLIP associates with adaptor protein FADD, pro-caspase 8 and pro-caspase 10 via its two DEDs, whereas it lacks the proteolytic activity of caspases. This competitive heterodimerization blocks pro-caspse8/10 recruitment to DISC by adaptor protein FADD, thus inhibiting Fas(CD95)- and TNFR1-induced apoptosis (Goltsev et al. 1997; Han et al. 1997; Hu et al. 1997; Irmler et al. 1997; Shu et al. 1997; Srinivasula et al. 1997; Rasper et al. 1998). The recruitment of c-FLIP protein to DISC and its anti-apoptotic activity is not affected by its ubiquitination (Poukkula et al. 2005; Kaunisto et al. 2009). c-FLIP$_L$, as well as its C-terminal p12 domain alone, also directly interacts with the death domain of DR5 to prevent the recruitment of FADD and the formation of a functional DISC (Jin et al. 2004). However, overexpression of c-FLIP$_L$ in certain cell lines does not promote survival but increase apoptosis (Inohara et al. 1997; Shu et al. 1997; Micheau et al. 2002). The mechanism of this phenotype is dubious, but it is someway caspase 8-dependent. As caspase 10 is the homologue of caspase 8 and only expressed in human, from here I will focus on the knowledge gained from animal study regarding the relationship of c-FLIP and caspase 8.

The structure of c-FLIP proteins lead to the difference in their function. c-FLIP$_L$, c-FLIP$_R$ and c-FLIP$_S$ all compete with pro-caspase 8 for association with FADD and pro-

caspase 8 itself. However, c-FLIP$_L$ interacts with pro-caspase 8 via both two DEDs and caspase-like C terminus, which leads to the complexity of the function of c-FLIP$_L$. The pro-caspase 8-c-FLIP$_L$ heterodimer exhibits proteolytic activity. As a consequence, partial auto-processed caspase 8, p43 fragments of caspase 8, is generated (Micheau et al. 2002). c-FLIP$_L$ is also cleaved by caspase 8 to generate the N-terminal p43FLIP and the C-terminal p12 fragment (Figure 5B). c-FLIP$_L$ and p43cFLIP recruit TRAF2 and RIP-1 to DISC, which subsequently activate pro-survival NF-κB signaling (Shu et al. 1997; Kataoka and Tschopp 2004; Dohrman et al. 2005a; Budd et al. 2006). The RIP-1 recruitment may also contribute to the c-FLIP$_L$-caspase 8 inhibition on necroptosis (Oberst et al. 2011) (will be introduced in the next section). In contrast, due to the lack of pro-caspase 8-like C-terminal domains, c-FLIP$_R$ and c-FLIP$_S$ solely functions as competitors for caspase 8 recruitment and homodimerization.

Overexpression of c-FLIP$_L$ increase the rate of apoptosis in some cell lines and T lymphocytes (Inohara et al. 1997; Shu et al. 1997; Dohrman et al. 2005b). This phenotype was explained by the mechanism that c-FLIP$_L$-caspase 8 heterodimer augments caspase activity to execute apoptosis. However, biochemistry data is lacked to show the mechanism of effector caspase activation by c-FLIP$_L$-caspase 8 heterodimer. The other question needing to be answered is whether physiological expression level of c-FLIP$_L$ is ever sufficient for this effect.

1.5.3 c-FLIP in the regulation of necroptosis

Recent study suggested that the long isoform of c-FLIP contributes to the suppression of necroptosis. Caspase 8 negatively regulates RIP-1 activity by cleaving the deubiquitinated RIP-1 (Lin et al. 1999; Rajput et al. 2011). Therefore, the association of caspase 8 and RIP-1 may lead to the reduction of functional RIP-1. Both c-FLIP$_L$ and p43FLIP recruit RIP-1 to caspase 8 in T lymphocytes, and p43FLIP binds to RIP-1 with higher affinity than do full-length c-FLIP$_L$ or pro-caspase 8 (Dohrman et al. 2005a). These data suggest that the long isoform of c-FLIP may contribute to the suppression of necroptosis via recruiting RIP-1 to caspase 8. Another possibility is that the pro-caspase 8-c-FLIP$_L$ heterodimer functions as the negative regulator of necroptosis by restraining RIP-1 activity.

The earliest direct evidence in the connection of c-FLIP and necroptosis was shown in cells dying through mixed types of cell death. Knockdown of c-FLIP by short hairpin RNA sensitized the resistant cell lines to FasL/IAP antagonist-induced cell death, which involves both apoptosis and necroptosis (Geserick et al. 2009). However, in this experimental setting c-FLIP$_L$ only marginally improved cell survival in the presence of z-VAD-fmk, suggesting the major protective effect of c-FLIP is the result of caspase inhibition. Interestingly, c-FLIP$_L$ was shown to interfere with RIP-1 recruitment to the death-inducing DISC and cytosolic complex II in FasL/IAP antagonist treated cells, which is contradict to the previous published results that c-FLIP$_L$ recruits RIP-1 to

caspase 8 (Dohrman et al. 2005a). More direct data regarding the role of c-FLIPL in necroptosis were obtained from the study of TNF-α-induced RIP-3-dependent necroptosis in MEFs and CrmA-expressing 3T3 cells. c-FLIPL expression is indeed required for the protection against necroptosis in this experimental setting (Oberst et al. 2011).

c-FLIP proteins exhibit both anti-apoptosis and anti-necroptosis function in the cells that lack c-IAPs activity. c-IAP antagonist (Smac mimetics) treatment following TLR3 stimulation leads to the formation of "Ripoptosome", a ~2MD intracellular death-inducing complex (Feoktistova et al. 2011). Genotoxic etoposide depletes XIAP and c-IAPs in cancer cells and also triggers the formation "Ripoptosome" (Tenev et al. 2011). The assembly of Ripoptosome requires the kinase activity of RIP-1. Assembled "Ripoptosome" contains RIP-1, FADD, caspase 8, and different isoforms of c-FLIP. Caspase 10 is detected in the TLR3-induced Ripoptosome, but not in the genotoxin-induced Ripoptosome. Interestingly, the cellular ratio of different isoforms of c-FLIP may determine the fate of the cells. c-FLIPL is a negative regulator of Ripoptosome formation, though it is a component of the Ripoptosome. c-FLIPs, conversely, promotes the assembly of Ripoptosome. Ectopic expression of c-FLIPs results in increased necrotic cell death in HaCaT cells upon TLR3 activation (Feoktistova et al. 2011). These two studies did not show clear evidence regarding whether c-FLIPL-caspase 8 in the Ripoptosome fails to suppress RIP-1 kinase activity and necroptosis, in other words,

whether those cells truly die from necroptosis in the absence of z-VAD-fmk (Feoktistova et al. 2011; Tenev et al. 2011). At least, it is evident that c-FLIP$_L$ and c-FLIP$_S$ have different potentials in regulating apoptosis and necroptosis, and c-FLIP$_S$ may be a necroptosis promoter under certain conditions.

1.5.4 c-FLIP in T lymphocyte

c-FLIP protein is widely expressed in organs with the exception of colon, placenta and testis. The expression level is particularly high in the heart, skeletal muscle and lymphoid tissues (Irmler et al. 1997; Rasper et al. 1998). c-FLIP$_L$ protein is detectable in naïve T lymphocytes and slightly increased upon TCR activation (Chau et al. 2005). Activated T lymphocytes then downregulates the expression of c-FLIP$_L$, which is correlated with their susceptibility of FasL-induced apoptosis (Irmler et al. 1997). Though the expression levels of c-FLIP$_S$ and c-FLIP$_R$ are much lower than that of c-FLIP$_L$ in the resting T cells, c-FLIP$_S$ and c-FLIP$_R$ are quickly upregulated upon TCR stimulation (Chau et al. 2005; Golks et al. 2005). The kinetic of c-FLIP protein level in cells suggests that they may protect activated T cells at the early stage of activation.

c-FLIP proteins have been shown to be essential for T cell development. As c-FLIP-deficiency causes embryonic lethality on day 10.5, the role of c-FLIP was studied in mouse model with specific deletion of c-FLIP in T-cell compartment or lethally irradiated recombination-activating gene 1 (Rag 1)-deficient mice reconstructed with c-FLIP-deficient embryonic stem cells (*rcFLIP*$^{-/-}$) (Chau et al. 2005; Zhang and He 2005b;

Zhang et al. 2008a). In the *rcFLIP*$^{-/-}$ mice, the thymocyte development is arrested at the CD25^{+}CD44^{+} (DN2) and CD25^{+}CD44^{-} (DN3) stage, resulting in a much smaller DP population compared to that in wild type mice. However, some thymocytes develop past those stages into DP and SP cells. The cellularity of *rcFLIP*$^{-/-}$ thymocytes is greatly reduced, which is cause by both impaired thymocyte development and a deficiency of B cells (Chau et al. 2005). Study in the T cell specific c-FLIP-deficient mice can avoid the interference of defective B cells. *c-Flip*$^{f/f}$ Lck-Cre^{+} mice show normal thymocyte development from the DN to DP stage, probably because c-FLIP deletion starts at the DN2 stage in this model and already expressed c-FLIP protein protects the thymocytes to pass to DP stage. The SP thymocytes population is impaired in *c-Flip*$^{f/f}$ Lck-Cre^{+} mice and *c-Flip*$^{-/-}$ SP cells fails to down-regulate their coreceptors (Zhang and He 2005b). In both model, the thymocytes defect is likely caused by enhanced apoptosis *in vivo*, as fresh isolated *c-Flip*$^{-/-}$ thymocytes showed increase apoptosis in the presence or absence of TCR/Fas stimulation (Zhang and He 2005b).

Survival of the mature T lymphocytes requires c-FLIP. c-FLIP protects mature T cell from TCR, Fas and TNF-α-induced cell death. Loss of *c-Flip* gene leads to severe TCR activation-induced apoptosis, while expression of either isoform of c-FLIP (c-FLIP$_L$ or c-FLIP$_R$) is sufficient to rescue the death phenotype (Zhang et al. 2008a). Importantly, deletion of *Tnf* gene failed to improve cell survival upon TCR stimulation and Fas mutation (*lpr*/*lpr*) only showed partial rescue in the enhanced TCR-induced apoptosis in

c-FLIP-deficient T lymphocytes (Zhang et al. 2008a). These results suggest that c-FLIP may play a role beyond extrinsic death pathway.

c-FLIP$_L$ is suggested to be involved in NF-κB activation and signal regulated kinase (ERK) signaling in T lymphocytes. c-FLIP$_L$ interacts with TRAF1/2, RIP-1 and Raf-1 in DISC (Kataoka et al. 2000). p43FLIP recruits TRAF2 and RIP-1 with higher affinity than full length of c-FLIP or caspase 8 (Kataoka and Tschopp 2004; Dohrman et al. 2005a). Ectopic expression of c-FLIP$_L$ increases TCR activation-induced proliferation in T lymphocytes (Lens et al. 2002). However, all those studies addressing the role of c-FLIP$_L$ in T cell signaling were conducted in overexpression models. Studies in T-cell specific c-FLIP deletion model later revealed that both NF-κB and ERK activation are intact in c-FLIP-deficient thymocytes and mature T cells. (Chau et al. 2005; Zhang and He 2005b). Moreover, JNK, p38 and IκB-a phosphorylation are slightly increased in c-FLIP$_L$-deficient T cells upon TCR activation (Zhang et al. 2008b). It is consistent with the findings from the study of primary human keratinocytes, that knockdown of c-FLIP enhances FasL- and TRAIL-induced NF-κB, JNK and p38 activation (Kavuri et al. 2011).

2. Materials and methodology

2.1 Animals

2.1.1 The generation of T-cell specific c-FLIP-deficient mice

c-Flip$^{f/f}$, c-FLIP$_L$ bacterial artificial chromosome (BAC) transgene and c-FLIP$_R$ BAC transgene mice were generated by a previous member in the laboratory (Zhang and He 2005b; Zhang et al. 2008b; Zhang et al. 2008a). In brief, the exon 1 of *c-Flip* gene was flanked by two *lox*P sites in *c-Flip*$^{f/f}$ mice (Figure 5A). c-FLIP$_R$ BAC transgene and c-FLIP$_L$ BAC transgene were generated by fusing mouse c-FLIP$_R$ cDNA and c-FLIP$_R$ cDNA, including their own stop codon and poly(A) tails, to the start codon in exon 1 of *c-Flip* gene respectively (Figure 5B). A 60kb BAC fragment that is 15kb downstream of the nearest 5′ gene (*Als2cr12*) and 10kb upstream of the nearest 3′ gene (*caspase-8*) was used for injection to create BAC transgenic mice. T cell specific c-FLIP$_L$-deficient mice were generated by crossing c-FLIP$_R$ BAC transgenic mice to *c-FLIP*$^{f/f}$ Lck-Cre$^+$ mice. *c-Flip*$^{f/f}$ littermates were used as wild type controls. The major advantage for using the BAC Tg approach is to have the expression of transgene controlled by its endogenous regulatory elements. c-FLIP conditional knockout mice were generated by crossing *c-Flip*$^{f/f}$ to ER-Cre$^+$ mice. Animals were used at 6-8 weeks of age. Animal usage was conducted according to protocols approved by the Duke University Institutional Animal Care and Use Committee (IACUC).

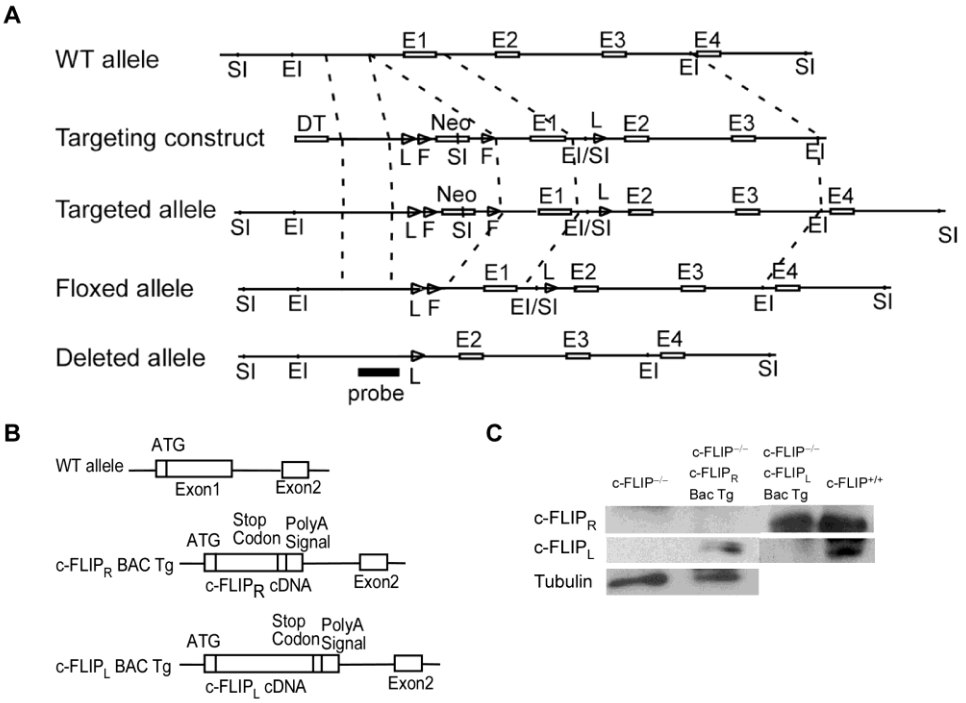

Figure 6: Generation of c-FLIP$_L$- and c-FLIP$_R$-deficient T cells

Figure 6. (A) Diagram of the c-FLIP targeting construct. E1-E4, exon 1 – exon 4 of the *c-FLIP* gene; EI, EcoRI; SI, SacI; L, *lox*P site; F, FRT site; DT, diphtheria toxin; Neo, neomycin-resistant gene. (B) Schematic of c-FIP$_R$ BAC transgene constructs. (C) Western blot analysis of c-FLIP expression in thymocytes of *c-Flip$^{f/f}$* Lck-Cre$^+$, *c-Flip$^{f/f}$* Lck-Cre$^+$ c-FLIP$_R$-BAC Tg$^+$, *c-Flip$^{f/f}$* Lck-Cre$^+$ c-FLIP$_L$-BAC Tg and wild type mice. Total thymocytes were blotted with anti-c-FLIP antibody (This figure is adapted from "An essential role for c-FLIP in the efficient development of mature T lymphocytes" Originally published in Journal of Experimental Medicine. 202:395-404. Figure 1A. And "The long isoform of cellular FLIP is essential for T lymphocyte proliferation through an NF-κB-independent pathway".

Association of Immunologists, Inc. Originally published in Journal of Immunology., 2008 Apr 15;180(8):5506-11. Figure 1).

2.1.2 Genotyping

Animals were genotyped by PCR. The reaction to determine the floxed and wild-type c-FLIP alleles was performed at 94°C for 30 s, 61°C for 30 s, and 72°C for 60 s for 35 cycles. The primers for the wild-type and floxed c-FLIP alleles were the same: forward, 5'-CAT GAG CAC TGA GGG ACA CAG CAC-3′; reverse, 5'-CGG AGT TTG CTA CAG GAA GGC CAC-3'. The product of the wild type allele was 340 bp, while the product of the floxed allele was 480 bp. The reaction to determine the c-FLIPL transgene and c-FLIPR transgene was performed at 94°C for 30 s, 52°C for 30 s, and 72°C for 120 s for 35 cycles. The primers for the c-FLIPL transgene and c-FLIPR were the same: forward, 5'-GAG GTT GAG GGA CTT GGC ATG-3′; reverse, 5'-TCA GCA GGA CCC TAT AAT CAG-3'. The reaction to determine the floxed and wild-type Atg3 alleles was performed at 94°C for 30 s, 60°C for 60 s, and 72°C for 60 s for 35 cycles. The primers for the wild-type and floxed Atg3 alleles were the same: forward, 5'- CGA TGG CAT CTT ATG CTG AGC AAT G-3′; reverse, 5'- AAC CAT AGC CGT GGT GTC TGG TAA-3'. The product of the wild type allele was 748 bp, while the product of the floxed allele was 866 bp. The reaction to determine the Lck-Cre alleles was performed at 94°C for 30 s, 57°C for 30 s, and 72°C for 45 s for 35 cycles. The primers were: forward, 5'- gca gga agt ggg taa cta gac taa c-3′; reverse, 5'- tct ccc acc gtc agt acg tga gat at-3'. The reaction to determine the ER-

Cre allele was performed at 94°C for 30 s, 61°C for 30 s, and 72°C for 45 s for 35 cycles. The primers were: forward, 5'- CCA CCA GCC AGC TAT CAA CT-3′; reverse, 5'- TGA ACC AGC TCC CTA TCT GC-3'. The reaction to determine the Bak knockout and wild-type alleles was performed at 94°C for 30 s, 55°C for 30 s, and 72°C for 120 s for 35 cycles. The forward primers for the wild-type and Bak knockout alleles were the same: 5'-GAG CCA TGA AGA TGT TTA GC-3′. The reverse primer for the wild type allele was: 5'-GGT GTC CAC ACT AGA GAA CTA CTC-3'; for the Bak knockout allele was: 5'-TCA GGA CAT AGC GTT GGC TAC-3'. The reaction to determine the Bax knockout and wild-type alleles was performed at 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s for 35 cycles. The forward primer for the wild-type allele was: 5'-CCG CTT CCA TTG CTC AGC GG-3′; for the Bax knockout alleles was: 5'-GTT GAC CAG AGT GGC GTA GG-3′. The reverse primers for the wild-type and Bak knockout alleles were the same: 5'-GAG CTG ATC AGA ACC ATC ATG-3'. The reaction to determine the Bim knockout and wild-type alleles was performed at 94°C for 30 s, 58°C for 60 s, and 72°C for 60 s for 35 cycles. The forward primer for the wild-type allele was: 5'-GTG CTA ACT GAA ACC AGA TTA-3′; for the Bim knockout alleles was: 5'-CTC AGT CCA TTC ATC AAC AG-3′. The reverse primers for the wild-type and Bak knockout alleles were the same: 5'-CAT TCT CGT AAG TCC GAG TCT-3'. The reaction to determine the lpr and wild-type Fas alleles was performed at 94°C for 30 s, 59°C for 30 s, and 72°C for 60 s for 35 cycles. The forward primers for the wild-type Fas and lpr allele were the same: 5'-GTA AAT AAT

TGT GCT TCG TCA G-3′. The reverse primer for the wild-type allele was: 5'-CAA ATC TAG GCA TTA ACA GTG-3', for the lpr alleles were the same: 5'-TAG AAA GGT GCA CGG GTG TG-3'.

2.2 Cell isolation and culture

2.2.1 Lymphocyte isolation

Single-cell suspensions were prepared from spleens or peripheral lymph nodes and re-suspended in ACK lysis buffer (0.15M NH_4CL, 10mM $KHCO_3$, 0.1mM EDTA pH7.4) for up to 3 minutes for red blood cell lysis. T lymphocytes were enriched using an EasySep™ mouse T cell negative enrichment kit from Stemcell™ Technologies according to the manufacturer's instructions.

2.2.2 Cell culture

Total splenocytes or enriched T lymphocytes were cultured in complete RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS) at 37°C in the presence of 5% CO_2 for indicated time. For naïve T cell culture, IL-7 (1 ng/ml; PeproTech) was added in the medium and re-added every 3 days. L929 cells were cultured in DMEM containing 2mM Glutamine and 10% FBS at 37°C in the presence of 5% CO_2. Semi-confluent cultures of L929 cells were spitted using 0.25% trypsin/EDTA. 10 μM z-VAD-fmk (Sigma), 10 μM z-IETD-fmk (BD Pharmingen), 10 μM z-LEHD-fmk (BD Pharmingen), 2 μg/ml anti-FasL (2MFL3, BioLegend), 100 nM necrostatin-1 (Enzo life science) and

10mM acetylcysteine (NAC) were add in lymphocyte cultures as indicated. For apoptosis induction, 10 μM etoposide or 500 nM staurosporine was added in the culture.

2.2.3 T cell activation

T lymphocytes within total splenocytes were stimulated with soluble anti-CD3 (2C11, 5 μg/ml, otherwise indicated in the figures). Anti-CD28 (clone 37.51, BioLegend, 2 μg/ml) was added to several cultures as indicated. Enriched T lymphocytes were activated with plate-bound anti-CD3 and anti-CD28 (pre-incubation the culture dish with 5 μg/ml anti-CD3 and 2 μg/ml anti-CD8 in PBS at 4°C overnight or at 37°C for 1 hour).

2.2.4 *In vitro* inducible deletion

Total splenocytes were cultured with 200 nM 4-hydroxy-tamoxifen (4-OHT; Sigma-Aldrich) for 3 day in the presence of IL-7 (1 ng/ml; PeproTech). Live cells were purified after deletion using Ficoll.

2.3 Flow cytometry analysis

Single-cell suspensions were prepared from spleens and lymph nodes after red blood cells lysis, or from T lymphocytes that were cultured for the indicated time. The cells were incubated with an FcR-blocking antibody (2.4G2), stained with FITC-, PE-, PE/Cy5-, APC-, APC-Cy7-, Pacific Blue or biotin-labeled mAbs on ice for 20 min, and washed with FACS buffer (2% FBS, 0.02% NaN_3 in PBS). Cells stained with biotin-labeled primary mAbs then stained with streptavidin-PE on ice for 20 min and washed.

A total of 0.5-20×10^5 events were collected on a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). All fluorescence-labeled Abs, including anti-CD3, anti-CD4, anti-CD8, anti-TCRβ, anti-CD19, anti-FasL, and anti-Fas, were obtained from BioLegend. To stain cell surface FasL, cells were cultured with 1 × protease inhibitor (Aprotinin, Sigma) for 4 hours immediately before staining.

2.4 T cell proliferation assay

After red blood cell lysis, splenocytes were incubated with 5 μM cell permeable carboxyfluorescein diacetate succinimidyl ester (CFDA-SE, Molecular Probes) in 5% FBS-PBS for 5 min at room temperature. Non-fluorescent CFDA-SE is processed by intracellular esterase, converting to fluorescent carboxyfluorescein succinimidyl ester (CFSE), which covalently binds to intracellular molecules. Cells were then washed with 5% FBS-PBS for 3 times to remove surface binding. CFSE-labeled splenocytes were stimulated with anti-CD3 (2C11, 5 μg/ml) in the presence or absence of anti-CD28 (clone 37.51; BioLegend, 2 μg/ml) for 2-3 days. Proliferation was determined by measuring CFSE dilution in live (7-AAD$^-$) CD4$^+$ or CD8$^+$ T cells by flow cytometry.

2.5 Apoptosis/necrosis assays

2.5.1 Annexin V staining.

Cells were first stained for surface markers following the methods described above. Then cells were re-suspended in Annexin V-binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and incubated with Annexin V-PE (BD Bioscience)

and 7-AAD (BD Bioscience) at room temperature in for 15 min. The cells were then diluted in Annexin V-binding buffer and analyzed by flow cytometry within one hour.

2.5.2 MOMP staining

Cells were incubated with TMRE (40 nM) in complete RPMI at 37°C for 20 min. After that, cells were stained with surface marker and 7-AAD (Invitrogen) in FACS buffer on ice for 15 min, and then washed with FACS buffer, stored on ice, and analyzed immediately.

2.5.3 Fragmented DNA release assay.

Four-week-old mice were injected *i.p*. with 1-1.5 mg 5-bromo-2'-deoxyuridine (BrdU) per animal per day for two weeks. Over 90% of the peripheral T lymphocytes were labeled with BrdU by the end of the injection period. The kinetics of fragmented DNA release were analyzed using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science) following the manufacturer's instruction. In brief, enriched naïve T lymphocytes were stimulated with plate-bound anti-CD3/anti-CD28 for the indicated time. Half of the supernatant was removed and stored at 4°C. T cells (both live and dead) are spin-down and lysed. Half of volume of the T cell lysates and the saved supernatants (for 1:1 supernatant/cells ratio) at different time points were subjected to ELISA for BrdU, which represented the relative quantity of fragmented DNA.

2.5.4 Cytochrome c release

T lymphocytes were enriched as 2.2.1 described. Cell purity was determined by flow cytometry to be >95%. Cytosol was released by digitonin in 80mM KCl buffer. The mitochondria/nuclei sample was prepared in the total cell lysis buffer (50 mM Tris-HCl pH 7.4], 150 mM NaCl, 2 mM EGTA, 2 mM EDTA, 0.2% Triton X-100, 0.3% NP-40 and 1 × Protease Inhibitors). Cytochrome c release was tested by western blot.

2.6 Western blot

T cells were purified using an EasySep™ mouse T cell enrichment kit (Stemcell Technologies) according to the manufacturer's instructions. For activated T cells, enriched T cells were cultured for 16-18 hours with plate bound anti-CD3. Cell purity was determined by flow cytometry to be >95%. Purified T cell lysates were prepared in sample buffer (50 mM Tris-HCl [pH 6.8], 50mM 2-ME, 2% SDS, 0.2% bromophenol blue, and 10% glycerol). The primary antibodies used for western blots were rat anti-c-FLIP (clone Dave-2; Alexis Biochemical), rabbit anti-full length caspase 8 (polyclonal, Cell Signaling), rabbit anti-active caspase 8 (polyclonal, Cell Signaling), mouse anti-RIP-1 (38/RIP, BD Bioscience), rabbit anti-RIP-3 (polyclonal, Axxora), hamster anti-Bcl-2 (polyclonal, BD pharmingen), rabbit anti-Bcl-x_L (polyclonal), rabbit anti-Mcl-1 (polyclonal, Rockland Immunochemicals), rabbit anti-Bim (polyclonal, Cell Signaling), rabbit anti-Bax (polyclonal, Cell Signaling), rabbit anti-Bak (polyclonal, Cell Signaling), rabbit anti-Bid (polyclonal, Abcam), mouse anti-α-Tubulin (B-5-1-2, Sigma) and goat

anti-β-Actin (polyclonal, Santa Cruz Biotechnology). For HRP-labeled western blot, the secondary antibodies were anti-rabbit IgG-HRP, anti-mouse IgG-HRP, anti-hamster IgG-HRP and anti-goat IgG-HRP (Jackson Immunoresearch). The development of the western blot was achieved with SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). For fluorescent western blot, the secondary antibodies were anti-rabbit IgG-Alexa Fluor 680, anti-mouse IgG-Alexa Fluor 680, and anti-goat IgG-Alexa Fluor 790 (Molecular Probes, Invitrogen).

2.7 Autophagy detection

2.7.1 LC3 fluorescence microscopy

Enriched primary T lymphocytes were first stained with anti-LC3 (P015, MBL International), then stained with Cy3-, FITC- and Pacific Blue-labeled, anti-rabbit IgG, anti-CD4, and anti-CD8. All images were captured with a custom-built Zeiss Observer D1 using a Zeiss 3100 objective lens and a 1.4 numerical aperture. Images were captured using a Photometrics Cool SNAP HQ2 and analyzed using Metamorph software. Images were deconvoluted and thresholded. Deconvolution was done blind at 40 iterations. LC3 positive staining is defined as >180% of background signal. LC3 puncta is defined as any enclosed LC3 positive staining area no smaller than 10 pixels.

2.7.2 LC3 detection by western blot

LC3-I/LC3-II ratio was analyzed by the western blot as described in 2.6. Anti-LC3 (polyclonoal P015, MBL International) was used to detect LC3 protein.

2.7.3 Transmission electron microscopy

Live naïve T lymphocytes were sorted into $CD4^+$ $CD44^{low}$ 7-AAD^- or $CD8^+$ $CD44^{low}$ 7-AAD^- populations. For sorting of activated T cells, total splenocytes were cultured with anti-CD3 (2C11, 5 μg/ml) for 18 hours before activated cells were sorted into $CD4^+$ 7-AAD^- or $CD8^+$ 7-AAD^- populations. Cells were fixed in 4% gluteraldehyde in 0.1 M sodium cacodylate overnight and then pelleted and embedded in 2.5% molten agar and transferred into fresh fixative. The embedded cells were incubated in fresh fixative for a period of one hour to overnight. The specimens were rinsed twice with 0.1 M sodium cacodylate buffer and post-fixed in 1% osmium tetroxide for 90 min. The specimens were then rinsed again in sodium cacodylate buffer and dehydrated through an ethanol series (50%, 70%, 95%, 95%, 100%, 100%) and placed in two changes of a transitional solvent (propylene oxide). The specimens were then placed in a 1:1 mixture of propylene oxide:epoxy resin 812 overnight before being placed in epoxy 812 for an hour and embedded. The blocks were polymerized in a 60°C oven overnight. Ultrathin sections (90 nm) were cut with a Reichert-Jung Ultracut E ultramicrotome, placed on copper grids, and stained with uranyl acetate and Reynolds' lead citrate. The specimens were viewed with a Phillips CM 12 transmission electron microscope equipped with an AMT XR-100 2Vu digital camera system. The intracellular double membrane structure was identified as autophagosomes.

2.8 RT-PCR.

Total RNA was extracted from sorted freshly isolated T cells and T cells cultured for 16-18 hours with plate bound-anti-CD3. The primers for RIP-1 are: 1:5'-GGAAGGAT AATCGTGGAGGC-3' (forward, exon4) and 5'–AAGGAAGCCACACCAAGATC-3' (reversed, exon5).

2.9 Reactive oxygen species (ROS) and mitochondria analysis

Splenocytes were cultured in the presence or absence of anti-CD3 (5 μg/ml, 2C11) for 16 hours before staining. For ROS analysis, single cell suspensions were incubated with 2.5 mM CM-H_2DCFDA (Invitrogen) in DPBS at 37°C for 15min or 2.5 mM dihydroethidium (DHE; Sigma-Aldrich) in RPMI 1640 medium at 37°C for 30 min. Mitochondria was stained by 100 nM MitoTracker® Green (Invitrogen, Molecular Probes) in RPMI 1640 medium for 30 min. The cells were washed with RPMI 1640 medium and then stained with anti-CD4-APC, anti-CD8-APC/Cy7, and 7-AAD, then washed with FACS buffer and analyzed by FACS immediately.

2.10 Statistics

Unpaired two-tailed Student's *t*-tests were used to compare the means of different samples.

3. The role of c-FLIP$_L$ in the survival of activated T lymphocytes

This chapter is adapted from "A role for c-FLIP$_L$ in the regulation of apoptosis, autophagy, and necroptosis in T lymphocytes", Ming-Xiao He and You-Wen He, Cell Death and Differentiation. 2013 Feb;20(2):188-97. doi: 10.1038/cdd.2012.148.

3.1 Introduction

The size of the T lymphocyte compartment is tightly controlled by carefully balanced T cell expansion and apoptosis. Apoptosis plays critical in the regulation of T cell response, as death receptor Fas (CD95) ligation to FasL results in the deletion of re-activated T cells through TCR activation-induced cell death (AICD) (Strasser et al. 2009). Recently, necroptosis was observed in activated T cells when the activity of caspase 8 is blocked (Bell et al. 2008; Osborn et al. 2010; Chen et al. 2011). Necroptosis was correlated with high level of autophagy in multiple types of cells including T lymphocytes, but whether excessive autophagy actually causes necroptosis remains controversial (Yu et al. 2004; Bell et al. 2008; Osborn et al. 2010; Ch'en et al. 2011b; Chen et al. 2011). Though several studies showed each of this process are involved in T cell response, the interplay of apoptosis, autophagy, and necroptosis in T lymphocytes remains poorly understood. c-FLIP is a potential regulator for all three of these pathways (apoptosis, autophagy, and necroptosis) in primary T lymphocytes.

c-FLIP proteins suppress extrinsic apoptosis pathway by inhibiting pro-caspase 8 homodimerization and activation. (Budd et al. 2006). However, when dimerized with c-FLIP$_L$, pro-caspase 8 retains partial proteolytic activity (Kataoka and Tschopp 2004). In contrast, the formation of c-FLIP$_R$-pro-caspase 8 heterodimers entirely inhibits pro-caspase 8 activation (Budd et al. 2006). c-FLIP$_L$ and c-FLIP$_R$ are both expressed in mature T lymphocytes, and the expression of either isoform is sufficient to protect resting T cells from apoptosis (Zhang et al. 2008a). Why T cells require the expression of both isoforms remains to be studied.

Previous reports suggested that c-FLIP$_L$ is involved in the regulation of necroptosis. c-FLIP$_L$ protects c-IAPs antagonist-treated cells from Fas-induced cell death, in which both apoptosis and necroptosis are involved (Geserick et al. 2009). Furthermore, c-FLIP$_L$ inhibits the formation of cell death inducing "Ripoptosome", which functions in TLR3-induced apoptosis and necroptosis (Feoktistova et al. 2011). Silencing of c-FLIP$_L$ by siRNA sensitizes cells to TNF-α-induced RIP-1/RIP-3-dependent necroptosis (Oberst et al. 2011). However, the role of c-FLIP$_L$ in necroptosis has yet to be studied in primary T cells in a genetically deficient model.

c-FLIP may also promote T cell survival though controlling threshold of autophagy. Overexpression of either isoform of c-FLIP protein suppresses autophagy, by the mechanism that c-FLIP competes with LC3 in Atg3 conjugation (Lee et al. 2009). On the other side, recent studies suggest a correlation between caspase 8 inhibition and

hyperactive autophagy (Yu et al. 2004; Bell et al. 2008; Wu et al. 2011). Whether c-FLIP regulates autophagy by itself or through controlling caspase 8 activity in primary cells has not yet been addressed. More importantly, whether enhanced autophagy leads to necroptosis in T lymphocytes remains controversial (Bell et al. 2008; Osborn et al. 2010; Ch'en et al. 2011). Thus, the role of c-FLIP in regulating autophagy and autophagy-related cell death in primary T cells has yet to be determined.

It was previously reported that c-FLIP$_L$-deficient T cells failed to respond to *Listeria monocytogenes* infection (Zhang et al. 2008b). In this chapter, it will be shown that c-FLIP$_L$-deficient T cells fail to proliferate upon TCR stimulation due to extensive Fas-dependent cell death. Both apoptosis and RIP-1-dependent necroptosis contribute to the loss of cellularity after T cell activation. Interestingly, c-FLIP$_L$-deficient T cells generate more active caspase 8 (p18) than wild type T cells, indicating that the apoptotic and anti-necrotic activities of caspase 8 may be independent. In addition, the autophagy induction in c-FLIP$_L$-deficient T cells is increased, which in fact promotes T cell survival. Together, our results identify c-FLIP$_L$ as a key regulator of apoptosis, necroptosis, and autophagy in primary T lymphocytes.

3.2 Result

3.2.1 c-FLIP$_L$-deficient T lymphocytes display enhanced cell death upon TCR stimulation.

c-FLIP$_R$ BAC transgenic mice were crossed to *c-Flip$^{f/f}$* Lck-Cre$^+$ mice to generate conditional knockout mice that only c-FLIP$_L$ is absent in T lymphocytes (referred to as c-

FLIP$_L^{-/-}$ mice). The study in c-FLIP$_L^{-/-}$ mice showed that c-FLIP$_L$ was dispensable for T cell development and naïve T cell survival. However, c-FLIP$_L^{-/-}$ mice failed to generate effector T-cell responses against *L. monocytogenes* infection *in vivo* (Zhang et al. 2008b). The impaired effector response in c-FLIP$_L^{-/-}$ mice was initially attributed to defective T cell proliferation. *In vitro* T cell proliferation assay showed that the proliferation of c-FLIP$_L$-deficient T cells is defective (Figure 7).

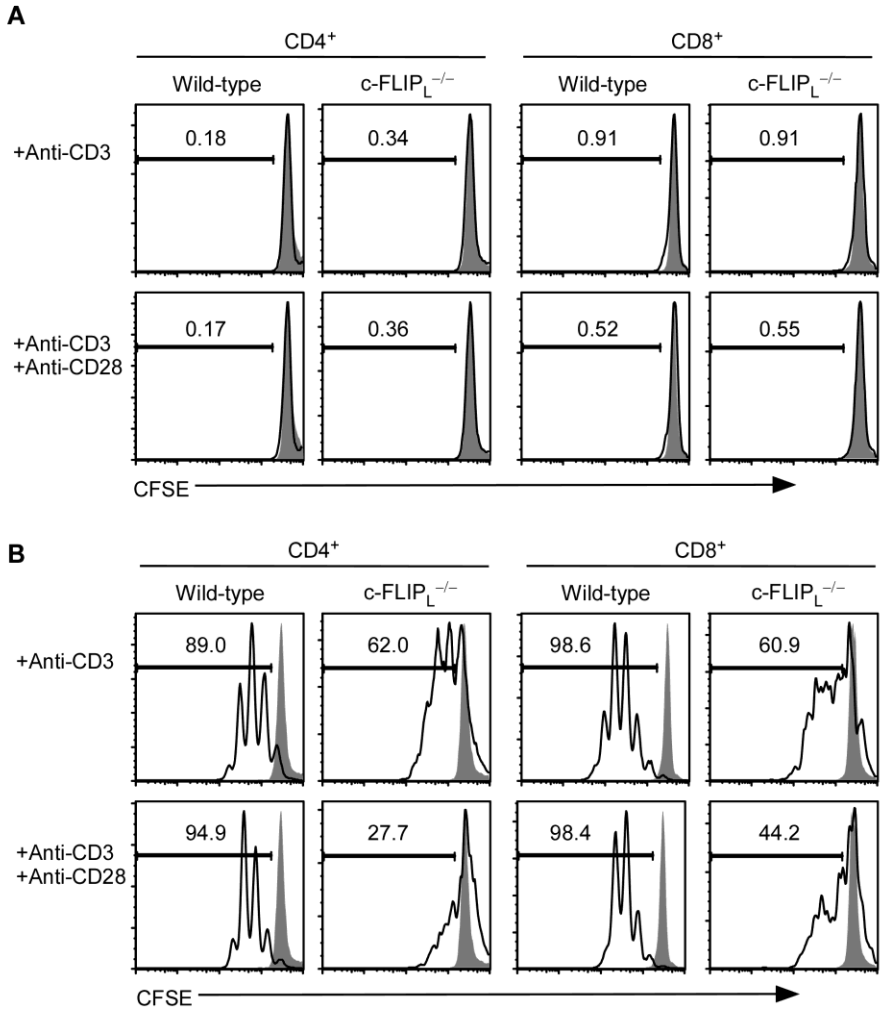

Figure 7: Defective proliferation upon TCR stimulation in c-FLIP$_L$-deficient T lymphocytes

Figure 7. T cell proliferation 24 hours (A) or 72 hours (B) after the initial TCR activation. Total splenocytes from c-FLIP$_{L}^{-/-}$ and wild type (*c-Flip*$^{f/f}$) mice were labeled with CFSE, stimulated with anti-CD3 in the absence or presence of anti-CD28. Cell proliferation was determined by flow cytometry after 24 hours (A) and 72 hours (B). Numbers represent the frequency of T cells that have undergone cell proliferation. Histograms were gated on the 7-AAD^{-} CD4^{+} or 7-AAD^{-} CD8^{+} populations. The grey area represents un-stimulated T cells. Data are representative of >3 experiments.

Because impaired proliferation could be caused by enhanced cell death, we then analyzed apoptosis and cell death by Annexin V /7-AAD staining. We found that the death rate of c-FLIP$_L$-deficient T cells upon TCR stimulation was dramatically increased when compared to that in wild type T cells. Regardless of the strength of the TCR signal, a majority of c-FLIP$_L$-deficient T cells (both $CD4^+$ and $CD8^+$) died within 36 hours upon simulation (Figure 8A). Fewer survived cells were detected at 40 hours after TCR stimulation (Figure 8B). We also examined cell death using mitochondrial outer membrane permeabilization (MOMP) staining and observed similar results (Figure 9) The decreased numbers of live c-FLIP$_L^{-/-}$ T were apparent less than 24 hours after TCR stimulation. This result suggested that this phenomenon was not attributable to impaired T cell proliferation, as T lymphocytes did not divide during the first 24 hours (Figure 7A). In contrast to these results, c-FLIP$_L$-deficient T cells displayed no survival defect in the absence of TCR stimulation (Figure 8 and 9), likely due to the protective effect of c-FLIP$_R$. Interestingly, we observed that c-FLIP$_L^{-/-}$ $CD4^+$ T cells consistently exhibited slightly better survival rate than wild type T cells in the absence of TCR stimulation (Figure 8 and 9). The cell survival defect after T cell activation was only observed in c-FLIP$_L$-deficient T cells, but not in c-FLIP$_R$-deficient T cells (Figure 10A), though both isoforms were expressed in mature T lymphocytes (Figure 10B).

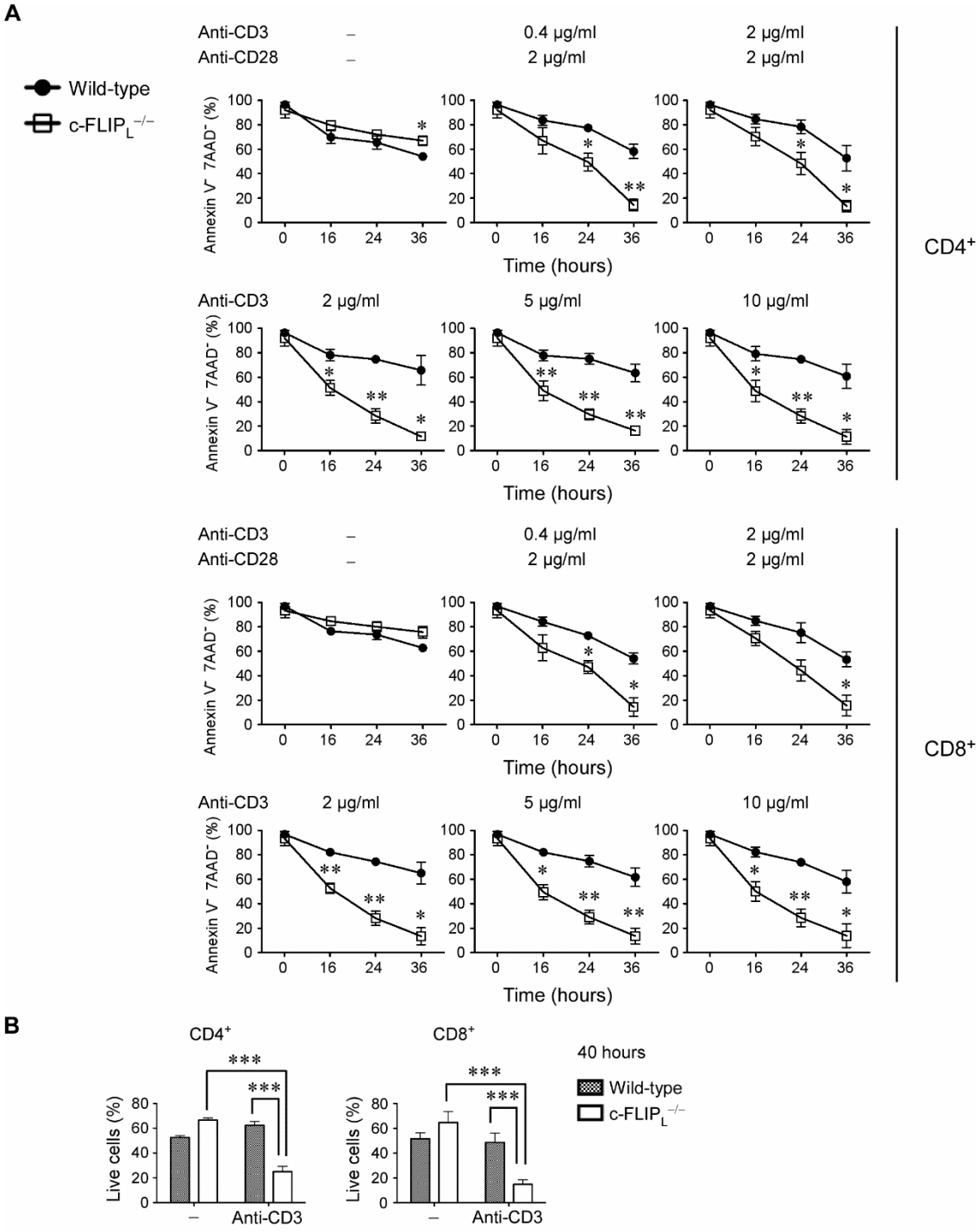

Figure 8: Enhanced cell death upon TCR activation in c-FLIP$_L$-deficient T lymphocytes

Figure 8. (A) Time course analysis of the survival rate of naïve and activated c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with different concentrations anti-CD3 in the presence or absence of anti-CD28 as labeled. The cell survival rates of $CD4^+$ and $CD8^+$ T cells were analyzed by flow cytometry at different time points after TCR activation. Live cells were analyzed by gating on the Annexin V^- 7-AAD^- population within the total $CD4^+$ or $CD8^+$ populations (n=4). (B) Cell death rate of naïve or activated c-FLIP$_L^{-/-}$ T cells after 40 hours of culture. Total splenocytes were cultured in the presence or absence of anti-CD3 (5 mg/ml) for 40 hours. Cell death rates were analyzed as described in A (n=6). All error bars represent the standard error of the mean (s.e.m.). * P<0.05, ** P<0.01, *** P<0.001.

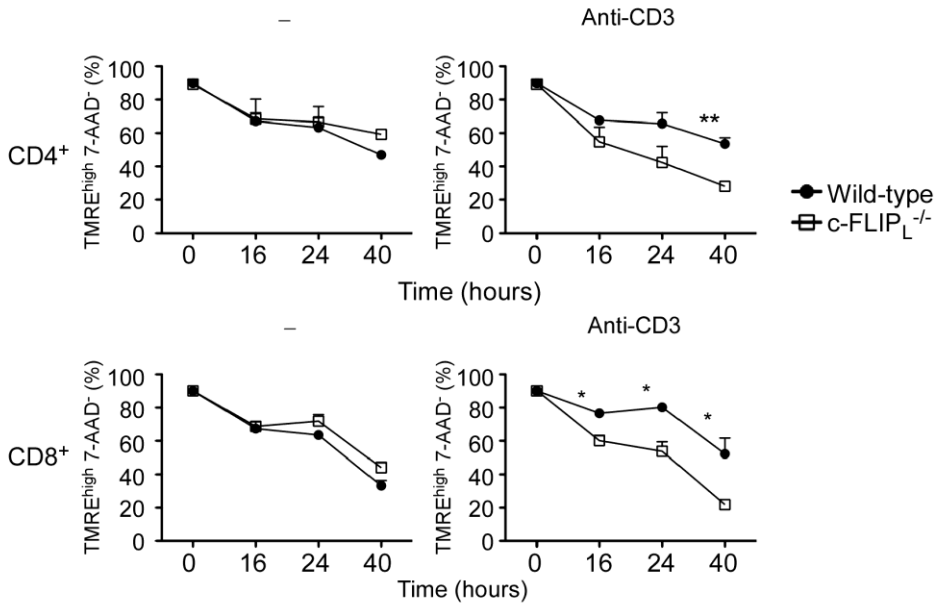

Figure 9: Enhanced MOMP after TCR stimulation in c-FLIP$_L$-deficient T lymphocytes.

Figure 9. TMRE staining of c-FLIP$_L^{-/-}$ T cells upon TCR stimulation. Total splenocytes were cultured in the absence or presence of anti-CD3 (5μg/ml) for 40 hours. At different time points after activation, the cells were incubated with TMRE for 30 min then stained with 7-AAD on ice for 15 min. Labeled cells were analyzed by flow cytometry. Live cells were analyzed by gating on the TMRE^{high} 7AAD$^-$ population within the total CD4$^+$ or CD8$^+$ populations (n=3). All error bars represent the standard error of the mean (s.e.m.). * P<0.05, ** P<0.01.

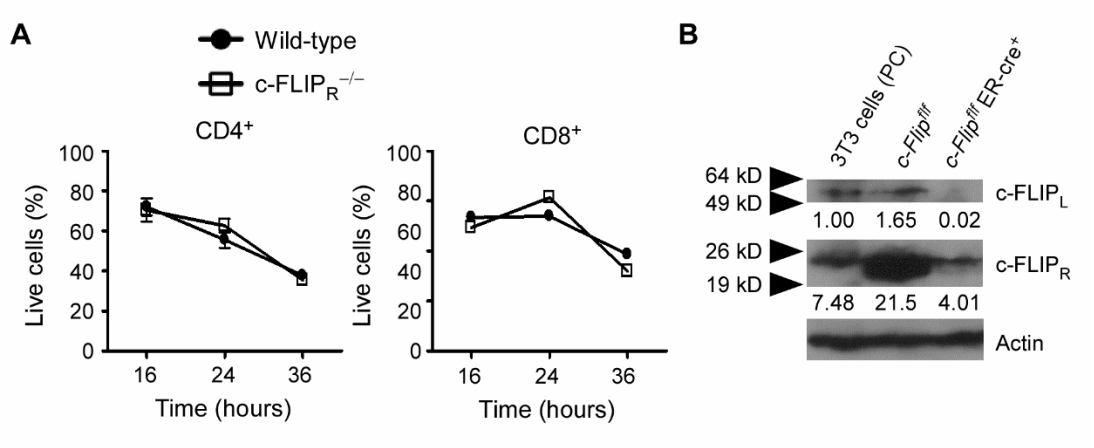

Figure 10: Normal survival after TCR stimulation in c-FLIP$_R$-deficient T lymphocytes

Figure 10. (A) Cell death upon TCR stimulation in c-FLIP$_L$-deficient T cells. Total splenocytes from *c-Flip$^{f/f}$* and *c-Flip$^{f/f}$* ER-Cre$^+$ *c-FLIP$_L$-Tg$^+$* mice were cultured for 3 days in the presence of 4-hydroxy-tamoxifen (0.2 μM) and IL-7 (1 ng/ml) to generate wild type and c-FLIP$_R^{-/-}$ cells. Live cells were isolated using Ficoll, stimulated with plate-bound anti-CD3, and cultured for 40 hours with IL-7 (1 ng/ml). Cell death was analyzed by flow cytometric analysis of 7-AAD staining. (B) c-FLIP$_L$ and c-FLIP$_R$ expression levels in T lymphocytes. Total splenocytes from *c-Flip$^{f/f}$* and *c-Flip$^{f/f}$* ER-Cre$^+$ mice were cultured as (A). Live T cells were enriched and then lysed with protein sample buffer. Numbers underneath each band show the relative expression level after normalized to β-Actin.

c-FLIP$_L$ functions downstream of death receptor signals (Budd et al. 2006). As the death receptor Fas and its ligand FasL are both expressed in activated T cells, c-FLIP$_L$ likely protects T cells from Fas-induced cell death upon TCR stimulation. Therefore, we tested whether blocking Fas signaling improved c-FLIP$_L$-deficient T lymphocyte survival. Indeed, anti-FasL mAb treatment completely rescued the survival defect in c-FLIP$_L^{-/-}$ CD4$^+$ T cells and partially rescued the survival defect in c-FLIP$_L^{-/-}$ CD8$^+$ T cells (Figure 11A). In contrast, anti-FasL mAb treatment did not further enhance cell survival in wild type T lymphocytes, as these cells are fully protected by endogenous c-FLIP. We further confirmed the role of Fas in the death of activated c-FLIP$_L^{-/-}$ T cells by crossing c-FLIP$_L^{-/-}$ mice to *lpr/lpr* mice, which do not express functional Fas on cell surfaces (Adachi et al. 1993). The survival of T cells from c-FLIP$_L^{-/-}$ *lpr/lpr* mice was comparable to that of T cells from wild type or *lpr/lpr* mice (Figure 11B). Importantly, rescuing the survival of c-FLIP$_L$-deficient T cell restored their ability to proliferate (Figure 11C), suggesting that the impaired effector T cell expansion upon TCR stimulation observed in c-FLIP$_L^{-/-}$ mice is a secondary effect of enhanced cell death. Interestingly, when the Fas-FasL interaction was blocked, c-FLIP$_L$-deficient T cells consistently proliferated slightly faster than wild type T cells, whereas anti-FasL mAb treatment showed no effect on wild type T cell proliferation at all. These data are consistent with the fact that NF-κB signaling is increased in c-FLIP$_L$-deficient T lymphocytes (Zhang et al. 2008b). Taken together, these

findings indicate that the enhanced cell death in activated c-FLIP$_{L}^{-/-}$ T cells requires Fas-FasL ligation.

We then tested whether c-FLIP$_{L}$-deficient cells expressed different levels of death receptors or death receptor ligands. Our results showed that both naïve and activated c-FLIP$_{L}$-deficient T lymphocytes expressed Fas at levels comparable to those in wild type T cells (Figure 12A). As FasL expression in naïve T cell is undetectable, we analyzed cell surface FasL 24 hours after TCR stimulation. Similar expressions of FasL were observed in wild type and c-FLIP$_{L}^{-/-}$ T lymphocytes (Figure 12B). These results show that the enhanced Fas-dependent cell death in c-FLIP$_{L}$-deficient T cells was not due to changes in the expression of Fas or FasL. Therefore, intracellular Fas signaling causes the cell death when the protection of c-FLIP$_{L}$ is lost.

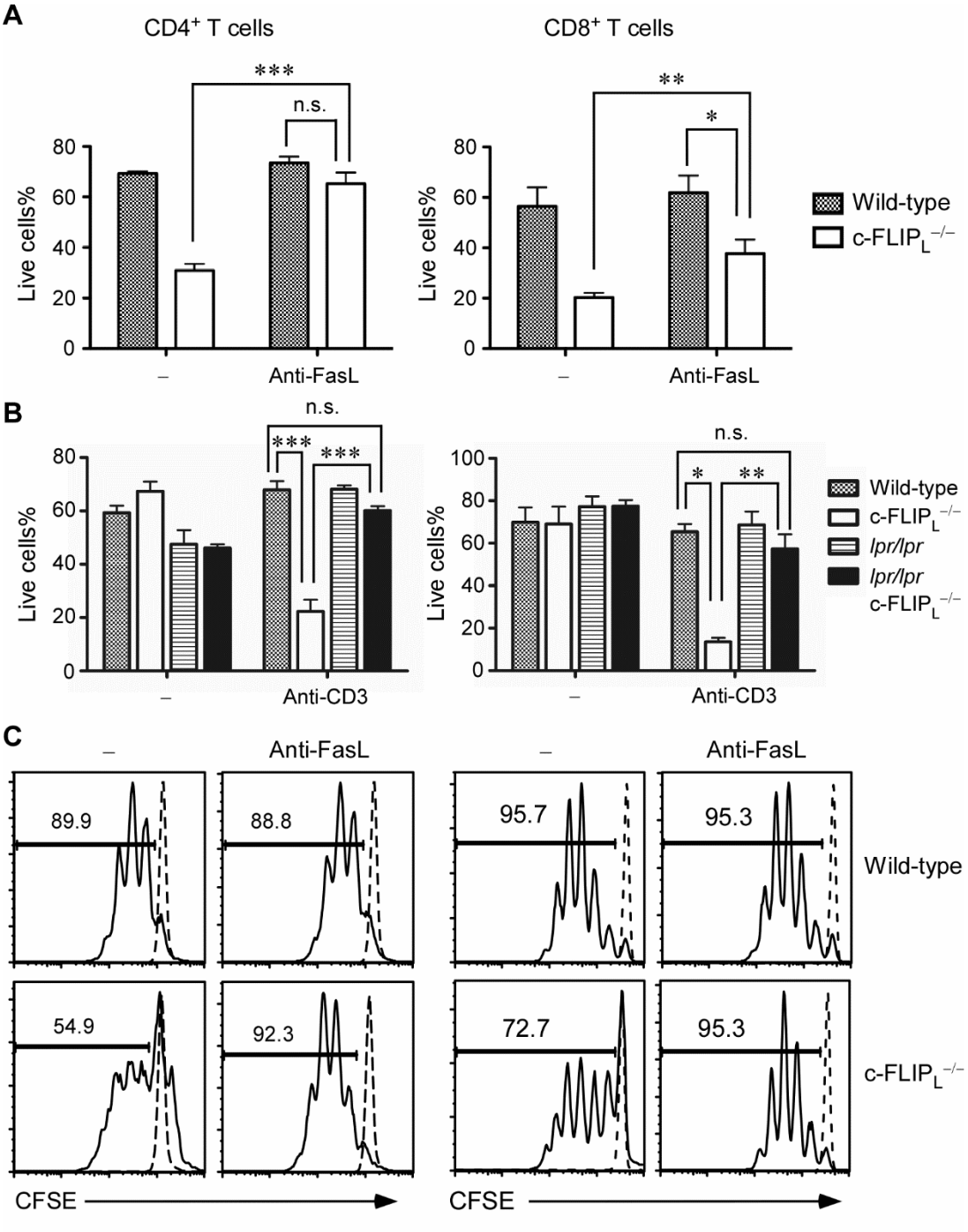

Figure 11: Fas-dependent increased cell death in activated c-FLIP$_L$-deficient T lymphocytes

Figure 11. (A) The effect of blocking FasL on cell death in TCR-stimulated c-FLIP$_{L}^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 (5 μg/ml) and cultured for 40 hours. Anti-FasL mAb was added in some cultures as indicated. The cell survival rates of T cells were analyzed by Annexin V/7-AAD staining. Live cells were gated on the Annexin V^{-} 7-AAD^{-} population within the total CD4^{+} or CD8^{+} populations (n=5). (B) Cell death of c-FLIP$_{L}^{-/-}$ T cells and c-FLIP$_{L}^{-/-}$ *lpr/lpr* T cells. (n=4). Cell death of CD4^{+} (left) and CD8^{+} (right) T cells was analyzed as described in (A). All error bars represent the standard error of the mean (s.e.m.). * P<0.05, ** P<0.01, *** P<0.001. (C) Effect of anti-FasL mAb on the proliferation of c-FLIP$_{L}$-deficient T cells. Total splenocytes from c-FLIP$_{L}^{-/-}$ and wild type (*c-Flip*$^{f/f}$) mice were labeled with CFSE, stimulated with anti-CD3, and cultured for 72 hours in the presence or absence of anti-FasL mAb. Histograms were gated on 7-AAD^{-} CD4^{+} (left) and 7-AAD^{-} CD8^{+} (right) populations. The data shown were representative of >3 independent experiments.

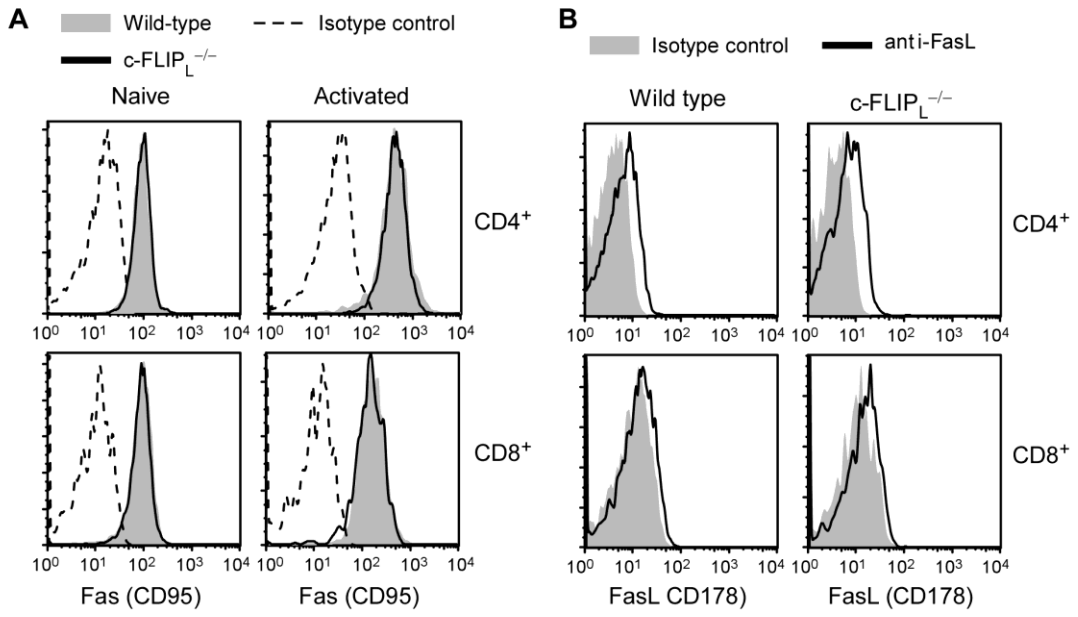

Figure 12: Comparable Fas and FasL expression between wild type and c-FLIP$_L^{-/-}$ T cells

Figure 12. (A) Fas expression on wild type and c-FLIP$_L^{-/-}$ T cells. Naïve and activated T lymphocytes (16 hours after anti-CD3 activation) were stained with anti-Fas-PE. The dashed line represents the isotype control. (B) FasL expression on activated wild type and c-FLIP$_L^{-/-}$ T cells. Total splenocytes were activated with anti-CD3 for 20 hours. FasL was stained after 4 hours of treatment with protease inhibitor. The grey area represents the isotype control. Data are representative of >3 experiments.

3.2.2 c-FLIP$_L$-deficient T cell death is partially mediated through caspase-dependent apoptosis.

As c-FLIP$_L$ inhibits death receptor-mediated caspase 8 activation (Budd et al. 2006), we next investigated whether the loss of c-FLIP$_L$ led to increased caspase activity and apoptosis in T lymphocytes. Active caspase 8 (p18) was not detected in naïve T cells regardless of c-FLIP$_L$ expression. However, after TCR stimulation, c-FLIP$_L$-deficient T cells generated large amount of active caspase 8 (p18), and blocking Fas-FasL interaction with the anti-FasL blocking antibody completely prevented active caspase 8 generation (Figure 13A). The accumulation of pro-caspase 8 upon blockade of FasL in both wild type and c-FLIP$_L^{-/-}$ T cells suggested Fas-dependent cleavage of pro-caspase 8 in activated T cells. However, active caspase 8 (p18) was not significantly generated in wild type T cells, probably because c-FLIP$_L$ suppresses complete cleavage of caspases 8. Pro-caspase 8 level was increased in activated T cells, likely because the catalytic activity of pro-caspase 8 is essential for T cell survival upon T cell activation (Leverrier et al. 2011). We further tested whether suppressing active caspase 8 generation and apoptosis could rescue the cell survival defect in c-FLIP$_L$-deficient T cells. Surprisingly, the caspase 8 inhibitor z-IETD-fmk only partially rescued the survival defect in c-FLIP$_L^{-/-}$ CD4$^+$ T cells and failed to rescue that of c-FLIP$_L^{-/-}$ CD8$^+$ T cells (Figure 13B). Similar results were obtained using the pan-caspase inhibitor z-VAD-fmk to block apoptosis (Figure 13B).

We next analyzed the kinetics of apoptosis in c-FLIP$_L$-deficient T cells. At early stages of apoptosis, phosphatidylserine on the cell surface can be stained by Annexin V.

We used Annexin V to detect cells undergoing apoptosis and 7-AAD to label cells that lost their plasma membrane integrity. Upon TCR stimulation, the frequencies of Annexin V^+ cells among 7-AAD^- cells were initially higher in c-$FLIP_L^{-/-}$ T cells (Figure 13C). During 12-16 hours after TCR stimulation, the rate of apoptotic cells became comparable in wild type and c-$FLIP_L^{-/-}$ T cells. At a later time point (32 or 40 hours), more apoptotic cells were detected in the c-$FLIP_L^{-/-}$ $CD4^+$ and $CD8^+$ T cells (Figure 13C and 13D). Meanwhile the death rate (7-AAD^+) steadily increased in c-$FLIP_L$-deficient cells (Figure 14). Although apoptosis was enhanced in c-$FLIP_L$-deficient T cells, inhibiting caspases fails to rescue the enhanced cell death, suggesting that a non-apoptotic cell death might contribute to the enhanced death observed in c-$FLIP_L$-deficient T cells.

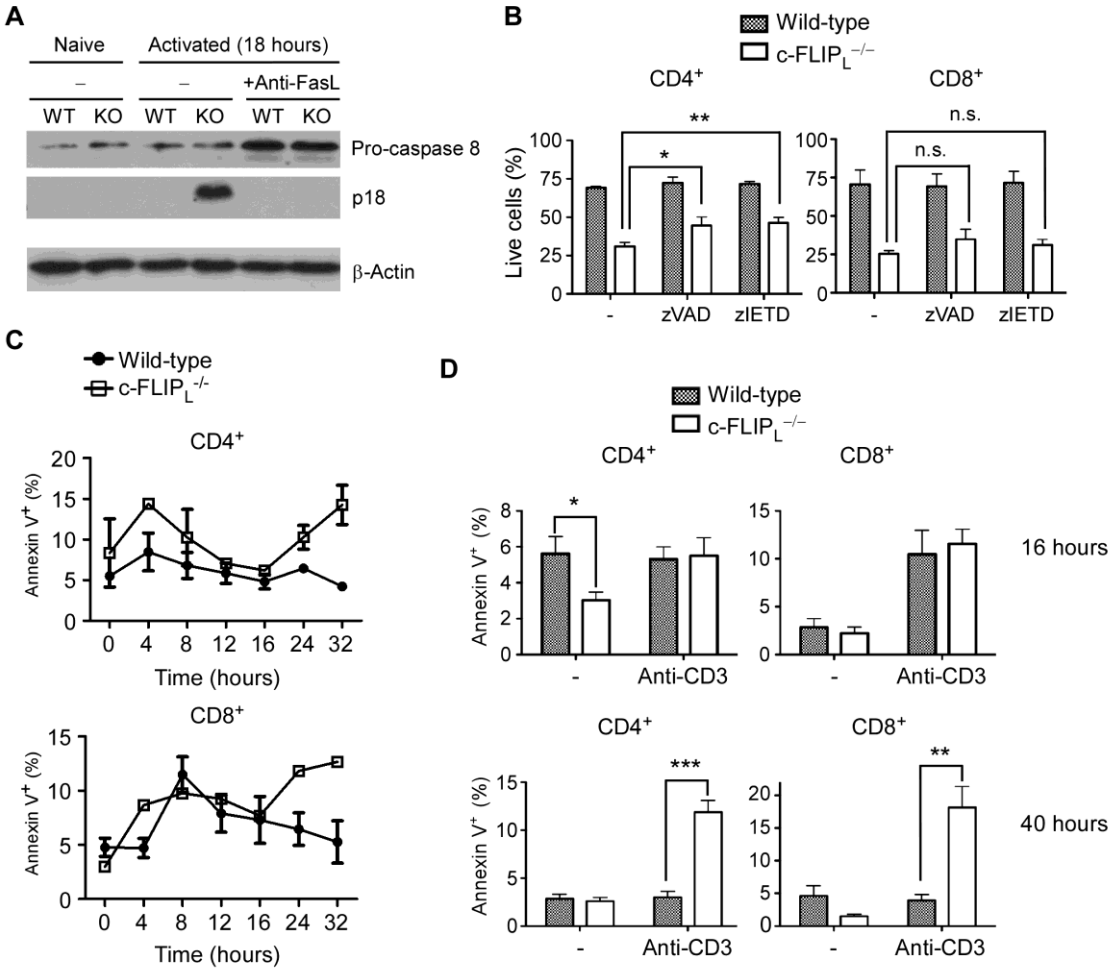

Figure 13: Enhanced apoptosis in c-FLIP$_L$-deficient T lymphocytes upon TCR stimulation

Figure 13. (A) Western blot analysis of caspase 8 activation in c-FLIP$_L$-deficient T cells. Total splenocytes from c-FLIP$_L^{-/-}$ and wild type mice were activated with anti-CD3 for 18 hours. T lymphocytes were enriched (>95%) and lysed for blotting. The data shown are representative of 3 independent experiments. (B) The effect of caspase inhibitors on cell death in c-FLIP$_L$-deficient T cells. Total splenocytes were stimulated with anti-CD3 (5 μg/ml) and cultured for 40 hours in the presence of z-VAD-fmk or z-IETD-fmk, and cell survival was measured by Annexin V and 7-AAD staining. Live cells were analyzed by gating on the Annexin V$^-$ 7-AAD$^-$ population within the total CD4$^+$ or CD8$^+$ populations (n=6). (C) The kinetics of apoptotic rates in c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 and analyzed by flow cytometry at different time points (n=2). Annexin V$^+$ % was calculated as follows: the number of Annexin V$^+$ 7-AAD$^-$ CD4$^+$ (or CD8$^+$) cells divided by the total number of 7AAD$^-$ CD4$^+$ (or CD8$^+$) cells. (D) Apoptotic rates 16 hours (n=6) and 40 hours (n=4) after TCR stimulation. All error bars represent the standard error of the mean (s.e.m.). * P<0.05, ** P<0.01, *** P<0.001.

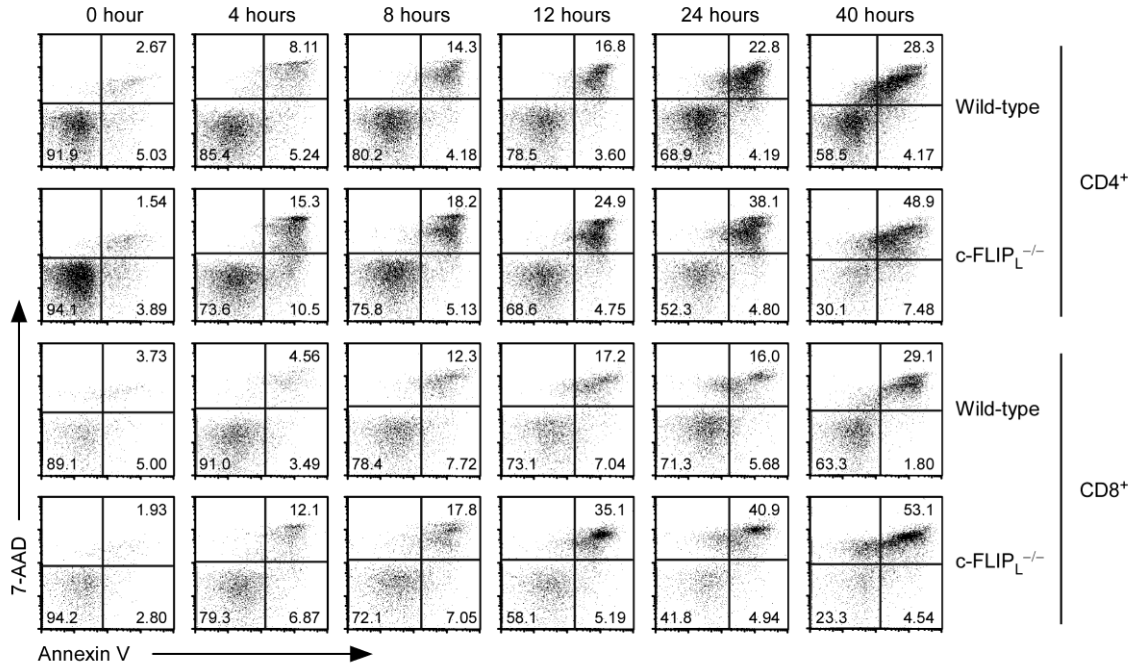

Figure 14: Kinetics of cell death after TCR stimulation in c-FLIP$_L$-deficient T lymphocytes

Figure 14. Time course of the survival rate of activated c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3. The cell survival rates of CD4$^+$ and CD8$^+$ T cells were analyzed by flow cytometry at different time points after TCR activation. Data are representatives of 2 independent experiments.

3.2.3 c-FLIP$_L$-deficient T cells undergo necroptosis upon TCR stimulation.

Because our results suggested that c-FLIP$_L$-deficient T lymphocytes might die through a non-apoptotic pathway upon TCR stimulation, we then analyzed whether activated c-FLIP$_L^{-/-}$ T cells died through necrosis. Apoptosis is characterized by the generation of mononucleosomal and oligonucleosomal DNA fragments, which are released first to the cytosol during early stages of apoptosis and then outside the cells at the late stage of apoptosis (Wyllie 1980). In contrast, necrotic cells directly release randomly digested DNA into the culture medium as they lose membrane integrity. We confirmed these two fragmented DNA releasing patterns in typical necroptotic (z-VAD-fmk treated) and apoptotic (etoposide treated) L929 cells by ELISA (Bonelli et al. 1996; Yu et al. 2004). z-VAD-fmk treated L929 cells immediately released fragmented DNA to the supernatant; whereas etoposide treated L929 cells showed a pattern of DNA release typical of apoptosis: the fragmented DNA was first detected in cytosol before releasing to supernatant (Figure 15A). We thus assessed the release of fragmented DNA in wild type and c-FLIP$_L^{-/-}$ T cells upon TCR stimulation. The fragmented DNA release in wild type T cells was similar to the pattern of apoptosis. In contrast, c-FLIP$_L$-deficient T cells quickly released fragmented DNA to the supernatant, which was a sign of ongoing necrosis. Remarkably, certain level of fragmented DNA was detected in the cytosol of activated c-FLIP$_L^{-/-}$ T cells, probably because some of cells died from apoptosis in the meantime (Figure 15B). We also test whether cell death observed in c-FLIP$_L$-deficient T

cells is caspase 3-dependent. In this assay, colorless NucView™ 488 caspase 3 substrate and EthD-1 were added in cell culture and those cells were imaged by live microscopy after TCR stimulation. Cell membrane-permeable NucView™ 488 caspase 3 substrate is cleaved by intracellular active caspase 3 to release a high-affinity fluorescent DNA dye (488nm). EthD-1 is impermeable for live cells with intact cell membrane. When cell membrane is ruptured, EthD-1 enters the cells, binds to nuclear DNA and emits red fluorescence (617nm). Therefore, the kinetic of green and red fluorescence represents whether the occurring cell death is caspase 3-dependent (Figure 15C). Not surprisingly, wild type T cells showed plasma membrane rupture over 15 minutes after caspase 3 activation. However, more than a quarter of c-FLIP$_L$-deficient T cells lost their cell membrane integrity before caspase 3 activation. Another 28% c-FLIP$_L$-deficient T cells showed shortened time frame between caspase 3 activation and plasma membrane rupture (Figure 15D). Taken together, these results suggest that at least a portion of c-FLIP$_L$-deficient T cells die through caspase 3-independent necrosis.

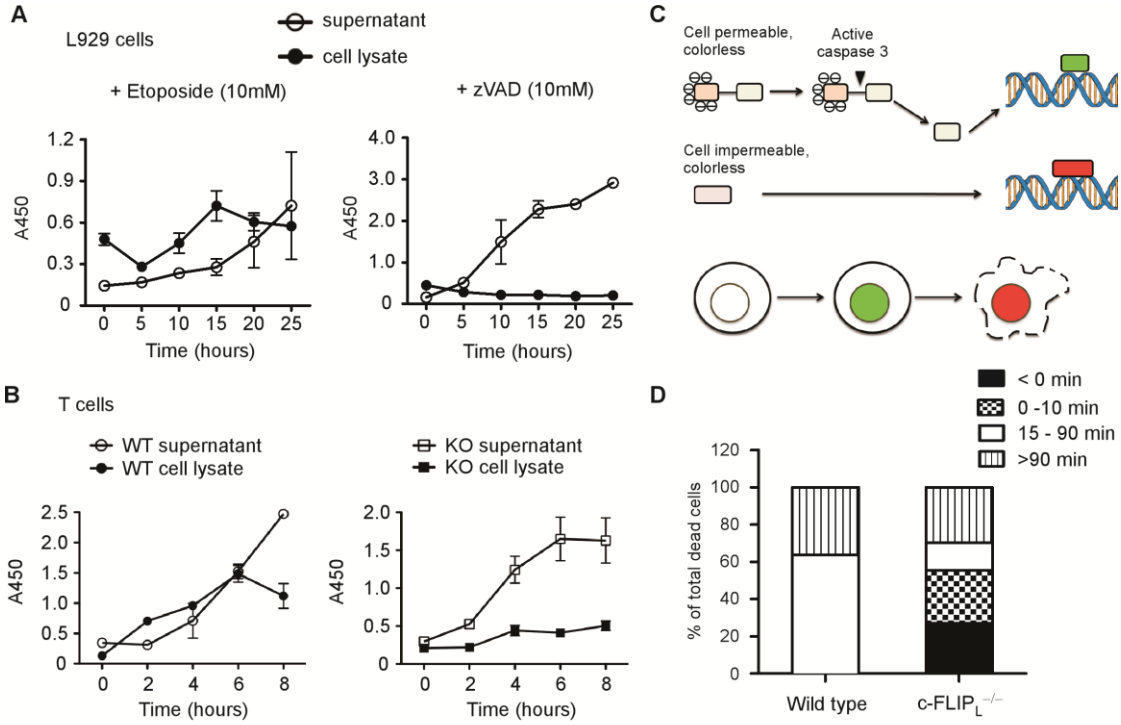

Figure 15: Necrosis in c-FLIP$_L$-deficient T lymphocytes after TCR stimulation

Figure 15. (A) Release of fragmented DNA in L929 cells. L929 cells were labeled with BrdU *in vitro*. Apoptosis and necroptosis were induced by etoposide and z-VAD-fmk, respectively. (B) Release of fragmented DNA in T lymphocytes upon TCR activation. T cells in c-FLIP$_{L}^{-/-}$ and control mice were labeled *in vivo* by *i.p.* BrdU injection and stimulated *in vitro* with plate-bound anti-CD3 for the indicated periods of time. BrdU-labeled fragmented DNA was measured in cell lysates and supernatants by ELISA. (C) Illustration of caspase 3 activation and plasma membrane rupture kinetic assays. Bottom is the model of a cell die in a caspase 3-dependent pathway. (D) Caspase 3-independent death in c-FLIP$_{L}$-deficient T cells. Naïve T cells were stimulated by anti-CD3 and imaged by live fluorescent microcopy for 8 hours. The time point of caspase 3 activation (Green^{+}) and cell death (EthD-1^{+}) were recorded for each cell that is EthD-1^{+} by the end of the 8 hours. The time frame between caspase 3 activation and cell death was calculated as $t_n = T_{cell\ deah} - T_{caspase\ 3+}$. More than 50 cells were examined in each group.

Previous data suggested that c-FLIP$_L$ might recruit RIP-1 to the DISC and lead to caspase 8-dependent RIP-1 cleavage (Dohrman et al. 2005a; Rajput et al. 2011). Therefore, we investigated whether RIP-1 is involved in the death of c-FLIP$_L$-deficient T lymphocytes. The mRNA level of RIP-1 was not increased in c-FLIP$_L^{-/-}$ T cells before and after TCR stimulation (Figure 16A). However, the protein level of RIP-1 was higher in naïve c-FLIP$_L^{-/-}$ T cells than controls. Sixteen hours after TCR stimulation, the difference was more obvious (Figure 16B). The level of RIP-1 became comparable between c-FLIP$_L^{-/-}$ and wild type T cells 24 hours after TCR stimulation, probably due to the excessive death of cells with high level of RIP-1. The expression of RIP-3, another protein essential for necroptosis induction, was slightly increased without c-FLIP$_L$ in T lymphocytes (Figure 16B). Moreover, the RIP-1 inhibitor necrostatin-1 (referred as Nec-1) partially rescued the enhanced cell death (Figure 16C) and completely restored the defective proliferation capacity in c-FLIP$_L$-deficient T cells (Figure 16D). More importantly, combined treatment with the caspase 8 inhibitor z-IETD-fmk and necrostatin-1 completely rescued the enhanced cell death observed in c-FLIP$_L^{-/-}$ CD4$^+$ T cells (Figure 16C). Taken together, these results indicate that besides apoptosis, c-FLIP$_L$-deficient T cells also undergo RIP-1-dependent necroptosis upon TCR activation.

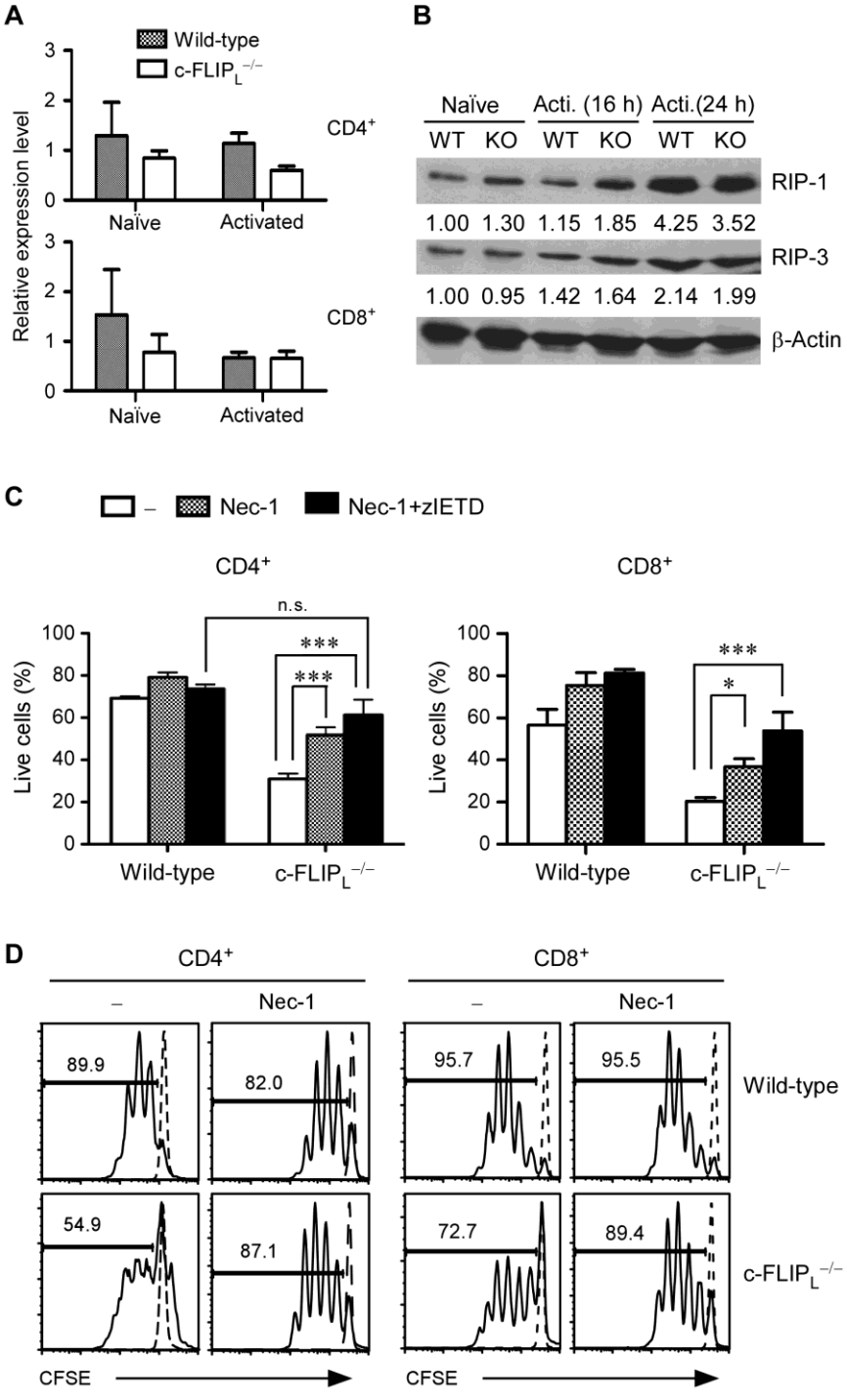

Figure 16: RIP-1-dependent necroptosis in c-FLIP$_L$-deficient T lymphocytes

Figure 16. (A) RIP-1 mRNA levels in resting or activated c-FLIP$_L^{-/-}$ T cells. For naïve T cells, live CD44^{low} CD4$^+$ or CD8$^+$ T cells were sorted from freshly harvested splenocytes. For activated T cells, total splenocytes were activated with anti-CD3 for 18 hours, and live CD4$^+$ or CD8$^+$ T cells were sorted. (B) RIP-1 and RIP-3 protein expression in resting or activated c-FLIP$_L^{-/-}$ T cells. Live T lymphocytes were enriched (>95%) for blotting. The numbers underneath each band represent the relative expression level after normalization to β-Actin. (C) Effect of necrostatin-1 and z-IETD-fmk on the cell death of c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 (5 μg/ml) and cultured in the presence or absence of necrostatin-1 (Nec-1, 100nM) and z-IETD-fmk (10 μM) for 40 hours. Cell survival was tested by flow cytometry. Live cells were analyzed by gating on the Annexin V$^-$ 7-AAD$^-$ population within the total CD4$^+$ or CD8$^+$ populations. All error bars represent the standard error of the mean (s.e.m.). * P<0.05, *** P<0.001. (D) Effect of necrostatin-1 on the proliferation of c-FLIP$_L^{-/-}$ T cells. Total splenocytes from c-FLIP$_L^{-/-}$ and wild type (*c-Flip$^{f/f}$*) mice were labeled with CFSE, stimulated with anti-CD3, and cultured for 24 hours. Nec-1 was added at the beginning of the culture period. Histograms were gated on 7-AAD$^-$ CD4$^+$ populations. The data shown were obtained in 3 independent experiments.

3.2.4 Autophagy is enhanced in c-FLIP$_L$-deficient T lymphocytes

A correlation of necroptosis and excessive autophagy was observed in several researches (Yu et al. 2004; Bell et al. 2008; Chen et al. 2011). Hence, we investigated whether the c-FLIP$_L$ deficient T lymphocytes displayed altered levels of autophagy. LC3-I/LC-3 II conversion in mammalian cells is a reliable marker for autophagy induction (Klionsky et al. 2008). After TCR stimulation (18 hours), LC3-II:LC3 ratio increased in both wild type and c-FLIP$_L^{-/-}$ T cells; however, LC3-II levels were much higher in activated c-FLIP$_L^{-/-}$ T cells than in wild type T cells (Figure 17A). We next counted the number of LC3 puncta per cell as a measure of the number of autophagosomes (Klionsky et al. 2008; McLeod et al. 2011). Naïve and activated c-FLIP$_L^{-/-}$ T cells displayed significantly more LC3 puncta than did wild type T cells. Additionally, the increase in LC3 puncta in c-FLIP$_L$-deficient T cells was more dramatic after TCR stimulation (Figure 17B). We further analyzed the intracellular structures in wild type and c-FLIP$_L^{-/-}$ T cells by transmission electron microscopy (TEM). Activated c-FLIP$_L^{-/-}$ T cells contained more intracellular double-membrane vacuoles (autophagosomes) than wild type T cells (Figure 17C, D), and the percentage of cells containing autophagosomes was much higher in activated c-FLIP$_L^{-/-}$ T cells than in wild type T cells (Figure 17E). Additionally, autophagy levels were also higher in naïve c-FLIP$_L^{-/-}$ T cells than in wild type T cells (Figure 17F, G). Interestingly, we observed an accumulation of membrane-containing autophagosomes in naïve c-FLIP$_L^{-/-}$ T lymphocytes, which were entirely

absent in naïve wild type T cells (Figure 17F, H). The significance of these structures remains unclear. Together, these data demonstrate that c-FLIPL-deficient T cells display enhanced autophagy induction, especially after T cell activation.

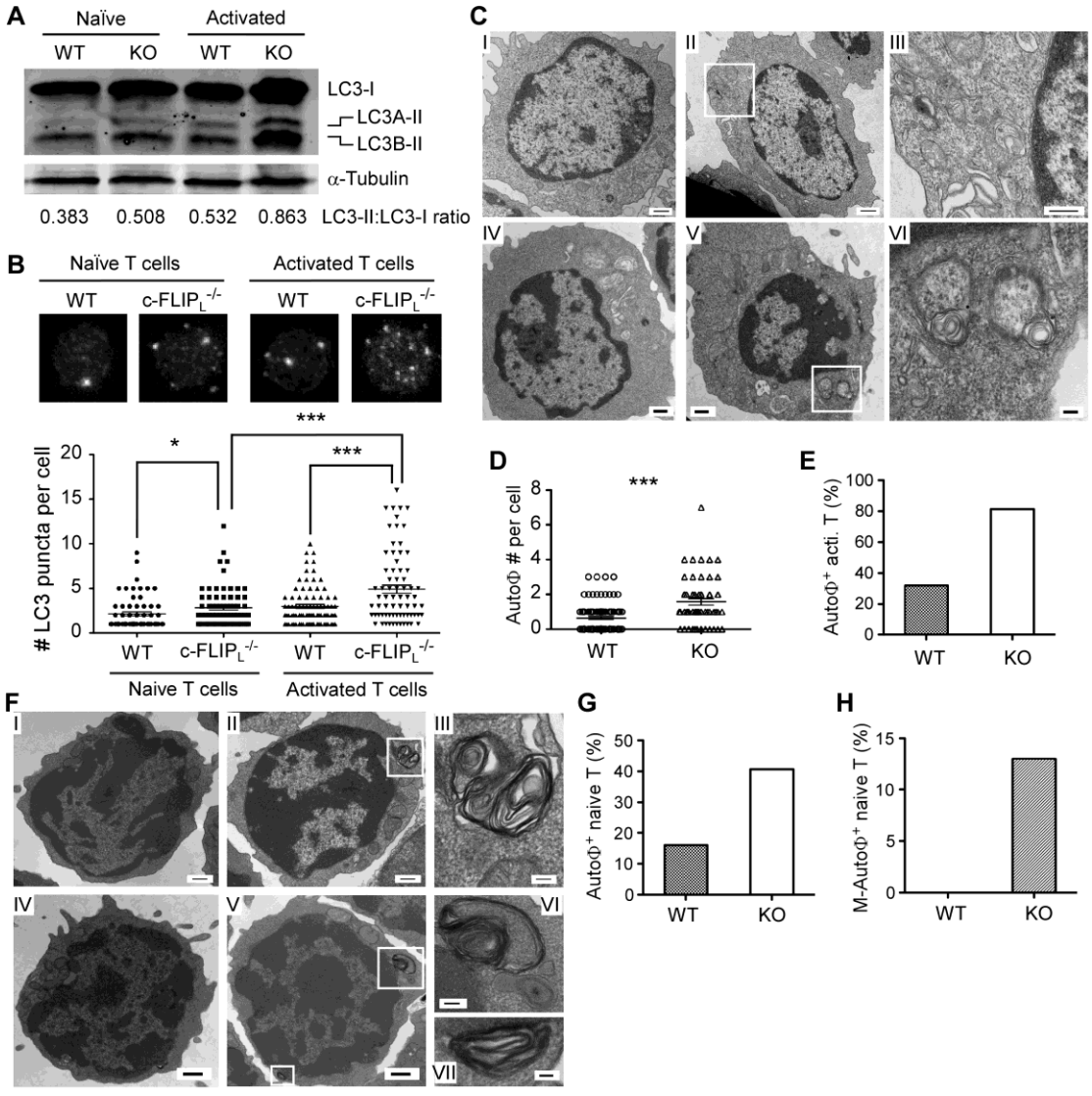

Figure 17: Enhanced autophagy in c-FLIPL-deficient T lymphocytes

Figure 17. (A) Western blot analysis of LC3 in resting and activated c-FLIP$_{L}^{-/-}$ T cells. T cells were activated with anti-CD3 for 18 hours, and LC3-I/LC3-II conversion was measured by western blot. (B) The numbers of LC3 puncta in c-FLIP$_{L}^{-/-}$ T cells. Activated T cells were intracellularly stained with anti-LC3 and analyzed for LC3 puncta by fluorescent microscopy. (C) TEM images of c-FLIP$_{L}^{-/-}$ T cells. Total splenocytes were activated with anti-CD3 for 18 hours. Activated wild type CD4^{+} (I) or CD8^{+} (IV) and c-FLIP$_{L}^{-/-}$ CD4^{+} (II, III) or CD8^{+} (V, VI) T cells were sorted and analyzed by TEM. III and VI are enlarged images of II and V, respectively. Bars represent 0.5 μm in I-V and 100 nm in VI. Shown are representative micrographs. (D) Numbers of autophagosomes per cell in activated T lymphocytes (n≥60). (E) Percentages of activated T cells containing autophagosomes among all activated T cells counted in D. (F) Accumulated membrane inside autophagosomes in naïve c-FLIP$_{L}^{-/-}$ T cells. Naive wild type CD4^{+} (I) or CD8^{+} (IV) and c-FLIP$_{L}^{-/-}$ CD4^{+} (II, III) or CD8^{+} (V, VI, VII) T cells were sorted (CD44^{low}) and analyzed by TEM. III is an enlarged image of II. V and VII are enlarged images of IV. Bars represent 0.5 μm in I-II and IV-V, 100 nm in III, VI and VII. Shown are representative micrographs. (G) Percentages of naive T cells containing autophagosomes among all naïve T cells (n≥50). (H) Percentages of naïve T cells in which membrane-autophagosomes were observed among all naïve cells. AutoΦ stands for autophagosomes; M-AutoΦ stands for membrane-containing autophagosomes. All error bars represent the standard error of the mean (s.e.m.). * $P<0.05$, *** $P<0.001$.

Over-expression of c-FLIP protein inhibits autophagy in several cell lines (Lee et al. 2009). However, whether c-FLIP expression in primary T cells has a similar effect remains unclear. We thus assessed autophagy level in activated T cells expressing c-FLIP isoform-specific BAC transgenes (referred to as c-FLIP$_R$-Tg and c-FLIP$_L$-Tg). Both c-FLIP$_R$-Tg and c-FLIP$_L$-Tg inhibited LC3 puncta formation upon TCR stimulation; however, c-FLIP$_R$-Tg inhibited autophagy more efficiently than c-FLIP$_L$ (Figure 18). The difference in the ability of c-FLIP$_R$-Tg and c-FLIP$_L$-Tg to inhibit autophagy in activated T cells is likely due to post-transcriptional regulation in c-FLIP proteins. c-FLIP$_R$ is preferentially expressed in T cells (Figure 10B), resulting in higher protein levels of c-FLIP$_R$-Tg than c-FLIP$_L$-Tg. These data suggest that c-FLIP can modulate the level of autophagy in activated T lymphocytes.

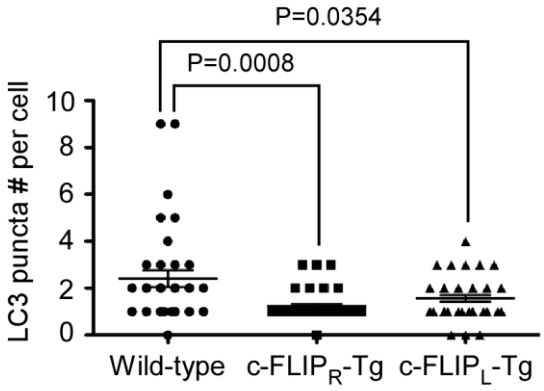

Figure 18: c-FLIP$_L$ and c-FLIP$_R$ inhibit autophagy induction in T lymphocytes.

Figure 18. Reduced autophagy induction in c-FLIP$_L$- BAC transgene or c-FLIP$_R$-BAC transgene expressing T lymphocytes upon TCR stimulation. Enriched T cells were activated with plate-bound anti-CD3 for 18 hours and then intracellularly stained with anti-LC3. LC3 puncta were analyzed by florescent microscopy. All error bars represent the standard error of the mean (s.e.m.).

3.2.5 Enhanced autophagy plays a cytoprotective role in c-FLIP$_L$-deficient T lymphocytes.

Conflicting findings have been reported regarding the role of caspase inhibition-mediated autophagy in cell death. Previous studies have concluded that autophagy either causes (Yu et al. 2004; Yu et al. 2006; Chen et al. 2011) or protects cells from z-VAD-fmk-induced necrotic cell death (Wu et al. 2008). Studies of T lymphocytes suggest that autophagy may be the cause of the RIP-1-dependent cell death observed in dominant negative FADD-expressing T lymphocytes, but not of that observed in *Fadd*$^{-/-}$ or *caspase-8*$^{-/-}$ T lymphocytes (Bell et al. 2008; Osborn et al. 2010; Ch'en et al. 2011). We therefore tested the role of autophagy in the death of c-FLIP$_L$-deficient T lymphocytes. Atg3 plays an essential role in autophagosome formation by catalyzing LC3 processing (Levine et al. 2011). Inducible short term deletion of Atg3 in T lymphocytes resulted in reduced autophagy induction (Figure 19A), but did not cause enhanced cell death (Jia and He 2011) (Figure 19B). Therefore, we crossed c-FLIP$_L$-deficient (*c-FLIP*$^{f/f}$ *c-FLIP*$_R$*-BAC Tg*) mice to *Atg3*$^{f/f}$ ER-Cre$^+$ mice. In this model, c-FLIP and Atg3 are successfully deleted *in vitro* upon 4-hydroxy-tamoxifen treatment (Zhang et al. 2008a; Jia and He 2011). We found that the loss of Atg3 further decreased cell survival in c-FLIP$_L$-deficient T cells, especially upon TCR stimulation (Figure 19C). These data suggest that autophagy plays a cytoprotective role in activated c-FLIP$_L^{-/-}$ T lymphocytes.

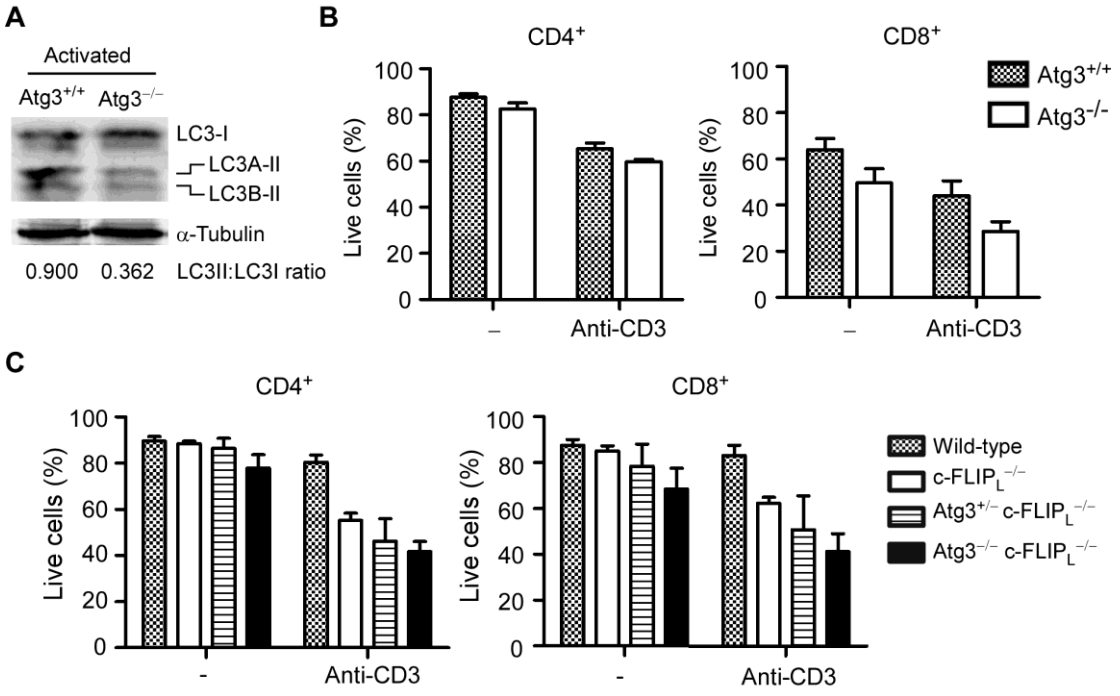

Figure 19: The cytoprotective role of autophagy in c-FLIPL-deficient T lymphocytes

Figure 19. (A) Reduced autophagy induction in *Atg3*$^{-/-}$ T cells. Total splenocytes from *Atg3*$^{f/f}$ and *Atg3*$^{f/f}$ ER-Cre^{+} mice were cultured for 3 days in the presence of 4-hydorxy-tamoxifen and IL-7 to induce deletion, resulting in wild type and *Atg3*$^{-/-}$ lymphocytes. T cells were negatively enriched by selection kit and activated with plate bound anti-CD3 for 18 hours. LC3-I/LC3-II conversion was measured by western blot. **(**B) Cell death upon TCR stimulation in *Atg3*$^{-/-}$ T cells (n>=3). (C) The effect of autophagy inhibition in the survival of c-FLIP$_{L}$-deficient T cells (n=5). Total splenocytes from Atg3$^{f/f}$ and *Atg3*$^{f/f}$ ER-Cre^{+} mice (B) or *c-Flip*$^{f/f}$ *Atg3*$^{f/f}$, *c-Flip*$^{f/f}$ *c-FLIP*$_{R}$*-Tg*$^{+}$ ER-Cre^{+}, *c-Flip*$^{f/f}$ *Atg3*$^{f/+}$ *c-FLIP*$_{R}$*-Tg*$^{+}$ ER-Cre^{+} and *c-FLIP*$^{f/f}$ *c-FLIP*$_{R}$*-Tg*$^{+}$ *Atg3*$^{f/f}$ ER-Cre^{+} mice (C) were cultured for 3 days in the presence of 4-hydorxy-tamoxifen and IL-7 to induce deletion, resulting in wild type and Atg3$^{-/-}$ (B) or wild-type, c-FLIP$_{L}^{-/-}$, c-FLIP$_{L}^{-/-}$Atg3$^{+/-}$ and c-FLIP$_{L}^{-/-}$Atg3$^{-/-}$ cells (C). Live cells were isolated using Ficoll, stimulated with plate-bound anti-CD3, and cultured for 40 hours with IL-7 (1 ng/ml). Live cells were analyzed by gating on the 7-AAD^{-} population within the CD4^{+} or CD8^{+} populations (n>=4).

3.2.6 Necroptosis in c-FLIP$_L$-deficient T cells involves reactive oxygen species (ROS).

Reactive oxygen species contribute to the execution of necroptosis (Yu et al. 2006; Chen et al. 2011). Cell-permeate 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) staining showed that the hydrogen peroxide is slightly increased in c-FLIP$_L$-deficient T cells under both resting and activated conditions (Figure 20A). However, the level of superoxide radicals (stained by dihydroethidium, DHE) in c-FLIP$_L$-deficient T cells seems to be comparable that in wild type T cells, under both resting and activated conditions (Figure 20B). Antioxidant acetylcysteine (NAC) partially inhibited TCR-induced cell death in both wild type and c-FLIP$_L^{-/-}$ T cells. Moreover, adding RIP-1 inhibitor Nec-1 to NAC treated cells did not improve the cell survival (Figure 20C). Therefore, the production of ROS likely contributes to necroptosis in c-FLIP$_L$-deficient T cells.

The major cellular reservoir of ROS production is the mitochondria (Hildeman et al. 2003). In T lymphocytes, the numbers of mitochondria are tightly regulated by autophagy (Jia and He 2011). However, the enhanced autophagy seems not to change the mitochondrial level in naïve and activated c-FLIP$_L^{-/-}$ T cells (Figure 21). RIP-1/RIP-3 activation may also lead to increased ROS production (Yu et al. 2006; Van Herreweghe et al. 2010). Additionally, excessive autophagy causes ROS accumulation by degradation of catalase, the major ROS scavenger (Yu et al. 2006).

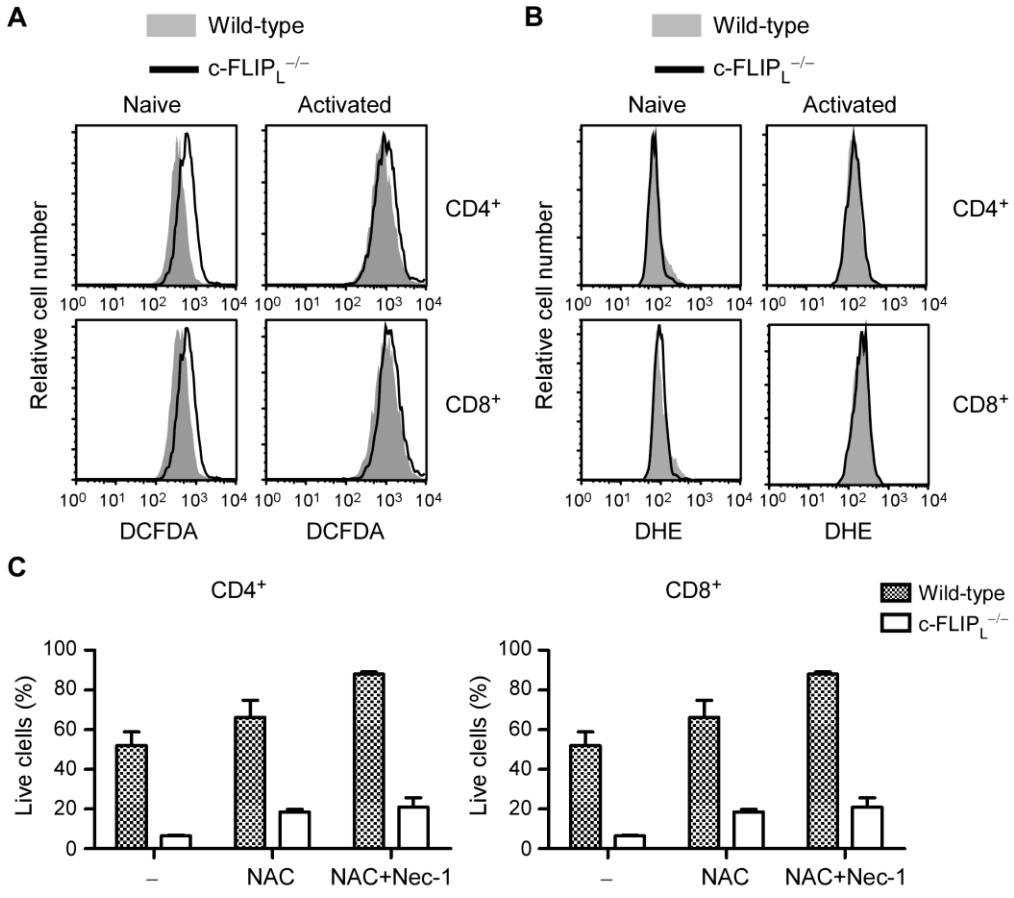

Figure 20: Increased ROS contributes to the necroptosis in c-FLIP$_L$-deficient T cells.

Figure 20. (A) CM-H_2DCFDA staining in c-$FLIP_L^{-/-}$ T cells. (B) DHE staining in c-$FLIP_L^{-/-}$ T cells. Naïve and activated T lymphocytes (16 hours after anti-CD3 stimulation) were stained with CM-H_2DCFDA (A) or DHE (B) and analyzed by flow cytometry. Data are representative of >3 experiments. (C) Antioxidant NAC improved the survival of c-$FLIP_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 and cultured for 40 hour in the presence or absence of NAC and Nec-1. Cell death rates of T cells were analyzed by flow cytometry. Cell survival was tested by flow cytometry. Live cells were analyzed by gating on the Annexin V^- 7-AAD^- population within the total $CD4^+$ or $CD8^+$ populations.

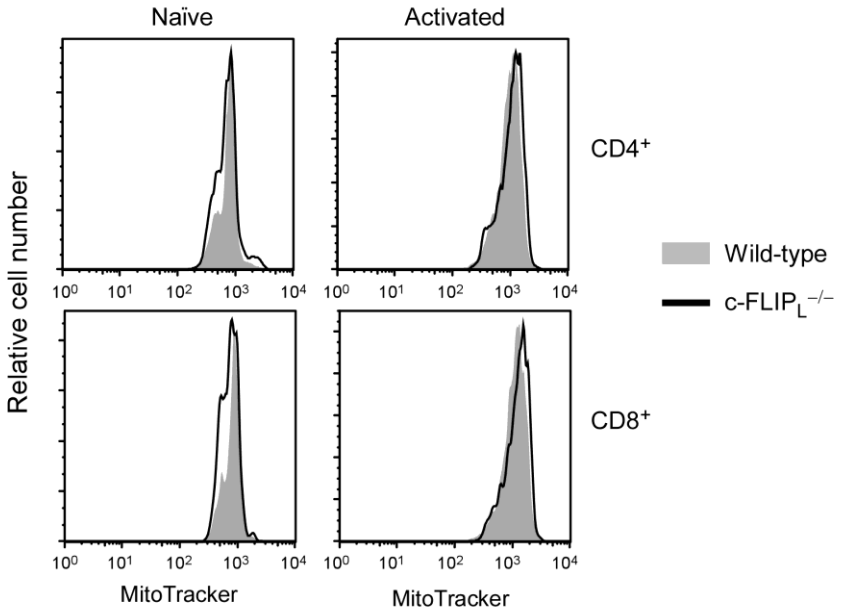

Figure 21: Unchanged mitochondrial volume in c-FLIP$_L$-deficient T cells.

Figure 21. Mitochondria volume in wild type and c-FLIP$_L^{-/-}$ T cells. The mitochondrial contents of naïve and activated T lymphocytes (16 hours after anti-CD3 activation) were analyzed by flow cytometry based MitoTracker staining. Data are representative of >3 experiments.

3.3. Conclusion and Discussion

3.3.1 The anti-necroptotic role of c-FLIP$_L$ in activated T cells

Deletion of c-FLIP$_L$ in T cells resulted in dramatic cell death upon TCR stimulation. Although c-FLIP$_L$ has long been considered as an anti-apoptotic protein, activated c-FLIP$_L^{-/-}$ T cells showed extensive necroptosis. In contrast, naïve c-FLIP$_L^{-/-}$ T cells showed no defects in cell survival. More interestingly, though previous studies showed the occurrence of necroptosis when caspase 8 activity was totally blocked, we observed both necroptosis and enhanced caspase 8 cleavage in c-FLIP$_L$-deficient T cells. Our results suggest that the anti-necrotic and pro-apoptotic activities of caspase 8 might be separately regulated. Moreover, the increased apoptosis and necroptosis observed in c-FLIP$_L$-deficient T cells were both Fas-dependent.

Necroptosis in c-FLIP$_L$-deficient T lymphocytes upon TCR stimulation occurs downstream of Fas signaling. This finding is consistent with several recent publications showing that death receptor-induced necroptosis in *Cyld*$^{-/-}$ MEFs, *caspase-8*$^{-/-}$ hematopoietic cells and *Fadd*$^{-/-}$ keratinocytes (Bonnet et al. 2011; Kaiser et al. 2011; O'Donnell et al. 2011). Caspase 8- or FADD-deficient T lymphocytes also show remarkable necroptosis upon TCR stimulation (Osborn et al. 2010; Ch'en et al. 2011), but whether Fas signaling induces necroptosis in these knockout models remains to be determined. Besides death receptor signaling, alternative necroptosis inducers may also exist. For example, TLR3 activation is shown to induce necroptosis in HaCaT

keratinocytes treated by z-VAD-fmk (Feoktistova et al. 2011). Therefore, future studies should focus on deciphering the signal pathway of necroptosis induction under physiological conditions and the biological implications of programmed necrosis.

Necroptosis usually occurs when caspase 8 activity and apoptosis are blocked. Here, we reported the existence of necroptosis in cells with enhanced apoptosis. Our results provide important information for the understanding of the cellular mechanism of necroptosis regulation. Both enhanced apoptosis and necroptosis are downstream of Fas-FasL ligation in c-FLIP$_L$-deficient T cells. When Fas is activated, multiple proteins, including c-FLIP$_L$ and pro-caspase 8, are recruited to the DISC (Budd et al. 2006). The fact that c-FLIP$_L$, caspase 8, and FADD are all involved in necroptosis regulation strongly suggests that the inhibition of RIP-1/RIP-3 occurs in the DISC (Osborn et al. 2010; Ch'en et al. 2011). The interaction between pro-caspase 8 and c-FLIP$_L$ in the DISC leads to the cleavage of c-FLIP$_L$ to generate p43FLIP (Budd et al. 2006). Both p43FLIP and c-FLIP$_L$ recruit RIP-1 to the caspase 8/c-FLIP/RIP-1 complex; and p43FLIP binds RIP-1 with higher affinity than do full-length c-FLIP$_L$ or caspase 8 in T lymphocytes (Dohrman et al. 2005a). Studies in human fibroblasts showed that caspase 8 mediates the cleavage of RIP-1 to produce an inhibitory RIP-1 fragment (Rajput et al. 2011). It is possible that c-FLIP$_L$ contributes to the RIP-1 cleavage and inactivation in T lymphocytes. This hypothesis is supported by the accumulation of RIP-1 protein in c-FLIP$_L$-deficient T cells, while RIP-1 mRNA level is unchanged. c-FLIP may regulate RIP-

1 activity by recruiting RIP-1 to the DISC for caspase 8-dependent degradation. Without c-FLIP$_L$, the recruitment of RIP-1 is blocked. Therefore, RIP-1 cannot be regulated by caspase 8 cleavage, leading to increased RIP-1 activity and necroptosis. Another possibility is that c-FLIP$_L$-caspase 8 heterodimer, instead of active caspase 8 alone, is responsible for RIP-1 inhibition. These two hypotheses may be true at the same time. In the term of the regulation of apoptosis, the loss of c-FLIP$_L$ protein causes enhanced apoptotic caspase 8 activation (p18 generation) and a subsequent increase in apoptosis in c-FLIP$_L$-deficient cells (Figure 22).

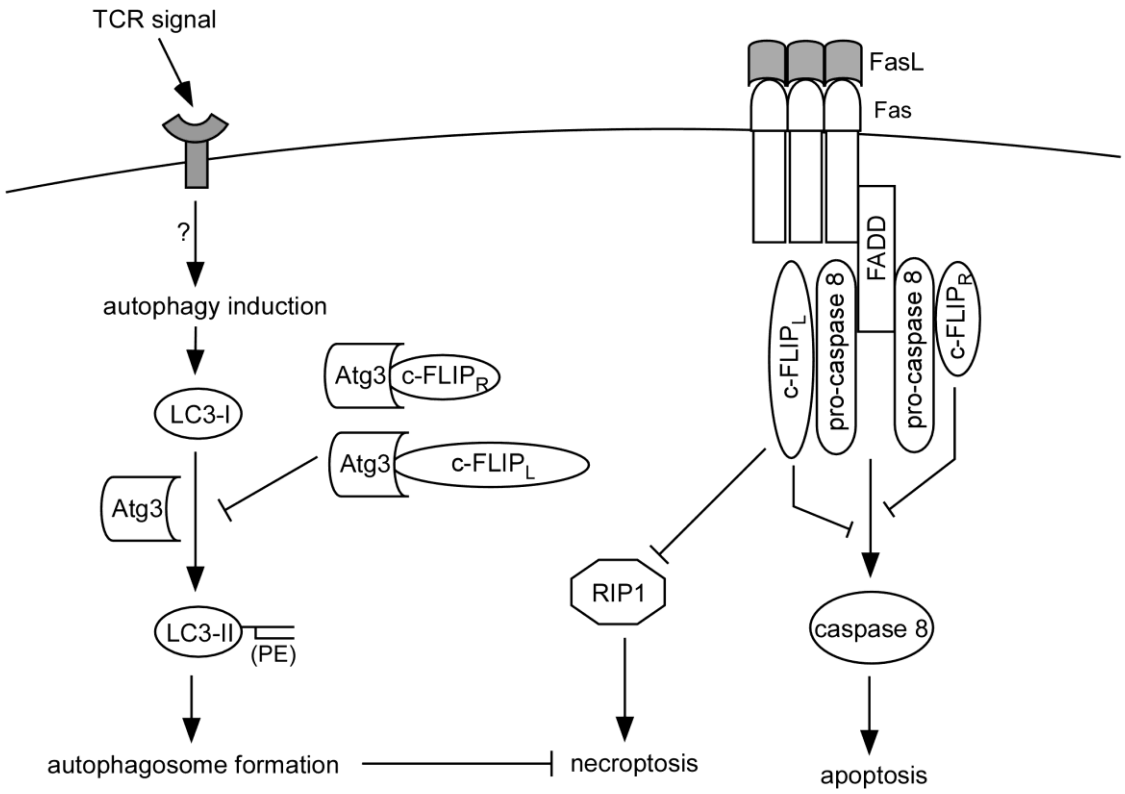

Figure 22: A proposed model for c-FLIP mediated regulation of apoptosis, necroptosis and autophagy

Figure 22. T lymphocytes upregulate the expression of Fas and FasL upon TCR-activation. The binding of Fas to FasL on the cell surface induces the formation of the DISC, where both c-FLIP$_L$ and c-FLIP$_R$ suppress the activation of pro-caspase 8 and downstream apoptosis. In addition, c-FLIP$_L$ and p43FLIP recruit RIP-1. c-FLIP$_L$ is required for inhibiting RIP-1 activity and necroptosis. Simultaneously, TCR activation induces autophagosome formation in T lymphocytes through an unknown mechanism. c-FLIP competes with LC3 for Atg3 binding, thereby reducing LC3 processing, and thus suppressing autophagy induction. In c-FLIP$_L$-deficient T cells, autophagy is enhanced and protects cells from RIP-1-dependent necrosis.

Our study reveals that excessive ROS contributes to cell death in c-FLIP$_L$-deficient T lymphocytes upon TCR stimulation, which is consistent with previous reports showing that ROS accumulation leads to necroptosis in z-VAD-fmk treated L929 cells (Yu et al. 2006; Chen et al. 2011). c-FLIP$_L$-deficient T cells showed increase in hydrogen peroxide (stained by CM-H$_2$DCFDA), but not in superoxide anion (stained by DHE). Cellular generation of hydrogen peroxide upon TCR stimulation is required for ERK phosphorylation upon TCR stimulations (Devadas et al. 2002). Therefore, enhance generation of hydrogen peroxide may be the reason that c-FLIP$_L$-deficient T cells exhibit elevated ERK signaling (Zhang et al. 2008b) and increased proliferation capacity when cell death is blocked. Mitochondria may serve as the production site of ROS due to increased Ca_2^+ flux and the alteration of metabolism, in which RIP-1 activity is involved (Van Herreweghe et al. 2010). Studies in L929 cells suggests that increased ROS is downstream of dysregulated autophagy (Yu et al. 2006; Chen et al. 2011). Whether enhanced autophagic flux in T cells leads to excessive ROS production remains to be determined. Our study showed that autophagy is generally cytoprotective for c-FLIP$_L$-deficient T cells. Nevertheless, it is possible that autophagy is upstream of both beneficial and harmful effects for cell survival and the combination of those effects determines the outcome.

3.3.2 The cytoprotective role of autophagy

Our findings demonstrate that c-FLIP$_L$ suppresses autophagy in both activated and resting T lymphocytes, and the enhanced autophagy is cytoprotective. Autophagy supports cell survival in many circumstances, especially upon nutrient withdrawal (Gordy and He 2012). In T lymphocytes, autophagy is indispensable not only for surviving growth factor withdrawal, but also for regulating mitochondria and endoplasmic reticulum (Pua and He 2009b; Jia and He 2011; Jia et al. 2011). The anti-apoptotic and anti-necroptotic protein c-FLIP$_L$ is involved in autophagy regulation. Previous report showed the various isoforms of c-FLIP controlled the threshold of autophagy when over-expressed in cells (Lee et al. 2009). Interesting, we observed autophagosomes enclosing multiple layers of membranes in c-FLIP$_L$ deficient T cells. These structures were absent in wild-type T cells. How autophagy promotes cell survival in c-FLIP$_L$-deficient T cells remains to be determined. In addition to clearing unwanted intracellular substances and providing energy, autophagy may benefit activated c-FLIP$_L^{-/-}$ T cells in a more direct way: studies in cells resistant to apoptosis revealed that autophagy actively removed active caspase 8. Whether T lymphocytes employ a similar mechanism must be addressed in further studies. In addition, it will be interesting to investigate whether the enhanced autophagy observed in naïve c-FLIP$_L^{-/-}$ T lymphocytes renders them more resistant to cell death.

3.3.3 The caspase 8-c-FLIP$_L$-FADD axel in the regulation of apoptosis, necroptosis and autophagy.

Apoptosis can be triggered when death receptors on the cellular membrane meet their ligands (Zhang et al. 2005). When sensing the death signals, death receptors activate caspase 8, which in turn triggers the apoptotic cascade. However, blocking caspase 8 activity does not protect cells from the fate of death. Loss of caspase 8 activity leads to excessive RIP1/RIP3-dependent necroptosis (Walsh and Edinger 2010). In T lymphocytes, pro-apoptosis factor caspase 8, anti-apoptotic factor c-FLIP$_L$ and their adaptor protein FADD are all involved in the suppression of necroptosis. *caspase-8*$^{-/-}$ and *Fadd*$^{-/-}$ T cells exhibit severe necroptosis, while c-FLIP$_L$-deficient T cells shows enhanced apoptosis and necroptosis (Ch'en et al. 2011a; He and He 2013b). c-FLIP$_L$ inhibits apoptosis by suppressing active caspase 8 generation and prevents necroptosis by promoting caspase 8-dependent RIP-1 cleavage.

A term "autophagic death" was used to describe the TNF-α induced cell death in L929 cells, which showed high number of autophagsomes (Yu et al. 2004). The name seemed to be reasonable at that time, as inhibition of autophagy induction rescued the cell death. However, contrary results later appeared, arguing that the enhanced autophagy is protective, rather than detrimental for the cell survival (Osborn et al. 2010; Ch'en et al. 2011). We examined whether enhanced autophagy is cytoprotective in c-FLIP$_L$-deficient T cell using genetically modified mouse models in which the autophagy machinery is disabled. Inducible deletion of Atg3 in c-FLIP$_L^{-/-}$ T cells further reduced the

survival of activated c-FLIP$_{L}^{-/-}$ T cells. Meanwhile, short term loss of Atg3 did not impair T lymphocyte survival. Previous work showed that autophagy in peripheral T cells functions to removed excessive mitochondria and endoplasmic reticulum (ER), and is indispensable for TCR-induced proliferation (Pua et al. 2009; Jia and He 2011; Jia et al. 2011). Here we found that in stressed cells (in which both apoptosis and necroptosis were induced), autophagy also played cytoprotective role.

Several independent groups reported necroptosis in activated T lymphocytes when the functional caspase 8 activity was compromised (Bell et al. 2008; Osborn et al. 2010; Ch'en et al. 2011a; Lu et al. 2011; He and He 2013b). Enhanced autophagy is usually detected in necroptotic cells, though whether it is the cause of cell death is debatable. The results from those models and our study are summarized in Table 1.

Table 1: The comparison of the phenotypes of caspase 8-, FADD- and c-FLIP$_L$-deficient T cells after TCR stimulation

Models	Necroptosis	Enhanced Autophagy	Apoptosis	Necroptosis rescued by Nec-1	Necroptosis rescued by *Rip-3*$^{-/-}$	Autophagy-dependent cell death
caspase-8$^{-/-}$	Yes	–	No	–	Yes	No
FADDdd	Yes	Yes	No	Yes	Yes	Yes
Fadd$^{-/-}$	Yes	–	No	Yes	–	No
c-Flip$^{-/-}$ *c-FLIP$_R$ Tg* (c-FLIP$_L^{-/-}$)	Yes	Yes	Yes	Yes	–	No

Combined with previously published data from other groups, we present a model describing how c-FLIP$_L$ and caspase 8 regulate the interplay of autophagy, apoptosis and necroptosis (Figure 23). Still, several questions need to be answered to resolve the twisted relationships between these three fundamental intracellular events. First, though multiple studies investigate the interplay of autophagy and apoptosis, it is still unclear that the mechanism of how autophagy antagonizes apoptotic signals in c-FLIP$_L$-deficient T cells. Second, little is known about whether and how autophagy protects the cells from necroptosis. Reactive oxygen species (ROS) contributes to the necroptosis in c-FLIP$_L$-deficient T cells. Autophagy regulates the major ROS resource, mitochondria (Pua et al. 2009). However, our results showed that the mitochondrial level were similar in wild type and c-FLIP$_L^{-/-}$ T cells. It is possible that autophagy may

contribute to remove damage organelles in c-FLIP$_{L}^{-/-}$ T cells to promote cell survival. Third, the mechanism of how necroptosis allies with enhanced autophagy needs to be addressed. Cell molecular study proved that c-FLIP proteins directly compete with LC3 for Atg3 through two DEDs (Lee et al. 2009). Here we showed that c-FLIP is also a negative regulator for necroptosis. Therefore, the alteration of the expression level of c-FLIP may correlate autophagy and necroptosis. However, the high level of autophagy in z-VAD-fmk treated L929 cells and in activated FADD-deficient T cells suggested that necroptotic conditions might induce autophagy that cannot be suppressed by endogenous c-FLIP. Other molecule must be altered during the necroptosis to promote autophagy induction.

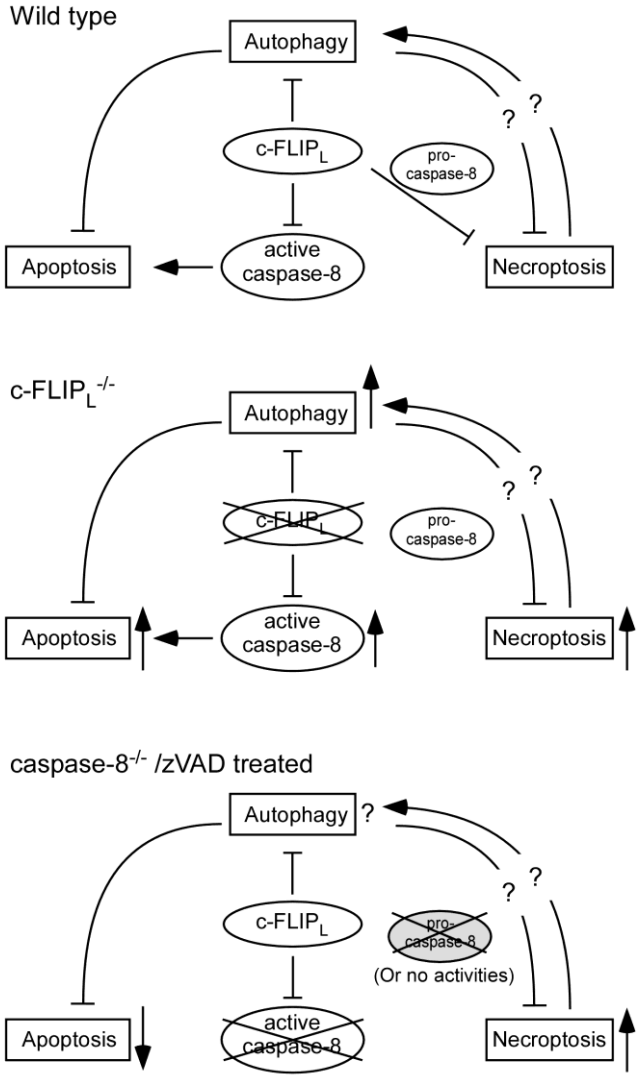

Figure 23: c-FLIP$_L$-mediated interplay of apoptosis, necroptosis and autophagy in T lymphocytes.

Figure 23. c-FLIP$_L$ controls apoptotic signaling (caspase 8) activities and downregulates autophagy. Together with pro-caspase 8 (with intact activity), c-FLIP$_L$ inhibits necroptosis induction in T cells. In the absence of c-FLIP$_L$, autophagy, necroptosis and apoptosis are all enhanced. When caspase 8 is deleted or its proteolytic activities are inhibited, apoptosis is blocked. However, necroptosis is induced in the cells, as the catalytic activity of pro-caspase 8 is required for RIP-1 inhibition. The autophagy levels in *caspase-8*$^{-/-}$ T lymphocytes and *Fadd*$^{-/-}$ cells have yet to be determined.

4. c-FLIP protects T lymphocyte from apoptosis in the intrinsic pathway

4.1 Introduction

c-FLIP has been shown to suppress death receptor-induced apoptosis. Death receptors ligation recruits pro-caspase 8 through adaptor protein (FADD or TRADD). The interaction between pro-caspase 8 molecules upon the recruitment leads to cleavage in their C-terminal and the release of the active caspase 8. Two isoforms of c-FLIP proteins are expressed in murine cells: 24 kD c-FLIP$_R$ and 55kD c-FLIP$_L$. Both c-FLIP$_L$ and c-FLIP$_R$ suppress apoptosis by forming heterodimer with pro-caspase 8 to inhibit its proper activation (Budd et al. 2006). c-FLIP-deficient T cells showed defective survival, especially upon TCR stimulation. (Zhang et al. 2008a). Blocking extrinsic apoptosis pathway by ablating Fas and/or TNF-α receptor expression results in limited improvement in the survival of c-FLIP-deficient T cells after TCR activation, which suggests that c-FLIP may protect cell survival in a death receptor-independent pathway. Therefore it is important to examine whether c-FLIP plays a role in regulating the intrinsic apoptosis pathway in T lymphocytes.

c-FLIP regulates autophagy and necroptosis, two processes involved in cell survival (He and He 2013a). Autophagy is essential for T cell homeostasis. Deficiency in any following autophagy machinery molecules, Atg5, Atg3, Atg7 or Beclin-1, leads to impaired T cell survival (Pua et al. 2007; Pua et al. 2009; Stephenson et al. 2009; Hubbard et al. 2010; Jia and He 2011). However, suppressing autophagy under certain conditions

is beneficial for the survival of T lymphocytes (Bell et al. 2008). c-FLIP overexpression represses autophagy and improves cell survival in NIH3T3, MEF and HCT116 cells (Lee et al. 2009). Immunoprecipitation results in HCT116 cells demonstrates that c-FLIP binds to Atg3 and prevents Atg3 interacting with LC3, a process essential for autophagosome formation. Necroptosis is a type of programmed necrotic cell death, which is suppressed by caspase 8 activity. Loss of the long isoform of c-FLIP leads to necroptosis in T lymphocytes upon TCR stimulation (He and He 2013b). Meanwhile, necroptosis has not been detected in naïve T lymphocytes. Whether c-FLIP protects mature T cells through regulating autophagy and necroptosis needs to be determined.

Previously we reported that c-FLIP conquers death receptor-induced apoptosis and primary TCR activation-induced cell death by inhibiting caspase 8 activation (Zhang et al. 2008a). Here we found that cell survival was impaired in naive c-FLIP-deficient T lymphocytes, especially after apoptosis induction by staurosporine (STS). Besides moderating caspase 8 activity, c-FLIP suppressed mitochondrion-mediated apoptotic signaling. Necroptosis was not detected in naïve T lymphocytes no matter c-FLIP proteins are expressed. V Autophagy was upregulated in c-FLIP-deficient T cells, but inhibition of autophagy did not improve cell survival in the absence of c-FLIP protein.

4.2 Results

4.2.1 Impaired survival of c-FLIP-deficient T lymphocytes

Conditional deletion of c-FLIP in the thymocytes leads to impaired survival and severe periphery T cells reduction (Zhang and He 2005b). The low cell number of c-FLIP-deficient T cells, along with the accumulative effect of the deletion, makes this model unsuitable for studying the role of c-FLIP in mature T lymphocytes. Therefore, we crossed *c-Flip*$^{f/f}$ mice with ER-Cre^{+} mice to generate inducible c-FLIP knockout mice. As *in vivo* deletion of c-FLIP causes lethality of the animals within 3 days, c-FLIP was deleted *in vitro* in T lymphocytes by 4-hydroxy-tamoxifen to generate c-FLIP$^{-/-}$ T cells (Zhang et al. 2008a). After deletion, live cells were purified and cultured with 1 ng/ml IL-7. The rate of apoptosis was analyzed after 16 hours in the absence or presence of apoptosis inducer staurosporine (STS) and etoposide (ETP). Staurosporine inhibits PKC kinase and induces caspase 3-dependent apoptosis in cells (Shimizu et al. 2004). Etoposide causes irreversible double-strand or single-strand breaks in DNA (Tenev et al. 2011). The survival rate of c-FLIP-deficient T cells was significantly reduced compared to that of wild type cells, once apoptosis is induced by staurosporine. The survival of c-FLIP-deficient T lymphocytes cultured in the absence of apoptosis-inducting drugs was also impaired compared to that of the control group, though the phenotype is much milder. However, genomic toxin etoposide did not trigger more cell death in c-FLIP-deficient T cells than in wild type T cells (Figure 24A, B).

c-FLIP has been shown to regulate necroptosis in activated T lymphocytes (He and He 2013b). Therefore, we first determined whether staurosporine treatment induces necroptosis in c-FLIP-deficient T cells. The induction of apoptosis involves the activation of a series of caspases, which can be inhibited by pan-caspase inhibitor z-VAD-fmk. Necroptosis induction requires the activity of RIP-1 kinase, which can be repressed by necrostatin-1 (Nec-1) (Degterev et al. 2008). The enhanced death rate of c-FLIP$^{-/-}$ T cells was partially rescued z-VAD-fmk, but not by Nec-1 (Figure 25). Addition of Nec-1 to z-VAD-fmk treated T cells did not further increase cell survival. These data suggest that staurosporine-treated T lymphocytes die from apoptosis, not necroptosis.

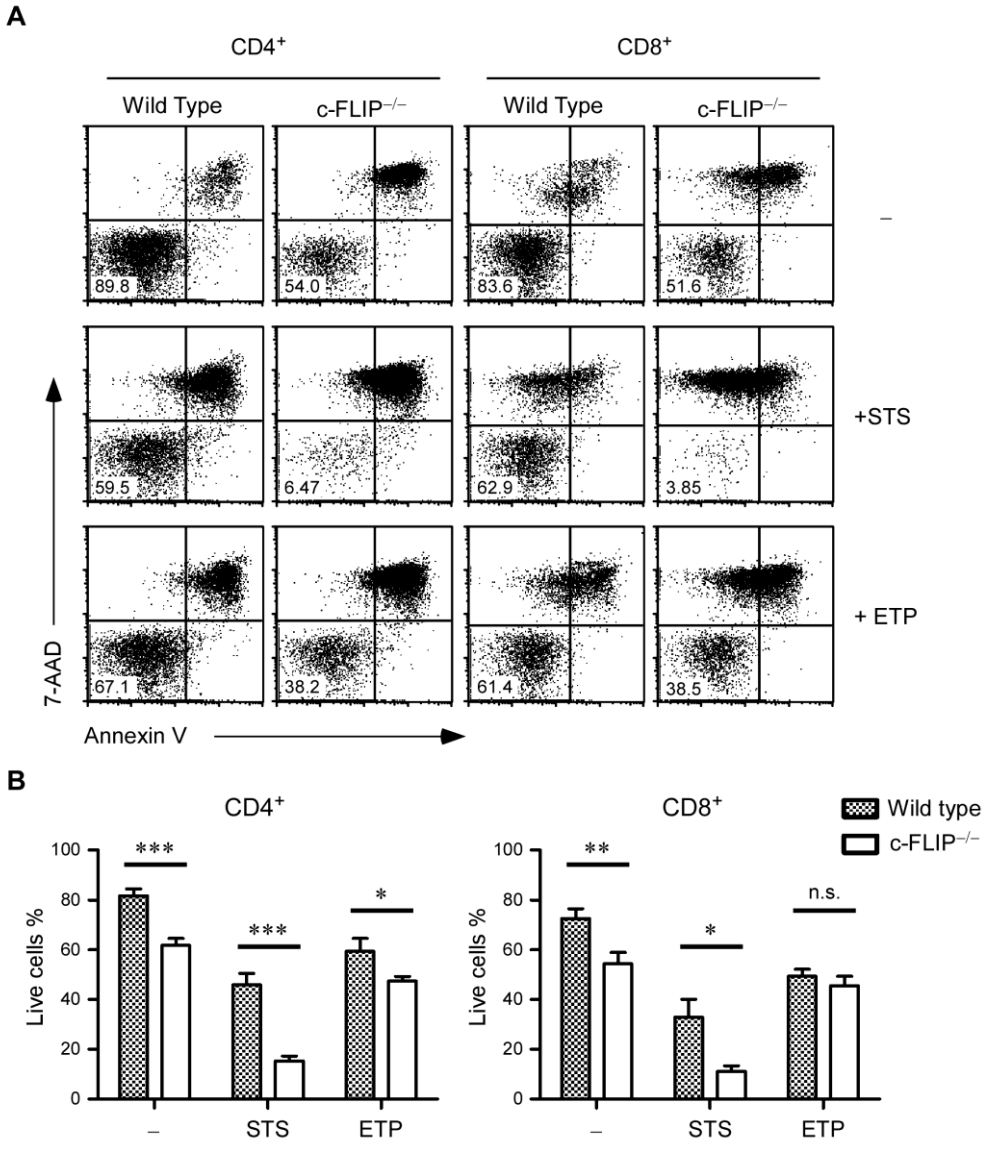

Figure 24: Enhanced apoptosis in c-FLIP-deficient T lymphocytes.

Figure 24. (A) The rate of apoptosis in c-FLIP-deficient T cells. Lymphocytes pooled from spleen and lymph nodes from *c-Flip*$^{f/f}$ and *c-Flip*$^{f/f}$ ER-Cre^{+} mice were cultured for 3 days with 4-hydroxy-tamoxifen for *in vitro* deletion. Live cells were enriched with Ficoll then cultured in the presence or absence of staurosporine (STS) or etoposide (ETP) for 16 hours. Cell death was measured by Annexin V and 7-AAD staining. (B) Statistics of (A). Live cells were analyzed by gating on the Annexin V^{-} 7-AAD^{-} population within the total CD4^{+} or CD8^{+} populations (n=6). All error bars represent the standard error of the mean (s.e.m.).

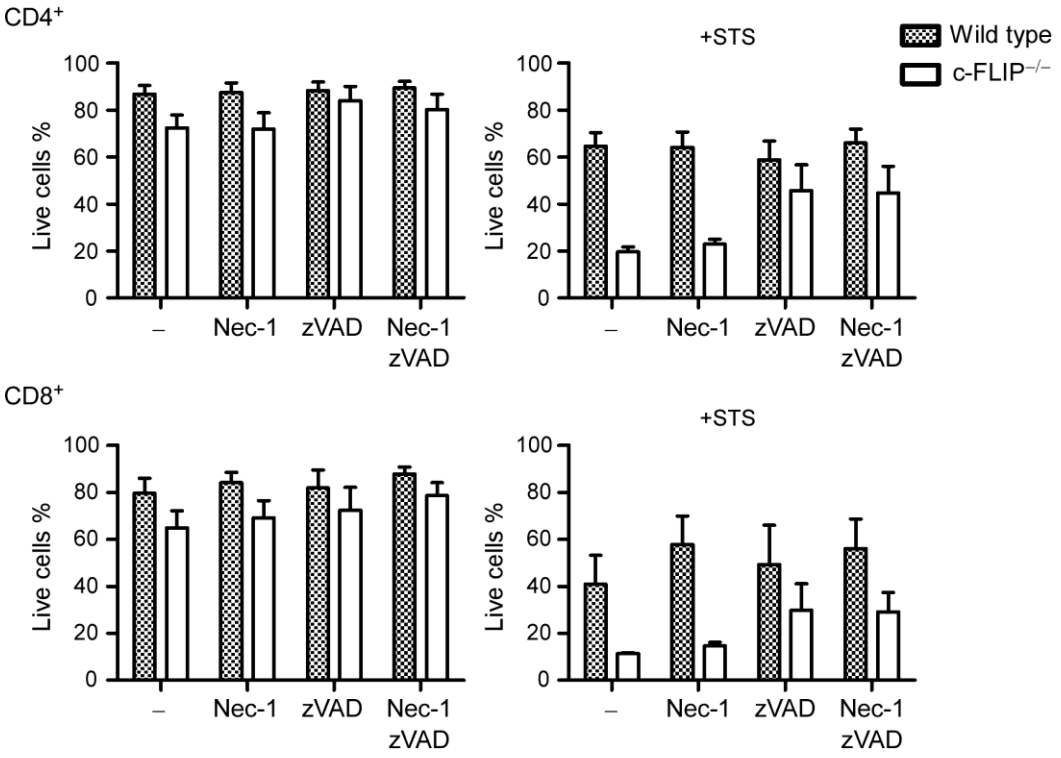

Figure 25: No necroptosis in the c-FLIP-deficient T cells

Figure 25. Effect of necrostatin-1 and z-VAD-fmk on the cell death of c-FLIP$^{-/-}$ T cells. Lymphocytes pooled from spleen and lymph nodes from *c-Flip$^{f/f}$* and *c-Flip$^{f/f}$* ER-Cre^{+} mice were cultured for 3 days with 4-hydroxy-tamoxifen for *in vitro* deletion. Live cells were enriched with Ficoll then cultured in the presence or absence of STS, z-VAD-fmk and Nec-1 for 16 hours. Apoptosis was measured by flow cytometry. Live cells were gated on the Annexin V^{-} 7-AAD^{-} population (n=6). All error bars represent the standard error of the mean (s.e.m.).

4.2.2 The protective role of c-FLIP in the intrinsic apoptosis pathway in naive T lymphocytes

Caspase 1 and caspase 3 activation are involved in the apoptosis induction by staurosporine, though the mechanism of how staurosporine activate caspases is unclear (Krohn et al. 1998; Chae et al. 2000). Here we used inhibitors of different caspases to test which apoptotic pathway is activated. Extrinsic pathway of apoptosis is triggered by death receptor ligation, followed by activation of caspases 8. Intrinsic pathway is characterized by cytochrome c released from mitochondria to cytosol and the activation of caspases 9 by apoptosome (Zhang et al. 2005). The enhanced apoptosis of c-FLIP-deficient T lymphocytes was partially rescued by both caspases 9 inhibitor z-LEHD-fmk and pan-caspase inhibitor z-VAD-fmk. Caspases 9 inhibitor z-LEHD-fmk showed better rescue effect, especially in $CD4^+$ T cells. Caspase 8 inhibitor z-IETD-fmk, however, failed to rescue the enhanced apoptosis in c-FLIP-deficient T lymphocytes (Figure 26A). Also, active form of caspase 8 could not be detected in either group of T cells (data not shown). The requirement of caspases 9 in staurosporine-induced apoptosis suggested the involvement of the intrinsic apoptotic pathway in c-FLIP-deficient T cells. Also, we detected cytochrome c release from mitochondria to cytosol in both wild type and c-FLIP-deficient T cells. The kinetic study showed that cytochrome c release was faster in c-FLIP-deficient T cells than wild type controls (Figure 26B). These data indicate that c-FLIP proteins protect T cells from also mitochondrial stress-induced apoptosis.

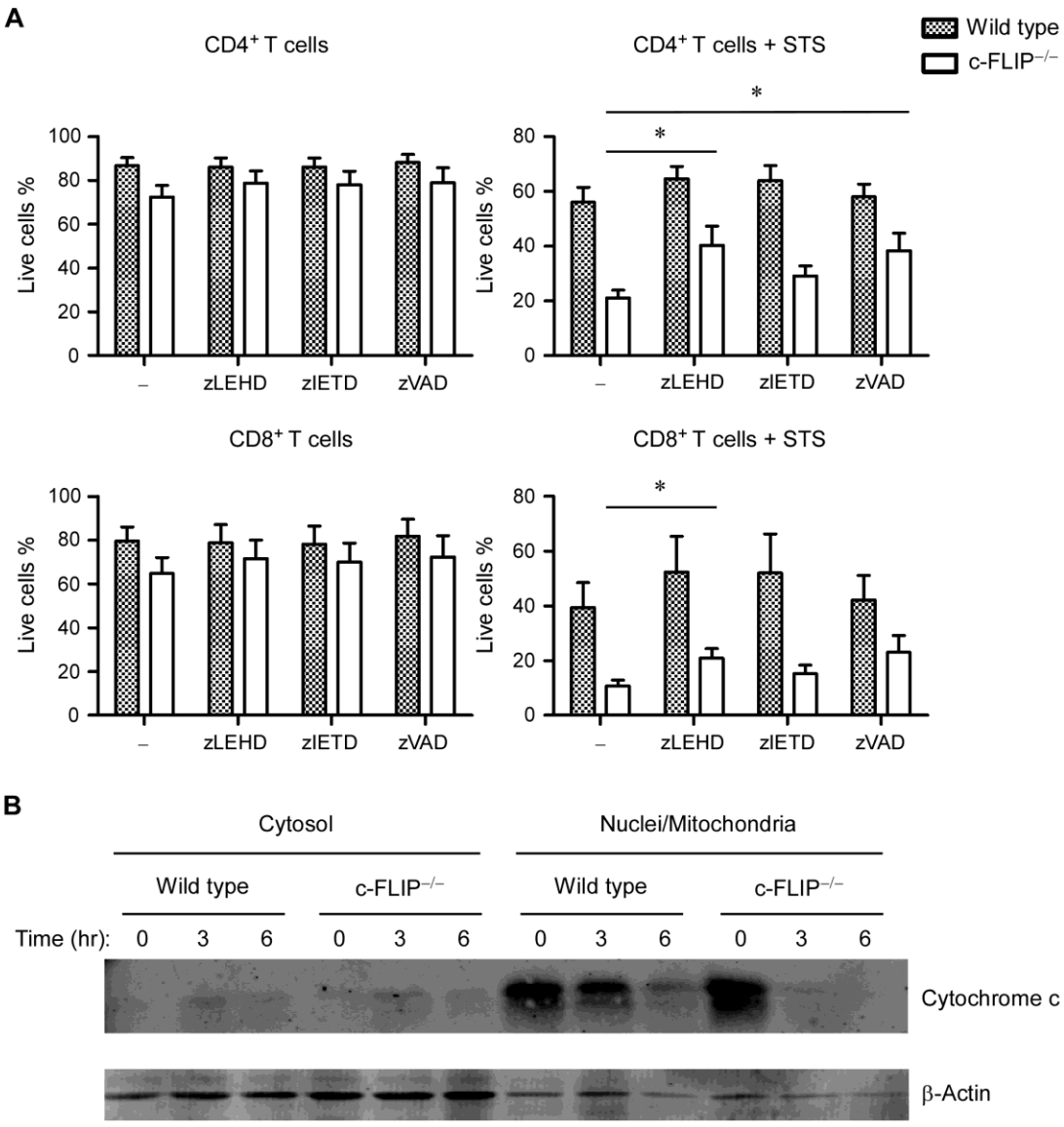

Figure 26: The regulatory role of c-FLIP in the intrinsic apoptosis pathway in T lymphocytes

Figure 26. (A) Effect of z-VAD-fmk, z-LEHD-fmk and z-IETD-fmk on the cell death of c-FLIP$^{-/-}$ T cells. Inducible deletion was conducted as in Figure 23. Live T cells were then cultured in the presence or absence of z-IETD-fmk, z-LEHD-fmk or z-VAD-fmk for 16 hours. Apoptosis was measured by flow cytometry based Annexin V/7-AAD staining. Live cells were analyzed by gating on the Annexin V$^-$ 7-AAD$^-$ population within the total CD4$^+$ or CD8$^+$ populations (n>4). All error bars represent the standard error of the mean (s.e.m.). (B) Cytochrome c release upon staurosporine. Live T cells were sorted after *in vitro* deletion of c-FLIP and cultured in the presence of staurosporine (STS) for indicated time. Cytosol was separated by digitonin in 80mM KCl buffer.

4.2.3 c-FLIP protects Bim-dependent apoptosis in naive T lymphocytes

To confirm that c-FLIP interacted with intrinsic apoptotic pathway, we tested whether deletion of Bcl-2 family pro-apoptotic proteins rescues the enhanced cell death. *c-Flip*$^{f/f}$ ER-Cre^{+} mice were crossed with *Bak*$^{-/-}$, *Bax*$^{-/-}$ and *Bim*$^{-/-}$ mice to generate double knockouts. During apoptosis induction in the intrinsic pathway, Bak and Bax form oligomeric complex in the outer membrane of the mitochondria to mediate the release of cytochrome c (Mikhailov et al. 2003; Dejean et al. 2005). Bax and Bak frequently compensate for each other in the process of MOMP. *Bak*$^{-/-}$*Bax*$^{-/-}$ cells show resistance to multiple death stimuli that mediates apoptosis through intrinsic pathway, while Bax- and Bak- deficient cells are susceptible to them (Wei et al. 2001). Deletion of Bak did not rescue the apoptosis phenotype of c-FLIP$^{-/-}$ T cells (Figure 27A). Bax deficiency seemed to reduce the increased spontaneous apoptosis in c-FLIP$^{-/-}$ T cells, but the result was not statistically significant (Figure 27B). Pro-apoptotic protein Bim responds to several stimulus and regulates the homeostasis of hematopoietic cells (Bouillet et al. 1999). Loss of Bim increased cell survival c-FLIP$^{-/-}$ CD4^{+} T cells, and fully rescued the cell death phenotype in c-FLIP$^{-/-}$ CD8^{+} T cells after staurosporine treatment. However, crossing to *Bim*$^{-/-}$ mice only slightly reduced the spontaneous apoptosis in c-FLIP-deficient T cells (Figure 27C). These results suggest that c-FLIP protects staurosporine-induced apoptosis in T cells in a Bim-dependent pathway, while spontaneous apoptosis in c-FLIP-deficient T cells may be mediated by other mechanisms.

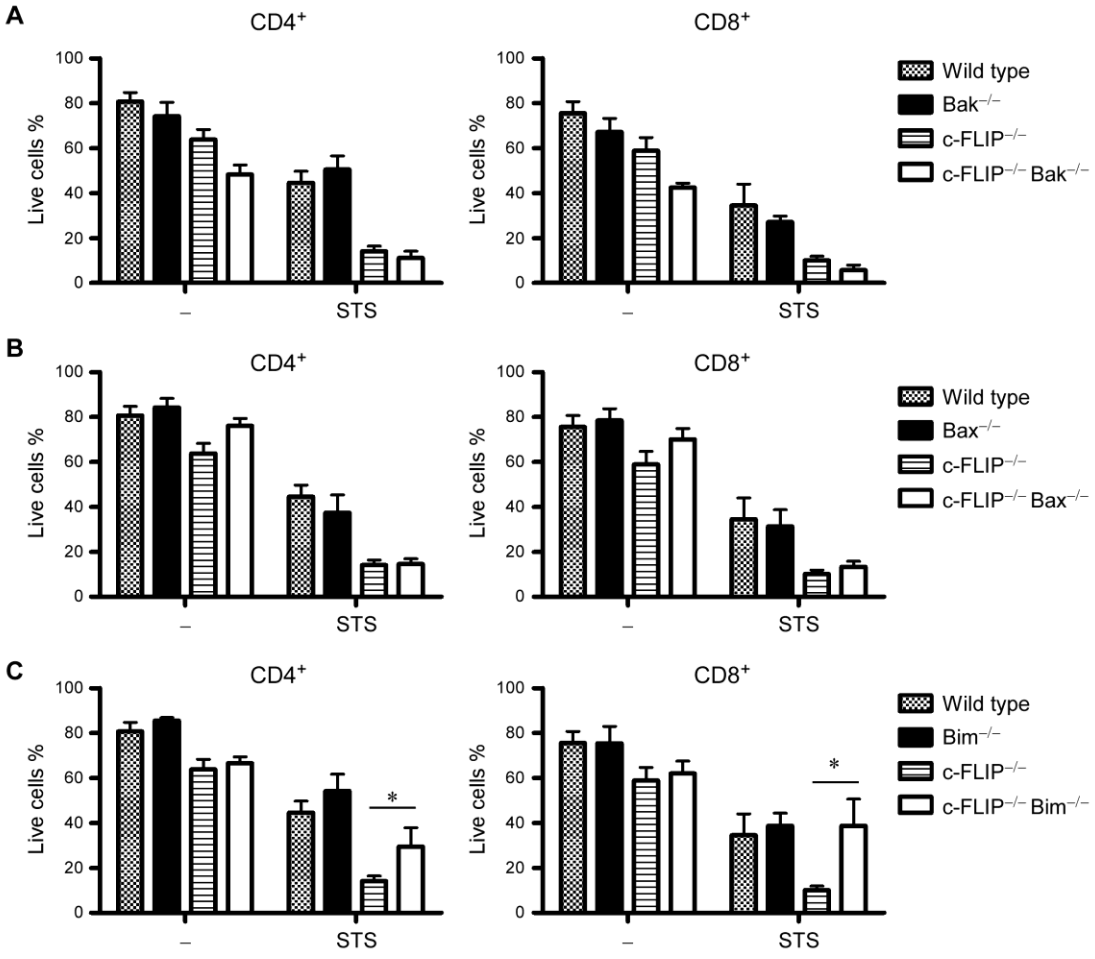

Figure 27: Bim-dependent apoptosis in c-FLIP-deficient T lymphocytes after staurosporine treatment

Figure 27. The effect of Bax, Bak and Bim deletion on the cell survival in c-FLIP-deficient T cells. Splenocytes from *c-Flip*$^{f/f}$, *c-Flip*$^{f/f}$ ER-Cre^{+}, *c-Flip*$^{f/f}$ *Bak*$^{-/-}$, *c-Flip*$^{f/f}$ ER-Cre^{+} *Bak*$^{-/-}$ (A), *c-Flip*$^{f/f}$ *Bax*$^{-/-}$, *c-Flip*$^{f/f}$ ER-Cre^{+} *Bax*$^{-/-}$ (B), *c-Flip*$^{f/f}$ *Bim*$^{-/-}$ and *c-Flip*$^{f/f}$ ER-Cre^{+} *Bim*$^{-/-}$ (C) mice were culture for 3 days with 4-hydroxy-tamoxifen for *in vitro* deletion to generate wild type, c-FLIP$^{-/-}$, Bak$^{-/-}$, c-FLIP$^{-/-}$Bak$^{-/-}$ (A), Bax$^{-/-}$, c-FLIP$^{-/-}$Bax$^{-/-}$ (B), Bim$^{-/-}$ and c-FLIP$^{-/-}$Bim$^{-/-}$ (C) T cells. Live cells were enriched then cultured in the presence or absence of staurosporine (STS) for 16 hours. The cell survival rates were analyzed by flow cytometry based Annexin V/7-AAD staining. Live cells were gated on the Annexin V^{-} 7-AAD^{-} population within the total CD4^{+} or CD8^{+} populations (n>=4). All error bars represent the standard error of the mean (s.e.m.).

We next examined the expression level of Bcl-2 family member in c-FLIP-deficient T cells. The expression levels of pro-apoptotic protein Bcl-2, Mcl-1 and Bcl-x_L were comparable between wild type and c-FLIP$^{-/-}$ T cells. After staurosporine treatment, Bcl-2 and Mcl-1 were downregulated in wild type cells, while the expression of Bcl-x_L was increased (Figure 28). c-FLIP-deficient T cells failed to upregulate Bcl-x_L, but showed higher expression of Mcl-1. Previous studied showed c-FLIP interacts with Bcl-x_L (Han et al. 1997). How this interaction regulates the function of Bcl-x_L remains to be studied. Interestingly, the survived wild type and c-FLIP-deficient T cell after apoptosis induction showed decreased level of Bax and Bim (Figure 28), which may be the consequence that cells expressing low level of pro-apoptotic proteins preferentially survived. These data suggest the crosstalk between c-FLIP and Bcl-2 family members in the regulation of apoptosis in T lymphocytes.

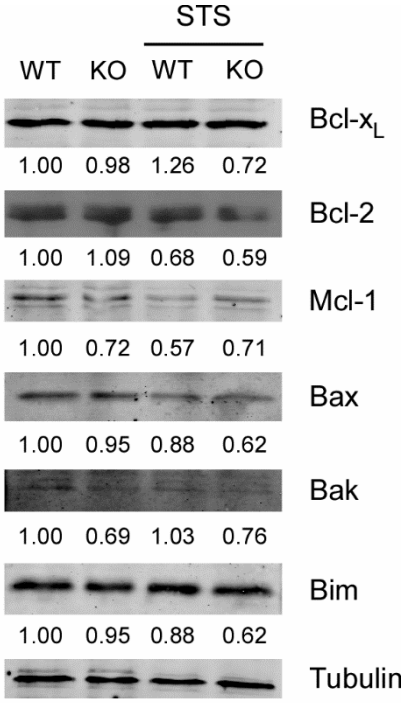

Figure 28: The expression of Bcl-2 family members in c-FLIP-deficient T cells

Figure 28. Western blot analysis of pro-apoptotic proteins in c-FLIP$^{-/-}$ T cells. Total splenocytes from *c-Flip$^{f/f}$* and *c-Flip$^{f/f}$* ER-Cre^{+} mice were cultured for 3 days in the presence of 4-hydorxy-tamoxifen and IL-7 to induce deletion, resulting in wild type (WT) and c-FLIP$^{-/-}$ (KO) lymphocytes. Live cells were purified by Ficoll and T cells were negatively enriched by selection kit. Enriched T cells were cultured in the absence or presence of STS for 6 hours, and then the cell lysate is analyzed by western bot. The expression level of each protein was quantified and normalized to the expression of Tubulin. The numbers underneath each band showed the relative expression level of each molecule compared to that in the wild type untreated T cells.

4.2.4 Autophagy is not involved in the enhanced apoptosis in c-FLIP-deficient T lymphocytes

We next tested whether c-FLIP modulated autophagy in T lymphocytes and whether autophagy was involved in the enhanced apoptosis in c-FLIP-deficient T cells. The conversion of LC3-I to LC3-II isoform and the consequent insertion of LC3-II in the isolation membrane are essential for autophagosome formation. Monitoring LC3 puncta formation inside the cells is wildly applied in autophagy detection (Klionsky et al. 2012). Here we tested the level of LC3-II in c-FLIP-deficient and wild type T cells by western blot. LC3-II was undetectable in both wild type and c-FLIP-deficient T cells in the absence of apoptosis stimuli. After staurosporine treatment, LC3-II was increased, especially in c-FLIP-deficient T cells (Figure 29A). We then intracellularly stained c-FLIP-deficient T cells and wild type cells with anti-LC3 to examine the LC3 puncta as the signs of autophagosome. The number of $LC3^{+}$ puncta in c-$FLIP^{-/-}$ T cells was not significantly higher than that in wild type T cells. However, c-FLIP-deficient T cells showed larger LC3 puncta and stronger fluorescent intensity, indicating larger autophagosome formed or higher LC3 protein level (Figure 29B). We then examined whether blocking autophagy alter the cell survival in c-$FLIP^{-/-}$ T lymphocytes. Loss of autophagy related gene *Atg3* in T lymphocyte leads to inhibition of autophagy (Jia and He 2011). Short term deletion of Atg3 does not cause increased apoptosis in naïve T cells (He and He 2013b). Therefore, *c-Flip*$^{f/f}$ ER-Cre^{+} mice were crossed to *Atg3*$^{f/f}$ mice to generate *Atg3*$^{f/f}$ *c-Flip*$^{f/f}$ ER-Cre^{+} mice. *Atg3*$^{f/f}$ *c-Flip*$^{f/f}$ littermates were used as controls.

Inducible deletion of c-FLIP and Atg3 was conducted successfully *in vitro* by 4-hydroxy-tamoxifen (He and He 2013b). We found that deletion of Atg3 showed no impact on the cell survival in the presence of staurosporine. In the absence of staurosporine, the spontaneous apoptosis in $CD4^+$ T cells was unchanged, while the spontaneous apoptosis of $CD8^+$ T cells was increased in $Atg3^{-/-}$c-$FLIP^{-/-}$ cells compared to that in $Atg3^{+/-}$c-$FLIP^{-/-}$ T cells (Figure 29B). These data suggests that autophagy is not the cause of the enhanced apoptosis of c-FLIP-deficient T lymphocytes.

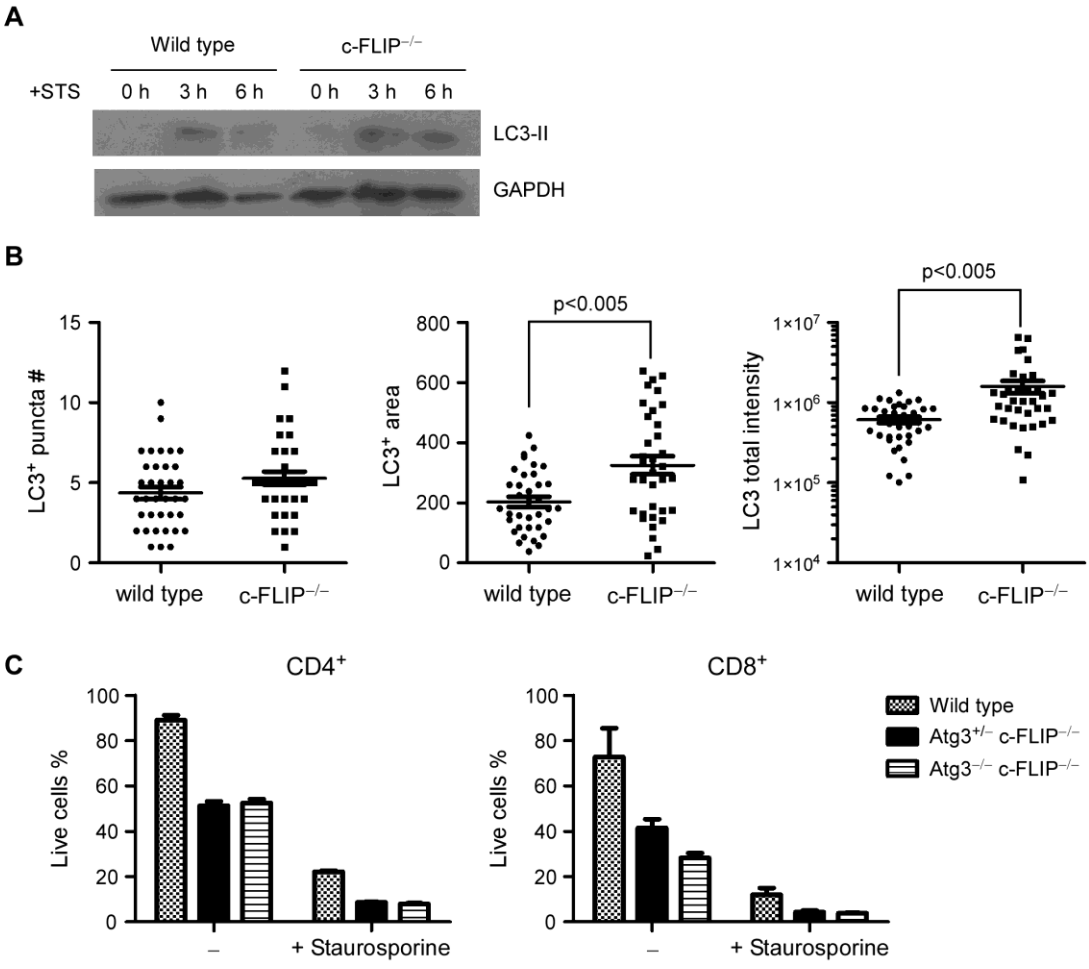

Figure 29: Enhanced autophagy and autophagy-independent cell death in c-FLIP-deficient T lymphocytes.

Figure 29. (A) Western blot analysis of LC3 in c-FLIP-deficient T cells. Total splenocytes from *c-Flip*$^{f/f}$ and *c-Flip*$^{f/f}$ ER-Cre^{+} mice were cultured for 3 days in the presence of 4-hydorxy-tamoxifen and IL-7 to induce deletion, resulting in wild type and c-FLIP$^{-/-}$ lymphocytes. Live cells were purified by Ficoll and T cells were negatively enriched by selection kit. Live T cells were treated with staurosporine (STS) for 0, 3 and 6 hours and lysed by sample buffer. The data shown are representative of 3 independent experiments. (B) LC3 puncta in c-FLIP-deficient T cells. Inducible deletion was induced as in (A) in T cells, and enriched live T cells were stimulated with anti-CD3 for 12 hours. Cells were intracellularly stained with anti-LC3 and examined by 3-D fluorescent microscopy. LC3 puncta represents LC3^{+} dots (>10 pixels). Area represents relative LC3 positive volume inside one cell. Total intensity represents the total LC3^{+} signal from each cell (n>30). (C) The effect of autophagy inhibition in the survival of c-FLIP$^{-/-}$ T cells. Total splenocytes from *c-Flip*$^{f/f}$ *Atg3*$^{f/f}$, *c-Flip*$^{f/f}$ *Atg3*$^{f/+}$ ER-Cre^{+} and *c-Flip*$^{f/f}$*Atg3*$^{f/f}$ ER-Cre^{+} mice were cultured for 3 days in the presence of 4-hydorxy-tamoxifen and IL-7 to generate wild type, c-FLIP$^{-/-}$Atg3$^{+/-}$ and c-FLIP$^{-/-}$Atg3$^{-/-}$ T cells. Live cells were isolated and cultured in the presence of absence of staurosporine for 16 hours with IL-7. Live cells were analyzed by gating on the 7-AAD^{-} population within the CD4^{+} or CD8^{+} populations. All error bars represent the standard error of the mean (s.e.m.) (n>=4).

4.2.5 The role of reactive oxygen species in the enhanced apoptosis in c-FLIP-deficient T lymphocytes

Staurosporine has been shown to rapidly increase intracellular reactive oxygen species (ROS) in HeLa cells. The high level of ROS contributes to caspase 3 activation and apoptosis induction (Shimizu et al. 2004). Notably, the long isoform of c-FLIP has been shown to promote cell survival by controlling ROS in activated T lymphocytes (He and He 2013b). Therefore, it was important to test whether ROS caused the increased apoptosis in staurosporine treated c-FLIP-deficient T lymphocytes. However, our results showed that loss of c-FLIP did not alter ROS production in naive T cells, in the presence or absence of staurosporine (Figure. 30A). Addition of antioxidant NAC moderately improves the cell survival after the staurosporine treatment in both wild type and c-FLIP-deficient T cells (Figure. 30B). This result is consistent with the previous publication showing ROS is involved in the apoptosis induction by staurosporine (Shimizu et al. 2004). However, the protection of NAC was not specific to c-FLIP-deficient T cell. These results suggest that c-FLIP protects naive T cells in a ROS-independent pathway.

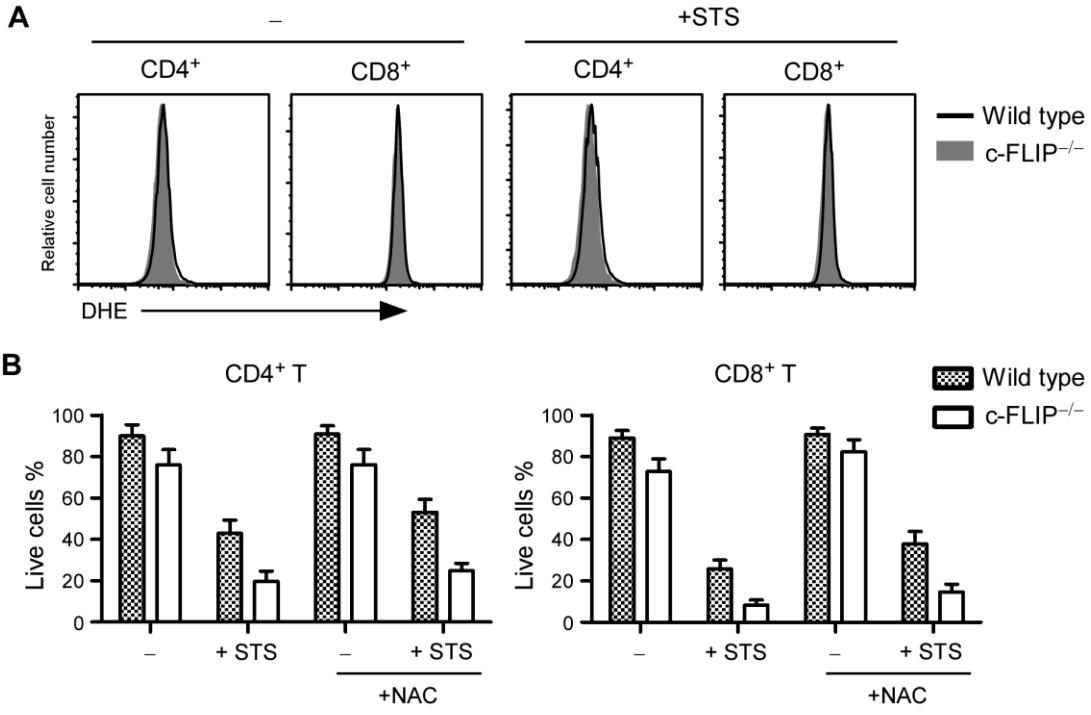

Figure 30: ROS-independent enhanced apoptosis in c-FLIP-deficient T cells.

Figure 30. (A) ROS levels in c-FLIP-deficient T cells. Live naïve T lymphocytes were cultured in the absence of presence of staurosporine for 16 hours. Cells were then stained with DHE and analyzed by flow cytometry. Data are representative of 3 independent experiments. (B) The effect of acetylcysteine on the survival of staurosporine treated T cells. Lymphocytes were cultured as in (A) in the presence or absence of NAC. Cell death rates of T cells were analyzed by flow cytometry. Live cells were analyzed by gating on the 7-AAD$^-$ population within the CD4$^+$ or CD8$^+$ populations. All error bars represent the standard error of the mean (s.e.m.) (n>=4).

4.3 Conclusion and Discussion

Here we showed higher rate of death receptor-independent spontaneous apoptosis in c-FLIP-deficient T cells in the presence of survival factor IL-7. The apoptosis phenotype is much more severe when apoptosis was induced by staurosporine. The enhanced apoptosis in c-FLIP-deficient T cells resulted from more rapid cytochrome c release and following caspase 9 activation. The autonomous activation of pro-caspase 8 was not the cause of the apoptotic phenotype in c-FLIP-deficient T lymphocytes. Notably, c-FLIP suppressed BH3-only pro-apoptotic protein Bim to regulate T cell survival upon apoptotic stimuli treatment. Deletion of Bim significantly improved the survival of c-FLIP$^{-/-}$ T cells in the presence of staurosporine. Though autophagy is enhanced in c-FLIP-deficient T cells, inhibition of autophagy failed to rescue the enhanced apoptosis.

c-FLIP-deficient T cells were susceptible to staurosporine-induced apoptosis, but showed similar apoptotic rate after etoposide treatment. The variable susceptibility of c-FLIP-deficient T cells in these conditions is probably the consequence that different apoptotic stimuli are sensed by different pro-apoptotic molecules in the cell. For example, Noxa targets Mcl-1 for proteasomal degradation in response to DNA damage (Ploner et al. 2008). Puma is shown to be the predominant mediator in γ-radiation-induced apoptosis in T lymphocytes (Erlacher et al. 2005). IL-7 deprivation-induced apoptosis, on the other side, is majorly mediated by Bim (Li et al. 2010). Etoposide and

staurosporine probably provoke apoptosis in distinct pathways. Etoposide induces apoptosis though generating double strand or single strand break in the genomic DNA (Tenev et al. 2011), while staurosporine inhibits PKC kinase and induces caspase 3-dependent apoptosis (Shimizu et al. 2004). Here we showed that c-FLIP protected T cells from staurosporine-induced apoptosis in a Bim-dependent pathway. Therefore, Bim is likely to be the responding sentinel upon staurosporine treatment. Our results provide insights in understanding the cellular process of staurosporine-induced apoptosis.

Enhanced intrinsic apoptotic signal in naïve c-FLIP-deficient T cells after staurosporine treatment is not the secondary effect of spontaneous caspase 8 activation. In Fas-induced apoptosis (extrinsic pathway), active caspase 8 cleaves Bid, a BH3-only pro-apoptotic protein, to generate the truncate form (tBid). tBid efficiently inserts into mitochondrial outer membrane and augments cytochrome c release (Li et al. 1998). It has been shown that c-FLIP-deficient T cells have higher caspase 8 activity after TCR activation (Zhang et al. 2008a). In the absence of death receptor signaling, loss of c-FLIP may result in higher level of caspase 8 autolysis. Our data suggests that caspase 8 activity is not the cause of increased apoptosis in c-FLIP-deficient T cells. The intracellular level of active caspase 8 in naïve T cells is too low to be detected (data not shown). Also, caspase 8 inhibitor z-IETD-fmk showed no rescue in the cell death phenotype of c-FLIP-deficient T cells. Therefore, though caspase 8 augments cytochrome c release upon Fas ligation via tBid, it is not likely to do so in naïve T cells in the absence

of death receptor ligands. These results are consistent with the previous findings that the full activation of initiator caspases requires the assembly of a multi-component complex (Riedl and Shi 2004). Caspase 9-activating apoptosome can be assembled in naïve T cells after Bax/Bak-mediated MOMP and cytochrome c release. However, caspase 8-activating DISC cannot be formed, due to the lack of extrinsic apoptotic signals.

Our results suggested a strong correlation of c-FLIP deficiency and intrinsic apoptotic signaling in naïve T cells. Caspase 9 inhibitor z-LEHD-fmk and pan-caspase inhibitor z-VAD-fmk showed remarkable protection in c-FLIP-deficient T cells upon staurosporine treatment. c-FLIP protected cells from apoptosis induction by antagonizing pro-apoptotic BH3-only protein Bim. Interesting, Bim deficiency did not improve the survival rate of untreated naïve c-FLIP$^{-/-}$ T cells. How c-FLIP regulates Bim is still unclear. On the other side, Bax deletion barely improved cell survival in naïve T cells. In HIV gp120 protein treated Jurkat cells, c-FLIP$_L$ suppresses apoptosis by promoting PKC protein expression and PKC-associated inactive Bax (Wang et al. 2009). However, our results showed that c-FLIP protected the survival of naïve T cells independent of Bax. One of the reasons may be that c-FLIP$_R$ is the dominant form of c-FLIP protein in naïve T cells instead of c-FLIP$_L$.

Staurosporine treatment induces superoxide production and subsequent cell death in hippocampal neurons (Krohn et al. 1998). Here we showed that ROS production also contributed to the enhanced apoptosis in staurosporine treated T

lymphocytes. Antioxidant NAC partially rescued the staurosporine-induced apoptosis. However, the cytoprotective effect of NAC was not specific to c-FLIP-deficient T cells. Our results showed that loss of c-FLIP did not increase the production of ROS in naïve T cells. Activated T cells, on the other side, require the long isoform of c-FLIP to regulate cellular ROS. Loss of c-FLIP$_L$ leads to increase ROS production and ROS-dependent cell death (He and He 2013b). These results suggest that c-FLIP proteins function differently in naïve and activated T lymphocytes.

Necroptosis was not detected in naïve c-FLIP$^{-/-}$ T cells. This result was consistent with previous publications showing necroptosis induction only in activated T lymphocytes (Bell et al. 2008; Osborn et al. 2010; Ch'en et al. 2011a; Lu et al. 2011). Why activated T cells are susceptible to necroptosis has yet to be studied. RIP-1 kinase plays an important role in T lymphocytes for proper TCR signaling. RIP-1-deficient T cells showed impaired TCR-induced phosphorylation of p65 NF-κB (Zhang et al. 2011). Activated T cells may upregulate RIP-1 for the benefit of cell signaling, while loss of adequate inhibition in RIP-1's kinase activity ends up killing the cells. In this scenario, the upregulation of RIP-1 is unlikely mediates by transcription, because necroptosis occur promptly after TCR activation. Our hypothesis is that there may be two "layers" of inhibition on RIP-1. TCR signaling release some inhibition on RIP-1, but the catalytic activity of caspase 8-c-FLIP$_L$ heterodimer is still sufficient to constrain RIP-1 phosphorylation. When caspase 8 activity is blocked or c-FLIP$_L$ is deleted, RIP-1 loses

the second layer of suppression and undergoes phosphorylation, probably with the aid of RIP-3. One potential player in the "first layer of inhibition" is deubiquitinating enzyme CYLD. CYLD functions in the proximal TCR signaling by promoting the recruitment of ZAP70 to deubiquitinated Lck (Reiley et al. 2006). CYLD is upregulated in activated T lymphocytes and deubiquitinates RIP-1, a necessary process for the phosphorylation of RIP-1 (Harhaj and Dixit 2012). Another possibility is that the intracellular translocation of RIP-1 contributes to TCR activation induced-RIP-1 kinase activity. Upon TCR stimulation, RIP-1 is recruited to caspase 8 and c-FLIP$_L$ (Dohrman et al. 2005a). This process provides a docking site for RIP-1 to interact with each other and potentially undergo autophosphorylation. Death receptor Fas may be involved in the recruitment of RIP-1 through mediating the formation of DISC. Our results showed that Fas was indeed required for the induction of necroptosis, at least in c-FLIP$_L$-deficient T lymphocytes. These two hypothesized mechanism do not contradict to each other and may both be true in the cells.

Altogether we showed that c-FLIP regulates apoptosis signaling in the Bim-dependent intrinsic pathway. The mechanism of the crosstalk of c-FLIP and Bim remains to be determined. Our results suggest that c-FLIP protein protects T lymphocytes survival through multiple pathways.

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Biography

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