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UNIVERSITY OF CALIFORNIA,
IRVINE

Regulation of Necroptosis and Autophagy in T cell Homeostasis and Function

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Biological Sciences

by

Jennifer Lu

Dissertation Committee:
Associate Professor Craig M. Walsh, Chair
Professor David A. Fruman
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2014

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ACKNOWLEDGMENTS

I would first like to thank Dr. Craig M. Walsh for his support and mentorship during my doctoral studies. I am grateful he gave me the opportunity to work in his lab, and for allowing me the freedom to pursue my own research ideas. Our weekly meetings provide extremely valuable insights and discussions, and I always leave our meetings mentally energized and refreshed. Under his guidance, I believe I have matured in my ability to think scientifically and critically, and am well prepared for my future endeavors.

My sincere thanks also go to members of the Walsh lab, past and present. I would like to thank Brian Weist for his guidance during my early graduate years, and for his helpful ideas and thoughtful discussions. My thanks also go to Jeniffer Hernandez and Ryan Newton, for their generous support and input on my research.

I would also like to thank Dr. David Fruman for being a member of my advancement and dissertation committee, and for our joint lab meetings. His suggestions have always been greatly appreciated and have aided the progress of my work.

My appreciation and thanks go to Dr. Olga Razorenova for being a part of my dissertation committee, and for her insights during our joint lab meetings.

I thank Dr. Naomi Morrisette, Dr. Tom Lane, and Dr. Lan Huang for serving on my advancement committee, and Dr. Aimee Edinger and Dr. Melissa Lodoen for their suggestions during our joint lab meetings.

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Lu, J.V., Walsh CM. 2014. Necrosome formation following T cell receptor ligation. Manuscript in preparation

Lu, J.V., Walsh CM. 2014. NF- κ B pathway regulates autophagy signaling. Manuscript in preparation.

Lu, J.V., Walsh CM. 2014. Autophagy is required for naïve T cell survival but not in activated T cells. Manuscript submitted.

Lu, J.V., Walsh CM. 2012. Programmed necrosis and autophagy in immune function. Immunological Reviews 249(1):205-17.

Lu, J.V., Weist BM, van Raam B., Marro B., Bell BD, Luhrs KA, Lane TE, Salvesen GS, Walsh CM. 2011. Complementary roles of FADD and RIPK3 in T cell development and homeostasis. Proceedings of the National Academy of Sciences, USA, 108(37):15312-7.

Duncan RE, Wang Y, Ahmadian M, **Lu, J.**, Sarkadi-Nagy E, Sul HS. 2010. Characterization of desnutrin functional domains: critical residues for triacylglycerol hydrolysis in cultured cells. J Lipid Res. 51(2):309-17.

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ABSTRACT OF THE DISSERTATION

Regulation of Necroptosis and Autophagy in T cell Homeostasis and Function

By

Jennifer Lu

Doctor of Philosophy in Biological Sciences

University of California, Irvine, 2014

Associate Professor Craig M. Walsh, Chair

Development of functional adaptive and innate immune responses requires strict regulation of programmed cell death signaling pathways. These signaling pathways are essential for shaping and maintaining the immune system, and ensuring functional immune responses. Programmed death is required for clonal deletion of lymphocytes during an infection, and dysregulation results in defective clearance of autoreactive T cells and autoimmune disease.

Death Receptor (DR) pathways regulate apoptotic signaling and are essential for immune homeostasis and tolerance. Ligation of DR, CD95/Fas/Apo-1, triggers formation of a cytosolic complex known as the “death-inducing signaling complex” (DISC), which includes adaptor molecule FADD (Fas associated with death domain), Receptor Interacting Protein (RIP) Kinase 1, and caspase-8. Caspase 8 is required for extrinsic apoptosis upon DR ligation, while promoting clonal expansion in T cells following T cell receptor stimulation. In the absence of caspase 8, cells succumb to a programmed necrosis-like death process facilitated by the generation of RIPK1-RIPK3 “necrosomes”. The aim of this dissertation is to elucidate the crosstalk between necroptosis and apoptosis in T lymphocytes in the context of caspase 8. By

generating RIPK3^{-/-} x FADD^{dd} double mutant mice, we show in Chapter 2 that FADD, caspase 8 and RIP kinases are all essential for clonal expansion, contraction, and antiviral responses.

FADD^{dd} T cells also display hyper-autophagy upon activation; thus, another aim of this dissertation is to uncover the role of autophagic signaling in T lymphocytes. Using mice genetically deficient in autophagy protein, Atg5, we demonstrate in Chapter 3 the differential roles for autophagy in naïve and proliferating T cells. Autophagy is required for clearing excess mitochondria in naïve T cells, while activated cells are capable of diluting mitochondria through rapid division. In Chapter 4, we characterize the platform(s) that promote recruitment of RIPK1-RIPK3 complex in T cells by identifying the kinetics, constituency, and subcellular localization of the necrosome. Understanding the paradigms of necroptosis and autophagy and defining the switch between apoptosis and necrosis will allow us to manipulate death pathways to modulate desired immune responses to various diseases and infections.

CHAPTER 1

INTRODUCTION

Programmed Necrosis and Autophagy in Immune Function

ABSTRACT

Death receptors (DR) of the tumor necrosis factor (TNF) superfamily regulate apoptotic death of various cell types in the immune system. Genetic mutations or blockade of DR result in defective clearance of activated lymphocytes leading to impaired lymphoid homeostasis, characterized by lymphoproliferative and autoimmune disease associated with Autoimmune Lymphoproliferative Syndrome (ALPS) [1]. Caspase 8 (casp8) and FADD are key molecules downstream of DR signaling. Paradoxically, T cells deficient in casp8 or FADD fail to proliferate in response to antigenic stimulation and succumb to a casp8-independent death termed “necroptosis” [2]. Additionally, germline deletion of casp8 or FADD results in embryonic lethality, which is rescued by deletion of serine/threonine receptor-interacting protein kinase-1 (RIPK1) or RIPK3 [3-7]. In the absence of casp8 activity, RIPK1 and RIPK3 associate to form a necrosis-inducing complex termed the “necrosome” [8-10]. Following antigenic stimulation, T cells lacking casp8 or FADD undergo hyper-autophagy, and fail to proliferate due to the induction of necroptosis. In TNF- α treated Jurkats and activated primary T cells, direct cleavage of RIPK1 by casp8 blocks necrosome formation to inhibit necroptosis [3]. Thus, the impaired expansion of casp8^{-/-} T cells is a consequence of excessive autophagy and necroptotic induction. We have demonstrated rescue of the proliferative defect in T cells expressing the dominant negative FADD (FADD^{dd}) using RIPK1 inhibitor, Nec-1 [2], as well as with genetic deletion of RIPK3. In addition to DR

ligands and TCR activation, a multitude of upstream signals, including innate immune signals, genotoxic stress, DNA-damaging agents, single-stranded viruses [8-12] have recently been demonstrated to trigger formation of the RIPK1-RIPK3 complex as well. In this study we aim to characterize the molecular intricacies of the necrosome and study the role of autophagic signaling in a T cell model system. Current understanding of the molecular mechanisms behind necroptotic signaling is unclear, and these studies will not only broaden our understanding of death regulation in T cells, but also provide general insight into the role of necroptosis and autophagy in coordinating various immune responses.

Apoptosis versus necrosis

For many years, the widely accepted form of cell death known to regulate embryonic development, immune homeostasis, and disease was apoptosis. Apoptotic death is described as an orderly, programmed event, characterized by an intact membrane until the late stages, activation of caspases, DNA fragmentation, and membrane blebbing [11]. Necrotic death is described as swelling of the endoplasmic reticulum, mitochondria, and cytoplasm, subsequently resulting in rupture of the plasma membrane and lysis of the cell, and is regarded as an unregulated and uncontrollable process [12]. Necrosis has long been viewed as an accidental death cause by physical or chemical injury promoting an immunogenic death modality in contrast with apoptosis, which generally induces a tolerogenic response. While caspase 8- apoptotic death has been well characterized, recent studies have now revealed that necrosis can also be regulated through specific intrinsic cellular programs.

Development of functional adaptive and innate immune responses requires strict regulation of programmed cell death signaling pathways. These signaling pathways are essential

for shaping and maintaining the immune system, and ensuring functional immune responses. Programmed death is required for clonal deletion of lymphocytes during an infection, and dysregulation results in defective clearance of autoreactive T cells, impaired tolerance, and autoimmune disease.

Death Receptor Signaling

The tumor necrosis factor receptor (TNF) receptor superfamily is composed of a twenty-three cell surface receptors that potentiate various functions *in vivo*. Receptors belonging to the TNF receptor family play an important role in immune regulation by controlling various responses in mammalian cells. Of particular interest is a subset called the death receptors (DR), many of which are essential for immune homeostasis and tolerance and participate in the clonal deletion of activated lymphocytes [1]. Death receptors share the feature of a death domain on their cytoplasmic tails, which promotes homotypic oligomerization the recruitment of adaptor molecules such as FADD, TRADD, and RIPK1 [13-16] all of which bear death domains (DD) for interaction with DRs. Thus, the DD serves as the crucial bridge between adaptor proteins and death receptors to transmit downstream apoptotic signals.

Death Inducing Signaling Complex, Complex I, and Complex IIb

Ligation of DR, CD95/Fas/Apo-1 triggers formation of a cytosolic complex known as the “death-inducing signaling complex” (DISC) [19], or complex II (**Figure 1.1**), which includes adaptor molecule FADD (Fas associated with death domain), RIPK1, caspase-8, and a caspase-8-like molecule that lacks proteolytic activity called c-FLIP [17]. Inactive Caspase-8 exists as monomers in the cytosol: Upon DR engagement, FADD is recruited to the DR by its DD, where

it then recruits and activates monomeric casp8 via its death effector domain (DED) interactions [18, 19]. Caspase-8 homodimerization is accompanied by auto-cleavage and induction of catalytic activity (autoactivation) [20], ultimately leading to complex II release to the cytosol to propagate apoptotic signaling.

Initiation of TNFR1 signaling by TNF is followed by recruitment of a different adaptor protein TNFR-associated death domain protein (TRADD) [21, 22], and ubiquitin ligases, TRAF2 and cIAP1 [23], to the membrane. RIPK1 is then recruited to form what is commonly referred to as “complex I” [24, 25] (Figure 1.1). Following a sequence of ubiquitination events modulated by cIAP1/2 [26-28] and the linear ubiquitin assembly complex (LUBAC) [29-31], pro-survival complex I, containing ubiquitinated RIPK1 as a scaffolding protein, recruits NF- κ B essential modulator (NEMO) and I κ B kinase (IKK) complex resulting in NF- κ B activation [32]. NF- κ B signaling drives cell survival and proliferation by upregulating synthesis of anti-apoptotic proteins, such as cFLIP and cIAP, and aids in the inflammatory response [27, 33]. When NF- κ B activation is blocked, and thus protein synthesis (cFLIP) is inhibited, assembly of complex II induces apoptosis. In response to TNFR1 engagement, adaptor protein TRADD recruits FADD to activate caspase 8, while following CD95/Fas/Apo-1 ligation, FADD binds directly to CD95 to activate caspase 8. Thus, the critical interaction for caspase activation is between FADD and Fas death domains, whereas the corresponding interaction responsible for TNF-induced caspase activation is between FADD and TRADD death domains.

The function of RIPK1 as a pro-survival or pro-death molecule is regulated by its ubiquitination status. Polyubiquitinated RIPK1 prevents the transition of the membrane-associated complex I to the cytosolic complex II [8, 9]. Depending on the cellular conditions and post-translational regulatory mechanisms, deubiquitylation of RIPK1 by enzyme cylindromatosis

(CYLD) permits internalization of complex I [34] and generation of complex II, resulting in caspase-8 activation and triggering of apoptosis [35].

Although initial research supported a relatively straightforward apoptotic signaling cascade downstream of death receptors [36], it is now evident that caspase-8 induced apoptosis is not the sole pathway in DR-induced cell death [37]. The first evidence of this was when an artificially multimerized mutation of FADD unexpectedly induced a caspase-independent form of cell death [38, 39]. Whereas the apoptotic effect of TNF is mediated by caspases, induction of necrotic death by TNF or other members of the TNF family is enhanced by caspase inhibitors. Disabling caspase-8 apoptotic machinery results in cell death resembling necrosis, termed “necroptosis” or “programmed necrosis”.

In addition to RIPK1, RIPK3 was also found to interact with the complex II [40] in response to DR-ligation or TCR stimulation leading to formation of necroptotic-inducing complexes, “necrosomes” or “complex IIb” [41]. When caspase-8 activity is compromised, RIPK3 is recruited to complex II by RIPK1 to form complex IIb (**Figure 1.1**). Association of RIPK1-RIPK3 complex promotes necroptosis, and necrosome assembly is blocked with RIPK1 inhibitor. RIPK1 kinase activity is required for propagating necrotic signaling, and caspase 8 negatively regulates necroptosis [3]. Direct cleavage of RIPK1 and RIPK3 by caspase 8 is the mechanism through which cells differentiate between apoptotic and necrotic death.

RIP Kinases are part of the RIP family of seven serine-threonine kinases that have been shown to be involved in innate and adaptive immunity. RIP kinases are characterized by their effector or protein interaction domains, which allow them to participate in various signaling pathways. RIPK1 contains a C-terminal death domain motif, an intermediate domain known as a RIP homotypic interaction motif (RHIM) [42], and an N-terminal kinase domain.

The kinase activity of RIPK1 is required for elaboration of programmed necrosis, and a family of molecules termed “necrostatins”, specifically Necrostatin-1, have been identified to bind to and inhibit the catalytic activity of RIPK1 [43, 44]. Addition of Nec-1 inhibited necroptotic death induced by DR signaling in several cell types. RIPK3 is structurally similar to RIPK1; the RHIM domain facilitates their interaction with each other, although RIPK3 lacks a DD motif, so it plays no role in the NF- κ B survival pathway. RIPK3 is recruited to RIPK1-containing necrosomes via its RHIM where a series of phosphorylation events occurs [8]. The kinase activity of RIPK1 is required to stabilize its interaction with RIPK3, and this interaction is blocked by necrostatin-1.

Fas-associated death domain protein (FADD)

To investigate of the role of DR-mediated death in T cells [45-47] a mutant form of FADD lacking a caspase-8 recruitment domain (FADD^{dd}) was ectopically expressed in T cells. This model unveiled the induction of RIPK1-dependent necroptosis in T cells following antigenic receptor signaling when caspase 8 activity is compromised. FADD^{dd} and caspase 8^{-/-} T cells harbor decreased numbers of CD8⁺ T cells in the periphery and displayed proliferative defects *in vitro* following TCR stimulation and *in vivo* in response to viral challenge, even though pro-survival NF- κ B activation remained intact [48, 49]. Extracellular blockade of DR ligation failed to rescue caspase 8^{-/-} or FADD^{dd}-expressing T cells, suggesting that the nucleation of RIPK1/RIPK3 necrosomes occurs in a manner independent of DR signaling. Upon activation, FADD^{dd} and Caspase 8^{-/-} T cells exhibit excessive autophagy [2] before succumbing to necroptosis, indicating the role of RIPK1 in regulating both processes. Nec-1 treatment rescued the expansion defect and blocked autophagy induction in caspase 8^{-/-} and FADD^{dd} T cells [2,

49]. Naïve cells lacking functional DISC proteins do not display this hyperautophagic phenotype, demonstrating that increased autophagy requires TCR activation.

Similar results observed in mice conditionally lacking caspase-8 or c-FLIP in T cells establish that these DISC proteins are also essential for T-cell clonal expansion [50, 51]. These studies demonstrate that DISC proteins are required for T cell expansion following antigenic stimulation. Germline deletions of caspase 8, RIPK1, cFLIP, or FADD in mice result in embryonic lethality, indicating a pro-survival role for DISC proteins. The death of FADD^Δ or caspase 8 deficient T cells is attributed to the loss of RIPK1-3 necroptotic regulation by casp8. Subsequent findings show that concurrent ablation of caspase 8 (or FADD^Δ) and RIPK3 completely rescues embryonic lethality resulting from developmental defects associated with caspase 8 deficiency [4-6].

Other roles of necroptosis in immune function

Another cytosolic complex, termed the “Ripoptosome” [52, 53] (**Fig. 1.2**), can activate both apoptosis and necroptosis through interactions between TLR domain-containing adaptor protein inducing interferon- β (TRIF) [54] and the RHIM of RIPK1. This ~2-megadalton signaling platform assembles in response to pattern recognition receptors (PRR) activation and genotoxic stress and is comprised of complex IIb proteins caspase 8, FADD, cFLIP, and RIPK1. The ripoptosome is a signaling platform that regulates caspase8 -apoptosis or RIPK1-necroptosis independent of TNF, TRAIL, CD95L, and mitochondrial pathways (MOMP) and requires RIPK1 kinase activity for complex stabilization. Caspase 8 and RIPK1 are also known to be recruited to the RNA sensor retinoic acid-inducible gene I (RIG1) complex [55], and cytosolic

DNA sensor, DNA-dependent activator of interferon regulatory factors (DAI) [56], which directly engage RIPK1 and RIPK3 through the RHIM to assemble a necrosome-like complex.

These findings uncover additional stimuli where necroptosis is induced, thus expanding the number of intracellular and extracellular signals that are under caspase 8 regulation.

Although various stimuli and different cell surface receptors (TNF, TCR, TLR3-4, genotoxic stress, RIG-1, DAI) activate death pathways and formation of necrotic complexes, the unifying mechanism of necroptosis is the recruitment of RIPK3 to these complexes.

Autophagy in T cells

Autophagy is a conserved process by which intracellular contents are degraded [57], and in the immune system, autophagy is important for clearing intracellular pathogens such as group A *Streptococcus* and *Mycobacterium tuberculosis* [58, 59]. Generally, autophagy is known as a cell survival mechanism, and is induced under low-nutrient or high-stress conditions. The formation of autophagosomes was observed in activated CD4⁺ T cells [60] indicating that autophagy occurs in T cells, and can be intrinsically linked to T cell activation and proliferation. [61] An essential role for autophagy was first established in activated T cells through the use of fetal liver reconstitution experiments using Atg5^{-/-} T cells. Upon TCR stimulation, Atg5^{-/-} T cells undergo inefficient clonal expansion accompanied by profound levels of cell death. Similar to the *in vivo* and *in vitro* phenotypes observed in Atg5^{-/-} mice, Atg7^{-/-} and Atg3^{-/-} mice displayed a significantly decreased number of peripheral T cells, and these were incapable of proliferating efficiently upon stimulation. Additionally, T cells lacking Atg3 contained increased mitochondrial and ER content. Mice deficient in Atg3, Atg5, and Atg7 also displayed greatly reduced T cell numbers in the periphery, suggesting a developmental defect or an inability to

maintain immune homeostasis. The thymocyte populations were unaffected and percentages of double negative (DN), double positive (DP), and single positive (SP) subsets remained constant.

Another characteristic of activated FADD^Δ T cells is an increase in autophagy levels, and to further investigate the relationship between Caspase8-dependent death and autophagy, [7] mice bearing a conditional Atg7 deletion were crossed with mice deficient in caspase 8. Deletion of Atg7 in combination with a caspase 8 deficiency exacerbated the loss of T cells, indicating the defects observed in T cells of mice lacking the capacity to induce autophagy are independent of RIPK1 activity. Given that autophagy is linked with RIPK1-mediated necroptotic death in activated T cells, it is surprising that autophagy plays a positive, homeostatic role for the survival of rapidly proliferating, highly anabolic T cells.

T cell Activation

Once a foreign pathogen invades the immune system it is engulfed, processed, and displayed as peptide fragments by antigen presenting cells in the context of MHC molecules. A circulating T cell with specificity for the foreign antigen binds to the peptide/MHC complex, resulting in various signaling cascades necessary for the proliferation and differentiation into an effector T cell. One of the first signaling events to take place is phosphorylation of the immunoreceptor Tyrosine base activation motifs (ITAMs) signaling motif on the cytosolic tails of the TCR/CD3 complex by the Src family kinase Lck, which is bound to the CD4 or CD8 co-receptors. Lck then recruits and activates Zap-70, which then phosphorylates LAT and SLP-76 forming a complex with other kinases, such as the inducible T cell kinase (ITK). Phospholipase C γ 1 (PLC γ 1) is subsequently phosphorylated by ITK resulting in the breakdown of phosphatidylinositol 4, 5-bisphosphate (PIP₂) into diacylglycerol (DAG) and inositol triphosphate (IP₃). DAG's role is to

activate PKC θ and the MAPK/Erk pathways leading to activation of the transcription factor NF- κ B. IP₃, on the other hand triggers the release of Ca²⁺ from the endoplasmic reticulum, resulting in entry of extracellular Ca²⁺ into the T cells through calcium release-activated Ca²⁺ (CRAC) channels. The influx of calcium leads to activation of calcineurin, which causes translocation of the transcription factor NFAT from the cytoplasm to the nucleus. Along with NF- κ B and other transcription factors, NFAT, results in transcription of many genes including the cytokine interleukin-2 (IL-2), which is essential for T cell proliferation.

Recent evidence suggests that autophagy functions as a critical process in mediating the events following TCR activation. Stimulation through the TCR results in an oxidative burst that may lead to generation of massive levels of toxic ROS. While ROS is beneficial for lowering T-cell activation thresholds through inhibition of phosphatase activity [62, 63], excess oxygen radicals can also be potentially toxic to T cells by inducing organelle damage through lipid peroxidation and other negative effects that promote T-cell death [64]. Thus, selective autophagy of mitochondria, or mitophagy, may be used to compartmentalize mitochondria producing ROS and protect cells from damaged mitochondria that would further amplify ROS production. High levels of ROS, increased mitochondrial content, and enhanced biosynthesis of mitochondria-specific genes was observed in naive Atg^{-/-} T cells [65, 66]. These observations are consistent with the idea that mitophagy plays a crucial role during T-cell development and activation. As T cells switch from oxidative phosphorylation to glycolytic metabolism following their activation they become less reliant on mitochondria for their source of energy. Therefore, the ability of T cells to induce autophagy is necessary for the removal of nonfunctional or unnecessary mitochondria that may release excess ROS or apoptosis-inducing mitochondrial proteins (e.g. cytochrome C) and trigger apoptotic or necrotic cell death.

As mentioned above, calcium signaling is also a consequence of T-cell activation, characterized by the opening of CRAC channels and subsequent entry of millimolar levels of calcium [65]. After TCR stimulation, ER calcium channels release calcium stores into the cytosol, leading to opening of CRAC channels and calcium flux from extracellular environment to intracellular space. Thus, the role of autophagy in ER homeostasis may indirectly impact calcium mobilization in T lymphocytes [66]. Consistent with the increased ER content observed in $Atg5^{-/-}$ and $Atg7^{-/-}$ T cells, [67] activated $Atg7^{-/-}$ T cells display impaired calcium influx and increased ER calcium stores. Increased ER content holds excess calcium, and the inability to deplete these stores results in deregulated calcium influx. These observations suggest the role of autophagy in proliferating T cells is not only to maintain organelle homeostasis, but also to regulate ER content and ensure calcium homeostasis.

Other studies suggest the requirement for autophagy in activated T cells is based on the known function of autophagy in degradation of cytosolic components to ensure bioenergetic output. After antigenic stimulation, naïve T cells rapidly transition to an activated state to respond to antigen and proliferate. T cell activation imposes significant bioenergetic demands required for transcriptional remodeling and activation of biosynthetic pathways [68]. Nutrient transporter proteins are dramatically upregulated in T cells during the first 48 hours of activation, and autophagy may be required during the early stages when access to extracellular nutrients is limited but metabolic reprogramming to support proliferation has already begun. In response to antigen, T cells upregulate autophagy for selective breakdown of intracellular macromolecules and recycling of amino acids, particularly glutamine, which plays a critical role in biosynthetic reactions, to generate sufficient energy to sustain their proliferation.

Autophagy has also been shown to play a role in mediating necroptotic death in T cells lacking caspase 8 [2]. However, as described above, deletion of autophagy genes impaired T cell activation and proliferation. Although the roles of autophagy have been characterized in various cellular contexts, its function in regulating T cell survival is still unclear. The goal of this thesis is to understand the interplay between apoptosis and necroptosis in T lymphocytes, and uncover critical roles for programmed necrosis during immune responses to potentially target these pathways in disease treatment. Caspase 8 and autophagy play essential roles in T cell survival, and uncovering their mechanism of action brings insight into the broader questions regarding overall regulation of the immune system.

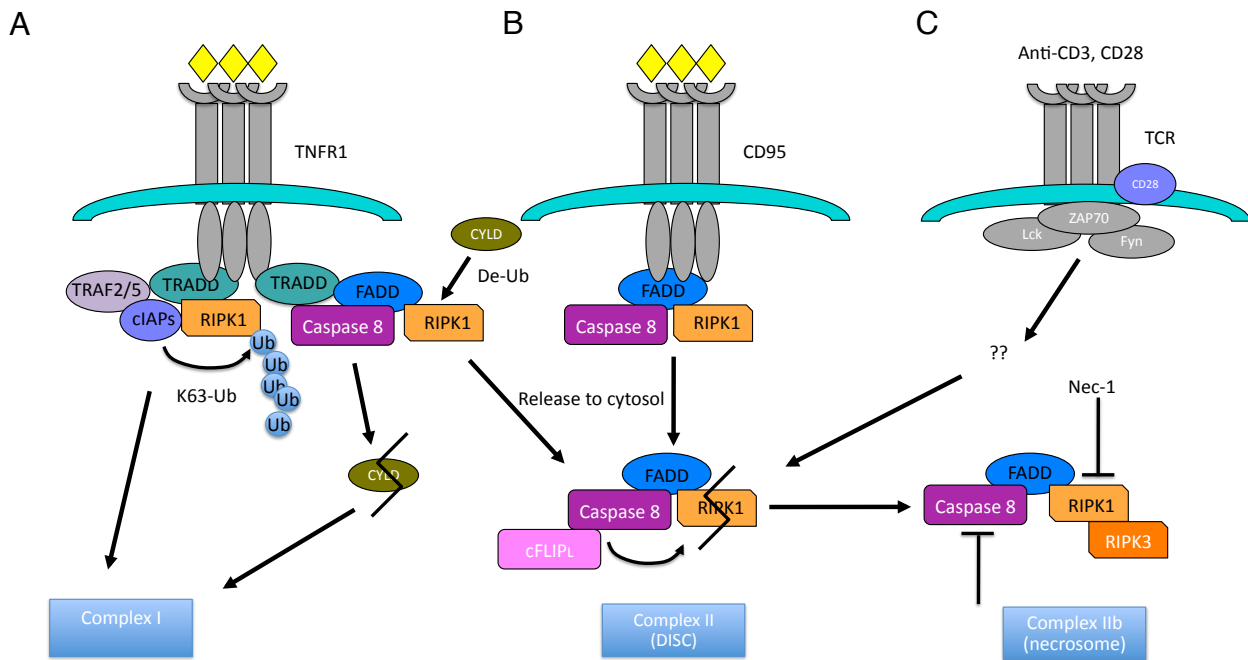


Figure 1.1 Modulation of necroptotic versus apoptotic cell death and TCR-mediated proliferation by caspase-8. Schematic of (A) TNF-induced cell death, (B) CD95L/Fas-induced cell death, and (C) TCR-induced necroptosis. Following ligation of TNFR1 by TNF α , TRADD recruitment leads to assembly of a receptor bound complex containing RIP1 and RIP3. In the presence of functional FADD and casp8, TNF or CD95L leads to assembly of complex I, or the DISC (A and B, respectively). For the TNFR1 complex 1, RIP1 is then deubiquitinated by CYLD, followed by assembly of complex II, which includes FADD, casp8 and cFLIPL. Casp8-mediated cleavage of CYLD and RIPK1 prevents recruitment of RIPK3 and activation of necroptosis. Similar signaling occurs following CD95L binding to CD95/Fas, except direct recruitment of FADD and casp8 to the receptor leads to activation of apoptosis via casp8 activity. For TCR stimulation (C), a RIPK1/RIPK3 containing necrosome occurs independent of death receptor ligation, possibly in the cytosol. In each case, casp8 activity is directed against RIPK1 and CYLD, preventing the assembly of a necrosome containing RIPK3.

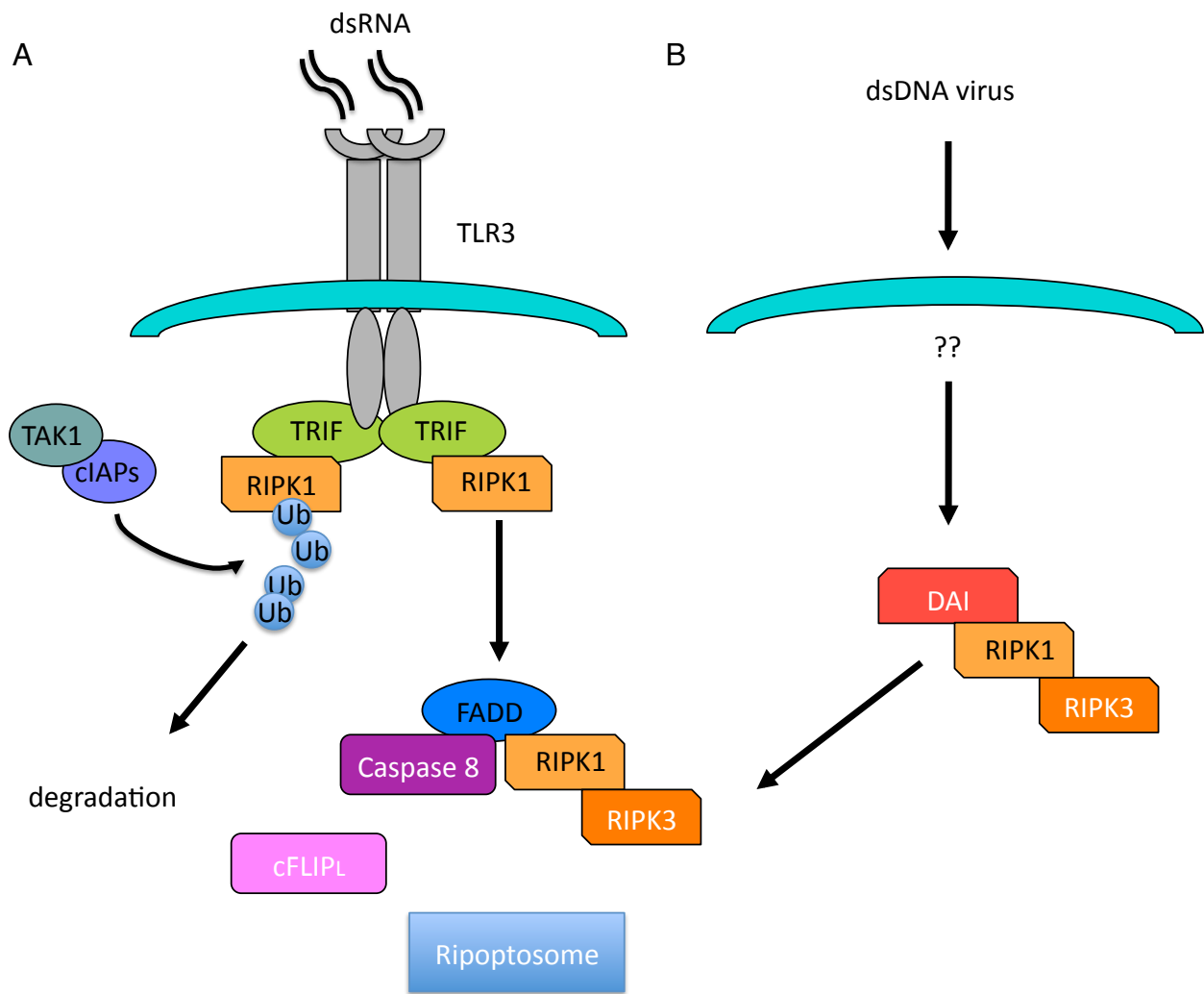


Figure 1.2 Induction of assembly of necrosomes following viral infection. (A) Binding of double-stranded RNA (dsRNA) to TLR3 leads to recruitment of TRIF, and RIPK1 via RHIM motif homotypic interactions. This leads to binding of FADD, casp8, RIP3 and possibly c-FLIPL. Cleavage of RIP1 by casp8 prevents recruitment of RIP3, and subsequent necroptosis. (B) dsDNA viruses activate DAI, a RHIM domain containing factor that binds to RIP1 and RIP3, leading to the generation of necrosomes.

References

1. Siegel, R.M., *Caspases at the crossroads of immune-cell life and death*. Nat Rev Immunol, 2006. **6**(4): p. 308-17.
2. Bell, B.D., et al., *FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells*. Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16677-82.
3. Lu, J.V., et al., *Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity*. Proc Natl Acad Sci U S A, 2011. **108**(37): p. 15312-7.
4. Kaiser, W.J., et al., *RIP3 mediates the embryonic lethality of caspase-8-deficient mice*. Nature, 2011. **471**(7338): p. 368-72.
5. Oberst, A., et al., *Catalytic activity of the caspase-8-FLIP(L) complex inhibits RIPK3-dependent necrosis*. Nature, 2011. **471**(7338): p. 363-7.
6. Zhang, H., et al., *Functional complementation between FADD and RIP1 in embryos and lymphocytes*. Nature, 2011. **471**(7338): p. 373-6.
7. Ch'en, I.L., et al., *Mechanisms of necroptosis in T cells*. J Exp Med, 2011. **208**(4): p. 633-41.
8. Tenev, T., et al., *The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs*. Mol Cell, 2011. **43**(3): p. 432-48.
9. Feoktistova, M., et al., *cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms*. Mol Cell, 2011. **43**(3): p. 449-63.
10. Upton, J.W., W.J. Kaiser, and E.S. Mocarski, *DAI/ZBP1/DLM-1 Complexes with RIP3 to Mediate Virus-Induced Programmed Necrosis that Is Targeted by Murine Cytomegalovirus vIRA*. Cell Host Microbe, 2012. **11**(3): p. 290-7.
11. Rajput, A., et al., *RIG-I RNA helicase activation of IRF3 transcription factor is negatively regulated by caspase-8-mediated cleavage of the RIP1 protein*. Immunity, 2011. **34**(3): p. 340-51.
12. Weber, A., et al., *Proapoptotic signalling through Toll-like receptor-3 involves TRIF-dependent activation of caspase-8 and is under the control of inhibitor of apoptosis proteins in melanoma cells*. Cell Death Differ, 2010. **17**(6): p. 942-51.
13. Strasser, A., L. O'Connor, and V.M. Dixit, *Apoptosis signaling*. Annu Rev Biochem, 2000. **69**: p. 217-45.

14. Yuan, J. and G. Kroemer, *Alternative cell death mechanisms in development and beyond*. Genes Dev, 2010. **24**(23): p. 2592-602.
15. Chinnaiyan, A.M., et al., *FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis*. Cell, 1995. **81**(4): p. 505-12.
16. Boldin, M.P., et al., *A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain*. J Biol Chem, 1995. **270**(14): p. 7795-8.
17. Hsu, H., J. Xiong, and D.V. Goeddel, *The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation*. Cell, 1995. **81**(4): p. 495-504.
18. Stanger, B.Z., et al., *RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death*. Cell, 1995. **81**(4): p. 513-23.
19. Kischkel, F.C., et al., *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor*. EMBO J, 1995. **14**(22): p. 5579-88.
20. Irmeler, M., et al., *Inhibition of death receptor signals by cellular FLIP*. Nature, 1997. **388**(6638): p. 190-5.
21. Donepudi, M., et al., *Insights into the regulatory mechanism for caspase-8 activation*. Mol Cell, 2003. **11**(2): p. 543-9.
22. Oberst, A., et al., *Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation*. J Biol Chem, 2010. **285**(22): p. 16632-42.
23. Pop, C., et al., *Role of proteolysis in caspase-8 activation and stabilization*. Biochemistry, 2007. **46**(14): p. 4398-407.
24. Ermolaeva, M.A., et al., *Function of TRADD in tumor necrosis factor receptor 1 signaling and in TRIF-dependent inflammatory responses*. Nat Immunol, 2008. **9**(9): p. 1037-46.
25. Harper, N., et al., *Fas-associated death domain protein and caspase-8 are not recruited to the tumor necrosis factor receptor 1 signaling complex during tumor necrosis factor-induced apoptosis*. J Biol Chem, 2003. **278**(28): p. 25534-41.
26. Wajant, H., K. Pfizenmaier, and P. Scheurich, *Tumor necrosis factor signaling*. Cell Death Differ, 2003. **10**(1): p. 45-65.
27. Ting, A.T., F.X. Pimentel-Muinos, and B. Seed, *RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis*. EMBO J, 1996. **15**(22): p. 6189-96.

28. Hsu, H., et al., *TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex*. Immunity, 1996. **4**(4): p. 387-96.
29. Vince, J.E., et al., *TRAF2 must bind to cellular inhibitors of apoptosis for tumor necrosis factor (tnf) to efficiently activate nf- κ b and to prevent tnf-induced apoptosis*. J Biol Chem, 2009. **284**(51): p. 35906-15.
30. Bertrand, M.J., et al., *cIAP1 and cIAP2 facilitate cancer cell survival by functioning as E3 ligases that promote RIP1 ubiquitination*. Mol Cell, 2008. **30**(6): p. 689-700.
31. Varfolomeev, E., et al., *c-IAP1 and c-IAP2 are critical mediators of tumor necrosis factor alpha (TNFalpha)-induced NF-kappaB activation*. J Biol Chem, 2008. **283**(36): p. 24295-9.
32. Haas, T.L., et al., *Recruitment of the linear ubiquitin chain assembly complex stabilizes the TNF-R1 signaling complex and is required for TNF-mediated gene induction*. Mol Cell, 2009. **36**(5): p. 831-44.
33. Rahighi, S., et al., *Specific recognition of linear ubiquitin chains by NEMO is important for NF-kappaB activation*. Cell, 2009. **136**(6): p. 1098-109.
34. Gerlach, B., et al., *Linear ubiquitination prevents inflammation and regulates immune signalling*. Nature, 2011. **471**(7340): p. 591-6.
35. Ea, C.K., et al., *Activation of IKK by TNFalpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO*. Mol Cell, 2006. **22**(2): p. 245-57.
36. Hacker, H. and M. Karin, *Regulation and function of IKK and IKK-related kinases*. Sci STKE, 2006. **2006**(357): p. re13.
37. He, S., et al., *Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha*. Cell, 2009. **137**(6): p. 1100-11.
38. Cho, Y.S., et al., *Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation*. Cell, 2009. **137**(6): p. 1112-23.
39. Schneider-Brachert, W., et al., *Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles*. Immunity, 2004. **21**(3): p. 415-28.
40. Wang, L., F. Du, and X. Wang, *TNF-alpha induces two distinct caspase-8 activation pathways*. Cell, 2008. **133**(4): p. 693-703.
41. Ashkenazi, A. and V.M. Dixit, *Death receptors: signaling and modulation*. Science, 1998. **281**(5381): p. 1305-8.

42. Walsh, C.M., K.A. Luhrs, and A.F. Arechiga, *The "fuzzy logic" of the death-inducing signaling complex in lymphocytes*. J Clin Immunol, 2003. **23**(5): p. 333-53.
43. Kawahara, A., et al., *Caspase-independent cell killing by Fas-associated protein with death domain*. J Cell Biol, 1998. **143**(5): p. 1353-60.
44. Vercammen, D., et al., *Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor*. J Exp Med, 1998. **187**(9): p. 1477-85.
45. Feng, S., et al., *Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain*. Cell Signal, 2007. **19**(10): p. 2056-67.
46. Declercq, W., T. Vanden Berghe, and P. Vandenabeele, *RIP kinases at the crossroads of cell death and survival*. Cell, 2009. **138**(2): p. 229-32.
47. Sun, X., et al., *Identification of a novel homotypic interaction motif required for the phosphorylation of receptor-interacting protein (RIP) by RIP3*. J Biol Chem, 2002. **277**(11): p. 9505-11.
48. Degterev, A., et al., *Identification of RIP1 kinase as a specific cellular target of necrostatins*. Nat Chem Biol, 2008. **4**(5): p. 313-21.
49. Degterev, A., et al., *Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury*. Nat Chem Biol, 2005. **1**(2): p. 112-9.
51. Penninger, J.M. and G. Kroemer, *Mitochondria, AIF and caspases--rivaling for cell death execution*. Nat Cell Biol, 2003. **5**(2): p. 97-9.
52. Chipuk, J.E., et al., *The BCL-2 family reunion*. Mol Cell, 2010. **37**(3): p. 299-310.
53. Kantari, C. and H. Walczak, *Caspase-8 and bid: caught in the act between death receptors and mitochondria*. Biochim Biophys Acta, 2011. **1813**(4): p. 558-63.
50. Newton, K., et al., *A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes*. EMBO J, 1998. **17**(3): p. 706-18.
51. Walsh, C.M., et al., *A role for FADD in T cell activation and development*. Immunity, 1998. **8**(4): p. 439-49.
52. Zornig, M., A.O. Hueber, and G. Evan, *p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice*. Curr Biol, 1998. **8**(8): p. 467-70.
53. Arechiga, A.F., et al., *Cutting edge: FADD is not required for antigen receptor-mediated NF-kappaB activation*. J Immunol, 2005. **175**(12): p. 7800-4.

54. Ch'en, I.L., et al., *Antigen-mediated T cell expansion regulated by parallel pathways of death*. Proc Natl Acad Sci U S A, 2008. **105**(45): p. 17463-8.
55. Salmena, L., et al., *Essential role for caspase 8 in T-cell homeostasis and T-cell-mediated immunity*. Genes Dev, 2003. **17**(7): p. 883-95.
56. Chau, H., et al., *Cellular FLICE-inhibitory protein is required for T cell survival and cycling*. J Exp Med, 2005. **202**(3): p. 405-13.
57. Klionsky, D.J. and S.D. Emr, *Autophagy as a regulated pathway of cellular degradation*. Science, 2000. **290**(5497): p. 1717-21.
58. Nakagawa, I., et al., *Autophagy defends cells against invading group A Streptococcus*. Science, 2004. **306**(5698): p. 1037-40.
59. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages*. Cell, 2004. **119**(6): p. 753-66.
60. Li, C., et al., *Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death*. J Immunol, 2006. **177**(8): p. 5163-8.
61. Pua, H.H., et al., *A critical role for the autophagy gene Atg5 in T cell survival and proliferation*. J Exp Med, 2007. **204**(1): p. 25-31.
62. Reyes, B.M., et al., *Redox equilibrium in mucosal T cells tunes the intestinal TCR signaling threshold*. J Immunol, 2005. **175**(4): p. 2158-66.
63. Kamata, H., et al., *Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases*. Cell, 2005. **120**(5): p. 649-61.
64. Hildeman, D.A., et al., *Reactive oxygen species regulate activation-induced T cell apoptosis*. Immunity, 1999. **10**(6): p. 735-44.
65. Pua, H.H., et al., *Autophagy is essential for mitochondrial clearance in mature T lymphocytes*. J Immunol, 2009. **182**(7): p. 4046-55.
66. Stephenson, L.M., et al., *Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes*. Autophagy, 2009. **5**(5): p. 625-35.
67. Cahalan, M.D., et al., *Molecular basis of the CRAC channel*. Cell Calcium, 2007. **42**(2): p. 133-44.

68. Bell, B.D. and C.M. Walsh, *Coordinate regulation of autophagy and apoptosis in T cells by death effectors: FADD or foundation*. Autophagy, 2009. **5**(2): p. 238-40.
69. Jia, W., et al., *Autophagy regulates endoplasmic reticulum homeostasis and calcium mobilization in T lymphocytes*. J Immunol, 2011. **186**(3): p. 1564-74.
70. Jones, R.G., et al., *The proapoptotic factors Bax and Bak regulate T Cell proliferation through control of endoplasmic reticulum Ca(2+) homeostasis*. Immunity, 2007. **27**(2): p. 268-80.

CHAPTER 2

Complementary Roles of FADD and RIPK3 in T cell Homeostasis and Function

Abstract

Caspase-8 (casp8) is required for extrinsic apoptosis, and mice deficient in casp8 fail to develop and die in utero while ultimately failing to maintain the proliferation of T cells, B cells and a host of other cell types. Paradoxically, these failures are not due to a defect in apoptosis, but to a presumed proliferative function of this protease. Indeed, following mitogenic stimulation, T cells lacking casp8 or its adaptor protein FADD develop a hyper-autophagic morphology, and succumb to a programmed necrosis-like death process termed necroptosis. Recent studies have demonstrated that RIP kinases RIPK1 and RIPK3 together facilitate TNF-induced necroptosis, but the precise role of RIPKs in the demise of T cells lacking FADD or casp8 activity is unknown. Here we demonstrate that RIPK3 and FADD have opposing and complementary roles in promoting T cell clonal expansion and homeostasis. We show that the defective proliferation of T cells bearing an interfering form of FADD (FADD^{dd}) is rescued by crossing with RIPK3^{-/-} mice, though such rescue ultimately leads to lymphadenopathy. Enhanced recovery of these double-mutant T cells following stimulation demonstrates that FADD, casp8 and RIPK3 are all essential for clonal expansion, contraction, and anti-viral responses. Finally, we demonstrate that caspase-mediated cleavage of RIPK1-containing necrosis inducing complexes (necrosomes) is sufficient to prevent necroptosis in the face of death receptor signaling. These studies highlight the two-faced nature of casp8 activity, promoting clonal expansion on the one hand and apoptotic demise on the other.

Introduction

Following ligation of death receptors (DR), death domain-containing members of the TNF receptor superfamily recruit proteins that are essential for promoting DR-induced apoptosis [1]. These include caspase-8 (casp8), a non-catalytic paralog of casp8 called c-FLIP, and the adaptor protein FADD. Curiously, loss of any of these proteins leads to early embryonic lethality and significant defects in hematopoiesis and activated lymphocyte survival [2]. Furthermore, T cell specific expression of an interfering form of FADD containing only the death domain of this adaptor (FADD^{ddd}) leads to defective T cell clonal expansion and altered thymopoiesis [3-5]. These findings suggest that the signaling molecules that promote apoptosis following DR function serve additional roles that are linked, but unrelated to apoptosis. Recently, it was discovered that the defective survival of T cells lacking active casp8 is associated with a hyperautophagic morphology, and that such T cells die from an alternative form of cell death mediated by Receptor Interacting Protein Kinase-1 (RIPK1) [6, 7].

For several years, it has been known that triggering DRs in the absence of caspase activity can lead to a non-apoptotic form of cell death that resembles necrosis [8, 9] which involves the serine/threonine kinase activity of RIPK1 [10]. Using a small-molecule library, Junying Yuan and colleagues identified a family of molecules termed “necrostatins” that are capable of binding to RIPK1 and blocking DR-induced necrosis [11], a process defined as “necroptosis.” RNAi screening of genes responsible for DR-induced necroptosis validated that RIPK1 is required for this alternative form of cell death [12] by forming a complex with RIPK3 termed the “necrosome” [13] in the absence of casp8 function [14-16]. Thus, it is now clear that both RIPK1 and RIPK3 are functionally required for the elaboration of necroptotic signaling following DR ligation in cells lacking the capacity to activate caspases. As RIPK1 and RIPK3

have both been shown to be targets for casp8 activity [17], it has been suggested that failure in casp8-mediated cleavage of RIPK1 and RIPK3 may lead preferentially to necroptosis [14, 19].

While our previous work has demonstrated that FADD^{ddd}-expressing and caspase 8-deficient T cells succumb to RIPK1-dependent necroptosis, we wished to assess the potential involvement of RIPK3 in this process. Interestingly, although FADD is required, the classic DRs are unlikely to be involved in the demise of such mutant T cells, since antagonizing them failed to block the induction of caspase 8 activity following T cell mitogenic stimulation [20]. Thus, we sought to establish the *in vivo* impact of non-DR induced necroptosis to T cell mediated immune function in the context of T cells lacking the capacity to activate casp8. Importantly, since mice bearing a germline RIPK1 deletion succumb to perinatal lethality [21], we chose instead to cross FADD^{ddd}-expressing mice [4] with RIPK3^{-/-} mice, as the latter strain develops in an overtly normal fashion, and RIPK3^{-/-} T cells display no obvious activation defects [22]. We find that a RIPK3 deficiency acts as a second site suppressor mutation in the context of FADD^{ddd}-expressing T cells, and prevents acute necroptosis of these cells following mitogenic stimulation. This RIPK3 deficiency also restores *in vivo* T cell mediated anti-viral activity observed in FADD^{ddd} transgenic mice [23], but promotes development of lymphoproliferative disease. RIPK1 and RIPK3 both appear to be cleaved following T cell antigenic stimulation, and we demonstrate that blockade of RIPK1 processing is sufficient to promote DR-induced necroptosis.

Results

To investigate the role of RIPK3 in T cell development and homeostasis, spleens, lymph nodes, and thymi were harvested from wildtype mice, mice expressing FADD^{ddd} in thymocytes and T cells [4], RIPK3^{-/-} [22], and RIPK3^{-/-} x FADD^{ddd} mice for initial comparison of CD4⁺ and

CD8⁺ T cell ratios. Wildtype naïve splenocytes typically display a 2:1 CD4:CD8 ratio, whereas FADD^{dd} splenocytes display a 4:1 CD4:CD8 ratio due to defective CD8⁺ accumulation and homeostasis [23]. Loss of RIPK3 signaling in FADD^{dd} T cells restored the CD4:CD8 ratio to wildtype levels in spleen and lymph nodes (**Fig. 1a**), and rescued the diminished fraction of FADD^{dd} CD44^{High}/CD62L^{High} T cells or central memory T cells (T_{CM}) [24](**Fig. 1b**). These dual mutant mice also displayed an enhanced fraction of CD44^{High}/CD62L^{Low} effector T cells (**Fig. 1c**), potentially due to defective clearance of autoreactive T cells. We observed no significant differences in the fractions of CD4, CD8, CD4/CD8 double positive (DP) or CD4/CD8 double negative (DN) populations among the four genotypes (**Fig. 1d**). FADD^{dd} T cells display a partial block during thymopoiesis at the CD4⁺CD8⁺CD25⁺CD44⁺ (DN3) stage associated with expression of the pre-T α /TCR β complex [4]. Failure to express the pre-T α /TCR β complex leads to thymocyte death, whereas successful surface expression promotes proliferation and differentiation into the CD25⁺/CD44⁺ “DN4” stage [25, 26]. As expected, we observed an enhanced DN3 population and a paucity of DN4 cells in FADD^{dd} thymocytes, whereas wildtype, RIPK3^{-/-}, and FADD^{dd} x RIPK3^{-/-} thymocytes developed normal fractions of DN3 cells (**Fig. 1e and Supp. Fig. S1**).

Previous studies have demonstrated that FADD^{dd} or casp8^{-/-} T cells display defective clonal expansion following antigen receptor stimulation [2] due to the induction of necroptotic cell death [6, 7]. With recent evidence demonstrating that both RIPK1 and RIPK3 are involved in promoting necroptotic death following DR ligation [27], we were curious to determine if RIPK3 may participate in the necroptotic death of FADD^{dd} T cells. Splenocytes from FADD^{dd} x RIPK3^{-/-} mice were labeled with CFSE and cultured with α -CD3/-CD28 for three days to detect the T cell proliferative response. As observed previously, FADD^{dd} T cells had a

diminished proliferative response, as assessed by accumulation of live CD8⁺/CFSE^{Lo} T cells (**Fig. 2a**). In contrast, FADDdd x RIPK3^{-/-} T cells displayed an enhanced proliferative response, correlating with a reduced fraction of 7-actinomycin D (7AAD) high T cells demonstrating that the loss of RIPK3 rescued the defective clonal expansion and death of FADDdd T cells (**Fig. 2b**). While treatment with the RIPK1 inhibitor Nec-1 restored FADDdd T cell proliferation to levels comparable to RIPK3^{-/-} T cells, the loss of both FADD and RIPK3 signaling led to an appreciable enhancement in recovery of live proliferating T cells (**Fig. 2c**). Further assessment of the role of FADD in peripheral tolerance revealed that upon restimulation through the T cell receptor, FADDdd and FADDdd x RIPK3^{-/-} T cells are resistant to restimulation-induced cell death (RICD) (**Fig. 2d**). However, loss of RIPK3 signaling, alone or in the context of the FADDdd mutation, led to little enhanced recovery of live T cells upon restimulation, demonstrating that the death pathway induced during RICD is almost entirely FADD-directed, casp8-mediated apoptosis.

Mice and human subjects lacking functional Fas receptor (CD95) or Fas-ligand (CD95L) develop lymphoproliferative disease and autoimmune lymphoproliferative syndromes (ALPS), respectively, as well as an accumulation of CD4⁻, CD8⁻, CD3⁺, B220⁺ T lymphocytes. Similarly, older FADDdd x RIPK3^{-/-} mice displayed a slight splenomegaly with frank lymphadenopathy and enlarged thymii (**Fig. 2e**), coupled with a dramatic increase of CD4⁻, CD8⁻, CD3⁺, B220⁺ T lymphocytes (**Fig. 2f-g**). Histological analyses revealed lymphocyte infiltrates within livers of the DKO mice (**Supp. Fig. S2**). These findings demonstrate that while RICD drives a mostly apoptotic form of death, chronic loss of both DR-induced apoptosis *and* necroptosis leads to recapitulation of the lymphoproliferative phenotype observed in mice lacking functional Fas or FasL.

FADDdd mice are incapable of mounting an effective immune response against viral pathogens, including LCMV and Mouse Hepatitis Virus (MHV) [23]. To address the consequence of the loss of both RIPK3 and FADD signaling during an antiviral response, FADDdd x RIPK3^{-/-} mice were injected intraperitoneally with 2×10^5 PFU of MHV and sacrificed 7d post-infection. Tetramer staining of T cells in the liver showed infiltration of virus-specific T cells to the infected tissue, which was not evident in FADDdd mice as expected, but restored in FADDdd x RIPK3^{-/-} mice (**Fig. 3a**). To determine whether FADD and RIPK3 play a role in cytotoxic T-lymphocyte activity (CTL), *in vivo* and *in vitro* CTL assays were performed using target cells pulsed with non-specific (OVA) or virus-specific immunodominant (S510) peptides. To assess *in vivo* killing activity, C57BL56/J splenocytes were pulsed with low and high concentrations of CFSE, followed by peptide pulsing with S510 and OVA peptides, respectively. 1hr following injection of pulsed splenocytes into 7d mock- and MHV-infected mice, spleens were harvested to assess the recovery of S510 vs OVA pulsed target cells. While FADDdd mice failed to kill S510-pulsed target cells due to defective accumulation of effector cells, killing activity was similar between wildtype, RIPK3^{-/-}, and FADDdd x RIPK3^{-/-} mice (**Fig. 3b-c**). As with restoration of virus specific effector cells, deletion of RIPK3 in FADDdd mice rescued their defective clearance of LCMV [81] (**Fig. 3d**).

To address the T cell intrinsic role of both RIPK3 and FADD signaling during an antiviral response, we performed MHV infection studies in immunodeficient Rag2^{-/-} x IL2R γ c^{-/-} mice reconstituted with purified T cells from the four genotypes. 2.5×10^6 T cells were adoptively transferred into Rag2^{-/-} x IL2R γ c^{-/-} mice, and rested for 7d before injected intraperitoneally with 2×10^5 PFU of MHV. While overall splenocyte numbers 7d post-infection were roughly similar in uninfected mice, indicating no defect in homeostatic proliferation [23],

FADDdd x RIPK3^{-/-} possessed a greater number of splenocytes relative to other mice, consistent with a potential defect in lymphocyte homeostasis following viral infection (**Fig. 4a**). To evaluate the generation of virus specific T cells, IFN- γ production was analyzed after a 6h *in vitro* restimulation of splenocytes with the immunodominant CD8⁺ T cell epitope, the MHV S510 peptide. RIPK3^{-/-} and FADDdd x RIPK3^{-/-} mice developed comparable numbers of IFN- γ expressing splenic CTL as wildtype mice, while the recovery of these virus specific T cells was significantly diminished in FADDdd spleens (**Fig. 4b**). These findings demonstrate that the loss of RIPK3 rescued the expansion capacity of antiviral T cells expressing FADDdd. Consistent with this, the diminished fraction of effector/memory CD8⁺ (CD44^{High}/CD62L^{Low}) T cells observed post-infection in FADDdd splenocytes was restored to wildtype levels in FADDdd x RIPK3^{-/-} mice (**Supp. Fig. S3**). These results demonstrate that the defect in FADDdd T cells is due to T cell intrinsic RIPK3 activity, a consequence of necroptosis driven by the latter in the absence of FADD activity.

Current models suggest that processing of RIPK1 and RIPK3 by casp8, itself activated by FADD (and possibly cFLIP [86]), prevents the elaboration of a necroptotic response within cells [14, 19]. Considering that casp8 catalytic activity is required for T cell clonal expansion [20], we tested whether processing of RIPK1 or RIPK3, both of which are targets of casp8 [18, 29], occurred in response to T cell antigenic stimulation. We detected cleaved RIPK1 and RIPK3 in viable OT-I ovalbumin (OVA)-specific T cells following stimulation with OVA peptide or anti-Fas [30] (**Fig. 5a**). This led us to pose the broader question of whether processing of RIPK1 and RIPK3 may also occur during DR-induced apoptosis vs. necroptosis. We treated Jurkat T cells lacking FADD [31] known to be highly sensitive to DR-induced necroptosis [10] (**Supp. Fig. S4**), and FADD^{-/-} Jurkat cells transfected with full length FADD (FADD^{REC}) (**Fig. 5b**) with

TNF α or TRAIL and detected RIPK1 (and PARP1) cleavage fragments in FADD^{REC} cells (**Fig. 5c**).

To assess the potential that casp8-mediated processing of RIPK1 prevents the elaboration of a necroptotic response following DR ligation, we treated RIPK1-deficient Jurkats [32] stably reconstituted with cleavage-resistant RIPK1_D325A or kinase dead/cleavage-resistant RIPK1_M92G/D325A (**Supp. Fig. S5**) with TNF α , +/- Nec-1 or zVAD-FMK (**Fig. 5d**). RIPK1^{-/-} cells were sensitized to TNF-induced apoptosis, which was rescued with the addition of zVAD-FMK. In contrast, cells expressing non-cleavable RIPK1_D325A were sensitized to TNF-induced necroptosis, which was rescued by Nec-1. Blocking kinase activity of cleavage-resistant RIPK1 (M92G/D325A) diminished TNF-induced necroptotic death observed in RIPK1_D325A reconstituted RIPK1^{-/-} Jurkats. We note that ectopic expression of RIPK1_D325A in RIPK1-sufficient Jurkats failed to shift TNF-induced apoptosis to necroptosis (**Supp. Fig. S6**). These results indicate that uncleaved RIPK1 does not act in a dominant fashion to promote necroptosis. Presumably, only a small fraction of *activated* RIPK1, recruited via association with FADD or other adaptors, is necessary for induction of the necroptotic pathway in response to TNF. Taken together, our results provide evidence that direct inactivation of RIPK1 and/or RIPK3, through a FADD-dependent mechanism that requires apical caspase catalytic activity, is the mode through which cells differentiate between apoptosis and necroptosis, and RIPK1 activity is required to promote necroptosis.

Discussion

Our findings here demonstrate that RIPK3 acts in concert with RIPK1 in promoting the necroptotic demise of T cells lacking the non-apoptotic casp8 activity that is normally observed

following mitogenic signaling [6, 7, 20]. Thus, early casp8 catalytic activity within T cells acts to prevent the elaboration of signaling from a RIPK1/RIPK3 containing necrosome [14] in a manner similar to that observed following DR signaling. While T cell intrinsic casp8 and FADD activity are required for antiviral T cell responses, loss of RIPK3 restores the ability of FADD^{ΔΔΔ} T cells to control MHV infection, suggesting that casp8 and RIPK3 play additional opposing roles during antiviral immune responses.

Unlike the case for DR signaling, the process that leads to RIPK1/RIPK3-containing necrosome formation is currently unclear. We previously observed RIPK1 recruitment to complexes that likely exist on autophagosomal membranes [6, 33] and found that extracellular blockade of DR ligation fails to rescue casp8^{-/-} or FADD^{ΔΔΔ} expressing T cells [20]. This suggests that the nucleation of RIPK1/RIPK3 necrosomes occurs in a manner independent of DR signaling. Here, we demonstrate that RIPK3, like RIPK1 [6, 7], promotes necroptotic death of FADD^{ΔΔΔ} T cells following their activation. Our findings contrast with a recent publication that suggested RIPK3 signaling is not involved in the necroptotic demise of T cells lacking FADD [34] based on the fact that RIPK3 failed to co-immunoprecipitate with RIPK1. Given that our results demonstrate a requirement for RIPK3 in TCR-induced necroptosis, it is possible that while the upstream pathways that promote the nucleation of RIPK1/RIPK3 containing necrosomes may be unique following DR ligation vs T cell mitogenesis, the downstream pathways are likely similar.

We present novel data that demonstrate an essential role for DR-induced apoptotic death during reactivation-induced cell death, a process that has previously been shown to require Fas signaling [35]. We emphasize that loss of RIPK3 signaling had little impact on this; FADD^{ΔΔΔ} was highly effective in blocking T cell RICD. In contrast, the loss of RIPK3 in FADD^{ΔΔΔ} T cells

led to a significant increase of live proliferating T cells following mitogenic stimulation. While Nec-1 treatment also rescued the expansion of FADD^Δ T cells, it did not lead to embellished recovery of proliferating cells vs. wildtype T cells. This result suggests that RIPK3 may have independent functions in T cells that may have been previously overlooked. Alternatively, Nec-1 treatment may have off-target effects that limit T cell expansion.

Although RIPK3^{-/-} T cells develop and proliferate relatively normally in previous studies [22] and in our work presented here, Francis Chan and colleagues have found that RIPK3^{-/-} mice fail to promote efficient inflammatory responses to vaccinia virus infection [17]. This may be a likely consequence of the expression of the caspase inhibitory protein SPI-2, a protein related to poxvirus CrmA, and possible assembly of necrosomes in an anti-viral inflammatory environment. Upton et. al. have shown that murine cytomegalovirus (MCV) promotes RIPK3-dependent necroptosis, and that mice lacking RIPK3 possess defects in viral clearance [33]. However, we note here that RIPK3^{-/-} mice were capable of controlling murine hepatitis virus (MHV) infection. In previous studies, FADD^Δ expressing mice failed to adequately respond to viral infection, and this was likely a result of defective CD8⁺ T cell responses [23]. Supporting this conclusion, the T cell intrinsic loss of RIPK3 restored the ability of FADD^Δ mice to efficiently clear MHV infections (**Fig. 4**). Thus, the primary defect in FADD^Δ expressing mice to MHV infection is in the expansion/survival of MHV-specific T cells. Furthermore, since direct infection of RIPK3^{-/-} mice led to efficient viral clearance, our results call into question a general requirement for RIPK3 signaling in antiviral immunity. Importantly, the immune defects seen in FADD^Δ mice were rescued with a RIPK3 deficiency, though older FADD^Δ x RIPK3^{-/-} mice bear a phenotypic resemblance to Fas-deficient (*lpr/lpr*) mice. These data indicate casp8-

dependent apoptosis and RIPK1/3-dependent necroptosis are both necessary to maintain homeostasis within the adaptive immune system.

Our studies emphasize a primary role for RIPK3 in promoting TCR-induced necroptosis in T cells lacking catalytically active casp8 [20]. The simplest interpretation is that RIPK1 and/or RIPK3 are inactivated by casp8 directly [4, 36] when brought into close apposition. Supporting this, we observed cleavage of both RIPK1 and RIPK3 in antigenically stimulated primary T cells, but only RIPK1 cleavage was detected in Jurkat T cells treated with DR ligands (**Fig. 5**). Expression of cleavage resistant RIPK1 was sufficient to convert TNF-induced apoptosis into necroptosis, while kinase dead RIPK1 diminished TNF-induced necroptosis and apoptosis. Thus, a failure in caspase-mediated cleavage of RIPK1 is alone sufficient to promote necroptosis following DR-ligation. Following the submission of our manuscript, several other labs have reported parallel findings in the context of T cells, supporting our studies here [37-40]. Taken together, our findings show that casp8 activity is essential for controlling RIPK1/3-dependent necroptosis in activated T cells, and that RIPK1/3 processing orchestrates the switch between apoptotic vs. necrotic cell death. Furthermore, these findings demonstrate that RIPK3-induced necroptotic activity leads to the early demise of FADD^{def} T cells, and this blocks antiviral immunity and the development of lymphoproliferative disease in mice lacking FADD signaling in T cells.

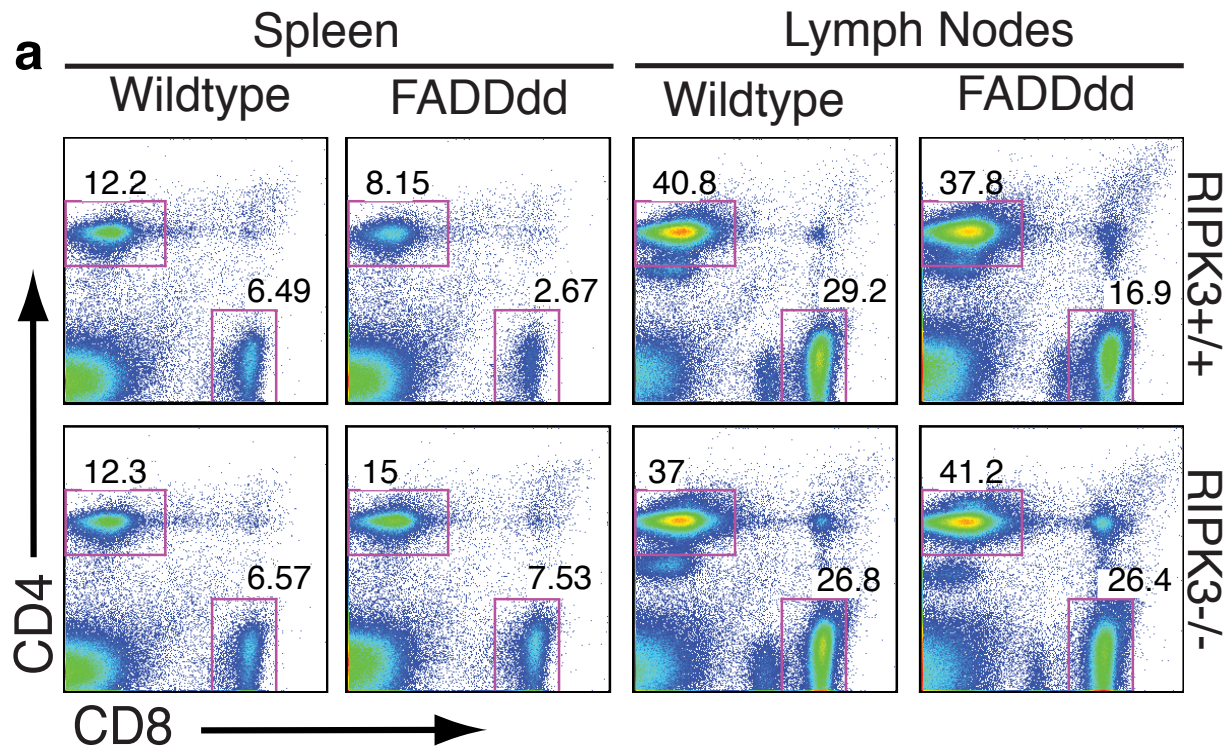


Figure 2.1 CD8⁺ T cell homeostasis restored in FADDdd x RIPK3^{-/-} mice. (a) Spleen, lymph node cells were analyzed by flow cytometry for CD4⁺ vs. CD8⁺ populations. Numbers represent percentage of cells staining in each gate.

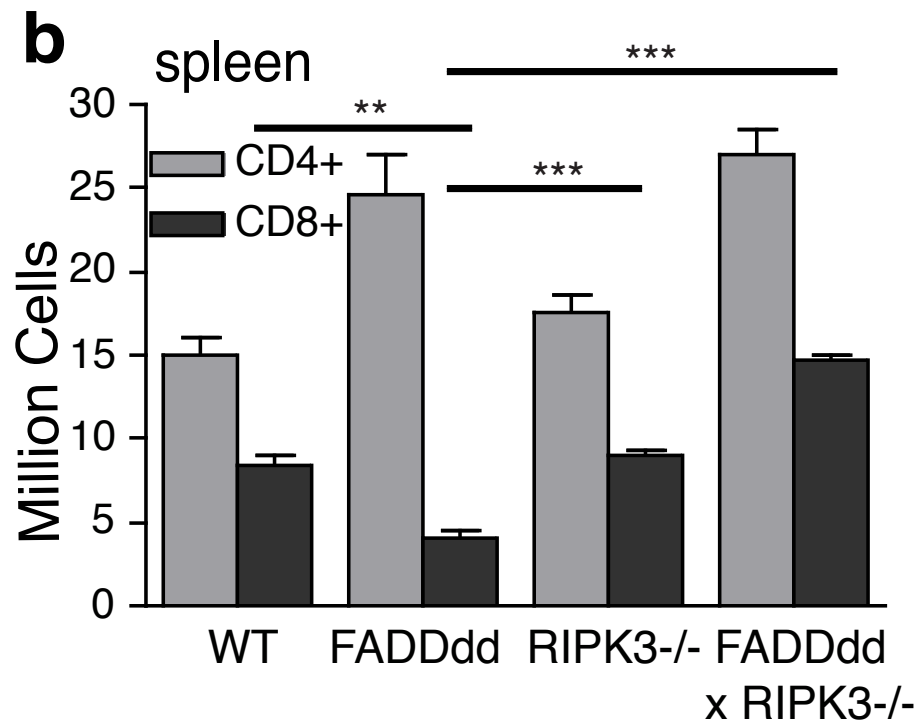


Figure 2.1 (b) Graph displays number of CD4+ and CD8+ cells in the spleen representative of three separate experiments. Considered a significant difference with respect to WT CD8+ (**, $p < 0.01$, **, $p < 0.001$). Error bars represent SEM.

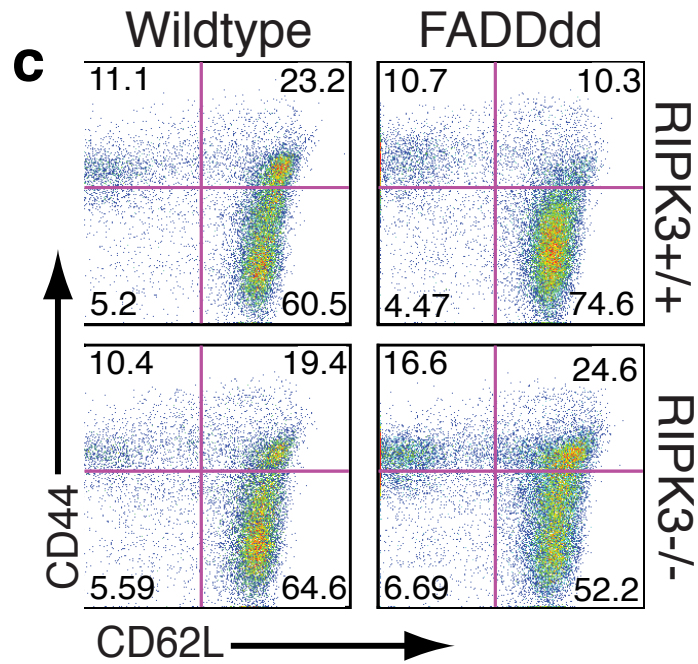


Figure 2.1 (c) Decreased CD8⁺ memory T cell population in FADD^{dd} mice restored with RIPK3 deficiency. CD8⁺ gated splenocytes analyzed for CD44⁺CD62L⁻.

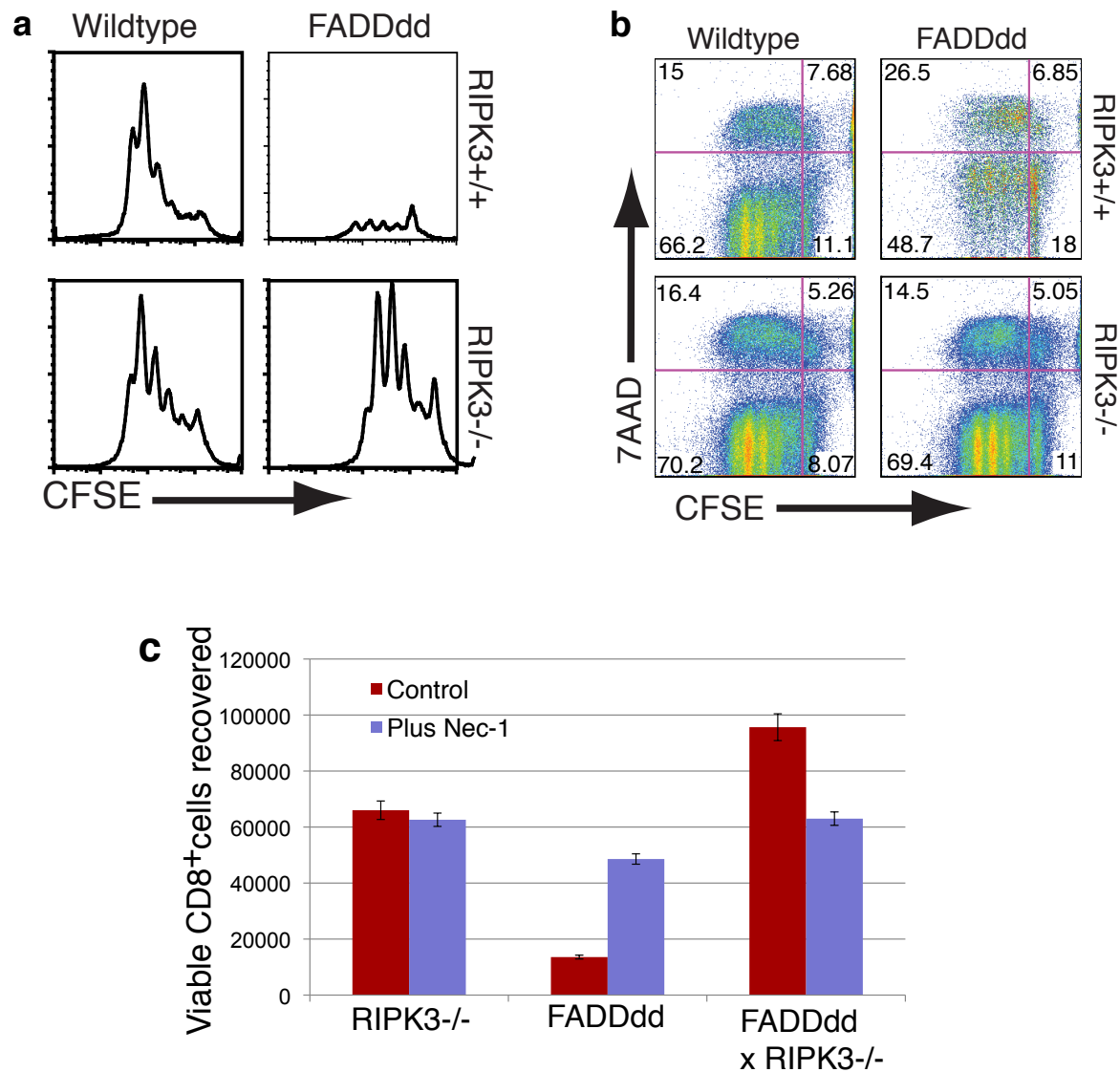


Figure 2.2 RIPK3 deficiency restores proliferation and survival of FADDdd CD8+ T cells. (a) CFSE analysis of CD8-gated T cells treated with plate-bound α -CD3 (145-2C11; 1 μ g/ml) plus α -CD28 (200 ng/ml). Splenocytes were stained and analyzed by flow cytometry after 3d of culture under these conditions. Shown are plots for CFSE vs. cell numbers of CD8+ splenocytes (b). Rescue of enhanced death phenotype of FADDdd CD8+ T cells by RIPK3 deficiency. Plots for CFSE vs. 7-actinomycin D (7-AAD) of CD8 populations; upper and lower left quadrants represent proportion of cultures that have divided and are dead or alive, respectively. (c) Enhanced recovery of live CD8+ T cells in FADDdd x $RIPK3^{-/-}$ vs. wildtype cultures following 3d stimulation. Activated splenocytes treated with or without 10 μ M Necrostatin-1 (Nec-1) added at start of culture. Graph represents percent of viable CD8+ cells recovered \pm SEM.

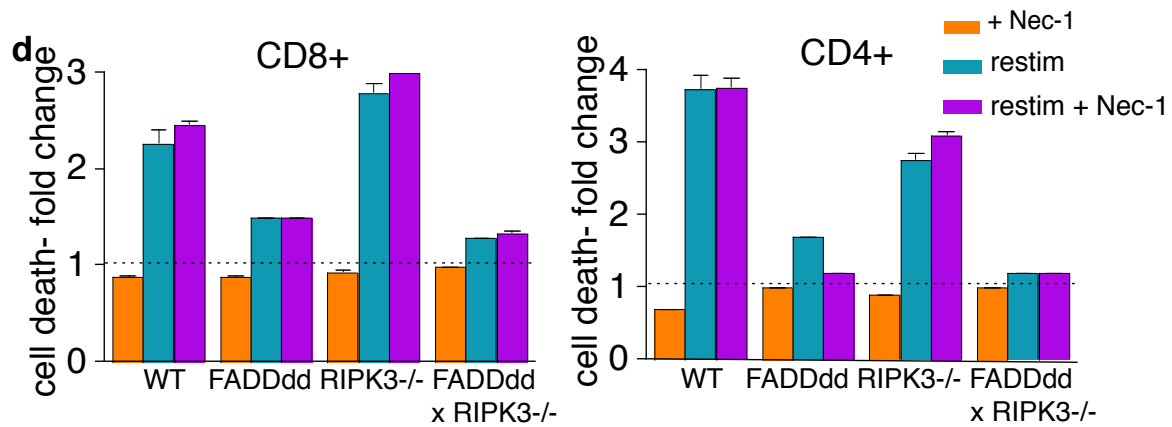


Figure 2.2 (d) Graphs display fold change in cell death of CD4+ or CD8+ cells upon restimulation with or without Nec-1 relative to death observed in activated cells ($1 \mu\text{g } 1 \times 10^5$ cells $\alpha\text{-CD3}$, $200 \text{ ng/ml } \alpha\text{-CD28}$) not subjected to restim. (dotted line). Error bars represent SEM. Considered a significant difference with respect to WT restim (*, $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

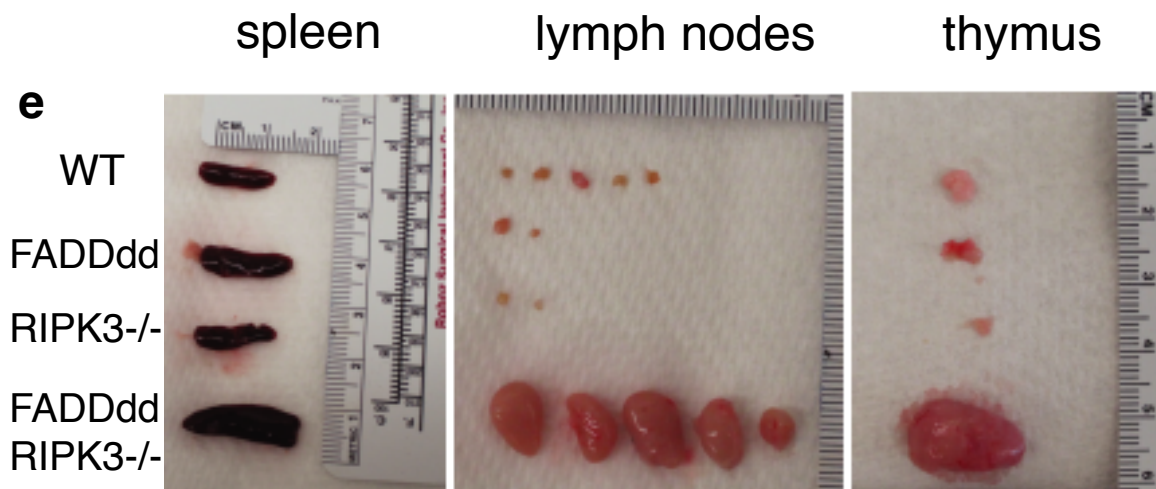


Figure 2.2 (e) Lymphoid tissue from 8-month-old mice of indicated genotypes.

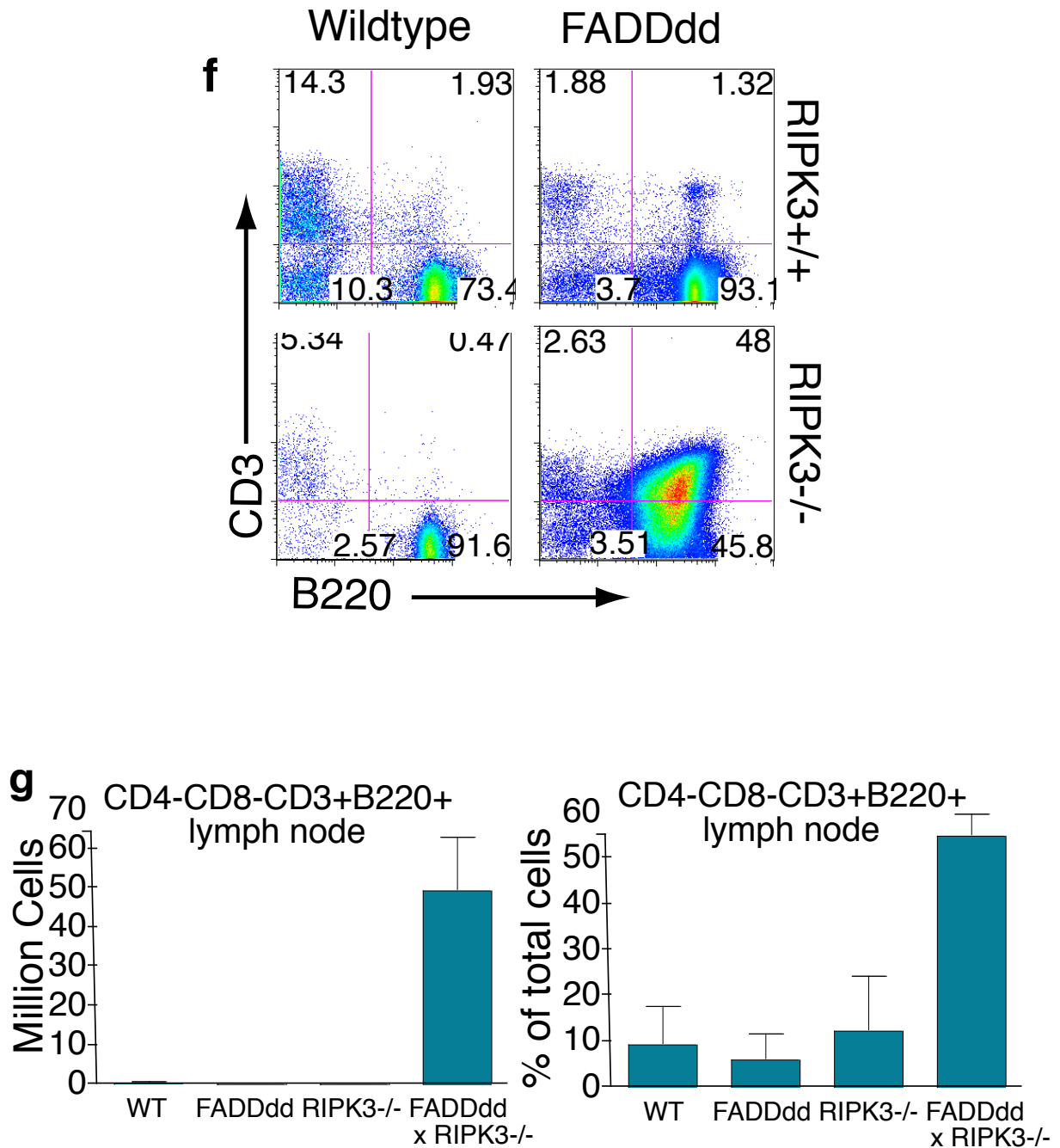


Figure 2.2 (f) FACS plots display percentage of double negative population that are CD3+ B220+ in mice of indicated genotypes. **(g)** Graphs represent counts or percentage of total live cells (after RBC lysis) that are CD4- CD8- CD3+ B220+. Error bars represent SEM, n = 3 of each genotype.

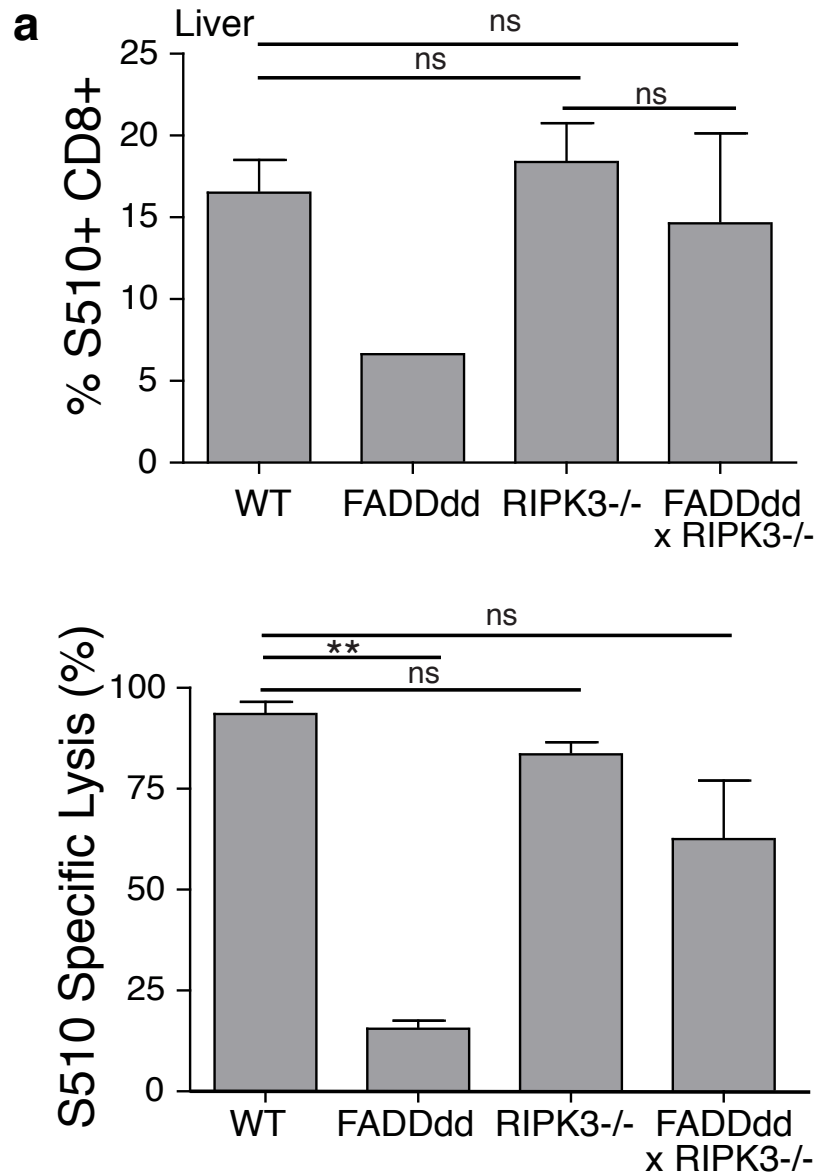


Figure 2.3 FADDdd x RIPK3^{-/-} mice exhibit normal immune response to Mouse Hepatitis Virus (MHV) infection. (a) T cells from livers of infected mice were stained with MHV-specific (S510) tetramer. Graph represents % S510 positive cells of total CD8⁺ cells.

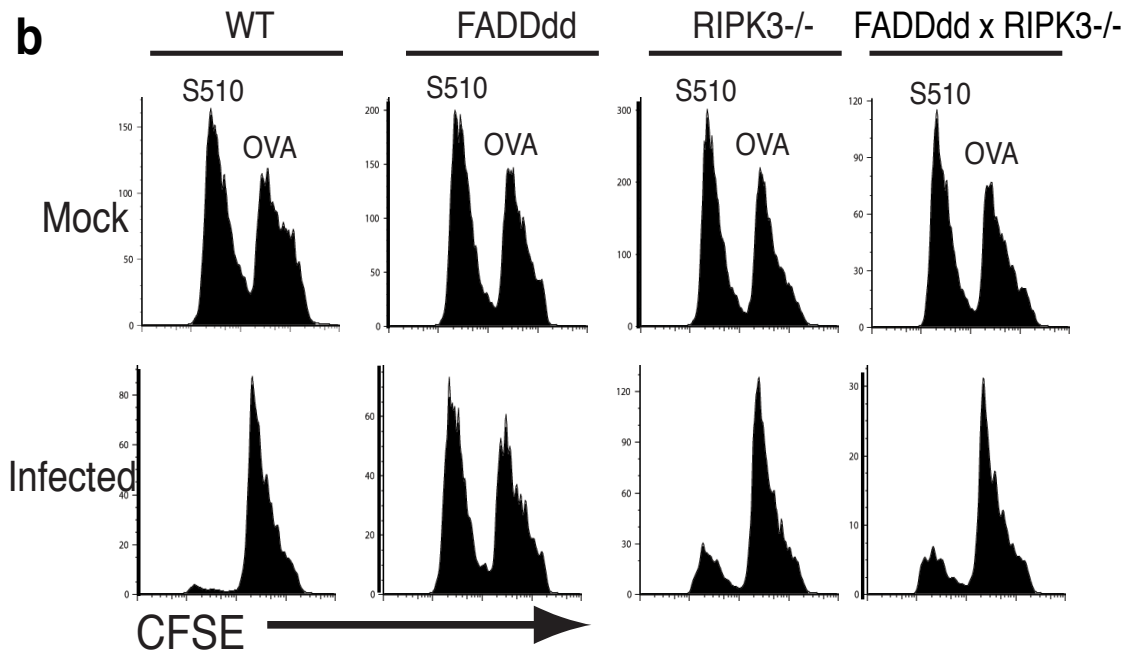


Figure 2.3 (b) Non-specific and specific target cells were labeled with CFSE^{hi/low} respectively and adoptively transferred into infected and sham mice for *in vivo* CTL analysis shown by FACS plots. Graph displays % specific lysis. Considered a significant difference at **, $p < 0.01$.

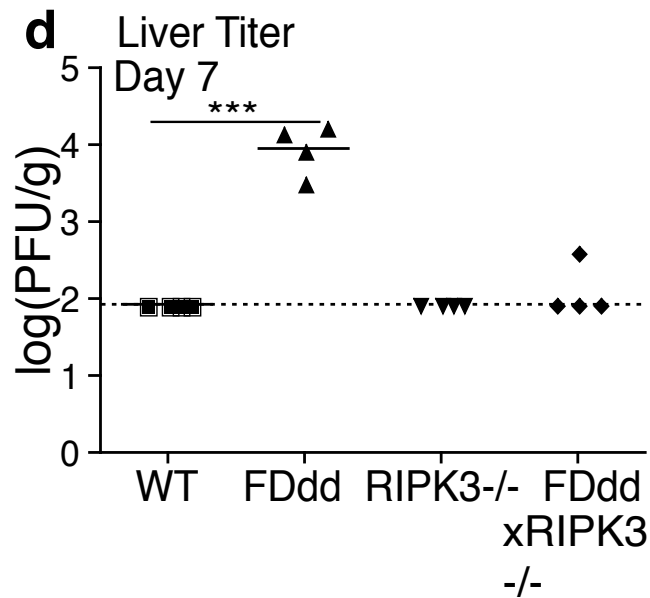
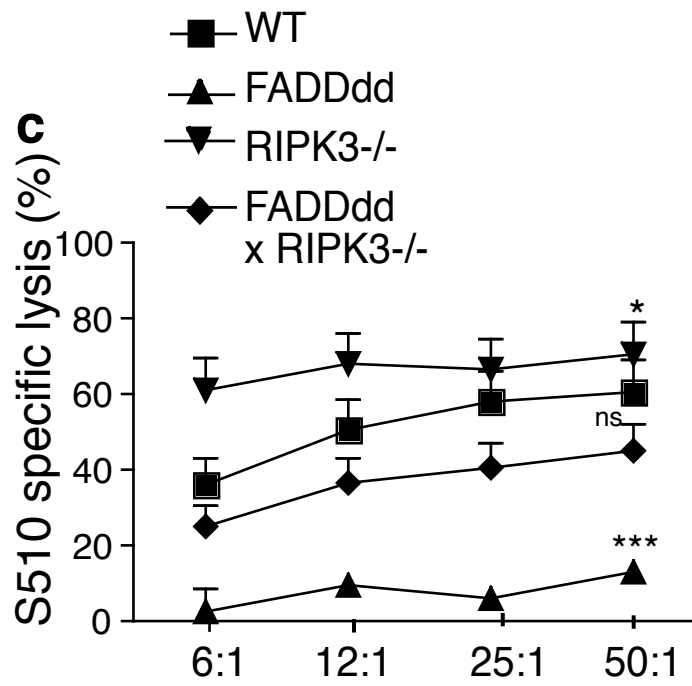


Figure 2.3 (c) Splenocytes from infected and sham mice were incubated with specific/non-specific target cells for 4h to measure *in vitro* CTL activity. Graph represents % specific lysis. Considered a significant difference with respect to WT specific lysis (*, $p < 0.01$, ***, $p < 0.001$). **(d)** Livers from infected mice were harvested 7d post-infection for viral titer analysis. Error bars represent SEM. Considered a significant difference at ***, $p < 0.001$.

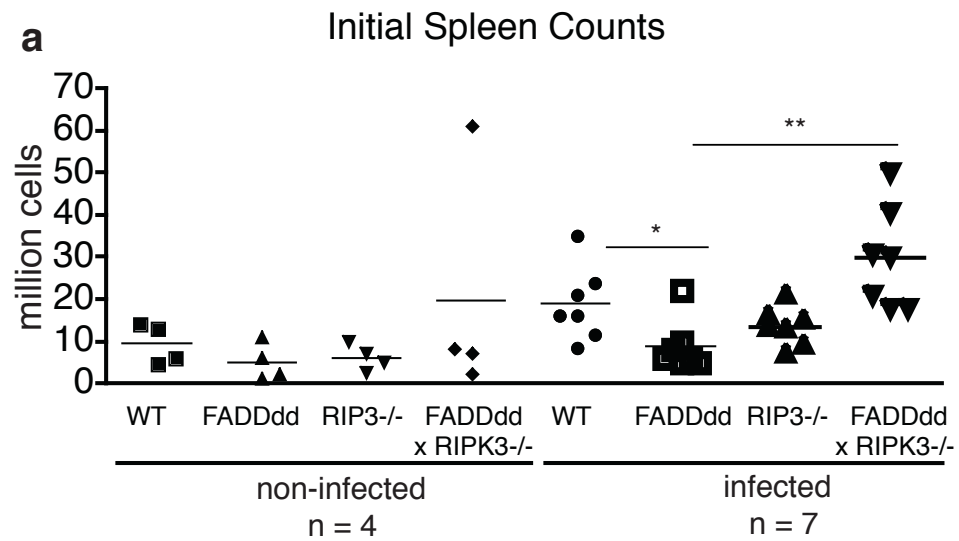


Figure 2.4 T cell intrinsic FADD and RIPK3 activity required for antiviral response to Mouse Hepatitis Virus (MHV) infection. (a) Initial splenocyte counts of infected and control mice in indicated genotypes. Considered a significant difference at *, $p < 0.05$, **, $p < 0.01$.

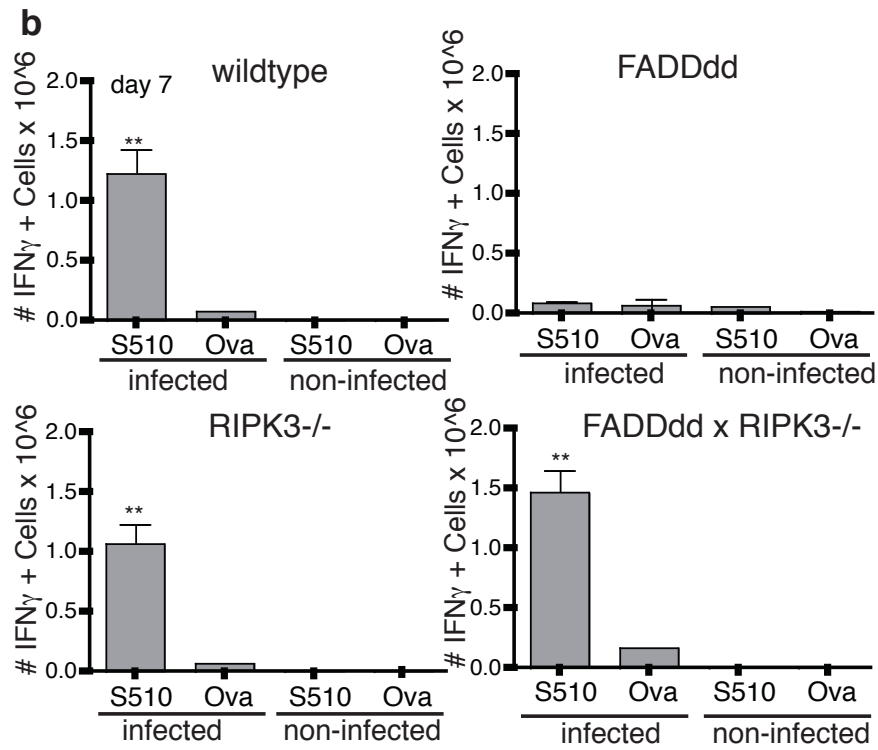


Figure 2.4 (b) FADDdd x RIPK3^{-/-} adoptive transfer mice and controls were infected intraperitoneally with MHV. IFN- γ levels in splenocytes of infected and control mice were determined by intracellular IFN- γ staining 7d post-infection. Splenocytes were restimulated 6h with S510 or OVA peptides and stained for CD8. IFN- γ + cells: (total splenocytes x % IFN- γ cells). Considered a significant difference with respect to infected FADDdd S510 (**, $p < 0.01$).

a

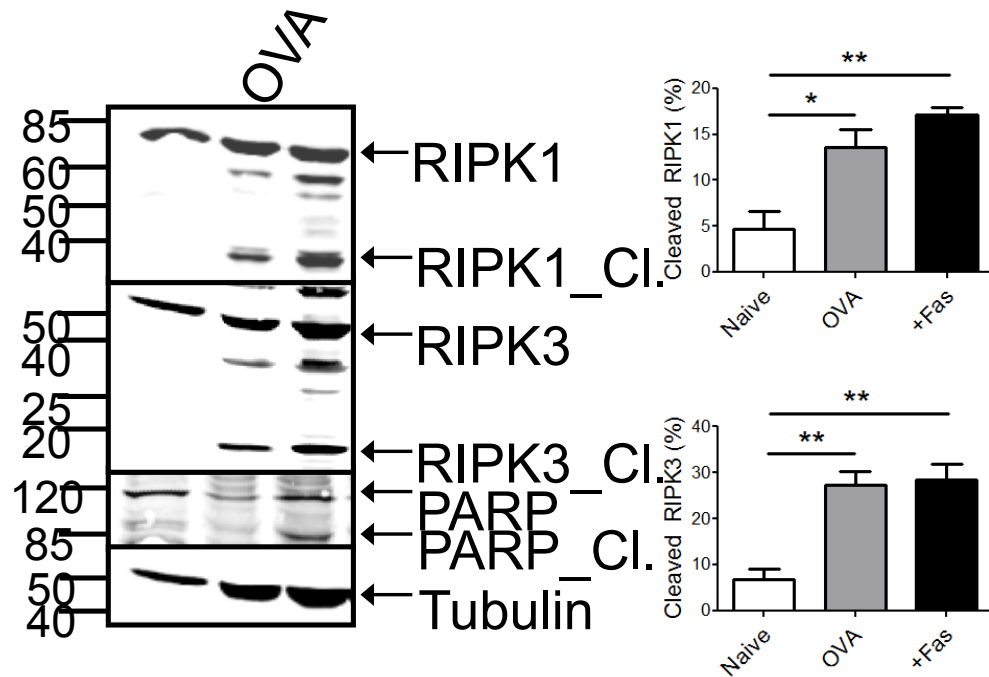


Figure 2.5 RIPK1/RIPK3 cleavage following TCR- vs. DR-ligation. (a) OT-I T cells [87] were stimulated with ovalbumin peptide (OVA) for 72h and stimulated without or with α -Fas or left untreated (naïve); Immunoblots were hybridized with α -RIPK1 and α -RIPK3 to detect processing. Graphs represent % RIPK1/3 cleavage. Considered a significant difference at *, $p < 0.05$, **, $p < 0.01$.

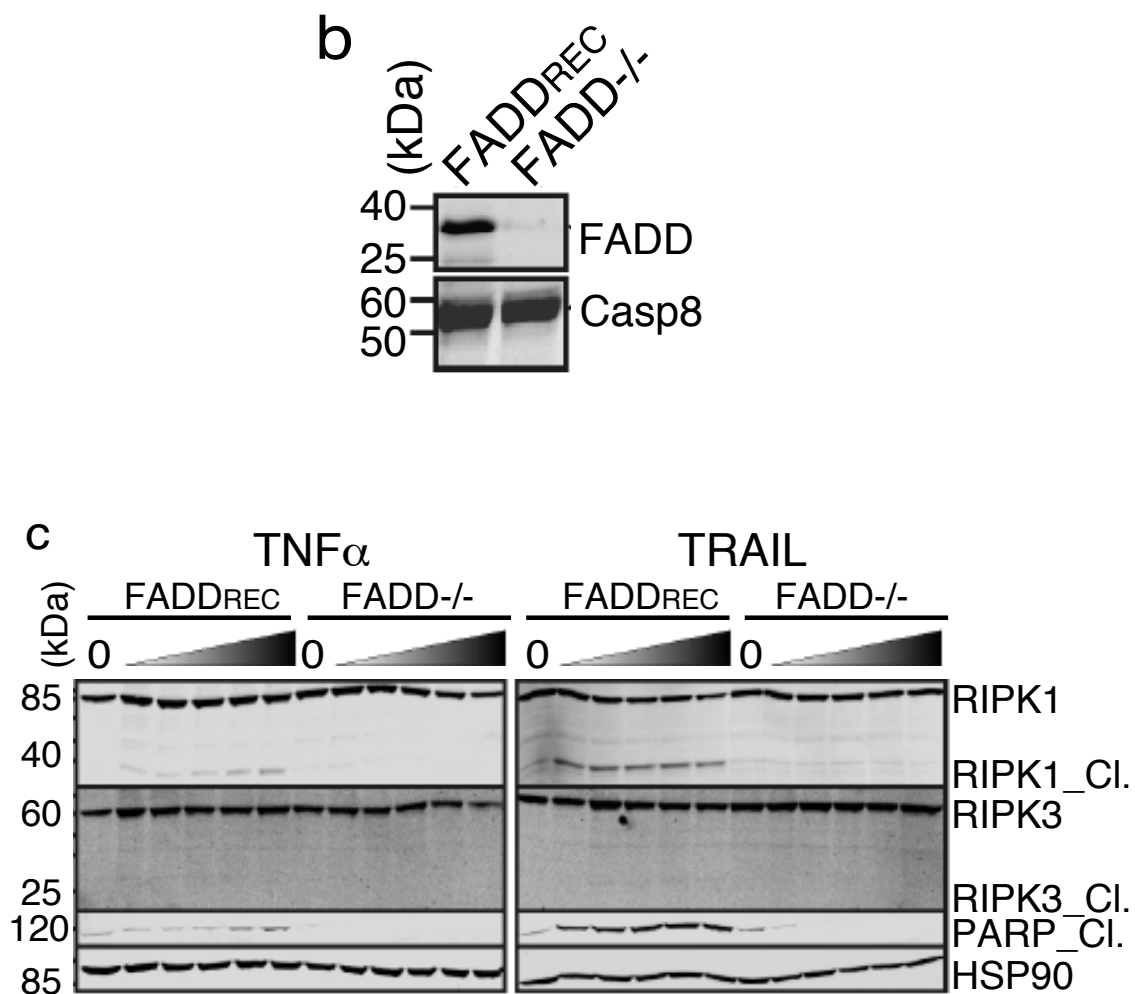


Figure 2.5 (b) Reconstitution of FADD-deficient Jurkats [88] (FADD^{-/-}) with full-length FADD (FADD^{REC}), blots of lysates probed with α -FADD and α -casp8. **(c)** Western blot of RIPK1 and RIPK3 cleavage in FADD^{-/-} and FADD^{REC} Jurkats; HSP90 used as loading control. RIPK1_Cl., PARP_Cl. = cleaved RIPK1 and PARP1, respectively.

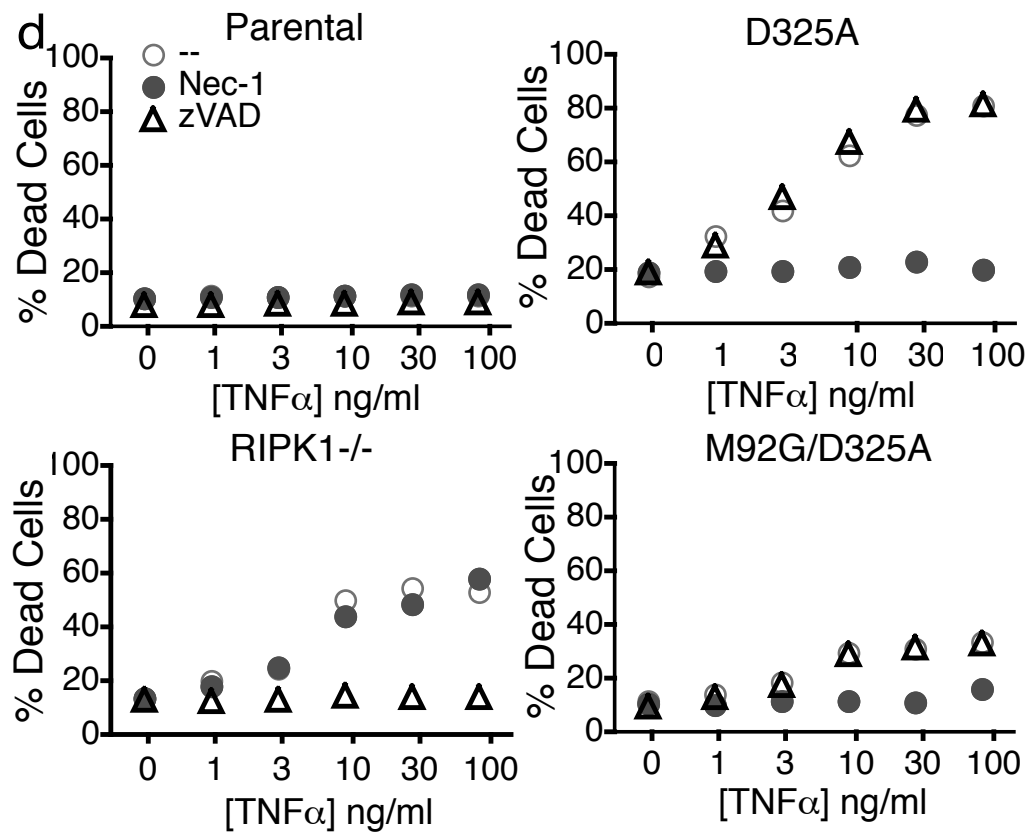
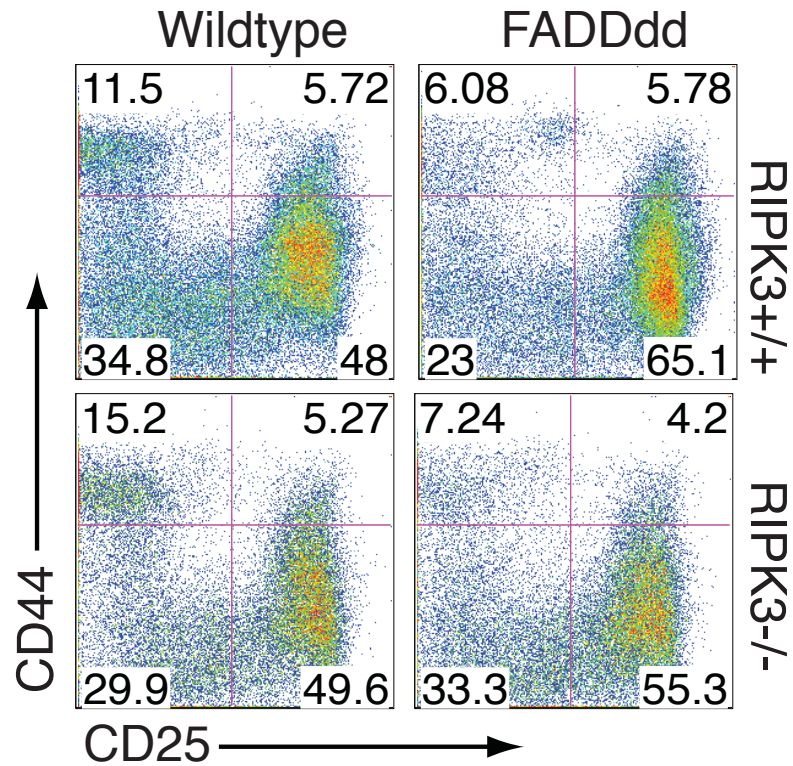


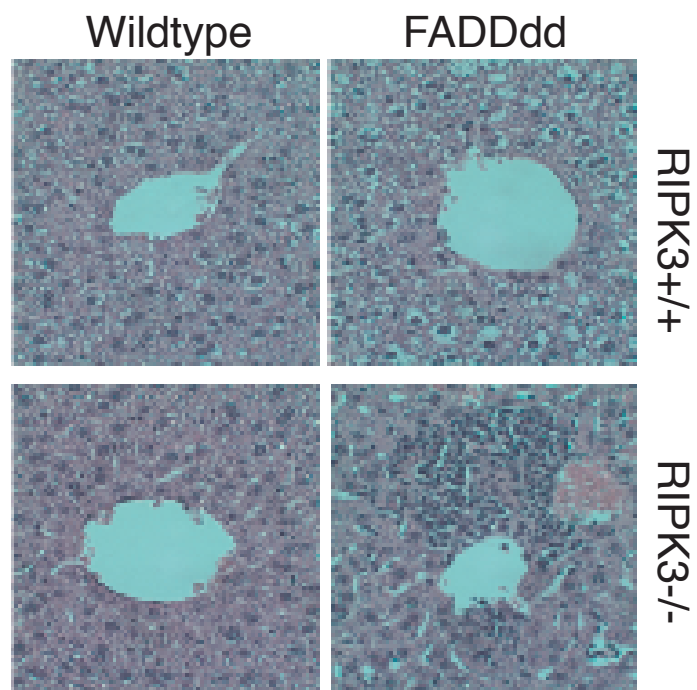
Figure 2.5 (d) Parental, RIPK1 $^{-/-}$, D325A RIPK1, M92G/D325A RIPK1 Jurkats treated with TNF α , Nec-1 (10 μ M), or z-VAD-FMK (20 μ M) and stained with 7AAD and annexin-V to detect death.

S1



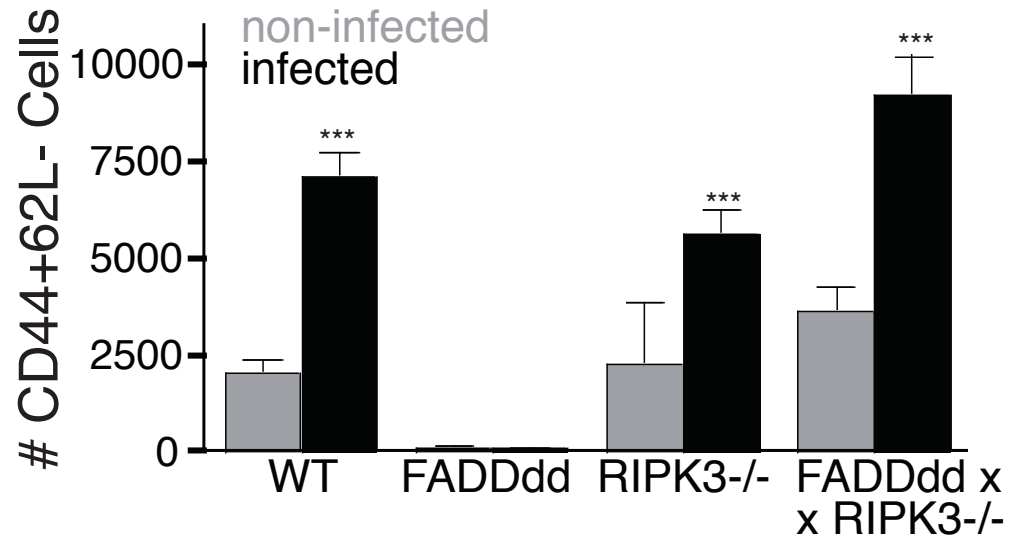
Supplementary Figure 2.S1 Rescue of DN4:DN3 ratio by a RIPK3 deficiency on the FADDdd genetic background. DN thymocyte populations defined by CD25 and CD44 expression reveals restored DN4 population in FADDdd x RIPK3^{-/-} mice. The data shown are representative of three separate experiments.

S2



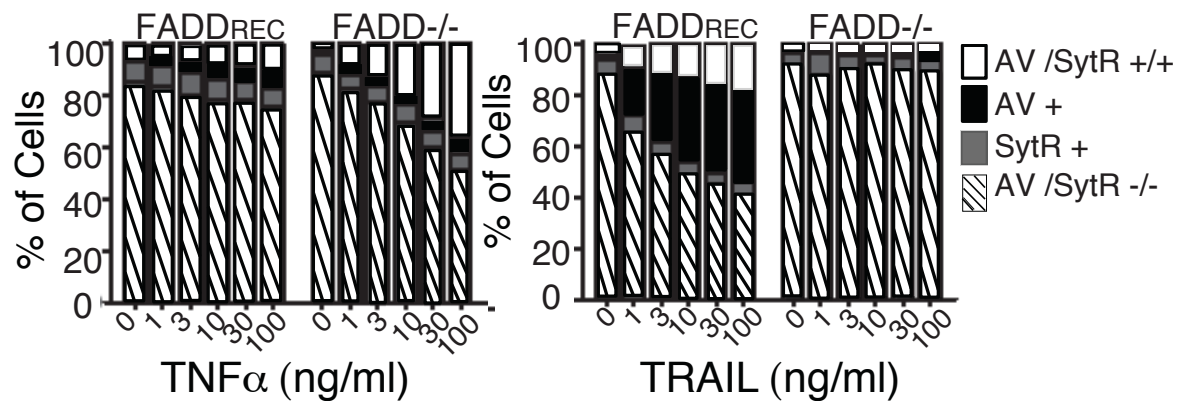
Supplementary Figure 2.S2 Liver inflammation in aged FADDdd x RIPK3-/- mice. H&E stained liver sections from 8-month-old mice of indicated genotypes.

S3

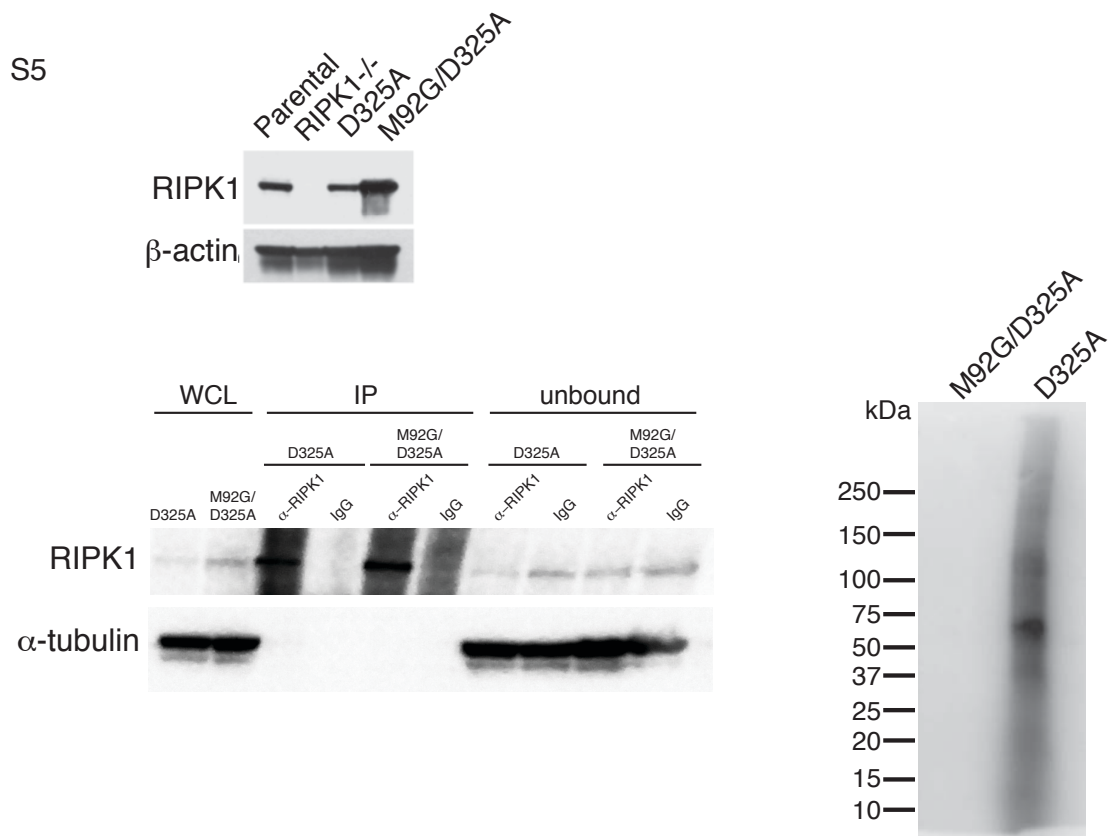


Supplementary Figure 2.S3 Restoration of antiviral effector/memory T cells in FADDdd x RIPK3-/- mice. Analysis of CD8+ effector/memory populations defined by CD44, CD62L 7d post- infection. Error bars represent SEM. Considered a significant difference with respect to infected FADDdd. (***, $p < 0.001$).

S4



Supplementary figure 2.S4 FADD^{-/-} and FADD^{REC} Jurkats were treated with TNF α or TRAIL for 18h at indicated concentrations followed by staining with annexin V (AV) and Sytox Red (SytxR) to detect death.

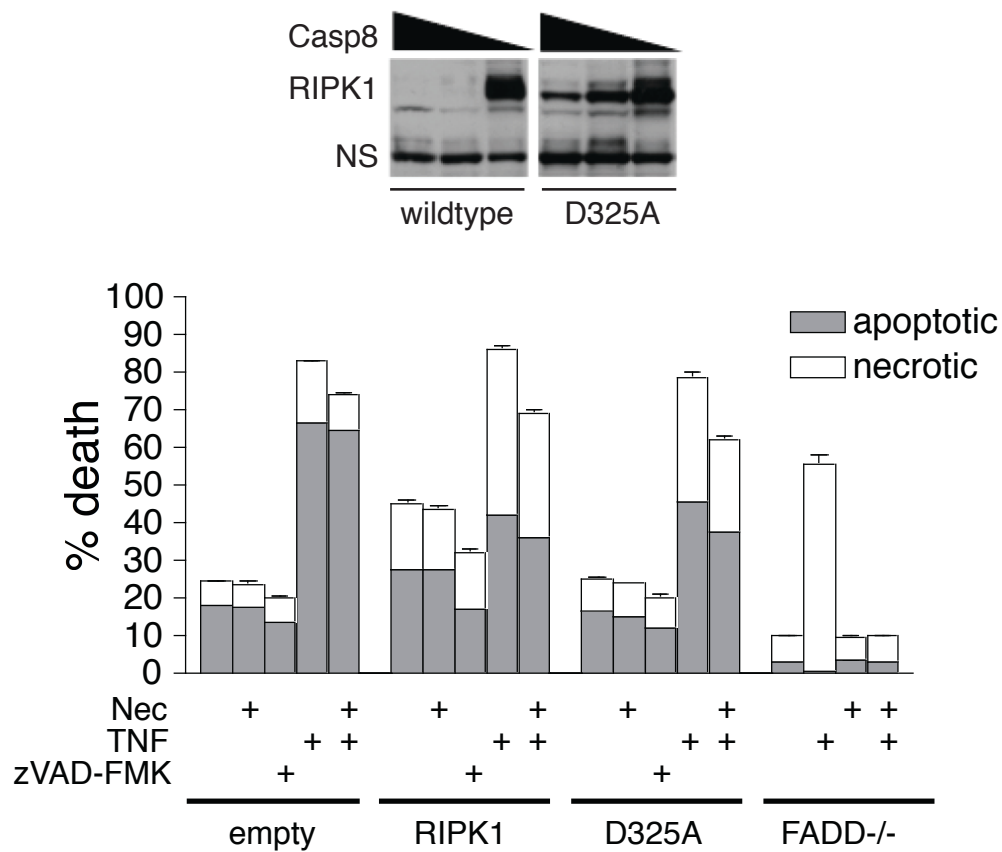


Supplementary Figure 2.S5 Top Panel: Reconstitution of RIPK1-deficient Jurkats (RIPK1-/-) with cleavage resistant (D325A RIPK1), kinase dead/cleavage resistant (M92G/D325A RIPK1); blots of lysates probed with anti-RIPK1.

Bottom Left Panel: RIPK1-/- Jurkats reconstituted with D325A RIPK1 or M92G/D325A RIPK1 were treated with TNF-α (40 ng/ml) for 2h before immunoprecipitation of RIPK1; blots of lysates probed with anti-RIPK1.

Bottom Right Panel: *in vitro* kinase assay of immunoprecipitated RIPK1 shows no autophosphorylation with M92G/D325A RIPK1 indicating that M92G mutation blocks RIPK1 kinase activity.

S6



Supplementary Figure 2.S6 Top Panel: Casp8 was co-transfected from high to low concentrations along with wildtype RIPK1 or RIPK1_D325A to show that the latter is resistant to cleavage. Bottom Panel: Parental Jurkats were infected retrovirally with pMit-empty, - RIPK1, and -RIPK1 D325A and treated with combinations of z-VAD-FMK (20 μ M), Nec-1 (10 μ M), TNF- α (20 ng/ml) for 14h. Cells were stained with 7AAD and annexin-V to detect apoptotic vs. necrotic cells.

Methods

Mice. FADDdd transgenic mice [71] were crossed with RIPK3^{-/-} mice [80], the latter provided by Kim Newton and Vishva Dixit at Genentech Corp. Rag2^{-/-} x γ c and C57BL6/J (“B6”) mice were obtained from Jackson Labs. Mice were bred and maintained in accordance with the institutional animal use and care committee at the University of California Irvine vivarium.

T cell activation. 24 well dishes were coated with 1 μ g plate-bound anti-CD3, plus 200 ng/ml soluble anti-CD28 in 1 ml RPMI media supplemented with 10% fetal calf serum (Omega Sciences), sodium pyruvate, 1X non-essential amino acids, 1X pen/strep/glutamine and beta mercaptoethanol (2×10^{-5} M). Splenocytes were isolated, treated to lyse erythrocytes, and plated in 24 well dishes at 2×10^6 per well. 10 μ M Nec-1 was added at start of culture.

Adoptive Transfer. CD4⁺ and CD8⁺ cells from B6, FADDdd, RIP3^{-/-}, and RIP3^{-/-} x FADDdd mice were isolated by depletion of B220⁺, CD11b⁺ and MHCII⁺ cells with magnetic microbeads (Miltenyi), and injected intravenously at a dose of 2.5×10^6 cells per Rag2^{-/-} x IL-2R γ c^{-/-} mouse.

Viral Infection. 2×10^5 PFU MHV (strain JHM-DM) was injected intraperitoneally into mice. PBS was injected into “mock” controls. Intracellular staining, viral titer plaque assays and statistical analyses were performed using methods reported previously [92].

RICD Assay. Restimulation was performed in 96-well flat-bottom plates coated with 1 $\mu\text{g/ml}$ $\alpha\text{-CD3}$ using an approach based on [93]. 1×10^5 cells were added per well to coated plates and kill assay was allowed to proceed overnight before staining with 7-AAD.

CTL Assay. *In vivo.* Mice were infected intraperitoneally with 2×10^5 PFU MHV, followed by an *in vivo* CTL activity assay based on [94]. B6 mice splenocytes were pulsed 1h 37°C with 5 μM OVA or S510 peptide as target cells and resuspended at $20 \times 10^6/\text{ml}$ for CFSE labeling. OVA/S510-pulsed cells were labeled with 2.5 and 0.5 μM CFSE respectively and mixed at 1:1 ratio to obtain a cell suspension of $100 \times 10^6/\text{ml}$. 100 μl target cells was adoptively transferred intraperitoneally into each infected mouse, and the assay was allowed to proceed 75 min. before harvesting spleens for FACS. % specific lysis: $100*[1-R(\text{Sham})/R(\text{infected})]$, $R = \text{CFSE}^{\text{hi}}/\text{CFSE}^{\text{lo}}$. ***In vitro.*** EL4 target cells were pulsed with peptides as stated above. OVA-pulsed/S510-pulsed EL4 cells were labeled with 2.5 μM CFSE and 5 μM EF670, respectively (10 min., 37°C ; eBioscience), and mixed at equal volumes. To prepare effectors, splenocytes of infected mice were RBC-lysed and plated in 96-well round-bottom plates at 100 $\mu\text{l}/\text{well}$ before adding 100 μl target cells. Plates were incubated at 37°C , 5% CO_2 for 4h before FACS analysis. % specific lysis: $100*[1-R(\text{infected})/R(\text{no effectors})]$, $R = \text{EF670}/\text{CFSE}$.

Retroviral Infection. Jurkats were infected with retroviral supernatants using methods reported previously (6). 2 days post-infection, cells were treated with Nec-1 (10 μM), zVAD-FMK (20 μM), CHX (5 $\mu\text{g/ml}$), $\text{TNF-}\alpha$ (20 ng/ml), and etoposide (50 μM). 14h post-treatment, cells were stained with annexin V, anti-Thy1, and 7-AAD for FACS analysis.

References

1. Ashkenazi A & Dixit V (1998) Death receptors: signaling and modulation. *Science* 281(5381):1305-1308.
2. Siegel RM (2006) Caspases at the crossroads of immune-cell life and death. *Nat Rev Immunol* 6(4):308-317.
3. Newton K, Harris A, Bath M, Smith K, & Strasser A (1998) A dominant interfering mutant of FADD/MORT1 enhances deletion of autoreactive thymocytes and inhibits proliferation of mature T lymphocytes. *EMBO J* 17(3):706-718.
4. Walsh C, *et al.* (1998) A role for FADD in T cell activation and development. *Immunity* 8(4):439-449.
5. Zornig M, Hueber AO, & Evan G (1998) p53-dependent impairment of T-cell proliferation in FADD dominant-negative transgenic mice. *Curr Biol* 8(8):467-470.
6. Bell BD, *et al.* (2008) FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells. (Translated from eng) *Proc Natl Acad Sci U S A* 105(43):16677-16682 (in eng).
7. Ch'en IL, *et al.* (2008) Antigen-mediated T cell expansion regulated by parallel pathways of death. (Translated from eng) *Proc Natl Acad Sci U S A* 105(45):17463-17468 (in eng).
8. Kawahara A, Ohsawa Y, Matsumura H, Uchiyama Y, & Nagata S (1998) Caspase-independent cell killing by Fas-associated protein with death domain. *J Cell Biol* 143(5):1353-1360.
9. Vercammen D, *et al.* (1998) Inhibition of caspases increases the sensitivity of L929 cells to necrosis mediated by tumor necrosis factor. *J Exp Med* 187(9):1477-1485.
10. Holler N, *et al.* (2000) Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* 1(6):489-495.
11. Degterev A, *et al.* (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nat Chem Biol* 1(2):112-119.
12. Degterev A, *et al.* (2008) Identification of RIP1 kinase as a specific cellular target of necrostatins. *Nat Chem Biol* 4(5):313-321.
13. Hitomi J, *et al.* (2008) Identification of a molecular signaling network that regulates a cellular necrotic cell death pathway. *Cell* 135(7):1311-1323.
14. Declercq W, Vanden Berghe T, & Vandenabeele P (2009) RIP kinases at the crossroads of cell death and survival. (Translated from eng) *Cell* 138(2):229-232 (in eng).

15. Zhang DW, *et al.* (2009) RIP3, an Energy Metabolism Regulator that Switches TNF-Induced Cell Death from Apoptosis to Necrosis. *Science*.
16. He SW, Lai; Miao, Lin; Wang, Tao; Du, Fenghe; Zhao, Liping; Wang, Xiaodong (2009) Receptor Interacting Protein Kinase-3 Determines Cellular Necrotic Response to TNF-alpha. *Cell* 137(6):1100-1111.
17. Cho YS, *et al.* (2009) Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. (Translated from eng) *Cell* 137(6):1112-1123 (in eng).
18. Lin Y, Devin A, Rodriguez Y, & Liu ZG (1999) Cleavage of the death domain kinase RIP by caspase-8 prompts TNF-induced apoptosis. *Genes Dev* 13(19):2514-2526.
19. Moquin D & Chan FK (2010) The molecular regulation of programmed necrotic cell injury. (Translated from eng) *Trends Biochem Sci* 35(8):434-441 (in eng).
20. Leverrier S, Salvesen GS, & Walsh CM (2010) Enzymatically active single chain caspase-8 maintains T-cell survival during clonal expansion. (Translated from Eng) *Cell Death Differ* (in Eng).
21. Kelliher M, *et al.* (1998) The death domain kinase RIP mediates the TNF-induced NF-kappaB signal. *Immunity* 8(3):297-303.
22. Newton K, Sun X, & Dixit VM (2004) Kinase RIP3 is dispensable for normal NF-kappaBs, signaling by the B-cell and T-cell receptors, tumor necrosis factor receptor 1, and Toll-like receptors 2 and 4. *Mol Cell Biol* 24(4):1464-1469.
23. Beisner DR, Chu IH, Arechiga AF, Hedrick SM, & Walsh CM (2003) The requirements for fas-associated death domain signaling in mature T cell activation and survival. *J Immunol* 171(1):247-256.
24. Sallusto F, Lenig D, Forster R, Lipp M, & Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. (Translated from eng) *Nature* 401(6754):708-712 (in eng).
25. Groettrup M, *et al.* (1993) A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor beta chain and a 33 kd glycoprotein. *Cell* 75(2):283-294.
26. Fehling HJ, Krotkova A, Saint-Ruf C, & von Boehmer H (1995) Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature* 375(6534):795-798.
27. Vandenabeele P, Declercq W, Van Herreweghe F, & Vanden Berghe T (2010) The role of the kinases RIP1 and RIP3 in TNF-induced necrosis. (Translated from eng) *Sci Signal* 3(115):re4 (in eng).

28. Pop C, *et al.* (2011) FLIP(L) induces caspase 8 activity in the absence of interdomain caspase 8 cleavage and alters substrate specificity. (Translated from eng) *Biochem J* 433(3):447-457 (in eng).
29. Feng S, *et al.* (2007) Cleavage of RIP3 inactivates its caspase-independent apoptosis pathway by removal of kinase domain. *Cell Signal* 19(10):2056-2067.
30. Hogquist K, *et al.* (1994) T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.
31. Juo P, *et al.* (1999) FADD is required for multiple signaling events downstream of the receptor Fas. *Cell Growth Differ* 10(12):797-804.
32. Ting AT, Pimentel-Muinos FX, & Seed B (1996) RIP mediates tumor necrosis factor receptor 1 activation of NF-kappaB but not Fas/APO-1-initiated apoptosis. *Embo J* 15(22):6189-6196.
33. Bell BD & Walsh CM (2009) Coordinate regulation of autophagy and apoptosis in T cells by death effectors: FADD or foundation. (Translated from eng) *Autophagy* 5(2):238-240 (in eng).
34. Osborn SL, *et al.* (2010) Fas-associated death domain (FADD) is a negative regulator of T-cell receptor-mediated necroptosis. (Translated from eng) *Proc Natl Acad Sci U S A* 107(29):13034-13039 (in eng).
35. Siegel RM, Chan FK, Chun HJ, & Lenardo MJ (2000) The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat Immunol* 1(6):469-474.
36. Van Herreweghe F, Festjens N, Declercq W, & Vandenabeele P (2010) Tumor necrosis factor-mediated cell death: to break or to burst, that's the question. (Translated from eng) *Cell Mol Life Sci* 67(10):1567-1579 (in eng).
37. Oberst A, *et al.* (2010) Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation. (Translated from eng) *J Biol Chem* 285(22):16632-16642 (in eng).
38. Kaiser WJ, *et al.* (2011) RIP3 mediates the embryonic lethality of caspase-8-deficient mice. (Translated from eng) *Nature* 471(7338):368-372 (in eng).
39. Zhang H, *et al.* (2011) Functional complementation between FADD and RIP1 in embryos and lymphocytes. (Translated from eng) *Nature* 471(7338):373-376 (in eng).
40. Ch'en IL, Tsau JS, Molkentin JD, Komatsu M, & Hedrick SM (2011) Mechanisms of necroptosis in T cells. (Translated from eng) *J Exp Med* 208(4):633-641 (in eng).

41. Ramos SJ, Hardison JL, L.N. S, T.E. L, & C.M. W (2007) Anti-viral effector T cell responses and trafficking are not dependent upon DRAK2 signaling following viral infection of the central nervous system. *Autoimmunity* 40(1):54-65.
42. Ramaswamy M, *et al.* (2007) Cutting edge: Rac GTPases sensitize activated T cells to die via Fas. (Translated from eng) *J Immunol* 179(10):6384-6388 (in eng).
43. Barber DL, Wherry EJ, & Ahmed R (2003) Cutting edge: rapid in vivo killing by memory CD8 T cells. (Translated from eng) *J Immunol* 171(1):27-31 (in eng).

CHAPTER 3

Autophagy is required for Naïve T cell homeostasis but not in activated T cells

Abstract

It has been suggested that the highly conserved self-degradation pathway known as autophagy is required for survival in proliferating T lymphocytes, as deficiency of an essential autophagy gene, *Atg5*, via CD4-Cre mediated deletion promotes death upon activation. However, single positive thymocytes from *Atg5^{fl/fl}* CD4-Cre mice exhibit an early defect, demonstrating that naïve T cells from these mice harbor a defect prior to TCR stimulation. *Atg5^{fl/fl}* CD4-Cre T cells adoptively transferred into RAG-/- or wild type mice display defective survival in both immunosufficient or immunodeficient hosts, indicating the death observed in response to TCR activation is an indirect result of their pre-existing defect. Naïve autophagy-deficient T cells display increased mitochondrial levels, while Tamoxifen-mediated inducible deletion of *Atg5* following T cell activation resulted in no discernable proliferative defects or mitochondrial accumulation. Our data suggests that naïve T cells rely on autophagy to regulate mitochondrial levels, while activated T cells partition excess mitochondria through rapid division.

Introduction

Autophagy is a fundamentally conserved process by which intracellular contents are degraded [1] and is induced under low-energy conditions as a cell survival mechanism. Formation of the double-membrane autophagosome is mediated by Atg proteins and the mature autophagosome, which encapsulates organelles and proteins, then fuses with the lysosome [95].

In the immune system, autophagy is important for clearing intracellular pathogens such as group A *Streptococcus* and *Mycobacterium tuberculosis* [3, 4]. Interestingly, Li et al. [5] observed the formation of autophagosomes in activated CD4⁺ T cells indicating that autophagy does indeed occur in T cells, and can be intrinsically linked to T-cell activation and proliferation. Under starvation conditions, autophagy was also observed in T cells in response to TCR activation, suggesting the process as a mode of regulating the metabolic pathways and requirements of T cell activation. Paradoxically, in Caspase 8- deficient T cells which have been shown to undergo necrosis in response to TCR stimulation, hyper-autophagy was observed in conjunction with cell death suggesting a pro-death mechanism when not properly regulated [6, 7]. However, mice harboring deletion of autophagy genes *Atg3*, *Atg5*, or *Atg7* display decreased peripheral T cells, characterized by increased mitochondrial content and enhanced apoptosis. Upon activation, autophagy-deficient T cells exhibit defective expansion, and this has been linked to the need for autophagy to provide sufficient bioenergetics for the processes involved in T cell activation [8] and for regulating mitochondrial numbers in dividing T cells [9, 10].

In an *Atg5^{fl/fl}* CD4-Cre T cell specific system as well as a Tamoxifen-inducible *Atg5^{fl/fl}* ER-Cre model, we show that autophagy serves differential roles in naïve and activated T lymphocytes. *Atg5^{fl/fl}* CD4-Cre mice are characterized by a lymphopenic environment, indicating a defect in maintaining T cell homeostasis. Maintaining homeostasis of naïve T cells is essential for ensuring a varied repertoire of T cells that is capable of responding to pathogen. The pool of naïve T cells is kept at a constant level by thymic egress and by contact with homeostatic cytokines and self peptide/MHC interactions in the periphery [11]. *Atg5^{fl/fl}* CD4-Cre T cells placed in an immuno-compromised environment fail to repopulate the peripheral compartments and also fail to survive in an immuno-sufficient host. We observed no defects in the ability of

naïve Atg5^{fl/fl} CD4-Cre T cells to recycle IL7R or respond to homeostatic cytokines indicating this defect is due to a failure in cellular survival independent of extracellular signals. Our proliferation studies indicate the death observed in response to TCR engagement is accumulated from the defect during the naïve stage. Although naïve T cells utilize autophagy for degradation of mitochondria, we demonstrate that autophagy is dispensable for the survival of activated lymphocytes when Atg5 is deleted post-activation.

Results

Recent evidence suggests that autophagy may function as a critical process in mediating the events following TCR activation. To investigate the role of autophagy in T lymphocyte development and function, mice genetically deficient in autophagy protein, Atg5, in T cells (Atg5^{fl/fl} CD4-Cre) were generated and Atg5 was successfully deleted in peripheral CD4⁺ and CD8⁺ T lymphocytes (**Fig. 3.1a**). Percentages of DN, DP, and SP populations were similar in Atg5^{fl/fl} CD4-Cre and wild type mice (**Fig. 3.1b**), and there were no differences in the subsets of the DN population defined by CD25 and CD44 (**Fig. 3.1c**), suggesting that Atg5 is dispensable for thymic development. The overall numbers of CD4⁺ and CD8⁺ T cells in the spleen were significantly reduced in Atg5^{fl/fl} CD4-Cre mice (**Fig. 3.1b**).

Previous studies showed a proliferation defect in Atg5^{fl/fl} CD4-Cre T cells in response to TCR stimulation. To assess the role of autophagy in activated T cells purified Atg5^{fl/fl} CD4-Cre T cells were labeled with proliferation dye CFSE and activated with high and low concentrations of plate-bound anti-CD3 and soluble anti-CD28 (**Fig. 3.1d**). Cells were harvested at Day 3 post activation for analysis of CFSE dilution by flow cytometry. Atg5^{fl/fl} CD4-Cre cells underwent defective proliferation compared to wild type cells following antigenic stimulation under both

high and low concentrations of stimulation. Addition of apoptotic inhibitor, Qvd, or necroptotic inhibitor, Nec-1, or the combination was unable to rescue the survival defect (**Fig. 3.1e**). Thus, it appears autophagy plays a role in survival of activated T cells. During T cell activation, there is a metabolic switch to ensure sufficient energy to promote proliferation and effector functions [12]. It has been suggested that autophagy is required to degrade cytosolic components to ensure sufficient bioenergetic output for the processes required during T cell proliferation. To test this, cultures were supplemented with methyl pyruvate (**Fig. 3.1e**), which is a cell permeable end product of glycolysis that has been shown to maintain the viability of nutrient-depleted cells with impaired autophagy [13] and no rescue was observed in autophagy-deficient cells.

Other studies have suggested that autophagy is required for the clearance of damaged organelles and excess mitochondria in activated T cells [14]. Excess mitochondria results in the release of excess reactive oxygen species and toxic factors such as cytochrome c that lead to apoptosis of the cell [15, 16]. Activated autophagy knockout cells were cultured in the presence of antioxidant BHA (**Fig. 3.1e**) and minimal rescue effect was observed. TCR engagement leads to activation of glycolysis as a result of ligation of CD28 followed by a subsequent increase in glucose uptake [17-19]. T cells can still conserve their function under glucose-restrictive conditions indicating that other mechanisms such as autophagy may cooperate with glycolysis to meet the bioenergetic demands that occurs during T cell activation. Atg5^{ff} CD4-Cre T cells were cultured under varying levels of glucose to determine whether autophagy-deficient T cells would be less susceptible to glucose deprivation compared to wild type cells (**Supp fig. 3.S1**). Atg5^{ff} CD4-Cre and wild type T cells displayed comparable levels of cell death induced by low levels of glucose.

Proliferation assays were performed using thymocytes and anti-CD3 and CD28 stimulation. CFSE dilution was analyzed at Day 3 post activation on single positive mature thymocytes in the presence or absence of Nec-1 and/or Qvd (**Supp fig. 3.S2**). Similarly, activated Atg5^{fl/fl} CD4-Cre thymocytes displayed increased death, and Nec-1, Qvd, or the combination provided minimal rescue. CD4⁺ and CD8⁺ populations of Atg5^{fl/fl} CD4-Cre splenocytes and thymocytes activated with plate-bound anti-CD3 and soluble anti-CD28 were analyzed by CFSE and 7AAD to further assess cell death (**Fig. 3.2a**). Atg5^{fl/fl} CD4-Cre T cells displayed an increased 7AAD^{high}/CFSE^{high} population, representative of undivided dead cells, indicating a majority of cell death observed in response to stimulation occurred during the initial activation stage.

We next characterized the profile of naïve Atg5^{fl/fl} CD4-Cre cells. Freshly isolated splenocytes from Atg5^{fl/fl} CD4-Cre mice displayed increased levels of cleaved caspase 3 (**Fig. 3.2b**), corresponding with increased apoptosis. To determine whether the increased death could be rescued with tonic survival signals, Atg5^{fl/fl} CD4-Cre T cells were rested with IL7 and IL15 [20] for 3 days to “rescue” the dying population before stimulating with anti-CD3 and CD28 (**Supp fig. 3.S3**). Pre-incubation with IL7 and IL15 promoted viability of activated wild type cells, but was unable to rescue the Atg5^{fl/fl} CD4-Cre cells. The activation profile of naïve Atg5^{fl/fl} CD4-Cre splenocytes was analyzed by staining with activation markers CD62L and CD44 (**Fig. 3.2c and supp fig. 3.S4**). Both CD4⁺ and CD8⁺ splenocytes from autophagy-deficient mice display a decreased percentage of naïve (CD62L^{high} CD44^{low}) cells as well as an enhanced CD62L^{low}/CD44^{high} population, characterized as activated effector memory cells. These data suggest that peripheral lymphocytes from a naïve Atg5^{fl/fl} CD4-Cre mouse are not truly naïve, and display a pre-activated phenotype. Cells were gated on the CD44^{hi} population and analyzed

using memory markers CD127 and KLRG (Fig. 3.2d). Within the activated cells, autophagy-deficient T cells had decreased KLRG^{low}/ CD127^{high} population, which is representative of the memory population [21].

Due to decreased T cell numbers in the periphery, we hypothesized that Atg5^{fl/fl} CD4-Cre T cells are constantly undergoing homeostatic proliferation to fill up the splenic compartment. To assess this, we performed in vivo BrdU labeling with Atg5^{fl/fl} CD4-Cre mice (Fig. 3.2e). Mice were injected intraperitoneally every 24 hours with BrdU and spleens and thymus were harvested at 48 hours for analysis of BrdU incorporation by flow cytometry. Atg5^{fl/fl} CD4-Cre mice displayed increased BrdU uptake indicating increased cell turnover corresponding with their lymphopenic environment.

To assess the ability of autophagy-deficient cells to homeostatically proliferate in a lymphopenic host, we purified and CFSE-labeled Atg5^{fl/fl} CD4-Cre T cells for adoptive transfer into RAG^{-/-} mice. 7 days post transfer; spleens were harvested for analysis of cell recovery. Atg5^{fl/fl} CD4-Cre T cells transferred into an immunodeficient RAG^{-/-} mouse failed to proliferate and little recovery was observed (Fig. 3.3a). To determine whether the presence of wild type cells could rescue the defect, wild type and Atg5^{fl/fl} CD4-Cre T cells were labeled with EF670 and CFSE respectively, mixed at a 1:1 ratio and co-transferred into RAG^{-/-} hosts. 7 days post-transfer, significantly decreased numbers of Atg5^{fl/fl} CD4-Cre donor cells was recovered (Fig. 3.3b).

We hypothesized that the constant signals to undergo homeostatic proliferation may cause the increased apoptosis observed in Atg5^{fl/fl} CD4-Cre mice. Perhaps placing the cells in an immunosufficient environment would provide a rescue effect. Atg5^{fl/fl} CD4-Cre cells were labeled and adoptively transferred into wild type hosts. 7 days post-transfer spleens were harvested for

analysis of cell recovery. Atg5^{fl/fl} CD4-Cre donor T cells displayed defective survival in wild type hosts, and diminished recovery was observed (**Fig. 3.3c**). Our observations indicate the defects observed in autophagy-deficient T cells manifest during the naïve stage.

Diminished recovery of transferred T cells could be attributed to a role for autophagy during trafficking to peripheral compartments. To test the requirement for autophagy in homing, Atg5^{fl/fl} CD4-Cre and wild type T cells were labeled with CFSE and EF670 respectively and mixed at an equal ratio. The mixture was transferred into wild type mice, and at 4 hours and 24 hours post-transfer, the percentages of Atg5^{fl/fl} CD4-Cre cells recovered from the spleen and lymph nodes were comparable to wild type (**Fig. 3.3d**).

To delete Atg5 post-activation, we utilized a retroviral Cre system. Purified T cells from Atg5^{fl/fl} mice were activated with anti-CD3 and CD28. 24 hours following stimulation Atg5^{fl/fl} T cells were retrovirally infected with MIG-Cre or MIG-empty control vector to acutely delete Atg5 (**Fig. 3.3e**). Deletion efficiency was confirmed by intracellular staining for Atg5 at Day 5 post-activation. At Day 3 and Day 5 post-activation, Cre-infected cells displayed no proliferation defect (**Fig. 3.3f**), suggesting that autophagy may not be necessary in activated T cells. Thus, the CD4-Cre driven Atg5 knockout mouse model does not accurately address the role of autophagy in activated T cells. To answer this question we bred Atg5^{fl/fl} mice with ER-Cre mice to generate a tamoxifen-inducible knockout mouse (Atg5^{fl/fl} ER-Cre). This model allowed us to delete Atg5 post activation and examine the effect of a direct deletion. Deletion was confirmed by flow cytometry at Day 5 following treatment with tamoxifen at the start of culture (**Fig. 3.3g**). Day 3 and 5 post-activation, deleting after activation did not have any impact on proliferation and survival (**Fig. 3.3h**).

From our findings that an acute deletion of autophagy has minimal impact on T cell death and division, we next determined the ability of acutely-deleted cells to survive in an immunodeficient host. Atg5^{fl/fl} cells were labeled, activated and infected with MIG-Cre (**Supp fig. 3.S5**). 3 days post-activation Cre-infected cells were transferred into RAG-/- mice (**Fig. 3.4a**). 7 days post-transfer, decreased numbers of Cre-infected cells was recovered from the spleen. Congenically marked mice (CD45.1) received sub-lethal irradiation to abolish endogenous T and B cells, followed by adoptive transfer of Cre-infected donor T cells (**Fig. 3.4b**). 7 days post-irradiation, spleens were harvested for analysis of recovery. Cre-infected cells displayed defective survival, and little recovery was observed.

Previous studies in Vps34-/- mice have indicated the importance of autophagy for internalizing and recycling of the IL7 receptor [22]. CD127 surface levels were assessed in naïve T cells, in the presence or absence of IL7 (**Fig. 3.4c**). IL7R expression was comparable in freshly isolated Atg5^{fl/fl} CD4-Cre and wild type T cells, and increased after 24 hours of *in vitro* culture. There were no defects in downregulation of CD127 expression in Atg5^{fl/fl} CD4-Cre cells when treated with exogenous IL7. Atg5^{fl/fl} CD4-Cre T cells were incubated with IL7 over several days and stained for Annexin V and 7AAD to determine the ability to respond to IL7. (**Fig. 3.4d**). IL7 greatly enhanced viability of wildtype T cells, and showed modest rescue of Atg5^{fl/fl} CD4-Cre T cells. Recent reports suggest continuous signaling promotes production of interferon gamma, resulting in cytokine-induced cell death [23]. Perhaps autophagy is required for maintaining survival of T cells when the IL7R is internalized, or autophagy-deficient T cells are more sensitive to cytokine-induced cell death. To test this, naïve Atg5^{fl/fl} CD4-Cre T cells were cultured for 7 days with IL7 in the presence or absence of α -IFN γ . Atg5^{fl/fl} CD4-Cre do not show enhanced

viability with α -IFN γ (**Fig. 3.4e**), suggesting the increased death of autophagy-deficient cells was not due to increased susceptibility to IL7-induced death.

Naïve splenocytes from Atg5^{f/f}CD4-Cre mice were stained with cell permeable mitochondria- specific staining reagent Mito-Tracker Green (**Fig. 3.5a**), and we observed increased mitochondria levels, consistent with previous reports that autophagy regulates mitochondrial numbers [9]. To determine whether autophagy was also required for mitochondrial homeostasis in activated T cells, activated splenocytes from Atg5^{f/f}ER-Cre mice were stained with Mito-Tracker Green (**Fig. 3.5b**) and mitochondrial membrane potential marker DioC6 (**Fig. 3.5c**). Mitochondrial levels and potential were unaffected when deletion was induced, suggesting autophagy is dispensable in activated T lymphocytes. We proposed that excess mitochondria in activated cells is diluted out through cell division. To test our hypothesis, activated Atg5^{f/f} CD4-Cre cells were treated with cell cycle inhibitor, nocodazole, to determine whether inhibiting expansion would lead to mitochondria build up and impact survival. Activated Atg5^{f/f}CD4-Cre T cells displayed lower overall mitochondrial membrane potential (**Supp fig. 3.S6**), and when mitochondrial levels were analyzed with cell division, addition of nocodazole led to increased mitochondria in non-dividing KO cells relative to mitotracker levels in non-treated cells (**Fig. 3.5d**). Non-dividing wild type T cells also display higher mitochondrial levels in the presence of nocodazole compared to non-treated cells, but these levels appeared to be decreasing. Using the inducible-deletion system, Atg5^{f/f} T cells were infected with MIG-Cre and nocodazole was added 24 hr post-activation in the presence or absence of Qvd. At day 5 post-activation, Cre-infected cells displayed similar levels of mitochondrial as control-infected cells. When cultured with nocodazole, empty-infected cells display dilution of mitotracker fluorescence intensity, while acutely-deleted cells retain a mitotracker-hi population. (**Supp fig. 3.S7**). When we compared

fold change in percentage of infected cells recovered relative to non-treated cells, viability of control-infected cells was similar even with nocodazole and Qvd treatment, while cre-infected cells displayed increased death with nocodazole, which was restored with Qvd (**Fig. 3.5e, supp fig. 3.S8**).

Discussion

It has been suggested that autophagy is required in activated T cells for elimination of damaged mitochondria or for T cell metabolism. However, our data call into question these proposed requirements for autophagy in activated T cells, as deficiency of an essential autophagy gene, Atg5, via CD4-Cre mediated deletion instead promotes early defects in single positive thymocytes.

Autophagy is upregulated in activated T cells, and we sought to determine the role of autophagy following TCR stimulation. We, and others have shown that upon TCR ligation, Atg5^{fl/fl} CD4-Cre T cells display defective proliferation characterized by increased apoptosis. This death in activated T cells cannot be rescued with necroptotic or apoptotic inhibitors, suggesting autophagy-deficient cells may have already committed to death early on. Autophagy has been suggested to be important in maintaining cellular components during proliferation and to break down cellular contents to supply the cell with sufficient bioenergetics for rapid proliferation. However, our data suggests autophagy is not critical for these cellular processes as addition of ROS scavengers or glycolytic intermediates were insufficient for restoring survival of activated Atg5^{fl/fl} CD4-Cre cells. Under starvation conditions, autophagy has been known to be upregulated to provide an alternative source of fuel. Thus, cells deficient in autophagy would be more impacted by low-energy conditions. We observed that Atg5^{fl/fl} CD4-Cre cells were equally

susceptible to glucose deprivation as wild type cells, demonstrating the survival defect is not likely due to metabolic issues. The majority of cell death occurs during the initial stage of TCR stimulation but resting Atg5^{fl/fl} CD4-Cre cells with homeostatic cytokines to enhance viability before stimulation provided no protective advantage.

Due to the lymphopenic environment in Atg5^{fl/fl} CD4-Cre mice, autophagy-deficient T cells display an increase in activated memory-like population, indicating an activated phenotype even under resting conditions. Because they are continuously receiving signals to expand, we hypothesized that transferring the cells from a lymphopenic environment to an immunosufficient compartment would promote their survival, however, we observed a decrease in survival. Our data indicates Atg5^{fl/fl} CD4-Cre T cells harbor a pre-existing defect during the naïve stage and uncovers a previously unknown role for the importance of autophagy in T cell homeostasis. Existing research on the requirement of autophagy in activated T cells have been conducted with mice lacking autophagy genes at the time of lymphocyte development. Atg5^{fl/fl} CD4-Cre T cells display an early, inherent defect, thus the proliferative defect observed following antigenic stimulation is likely a culmination from the pre-existing defect, and not a direct result of TCR stimulation. The Atg5^{fl/fl} CD4-Cre model does not consider the effect of losing autophagy in the naïve population as well as the extensive levels of apoptosis seen in freshly isolated splenocytes. Thus the Atg5^{fl/fl} ER-Cre and retroviral Cre inducible deletion system provides a relevant method to investigate the requirement for autophagy in activated T cells. When we induce deletion of Atg5 following TCR engagement, no defects were observed in the proliferative capacity or survival suggesting that autophagy is dispensable in activated T cells. Placing inducibly-deleted cells in a lymphopenic environment uncovers the survival defect indicating the necessity for autophagy for homeostatic proliferation and maintaining peripheral homeostasis. Similarly,

decreased recovery of inducibly-deleted cells in irradiated hosts suggest the defect is not caused by a skewed balance of homeostatic cytokines in the extracellular environment due to the lymphopenia. Although IL7R recycling is functional in the absence of autophagy, the defect could occur in the downstream IL7 signaling pathway.

Our data indicates a previously undiscovered and novel role for autophagy in T cell homeostasis. Naïve Atg5^{f/f} CD4-Cre T cells display accumulated mitochondria and increased mitochondrial potential, and are more sensitive to apoptosis. Our results suggest autophagy is essential for maintaining naïve T cell survival through regulation of mitochondrial levels. However, deleting Atg5 post-activation in the retroviral Cre and Atg5^{f/f} ER-Cre system resulted in no discernable effects on mitochondrial levels, survival, and proliferation. However, blockade of cell division in inducibly-deleted cells leads to accumulation of mitochondria and possibly other organelles, resulting in increased apoptosis [24] which can be blocked with Qvd. Our findings suggest that proliferating T cells do not rely on autophagy for clearing mitochondria because they partition out excess mitochondria through rapid cycling. Autophagy may not be exclusive to the removal of mitochondria, but also other cellular organelles, as well as proteins such as p62 (recognizes toxic cellular waste); and failure to do so leads to a survival disadvantage. More importantly, the effect of losing autophagy in naïve T cells would suggest a potentially interesting role of Atg proteins in the generation and maintenance of memory T cells following a viral infection. Although naïve Atg5^{f/f} CD4-Cre mice harbor an increased activated effector memory population, within that subset they display decreased memory cells. Thus, modulating the differential requirements for autophagy in different stages of T cell development is a potential therapeutic target in tolerance and autoimmune disease models.

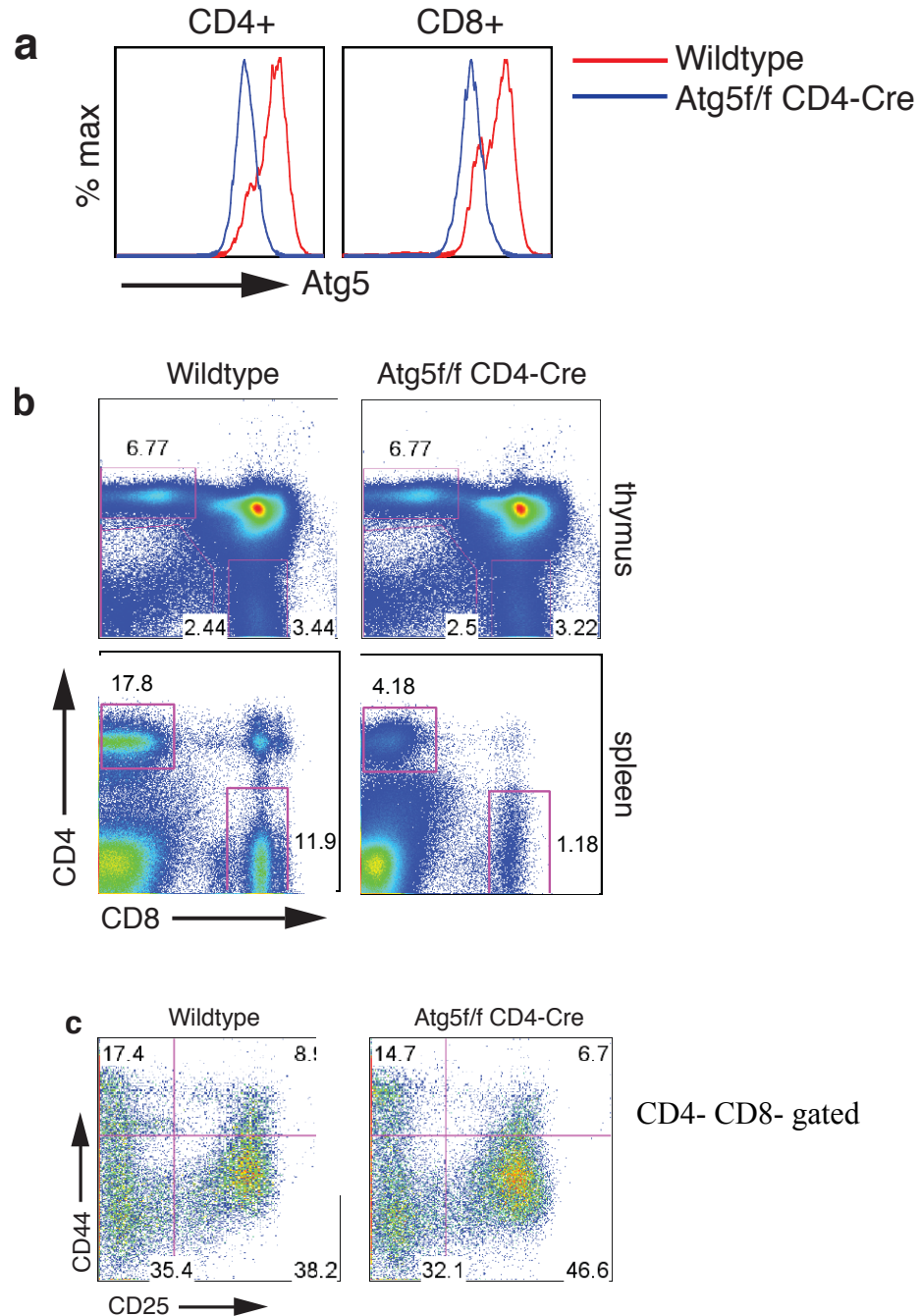


Figure 3.1 Activated Atg5^{-/-} T cell death is not rescued by blocking death or ROS. (a) Atg5 deletion successful in peripheral T cells. Freshly isolated splenocytes from Atg5^{f/f} CD4-Cre mice were stained for CD4+, CD8+ and Atg5 and analyzed by flow cytometry. **(b) Atg5^{-/-} mice display decreased peripheral T cells.** Freshly isolated thymocytes and splenocytes from Atg5^{f/f} CD4-Cre mice were stained for CD4+ and CD8+. **(c) Thymic DN subsets unaffected in Atg5^{-/-} mice.** Atg5^{f/f} CD4-Cre thymocytes were gated on the DN (CD4-CD8-) population and further analyzed by CD44 and CD25. Data representative of 3 separate experiments.

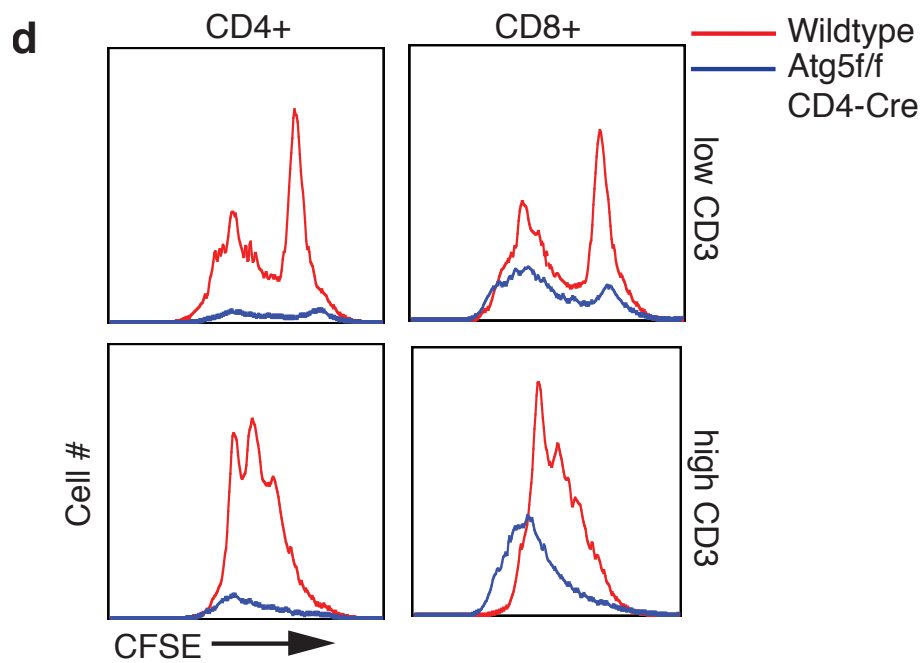


Figure 3.1 (d) Activated *Atg5*^{-/-} T cells display survival defect. Purified *Atg5*^{f/f} CD4-Cre splenocytes were stained with CFSE and activated with 50 ng (low) or 1 ug (high) plate-bound anti-CD3 and soluble CD28 and harvested at Day 3.

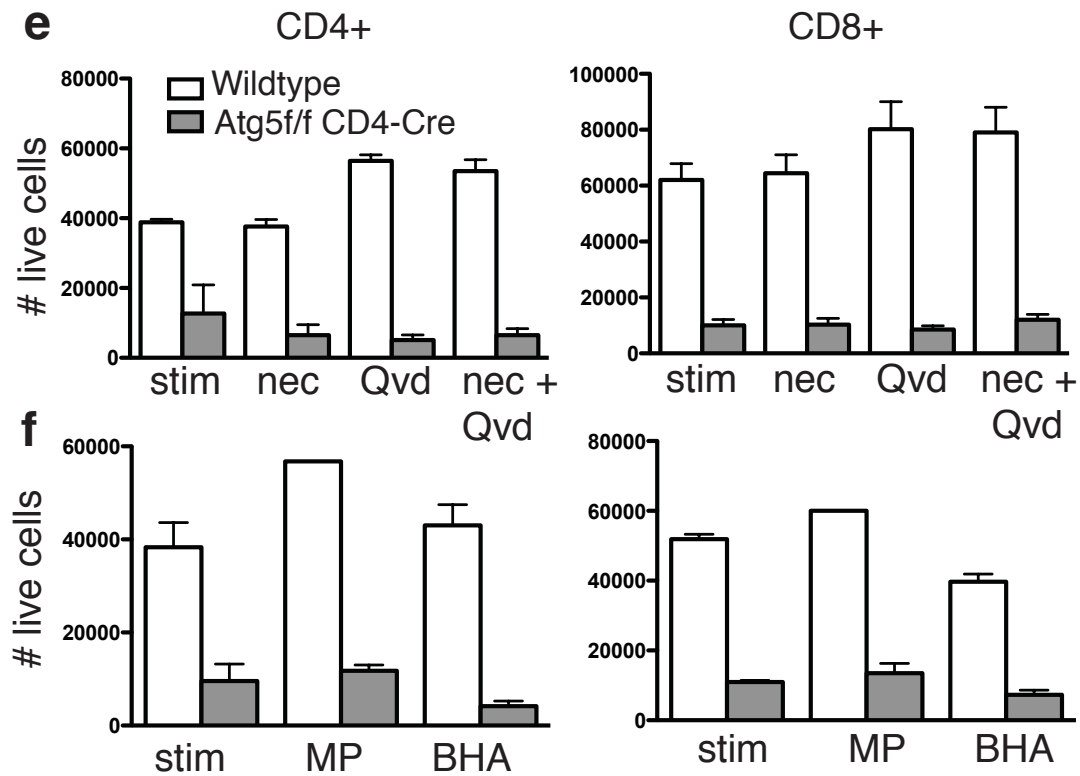


Figure 3.1 (e) Death inhibitors provide no rescue of activated *Atg5*^{f/f} T cells. *Atg5*^{f/f} CD4-Cre T cells were activated with 200 ng plate-bound CD3 and 200 ng/ml soluble CD28. Cells were harvested at Day 3 post-activation and graphed by # live cells (7AAD-). Nec-1, Qvd were added at the start of culture. **(f) ROS scavenger and methyl pyruvate do not rescue survival of activated *Atg5*^{f/f} T cells.** 3-day activated *Atg5*^{f/f} CD4-Cre T cells were treated with methyl pyruvate (MP) at the start of culture, BHA was added 24 hr post-activation. Graph represents # live cells.

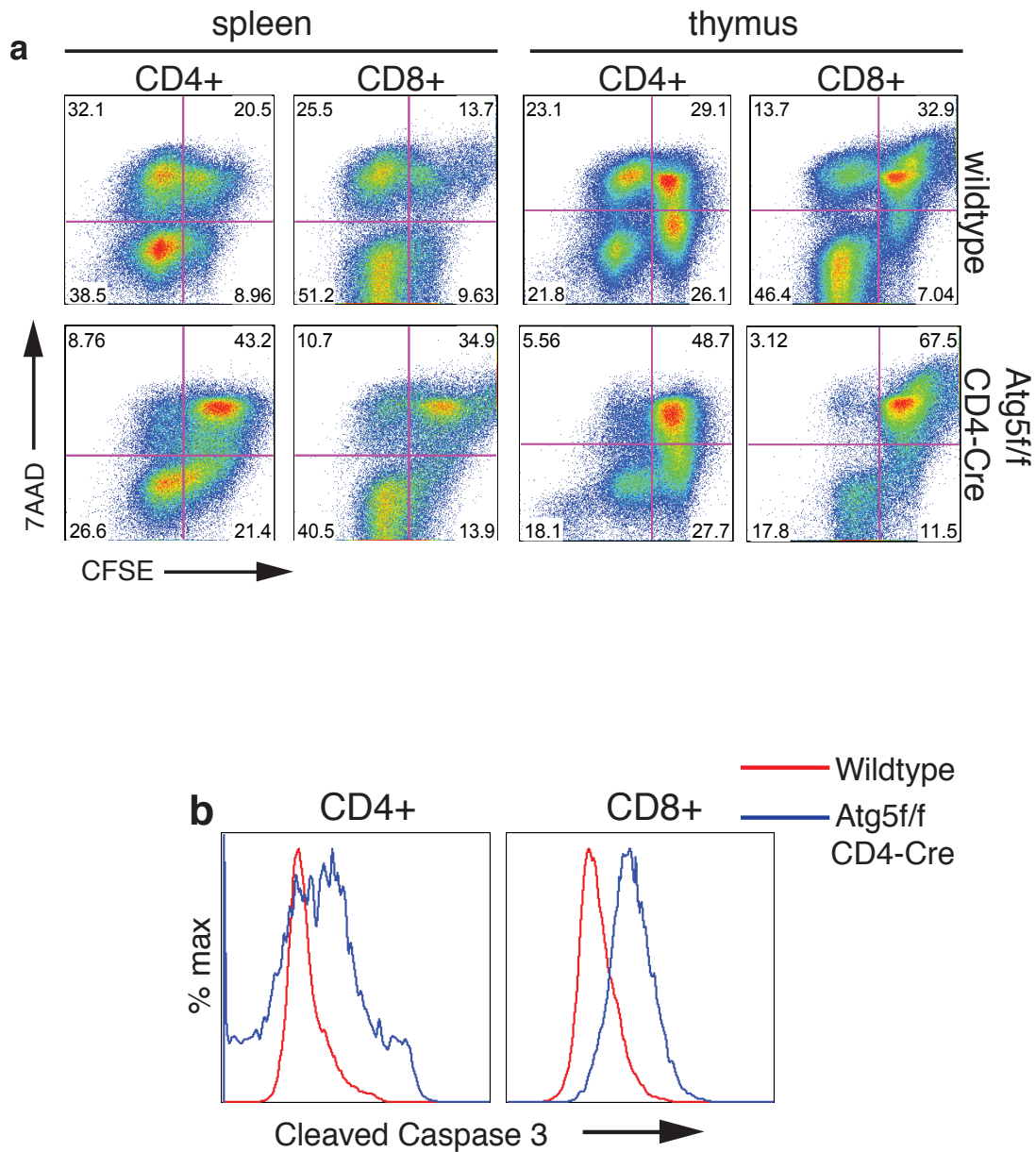


Figure 3.2 Naïve Atg5^{-/-} T cells display pre-activated state. (a) Activated Atg5^{-/-} T cell death occurs during initial TCR activation. 3-day activated Atg5^{f/f} CD4-Cre splenocytes or thymocytes were analyzed by CFSE and 7AAD. Numbers represent % of population in each quadrant. **(b) Enhanced apoptosis in naïve Atg5^{-/-} T cells.** Naïve Atg5^{f/f} CD4-Cre and wild type T cells were stained for cleaved caspase 3.

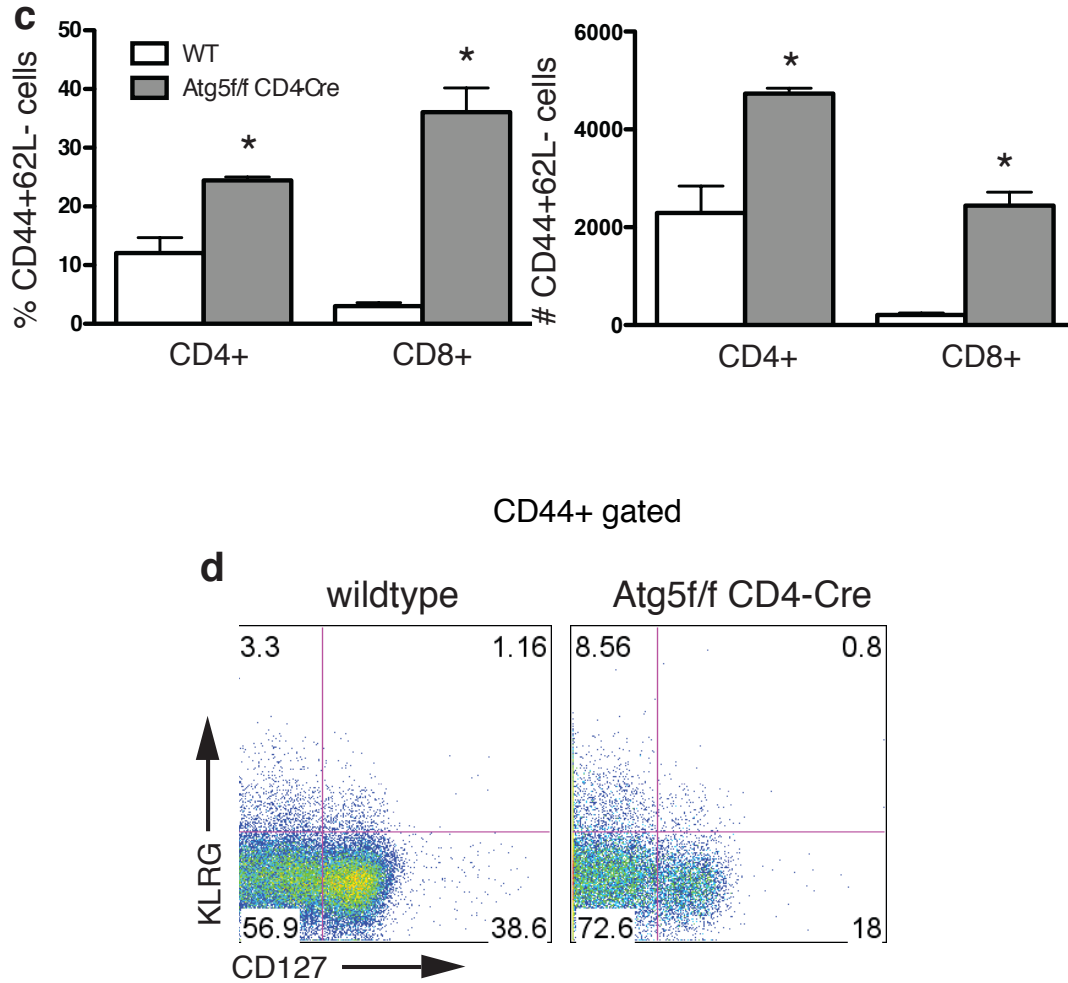


Figure 3.2 (c) Naïve *Atg5*^{-/-} T cells display activated phenotype. Naïve *Atg5*^{f/f} CD4-Cre splenocytes were stained for CD44 and CD62L. Graph represents percent of CD44+ 62L- cells. **(d) Decreased memory population in *Atg5*^{-/-} mice.** Cells were gated on CD44+ population and analyzed by KLRG and CD127.

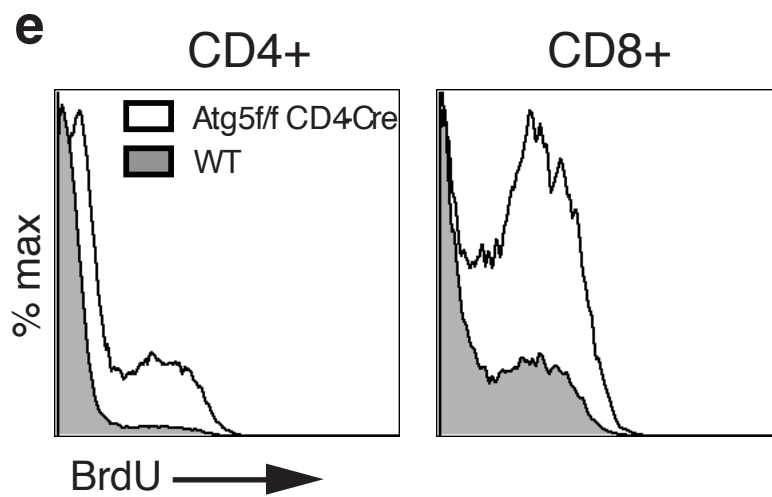


Figure 3.2 (e) *Atg5*^{-/-} mice display increased cell turnover. *Atg5*^{f/f} CD4-Cre and WT mice were injected intraperitoneally with 10 mg/ml BrdU every 24 hrs. Spleens were harvested at 48hrs and stained with CD4+, CD8+, and BrdU for FACS analysis.

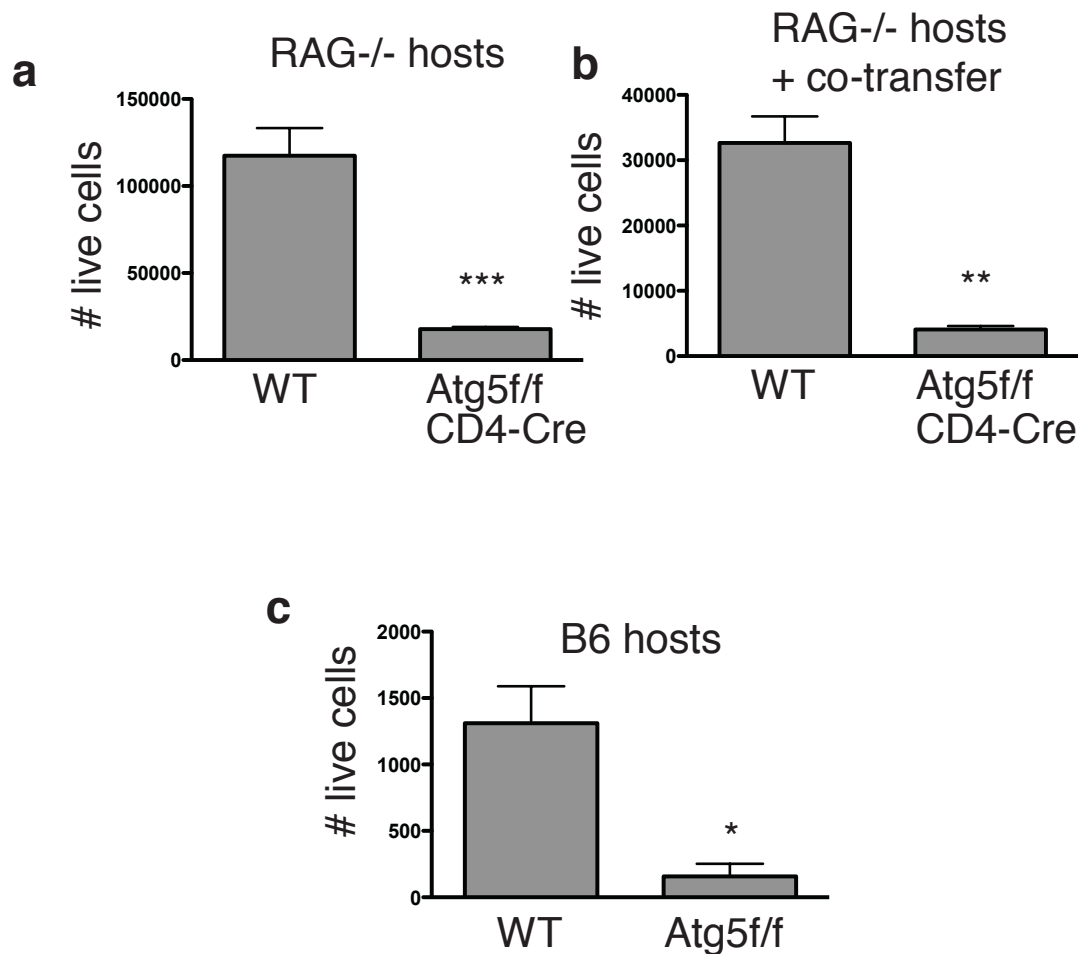


Figure 3.3 Atg5 is not required for proliferation or survival when deleted post-activation.
(a) Atg5^{-/-} T cells fail to survive in immunodeficient hosts. 2.5×10^6 Atg5^{f/f} CD4-Cre or WT purified T cells were labeled with CFSE and adoptively transferred into RAG^{-/-} mice. 7 days post-transfer, spleens were harvested and stained for CD4⁺ and CD8⁺. N = 6. **(b) Atg5^{f/f} CD4-Cre T cells and WT T cells were labeled with CFSE and EF670 respectively and mixed at a 1:1 ratio.** 2.5×10^6 cells were adoptively transferred into RAG^{-/-} mice. Spleens were harvested for FACS analysis 7 days post-transfer. N = 6. **(c) Atg5^{-/-} T cells fail to survive in immunosufficient hosts.** 2.5×10^6 Atg5^{f/f} CD4-Cre or WT purified T cells were labeled with CFSE and adoptively transferred into B6 mice. 7 days post-transfer, spleens were harvested and stained for CD4⁺ and CD8⁺. Considered a significant difference with respect to WT (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$). Error bars represent SEM.

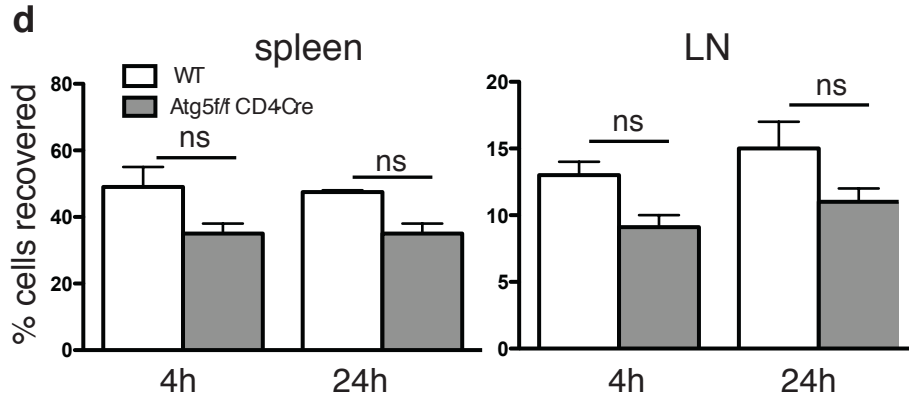


Figure 3.3 (d) *Atg5* is not necessary for cell trafficking. Wild type and *Atg5^{f/f}* CD4-Cre T cells were labeled with EF670 and CFSE respectively, and mixed at equal ratios. 6×10^6 mixed cells were adoptively transferred into B6 mice, and spleens and lymph nodes were harvested for FACS analysis at 4h and 24h post-transfer. Graph represents percent recovery of transferred cells. Error bars represent SEM.

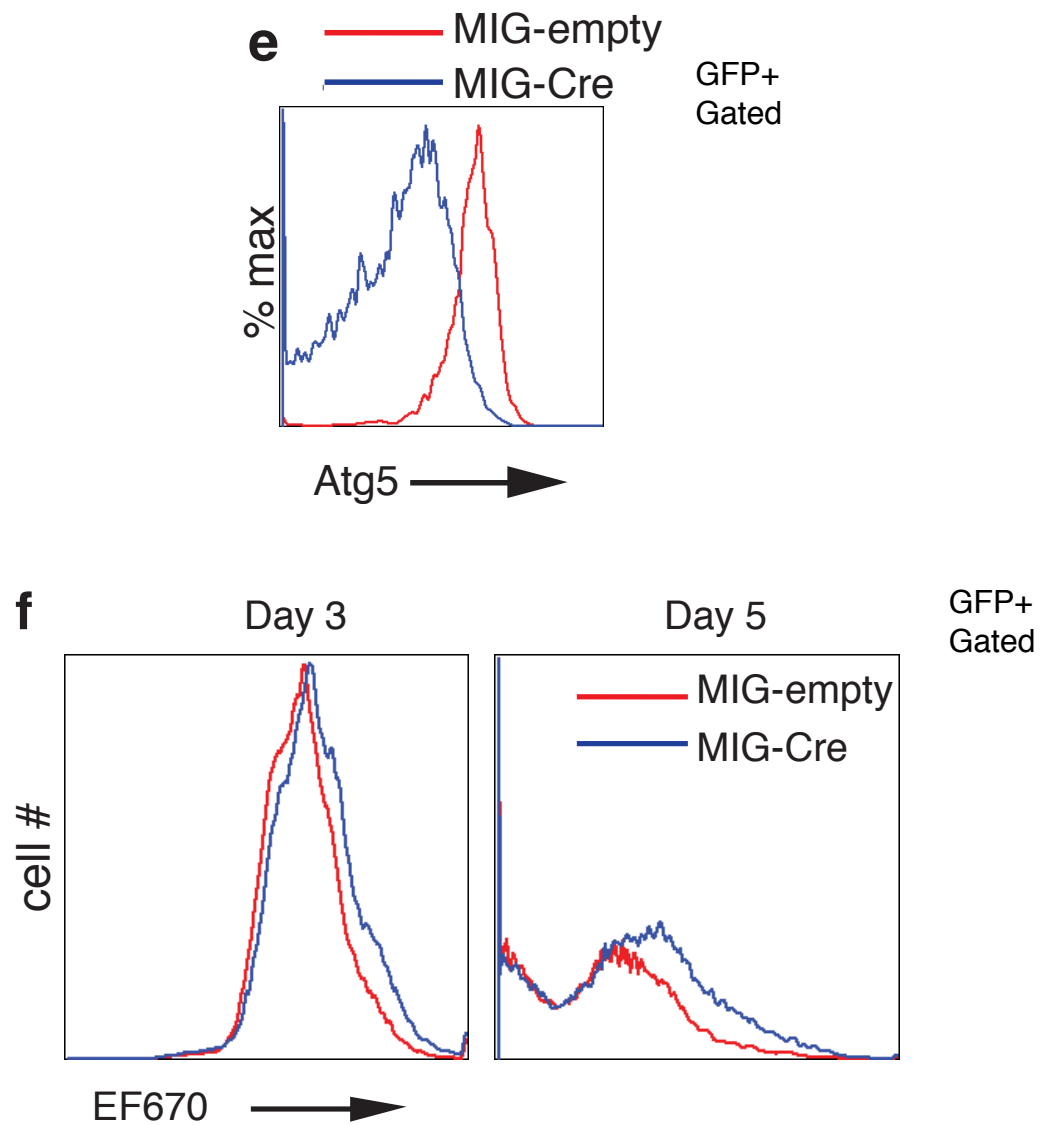


Figure 3.3 (e) Atg5 deletion is validated in MIG-Cre infected cells. Atg5^{f/f} and wild type T cells were labeled with EF670 and CD3/28 activated. At 24h and 48h post-activation, cells were retrovirally infected with MIG-Cre and MIG-empty and harvested at Day 3 and Day 5 post-activation. Deletion of Atg5 was confirmed by FACS, and **(f) Autophagy is not required in proliferating T cells.** Proliferation was analyzed on GFP^{high} (infected) population. Data representative of 3 separate experiments.

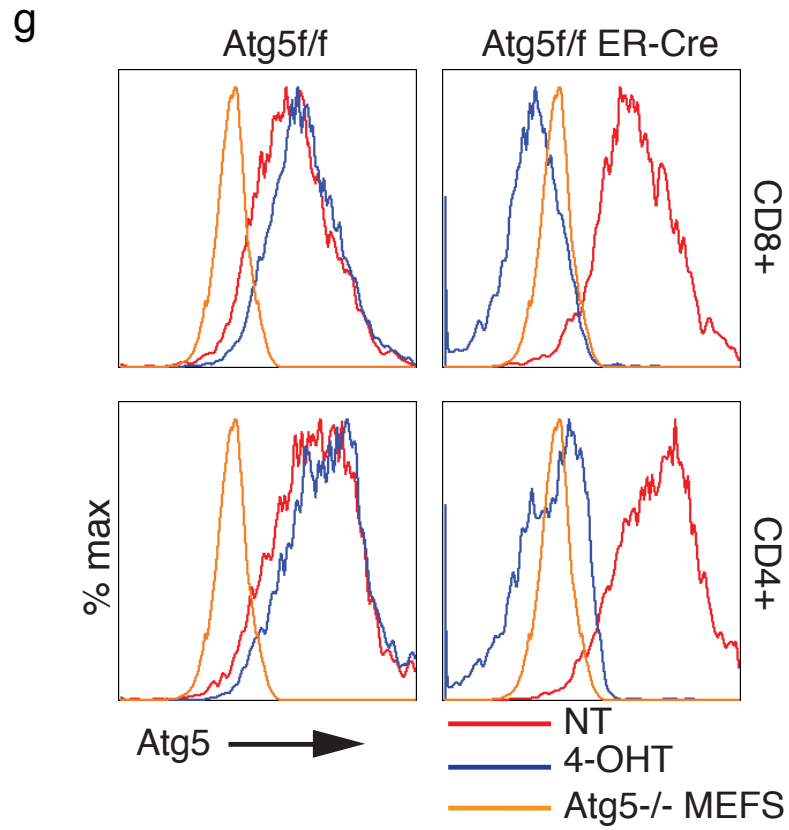


Figure 3.3 (g) Deletion of Atg5 validated in Atg5^{f/f} ER-Cre cells treated with 4-OHT. Atg5^{f/f} ER-Cre and Atg5^{f/f} T cells were activated with CD3-28 and treated with 4-OHT (25 nm) at the start of culture. Deletion was validated by FACS at Day 5 post-activation. Atg5^{-/-} MEFS were used as control.

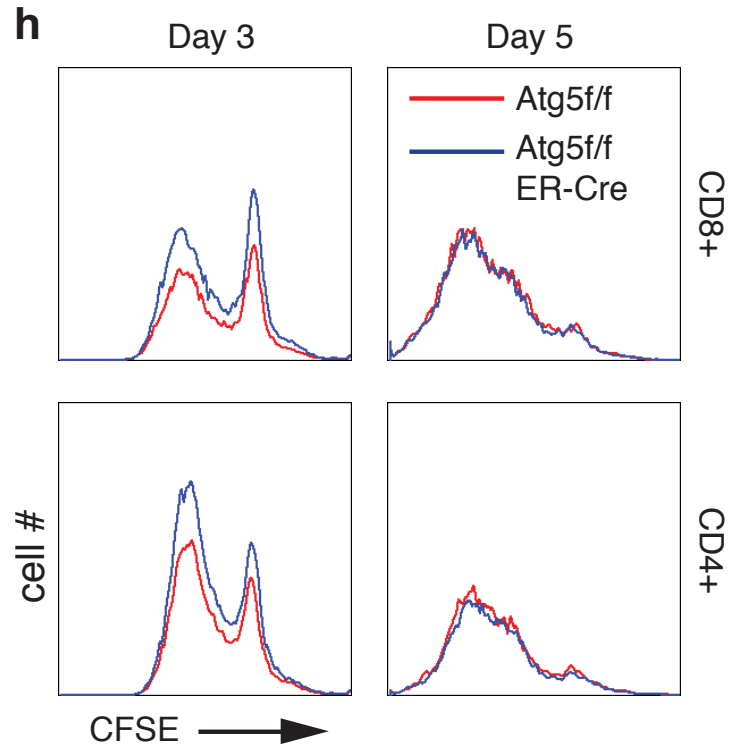


Figure 3.3 (h) Autophagy is not required in proliferating T cells. Atg5^{f/f} ER-Cre and Atg5^{f/f} T cells were activated with CD3-28 and treated with 4-OHT (25 nm). Plots display CFSE histograms at Day 5. Data representative of 3 separate experiments.

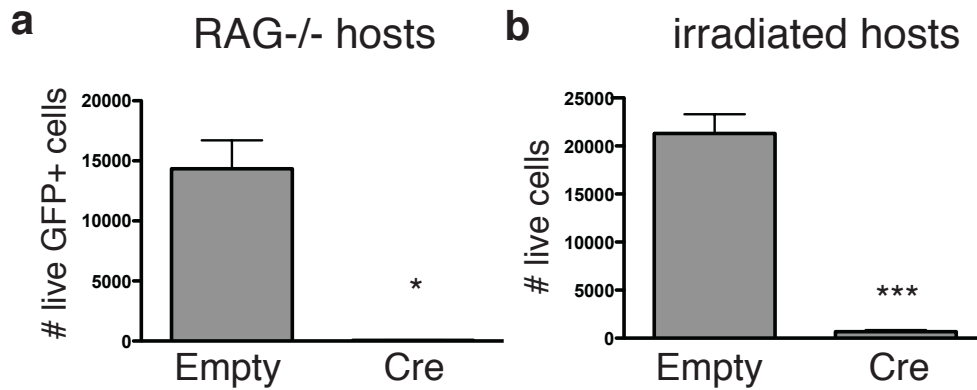


Figure 3.4 $Atg5^{fl/fl}$ CD4-Cre T cells display defect in maintaining homeostasis. (a) Autophagy is required for naïve T cell survival. $Atg5^{fl/fl}$ T cells were labeled with EF670, CD3/28 activated, and infected with MIG-Cre or MIG-empty retrovirus. Cells were harvested at Day 3 post-activation for adoptive transfer into RAG-/- hosts. Spleens were harvested 7 days post-transfer for analysis of transferred, GFP+ cells. Graph represents number of transferred cell recovered $n = 3$. **(b)** Congenically marked mice were irradiated with a sublethal dose of 600cGy, followed by adoptive transfer of 2.0×10^6 MIG-cre or MIG-empty infected cells. Spleens were harvested 7 days post-transfer for analysis of transferred cells. Graph represents number of transferred cell recovered. $n = 4$. Considered a significant difference with respect to empty control (*, $p > 0.05$, ***, $p < 0.001$). Error bars represent SEM.

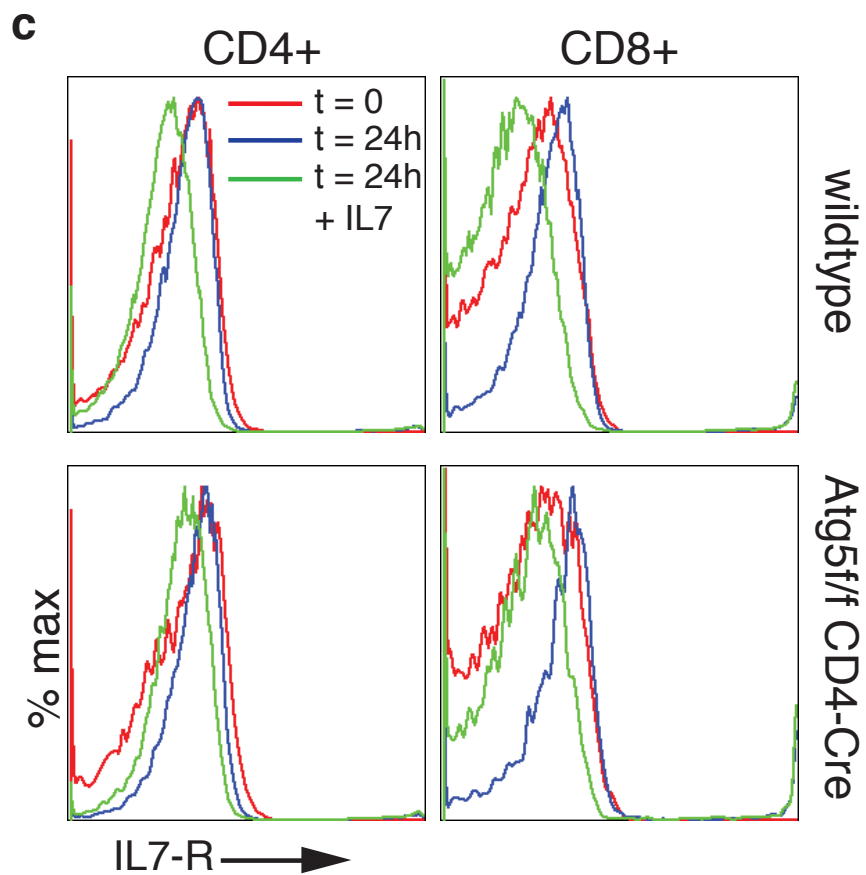


Figure 3.4 (c) *Atg5* is not required for IL7R recycling. Naïve *Atg5^{f/f}* CD4-Cre T cells were cultured +/- IL7 and stained for CD127 expression at t = 0 and t = 24h.

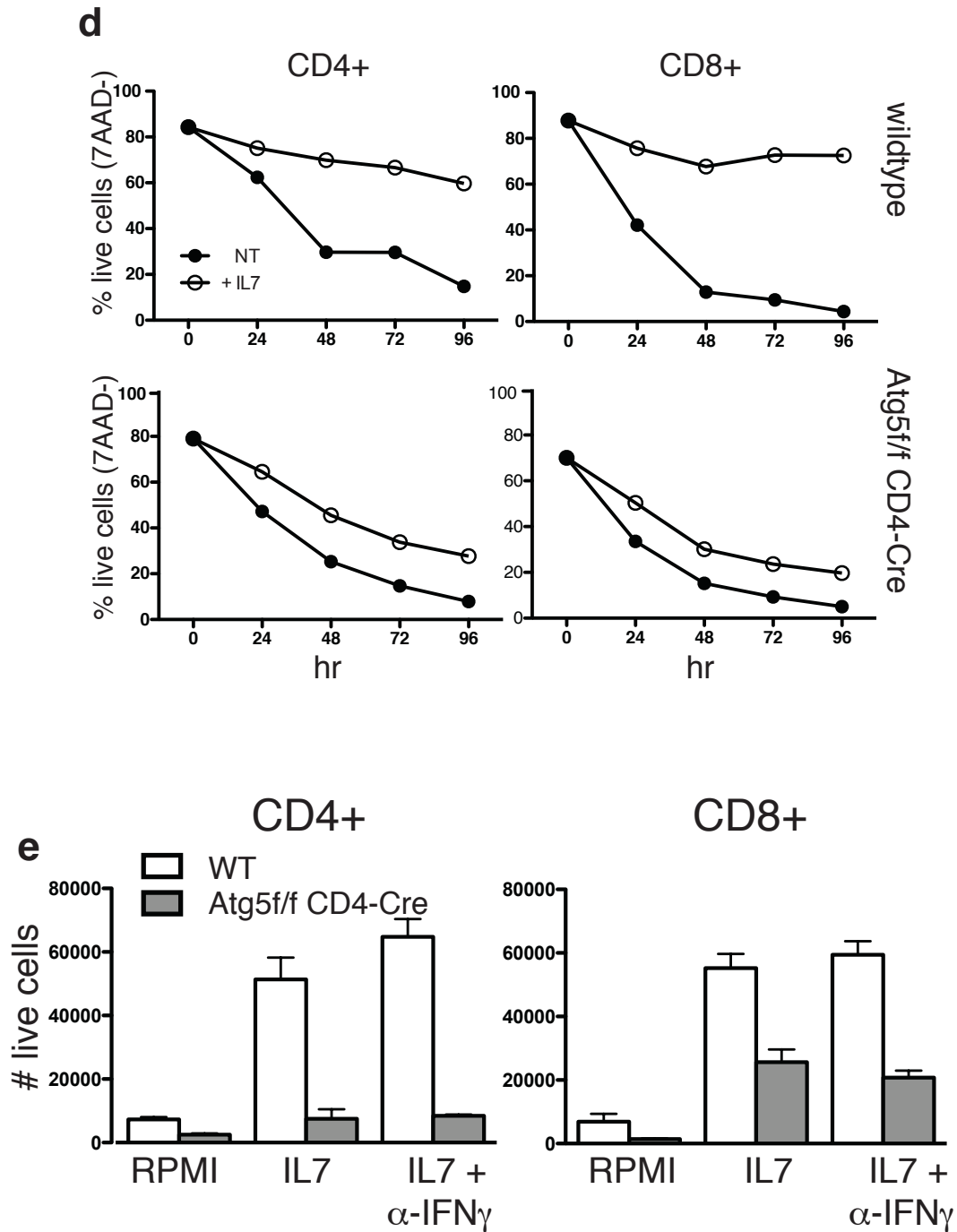


Figure 3.4 (d) IL7 is not sufficient to maintain *Atg5*^{-/-} T cell survival. Naïve *Atg5^{f/f} CD4-Cre* and wild type T cells were cultured +/- IL7 and viability was analyzed (7AAD- cells). Graph displays % live CD4+ or CD8+ T cells at each time point. **(e) *Atg5*^{-/-} T cell death not rescued with α -IFN γ .** Naïve *Atg5^{f/f} CD4-Cre* and wild type T cells were cultured with IL7 and +/- α -IFN γ over 5 days. Graph represents # of live CD4+ or CD8+ T cells.

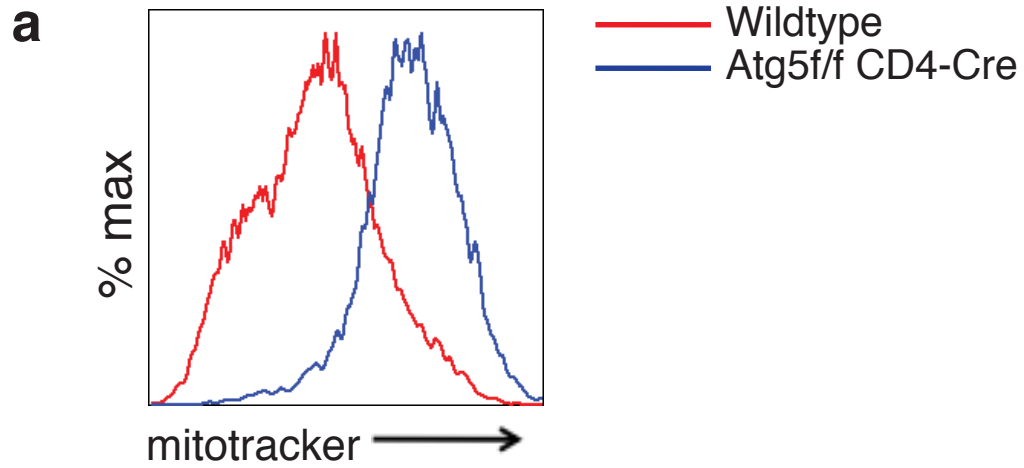


Figure 3.5 Activated, proliferating T cells clear excess mitochondria through cell division.
(a) Increased mitochondrial levels in naïve $Atg5^{f/f}$ CD4-Cre T cells. Freshly isolated $Atg5^{f/f}$ CD4-Cre and wild type T cells were stained with mitotracker. Histogram represents MFI of mitotracker.

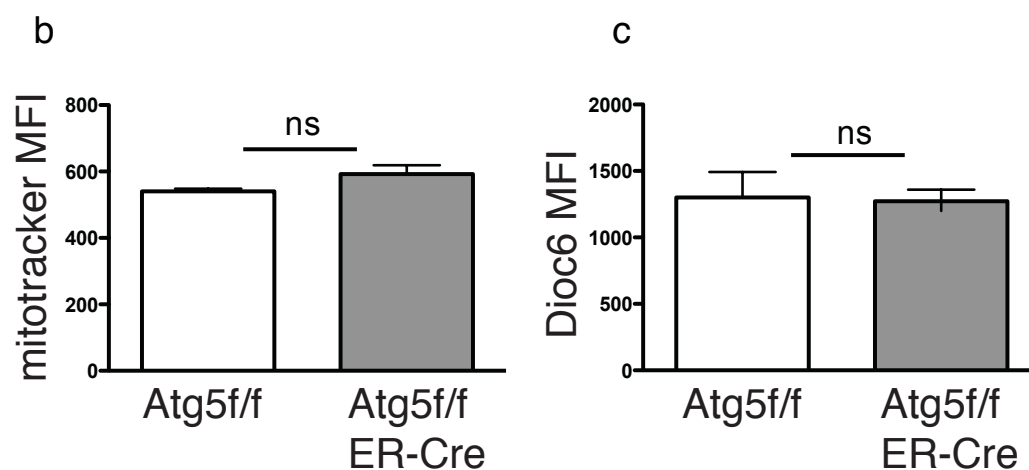


Figure 3.5 (b) Activated Atg5^{f/f} CD4-Cre T cells do not display increased mitochondria. ^{/f}
ER-Cre T cells were activated with CD3-28. 4-OHT was added at start of culture. Graph represents MFI of mitotracker or (c) Dioc6 at Day 5.

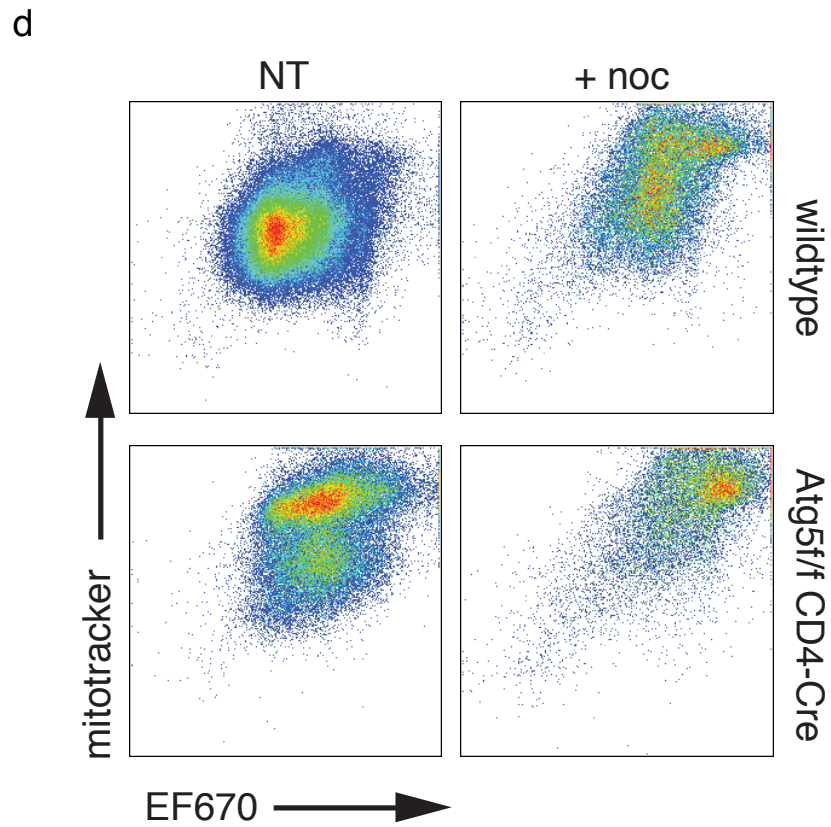


Figure 3.5 (d) Blocking cell cycle leads to increased mitochondria in *Atg5^{f/f}* CD4-Cre T cells. *Atg5^{f/f}* CD4-Cre T cells were activated with CD3-28. Nocodazole (0.25 ng/ml) was added 24h post-activation. Day 5 post-activation, cells were harvested and analyzed by EF670 and mitotracker. Data representative of 3 separate experiments.

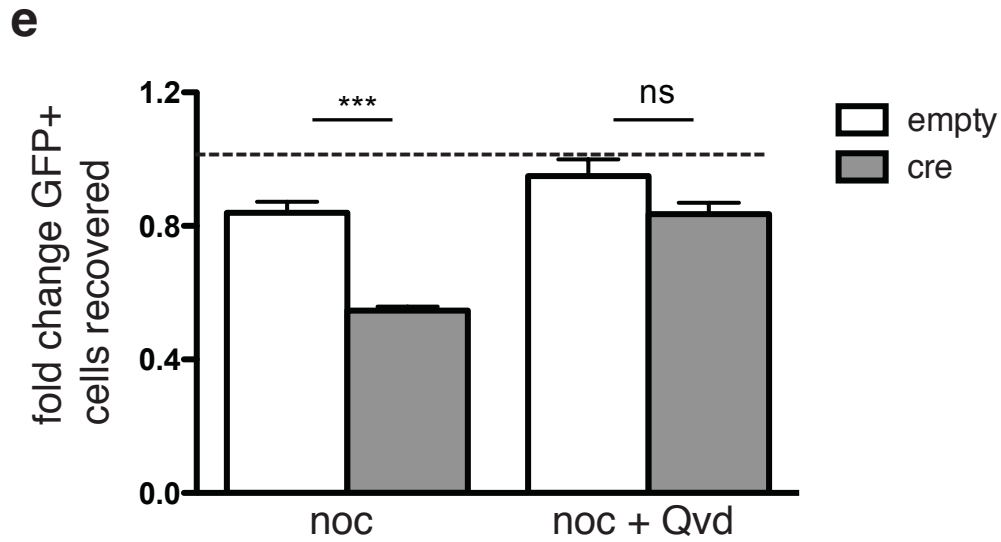
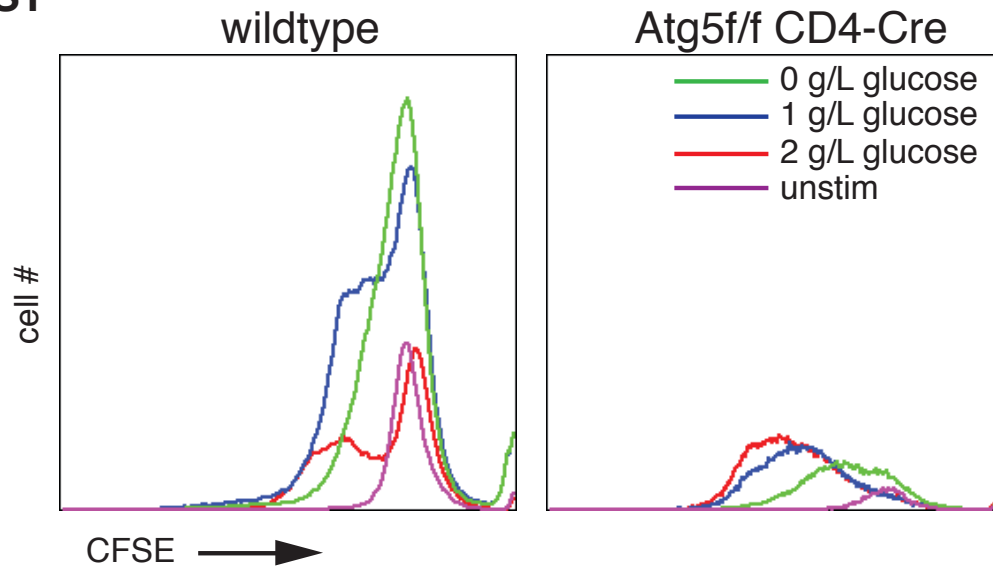
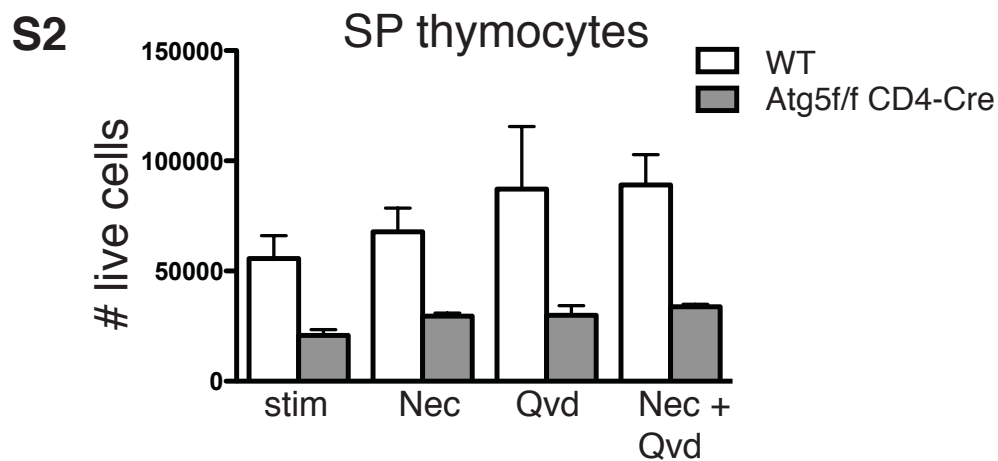


Figure 3.5 (e) Cell cycle blockade leads to enhanced death in autophagy-deficient T cells. *Atg5^{f/f}* T cells were activated with plate-bound anti-CD3 and CD28 and infected with MIG-Cre or MIG-empty. Nocodazole was added 24 hour post-activation and Qvd was added at the start of culture. Graph displays fold-change in live GFP+ cells relative to untreated control (dotted line). Considered a significant difference with respect to empty control (***, $p < 0.001$). Error bars represent SEM.

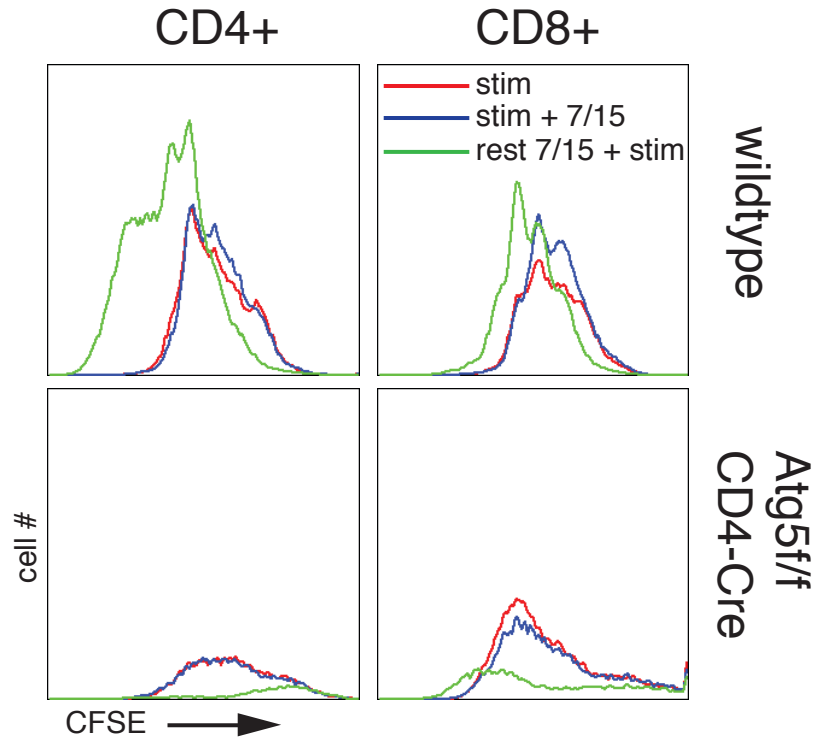
S1

Supplementary Figure 3.S1 *Atg5^{f/f}* CD4-Cre and wild type T cells were activated with 200 ng plate-bound CD3 and 200 ng/ml soluble CD28 in 0, 1, or 2 g/L glucose- containing media. Plots represent CFSE histogram at Day 3 post-activation.

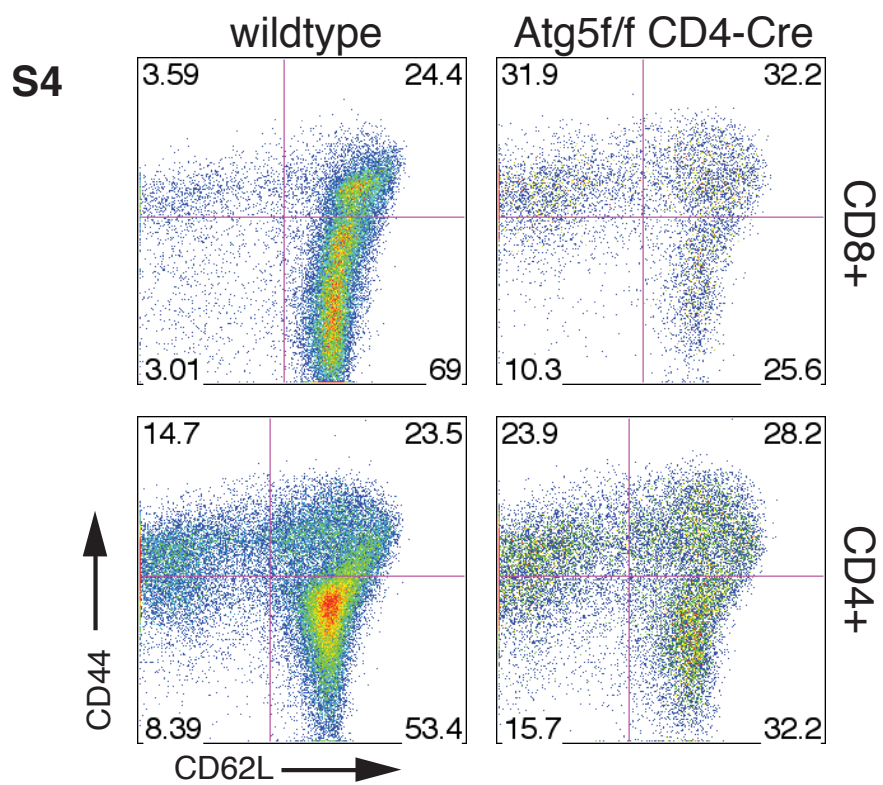


Supplementary Figure 3.S2 Atg5^{f/f} CD4-Cre and wild type thymocytes were activated with 200 ng plate-bound CD3 and 200 ng/ml soluble CD28 and treated with Nec-1, Qvd, or both. Graph represents number of live, single positive (SP) thymocytes at Day 3 post-activation.

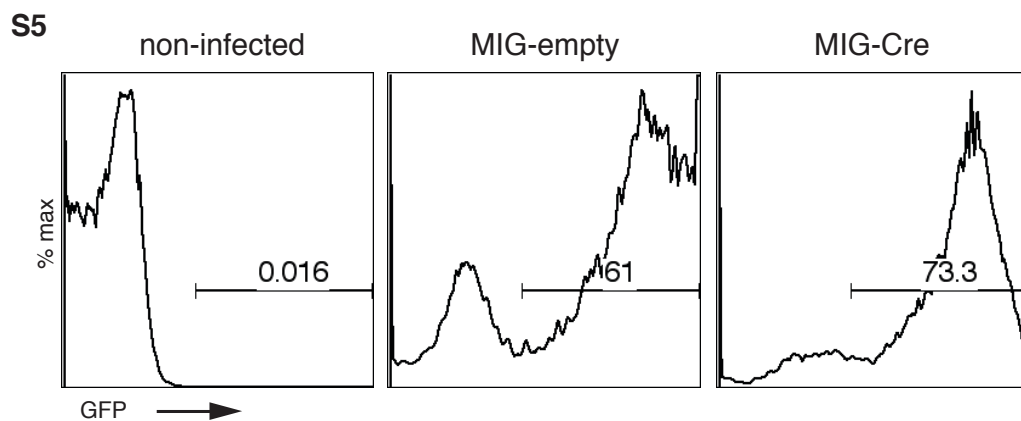
S3



Supplementary Figure 3.S3 *Atg5^{f/f}* CD4-Cre and wild type T cells were activated or rested with IL7+IL15 for 3 days prior to activation. Plots represent CFSE histogram Day 3 post-activation.

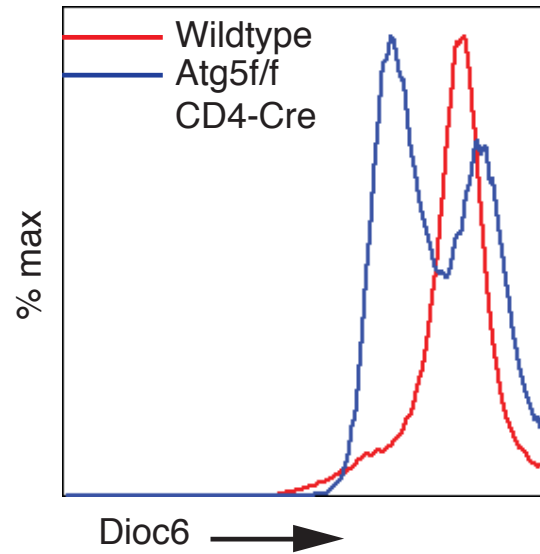


Supplementary Figure 3.S4 Naïve *Atg5^{f/f}* CD4-Cre and wild type T cells were stained with CD44 and CD62L. Number represents percent population in each quadrant.

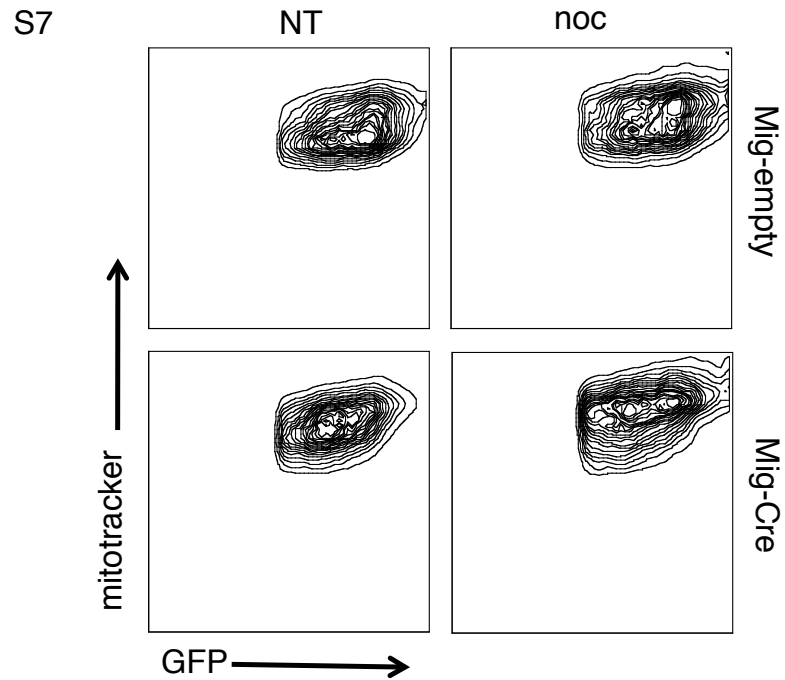


Supplementary Figure 3.S5 *Atg5^{fl/fl}* T cells were CD3/28 activated and retrovirally infected with MIG-empty and MIG-Cre at 24h post-activation. Plot represents % GFP+ (infected) cells prior to adoptive transfer at Day 3 post-activation.

S6

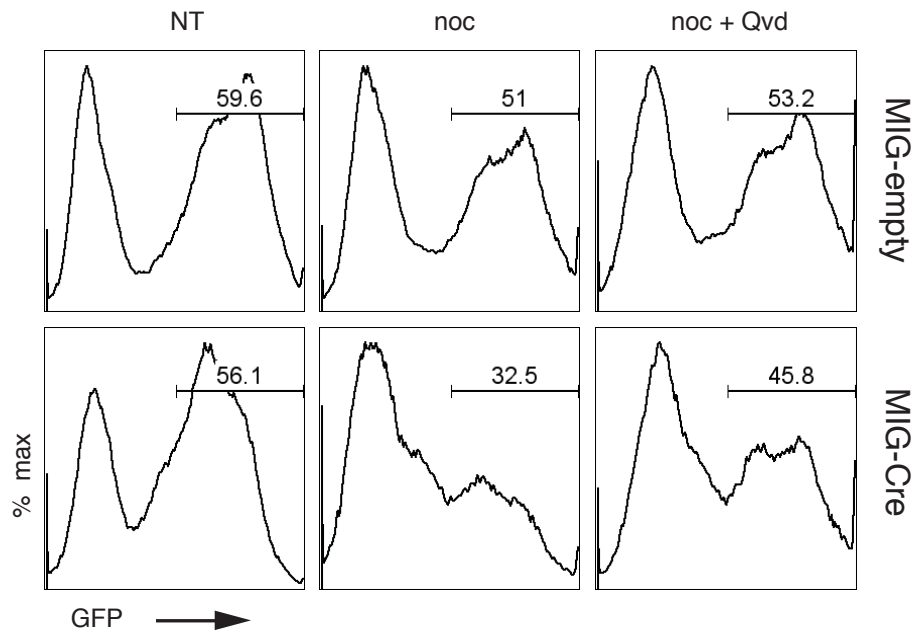


Supplementary Figure 3.S6 Atg5^{f/f} CD4-Cre and wild type T cells were activated with 200 ng plate-bound CD3 and 200 ng/ml soluble CD28. Histogram represents Dioc6 MFI 3 days post-activation.



Supplementary Figure 3.S7 *Atg5^{f/f}* T cells were CD3/28 activated and retrovirally infected with MIG-empty and MIG-Cre. Nocodazole was added at 24h and cells were gated on GFPhi population and analyzed by mitotracker and GFP 5 days post-activation.

S8



Supplementary Figure 3.S8 *Atg5^{+/+}* T cells were CD3/28 activated and retrovirally infected with MIG-empty and MIG-Cre. Nocodazole and Qvd were added at 24h. Number represents % of GFP+ (infected) cells 5 days post-activation.

Materials and Methods

Mice. Atg5^{f/f} mice were crossed with Rosa26-Cre-ER^{T2} mice. Rag2^{-/-} x gc, C57BL6/J (“B6”), B6.SJL-Ptprc^a Pepc^b/BoyJ, and Rosa26-Cre-ER^{T2} mice were obtained from Jackson Labs. Mice were bred and maintained in accordance with the institutional animal use and care committee at the University of California Irvine vivarium.

T cell activation. 24 well dishes were coated with 200 ng plate-bound anti-CD3, plus 200 ng/ml soluble anti-CD28 in 1 ml RPMI media supplemented with 10% fetal calf serum (Omega Sciences), sodium pyruvate, 1X non-essential amino acids, 1X pen/strep/glutamine and beta mercaptoethanol (2×10^{-5} M). Splenocytes were isolated, treated to lyse erythrocytes, and plated in 24 well dishes at 2×10^6 per well. 10 μ M Nec-1, 20 μ M QVD, methyl pyruvate (5.5 μ M) were added at start of culture. BHA (150 μ M), nocodazole (0.25 ug/ml) were added at 24 hrs post activation.

Adoptive Transfer. CD4⁺ and CD8⁺ cells from B6 and Atg5^{-/-} mice were isolated by depletion of B220⁺, CD11b⁺ and MHCII⁺ cells with magnetic microbeads (Invitrogen), and injected intravenously at a dose of 2.0×10^6 cells per mouse.

BrdU Incorporation. Mice were given I.P. injections of 200 μ l 10 mg/mL of BrdU every 24 hours. Mice were euthanized after 2 days and single cell suspensions were made from the spleen. T cells were surface stained with anti-CD4 and anti-CD8, followed by intracellular staining with anti-BrdU Abs.

Retroviral Infection. To generate retroviral supernatants, 20 mg pMIG-vector plus 20 mg psi-Eco retroviral packaging vector were co-transfected into 293T using 20mM CaCl₂ and HBS. 5 ml fresh DMEM complete was added 12 h later and then substituted for 6 ml RPMI complete another 12 h later. For the next 2 days, every 12 h, media-containing virus was collected and replaced by fresh RPMI complete. For infecting primary T cells, purified T cells were first labeled with 5 μ M CFDA-SE (Molecular Probes, Eugene, OR, USA) for 8 min and activated as described above for 24 h. Cells were then transduced with 1ml of viral supernatant plus 4mg/ml polybrene and spun at 1800 r.p.m. for 1 h at room temp. 24h post-infection, cells were re-plated with 50U/ml IL-2.

Irradiation. Mice received sub-lethal irradiation at a dose of 600 cGy using an x-irradiator.

Mitochondrial and ROS Staining. Splenocytes were incubated with 300 nm mito-tracker green, 40 μ M DioC6, or 370 nm HE for 20 min. in complete RPMI at 37C.

In vitro inducible Atg5 deletion. Splenocytes from Atg5^{fl/fl}ER-Cre mice were cultured with 50 nm 4-OHT (Sigma). Deletion was confirmed by intracellular staining using anti-Atg5 antibody (Novus).

References

1. Klionsky, D.J. and S.D. Emr, *Autophagy as a regulated pathway of cellular degradation*. Science, 2000. **290**(5497): p. 1717-21.
2. Geng, J. and D.J. Klionsky, *The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy*. 'Protein modifications: beyond the usual suspects' review series. EMBO Rep, 2008. **9**(9): p. 859-64.
3. Nakagawa, I., et al., *Autophagy defends cells against invading group A Streptococcus*. Science, 2004. **306**(5698): p. 1037-40.
4. Gutierrez, M.G., et al., *Autophagy is a defense mechanism inhibiting BCG and Mycobacterium tuberculosis survival in infected macrophages*. Cell, 2004. **119**(6): p. 753-66.
5. Li, C., et al., *Autophagy is induced in CD4+ T cells and important for the growth factor-withdrawal cell death*. J Immunol, 2006. **177**(8): p. 5163-8.
6. Bell, B.D., et al., *FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells*. Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16677-82.
7. Lu, J.V. and C.M. Walsh, *Programmed necrosis and autophagy in immune function*. Immunol Rev, 2012. **249**(1): p. 205-17.
8. Hubbard, V.M., et al., *Macroautophagy regulates energy metabolism during effector T cell activation*. J Immunol, 2010. **185**(12): p. 7349-57.
9. Pua, H.H., et al., *Autophagy is essential for mitochondrial clearance in mature T lymphocytes*. J Immunol, 2009. **182**(7): p. 4046-55.
10. Stephenson, L.M., et al., *Identification of Atg5-dependent transcriptional changes and increases in mitochondrial mass in Atg5-deficient T lymphocytes*. Autophagy, 2009. **5**(5): p. 625-35.
11. Sprent, J. and C.D. Surh, *Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells*. Nat Immunol, 2011. **12**(6): p. 478-84.
12. Fox, C.J., P.S. Hammerman, and C.B. Thompson, *Fuel feeds function: energy metabolism and the T-cell response*. Nat Rev Immunol, 2005. **5**(11): p. 844-52.
13. Lum, J.J., et al., *Growth factor regulation of autophagy and cell survival in the absence of apoptosis*. Cell, 2005. **120**(2): p. 237-48.
14. Jia, W. and Y.W. He, *Temporal regulation of intracellular organelle homeostasis in T lymphocytes by autophagy*. J Immunol, 2011. **186**(9): p. 5313-22.

15. Radoshevich, L., et al., *ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death*. Cell, 2010. **142**(4): p. 590-600.
16. Williams, M.S. and J. Kwon, *T cell receptor stimulation, reactive oxygen species, and cell signaling*. Free Radic Biol Med, 2004. **37**(8): p. 1144-51.
17. Schwartz, R.H., *T cell anergy*. Annu Rev Immunol, 2003. **21**: p. 305-34.
18. Greiner, E.F., M. Guppy, and K. Brand, *Glucose is essential for proliferation and the glycolytic enzyme induction that provokes a transition to glycolytic energy production*. J Biol Chem, 1994. **269**(50): p. 31484-90.
19. Frauwirth, K.A., et al., *The CD28 signaling pathway regulates glucose metabolism*. Immunity, 2002. **16**(6): p. 769-77.
20. Chetoui, N., et al., *Interleukin-7 promotes the survival of human CD4+ effector/memory T cells by up-regulating Bcl-2 proteins and activating the JAK/STAT signalling pathway*. Immunology, 2010. **130**(3): p. 418-26.
21. Wherry, E.J., et al., *Antigen-independent memory CD8 T cells do not develop during chronic viral infection*. Proc Natl Acad Sci U S A, 2004. **101**(45): p. 16004-9.
22. Willinger, T. and R.A. Flavell, *Canonical autophagy dependent on the class III phosphoinositide-3 kinase Vps34 is required for naive T-cell homeostasis*. Proc Natl Acad Sci U S A, 2012. **109**(22): p. 8670-5.
23. Kimura, M.Y., et al., *IL-7 signaling must be intermittent, not continuous, during CD8(+) T cell homeostasis to promote cell survival instead of cell death*. Nat Immunol, 2013. **14**(2): p. 143-51.
24. Mortensen, M., et al., *Loss of autophagy in erythroid cells leads to defective removal of mitochondria and severe anemia in vivo*. Proc Natl Acad Sci U S A, 2010. **107**(2): p. 832-7.

CHAPTER 4

Necrosome Formation Following T cell receptor ligation

Abstract

Necroptosis is a programmed form of death that is typically induced when conventional caspase-dependent apoptosis is blocked. When caspase 8 activity is compromised, RIPK1 recruits RIPK3 to the DISC, resulting in the generation of necroptotic-inducing complexes, “necrosomes” [1]. Currently, it is clear that necrosomes are formed not simply due to TNF ligation, but also in response to TCR ligation in dividing T cells [2]. We utilize the Proximity Ligation Assay (PLA) in our studies, which will reveal not only the kinetics of necrosome formation, but also the subcellular localization and constituency of these necrosomes. Using the PLA, we show preliminary data that suggest the necrosome assembles 24-36 hours post-activation in T cells, and this finding correlates with the induction of caspase 8 activity at 24 hours. Treatment with cell cycle inhibitors appears to block the generation of necrosomes, implicating an undiscovered role for necroptosis as a potential cell cycle checkpoint in proliferating T cells. These findings potentially extend to other conditions and cell types in which necrosomes are formed as well.

Introduction

DR pathways regulate apoptotic signaling and are essential for immune homeostasis and tolerance. Ligation of DR, CD95/Fas/Apo-1, triggers formation of a cytosolic complex known as the “death-inducing signaling complex” (DISC) [3], or complex II, which includes adaptor molecule FADD (Fas associated with death domain), RIPK1, caspase-8, and a caspase-8-like

molecule that lacks proteolytic activity called c-FLIP [4]. Upon DR engagement, FADD is recruited to the DR by its DD, where it then recruits and activates casp8 via its death effector domain (DED) interactions [5, 6]. Casp8 autocleavage and self-activation [7] ultimately releases complex II to the cytosol to propagate apoptotic signaling. In FADD^{-/-} Jurkats lacking caspase 8 activity [8], treatment with TNF α induces a rapid form of cell death that depends on the catalytic activity of RIPK1 [9], a serine-threonine kinase that contains a C-terminal death domain [10]. Yuan and colleagues developed a RIPK1-specific kinase inhibitor (Necrostatin-1/Nec-1) and have demonstrated that Nec-1 completely blocks the death of FADD-deficient Jurkats treated with TNF α [11, 12]. We and others found that the defective clonal expansion of casp8^{-/-} and FADD^{dd}-expressing T cells was rescued by Nec-1 treatment [13, 14] implicating RIPK1 in the necroptotic death of T cells lacking the ability to activate FADD and caspase 8 following antigenic stimulation [15]. Subsequently, three independent groups found that RIPK3, a RIPK1 homologous kinase, is also required for TNF α -induced necroptosis [16-18]. In the absence of caspase 8 activity, RIPK1 recruits RIPK3 resulting in the generation of a necroptotic-inducing complex, termed the necrosome, leading to cross-phosphorylation of the kinases, and subsequently transduce a downstream signal that promotes necroptosis [19]. The mechanism of necroptotic regulation involves cleavage of RIPK1 by caspase 8, as non-cleavable RIPK1 dominantly promotes the necroptotic pathway in Jurkats treated with TNF α [20].

The necroptotic death pathway can be activated through various stimuli and different cell surface receptors (TNF, TCR, TLR3-4, genotoxic stress, RIG-1, DAI), and these signaling pathways converge at the formation of the RIPK1-RIPK3-containing necrosome. Thus, characterizing the platform(s) that promote recruitment of RIPK1-RIPK3 complex in T cells by identifying the kinetics, constituency, and subcellular localization of the necrosome will help us

understand the cellular events where these complexes develop physiologically. Furthermore, these findings can be extended to other systems and cell types where RIPK1-RIPK3-driven necroptosis occurs.

Results

We have shown in Chapter 2 that RIPK1-RIPK3 complex forms in response to TCR stimulation to promote necroptotic signaling, and in this chapter, we determine the means of its assembly. The necrosome can similarly be induced under various circumstances and in differing cell types. However, this has only been characterized in the context of the Jurkat system in response to TNF α treatment, and in the absence of caspase 8 activity. The signals and mechanism that drive RIPK1-RIPK3 association during TCR stimulation are unknown, thus we aim to identify these events and characterize the molecular components of the necrosome.

We established that TCR stimulation recruits the necrosome in Chapter 2, thus an important question is the temporal kinetics of necrosome assembly in response to TCR stimulation. To define the temporal regulation of necrosome formation in activated lymphocytes, we determined the time point at which Nec-1 (RIPK1 inhibitor) must be added to block necroptosis. We added or washed away Nec-1 from activated FADD^{+/+} T cells, between 0h-48h post-activation, and observed the strongest rescue when Nec-1 was added 24h post-activation. (**Fig. 4.1**) Addition of Nec-1 prior to plate-bound CD3 activation resulted in less rescue compared to later treatment, which we do not believe is a result of Nec-1 instability in culture, but treatment at 48h after stimulation was too late to reverse necroptotic death. It has been suggested that casp8 is recruited to the Bcl-2/Carma/Malt-1 (BCM) complex following TCR signaling [21-23], which would indicate necrosome formation at around 30 minutes after

activation, although we did not observe any defect in TCR proximal NF- κ B activation [24]. Our initial findings suggest RIPK1-RIPK3 association may not associate with an early TCR signaling event.

To support our findings above, we utilized proximity ligation assays (PLA) [25] to study RIPK1 and RIPK3 interaction (**Fig. 4.2**). This method is more sensitive than co-immunoprecipitation (co-IP), and involves the use of fluorescence microscopy to detect protein interactions. Because it is conducted *in situ*, the subcellular localization of the complex can be determined and there are less artifacts compared to cell lysates. We activated OTI-FADD^{td} cells with OVA and monitored the progression of the necrosome from 0h to 48h post-activation. To validate that inhibition of RIPK1 activity prevents its interaction with RIPK3, we conducted these assays in the presence or absence of Nec-1. Over time, we were able to directly visualize the timepoint at which necrosomes are generated. Our preliminary data indicates the necrosome assembles between 24h and 48h post-activation, and is not seen when Nec-1 is added (**Fig. 4.3**). Recent reports from Chan et al. suggest RIPK1 and RIPK3 form beta-amyloid, filament-like structures [26]. Our data show the RIPK1/RIPK3 signal as punctate and cytosolic/peri-nuclear; however, Chan et al. utilized methods involving overexpression of RIP kinases in cells. The necrosome appears to be present throughout the cytosol and absent in the nucleus.

We next evaluated the involvement of TCR signaling pathways that could potentially regulate its formation. Our initial findings suggest that necroptosis is not initiated until 24h after TCR stimulation, which possibly corresponds with when T cells begin their initial burst of rapid expansion. In parallel, we have observed caspase 8 activity to increase in response to mitogenic stimulation [27] (using IETD-AFC fluorogenic cleavage assays) and activity levels are impacted with treatment of cell cycle inhibitors (**Fig. 4.4**). This finding suggests a potential role for

necroptosis during cell cycle transition, and necrosome formation at the 24h time point correlates with when T cells begin undergoing cell division. In support of our hypothesis, FADD has also been observed to interact with spindle fibers driven by CKI α following mitogenic stimulation [28].

To address the role of necroptosis in cell cycle progression, we treated OTI-FADD^{dd} T cells with various cell cycle inhibitors (nocodazole, CDK2 inhibitor, and pan-PI3K/mTOR inhibitor LY that induces G1 cell cycle arrest) and assessed their effects on necrosome formation. BrdU and 7AAD analysis indicates the inhibitors blocked cell cycle at the appropriate stages as defined by the quadrants (**Fig. 4.5a**), and PLA signal was not detectable in OTI-FADD^{dd} T cells when cell cycle is blocked (**Fig. 4.5b**).

Discussion

An important issue regarding the assembly of necrosomal components is related to the timing of this event following TCR/mitogenic stimulation. Given the aforementioned hypotheses about how necrosomes are formed following TCR stimulation, it is essential that the sequence of events be understood. For example, it has been suggested that caspase 8 is itself recruited to the TCR proximal Carma1/Bcl-10/Malt-1 (CBM) complex that is required for the activation of NF- κ B. In contrast to the findings of this study, we failed to observe any defect in TCR proximal NF- κ B activation [24]. Nevertheless, it might be possible that RIPK1 may similarly be recruited to this platform. The kinetics of TCR induced NF- κ B activation, with maximal activation seen within several minutes of TCR stimulation, would thus indicate recruitment of RIPK1 and casp8 during the first 30 min. or so following TCR stimulation. We have attempted to address this possibility by determining the point at which Nec-1 must be added to block necroptosis. We have

found that addition of Nec-1 between 24h and 48h gives full protection to FADD^Δ T cells following stimulation (**Fig. 4.2**). Late addition of Nec-1 appears to provide a more complete rescue from necroptosis than addition of this inhibitor prior to TCR stimulation by plate-bound anti-CD3. Other studies have proposed that early TCR-induced recruitment of RIPK1 and caspase 8 to the CBM complex leads to the generation of necrosomes, and we will address that by extending our PLA studies to include CMB complex members. Additional time course experiments are needed to narrow the time window at which we detect necrosome formation. As well, the limitations of the PLA in detecting lower levels of the complex are unknown.

We note that caspase 8 activity, as measured using IETD-AFC fluorogenic cleavage assays, is induced by mitogenic stimulation, and the levels of activated caspase 8 are impacted by cell cycle blockade, as well as by the pan-PI3K/mTOR inhibitor LY. In parallel, we observed the loss of necrosome formation by PLA in OTI-FADD^Δ T cells when treated with cell cycle inhibitors. These preliminary findings support the idea that cell cycle progression and signaling may be involved in the generation of necrosome platforms that recruit and activate casp8. This potentially correlates with the previous observation of FADD association with spindle fibers driven by CKI α following mitogen stimulation, an issue we will address by treating cells with CKI α inhibitors. Although the PLA is a useful tool in providing localization data of protein-protein interactions, our findings require additional PLA experiments for quantitation and significance purposes. We will also corroborate our PLA findings by performing co-immunoprecipitation experiments in conjunction.

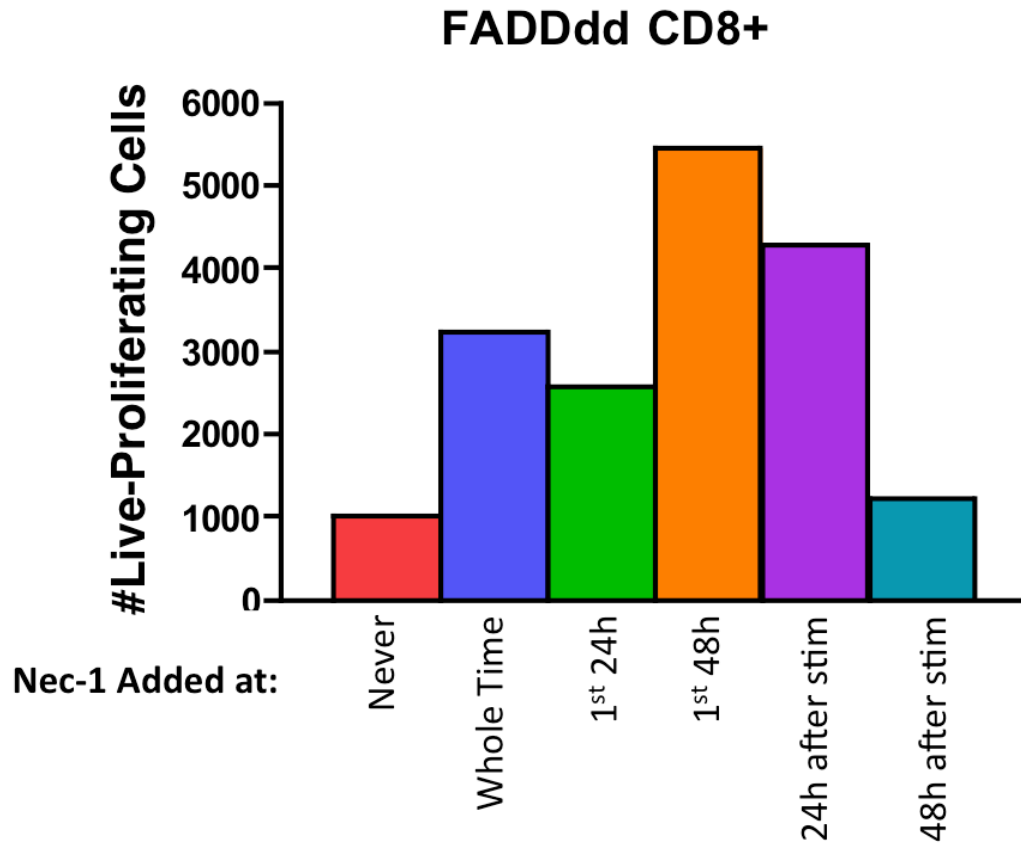


Figure 4.1 RIPK1 activity is maximal at 24h post activation. FADDdd CD8⁺ T cells were labeled with CFSE and then stimulated with anti-CD3 plus anti-CD28 for 72h. Nec-1 was added (10 μ M) for the indicated times. In some cases, Nec-1 was added and washed out at 24 or 48h, or added at 24 or 48h after stimulation.

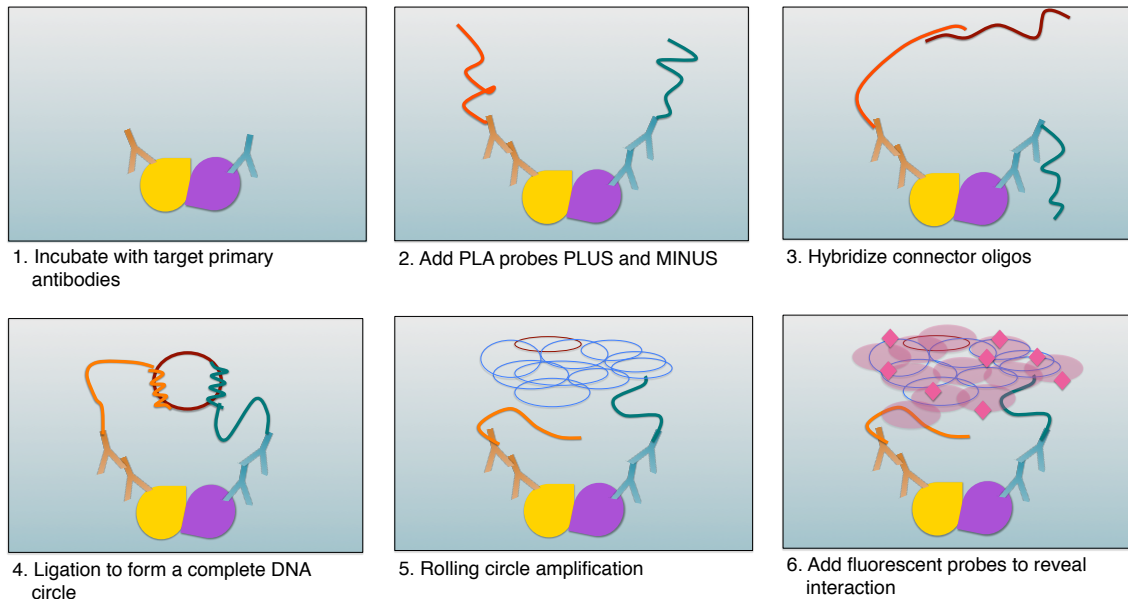


Figure 4.2 Proximity Ligation Assay (PLA) requires primary antibodies that recognize two proteins of interest, and tagged secondary antibodies that bind to the primary antibodies (in this case, RIPK1 and RIPK3). The secondary antibodies are tagged with short DNA “tag” oligos that have homology to “connector” oligos. If the two proteins (e.g. RIPK1 and RIPK3) are in proximity, the connector oligos are capable of hybridizing with the tag oligos, which then are ligated to form a DNA circle. Polymerase is added to induce rolling circle amplification, followed by the addition of short, fluorescently labeled oligo probes that hybridize with the amplified DNA. The amplification step greatly boosts the number of target sites for binding the fluorescently labeled oligos, leading to high sensitivity.

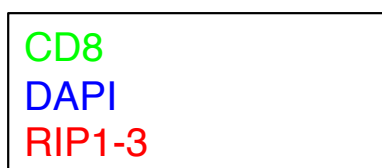
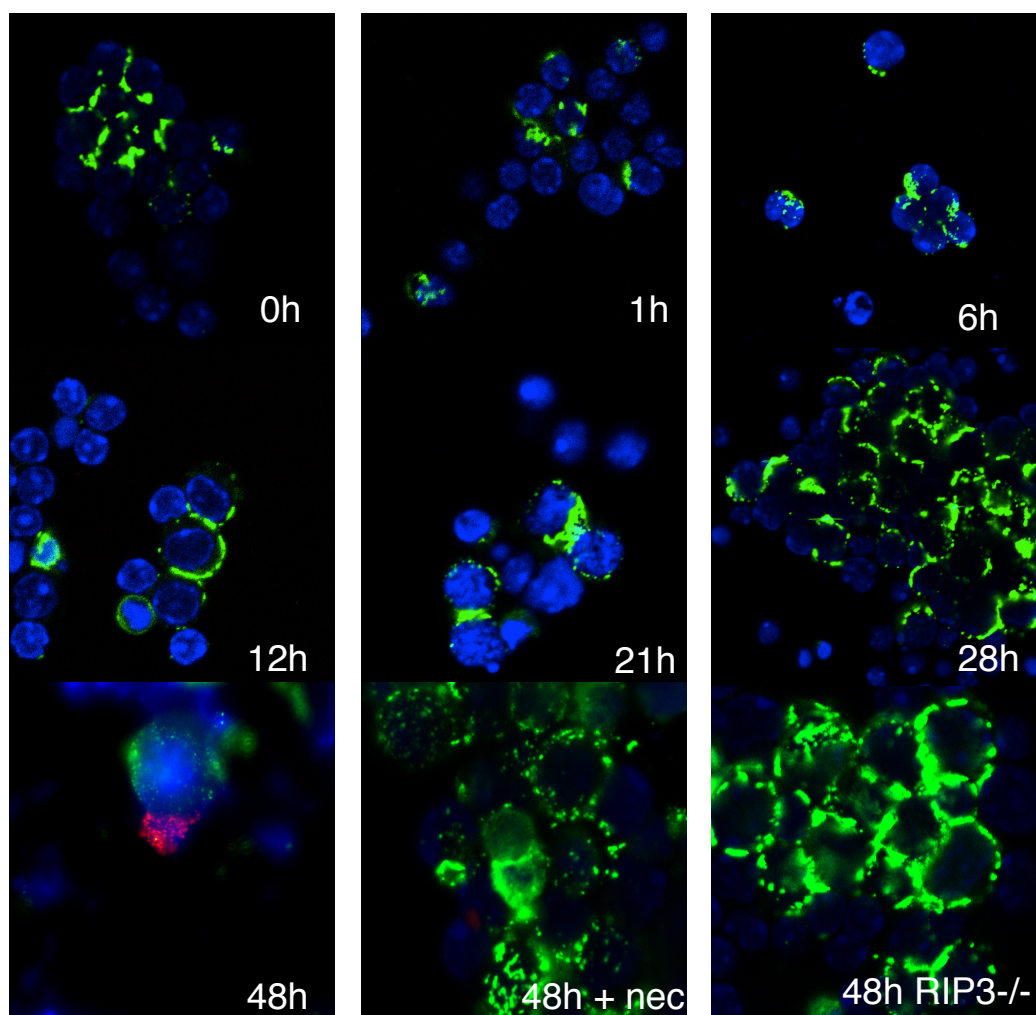


Figure 4.3 Assembly of mitogen-induced RIPK1-RIPK3 necrosomes at 48h post-activation. OTI-FADDdd T cells were activated with 1 μ M OVA +/- Nec-1 and harvested at various time points for PLA analysis. RIPK3^{-/-} T cells were used as control.

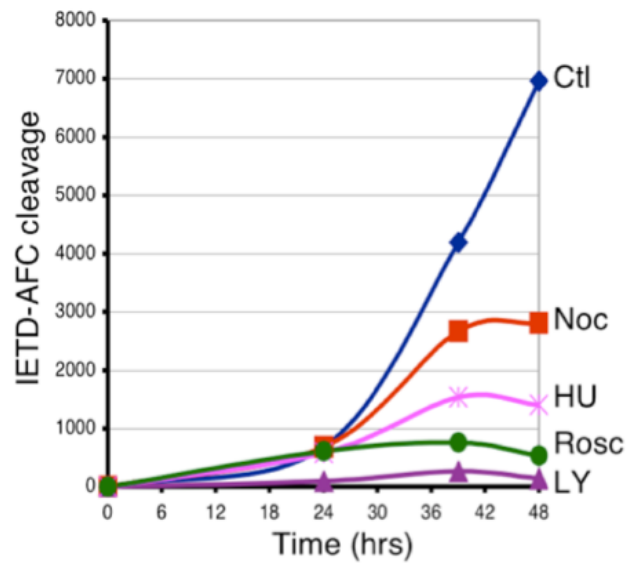


Figure 4.4 Blockade of mitogen-induced casp8 activity by cell cycle inhibitors. WT T cells were activated with anti-CD3 and anti-CD28 in the presence of inhibitors nocodazole (Noc), hydroxyurea (HU), roscovitine (Rosco), and LY. Cells were harvested at indicated times. (Leverrier, S.)

a

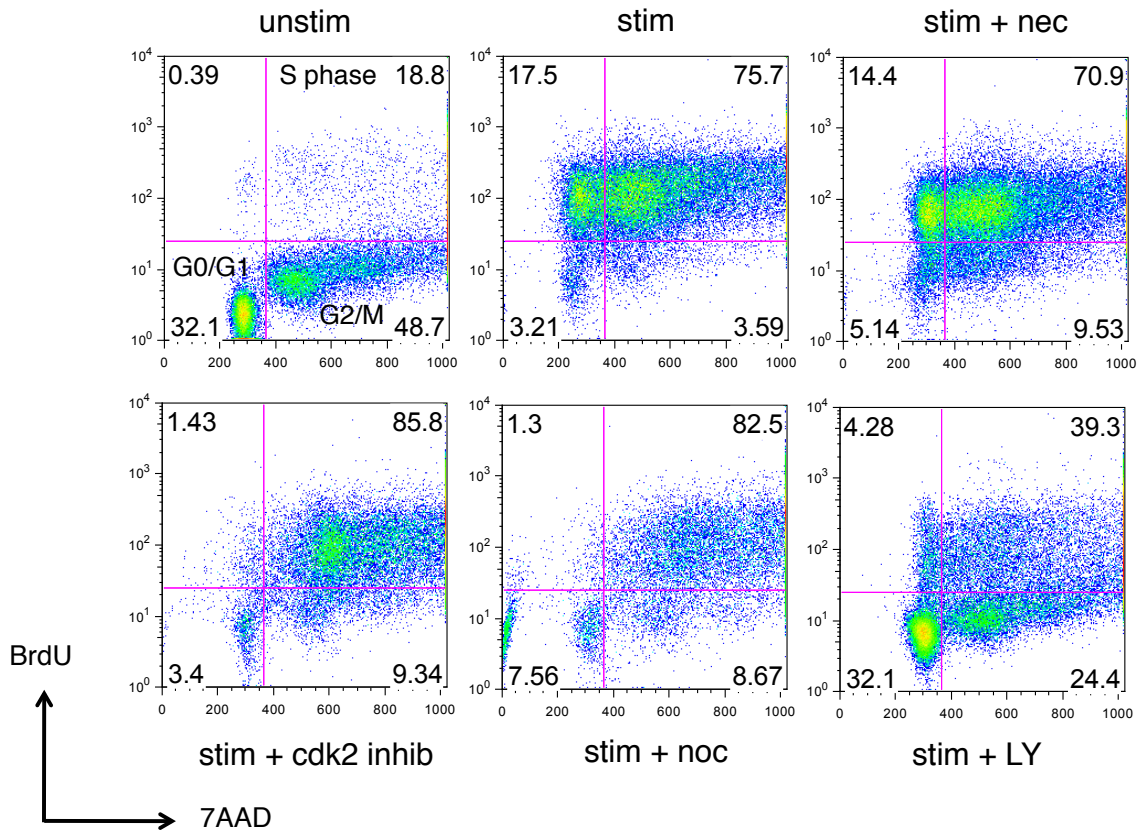


Figure 4.5 Blockade of mitogen-induced RIPK1-RIPK3 necrosome by cell cycle inhibitors.
(a) Cell cycle analysis with various cell cycle inhibitors. OTI-FADDdd T cells were activated with 1 μ M OVA in the presence of inhibitors nocodazole (Noc), LY, and Cdk2 inhibitor. Cells were harvested at 48hr and stained with BrdU and 7AAD for cell cycle analysis by FACS. Quadrants represent G0/G1, S, and G2/M stages as labeled.

b

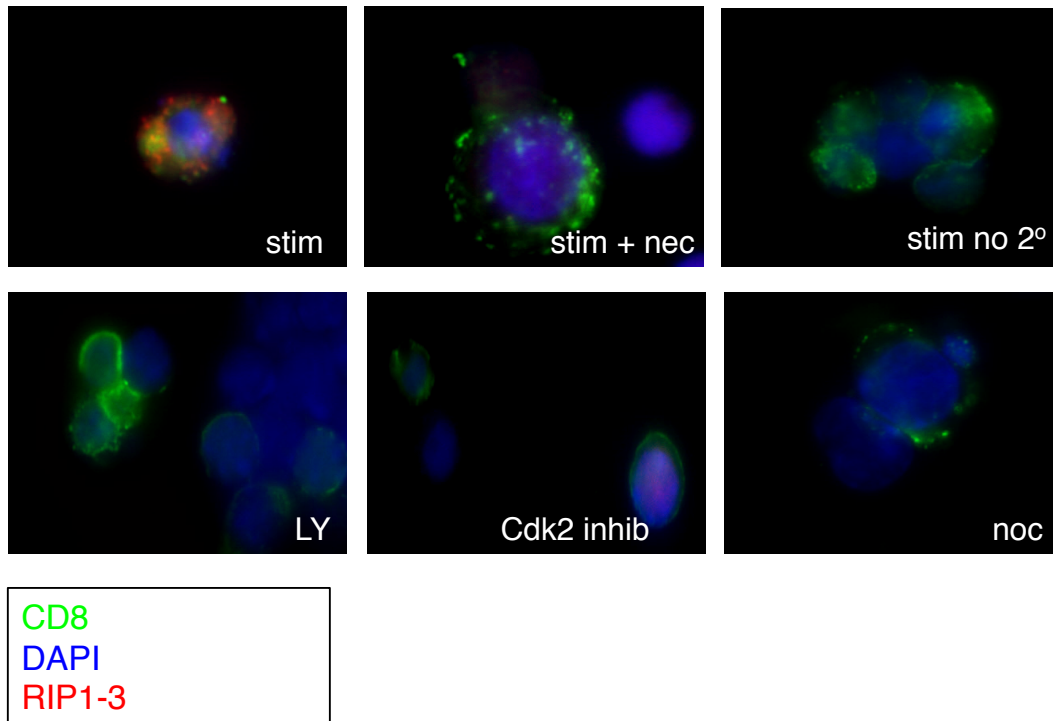


Figure 4.5 (b) Cell cycle inhibitors block RIPK1-RIPK3 PLA signal. OTI-FADDdd T cells were activated with 1 μ M OVA in the presence of inhibitors nocodazole (noc), LY, and Cdk2 inhibitor. Cells were harvested at 48hr for PLA analysis.

References

1. Declercq, W., T. Vanden Berghe, and P. Vandenabeele, *RIP kinases at the crossroads of cell death and survival*. Cell, 2009. **138**(2): p. 229-32.
2. Walsh, C.M. and A.L. Edinger, *The complex interplay between autophagy, apoptosis, and necrotic signals promotes T-cell homeostasis*. Imm. Rev., 2010. **236**: p. 95-109.
3. Kischkel, F.C., et al., *Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor*. EMBO J, 1995. **14**(22): p. 5579-88.
4. Irmeler, M., et al., *Inhibition of death receptor signals by cellular FLIP*. Nature, 1997. **388**(6638): p. 190-5.
5. Donepudi, M., et al., *Insights into the regulatory mechanism for caspase-8 activation*. Mol Cell, 2003. **11**(2): p. 543-9.
6. Oberst, A., et al., *Inducible dimerization and inducible cleavage reveal a requirement for both processes in caspase-8 activation*. J Biol Chem, 2010. **285**(22): p. 16632-42.
7. Pop, C., et al., *Role of proteolysis in caspase-8 activation and stabilization*. Biochemistry, 2007. **46**(14): p. 4398-407.
8. Juo, P., et al., *FADD is required for multiple signaling events downstream of the receptor Fas*. Cell Growth Differ, 1999. **10**(12): p. 797-804.
9. Holler, N., et al., *Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule*. Nat Immunol, 2000. **1**(6): p. 489-95.
10. Stanger, B.Z., et al., *RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death*. Cell, 1995. **81**(4): p. 513-23.
11. Degterev, A., et al., *Identification of RIP1 kinase as a specific cellular target of necrostatins*. Nat Chem Biol, 2008. **4**(5): p. 313-21.
12. Degterev, A., et al., *Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury*. Nat Chem Biol, 2005. **1**(2): p. 112-9.
13. Bell, B.D., et al., *FADD and caspase-8 control the outcome of autophagic signaling in proliferating T cells*. Proc Natl Acad Sci U S A, 2008. **105**(43): p. 16677-82.
14. Ch'en, I.L., et al., *Antigen-mediated T cell expansion regulated by parallel pathways of death*. Proc Natl Acad Sci U S A, 2008. **105**(45): p. 17463-8.

15. Bell, B.D. and C.M. Walsh, *Coordinate regulation of autophagy and apoptosis in T cells by death effectors: FADD or foundation*. Autophagy, 2009. **5**(2): p. 238-40.
16. Cho, Y.S., et al., *Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation*. Cell, 2009. **137**(6): p. 1112-23.
17. Zhang, D.W., et al., *RIP3, an Energy Metabolism Regulator that Switches TNF-Induced Cell Death from Apoptosis to Necrosis*. Science, 2009.
18. He, S.W., Lai, Miao, Lin; Wang, Tao; Du, Fenghe; Zhao, Liping; Wang, Xiaodong, *Receptor Interacting Protein Kinase-3 Determines Cellular Necrotic Response to TNF-alpha*. Cell, 2009. **137**(6): p. 1100-1111.
19. Vandenabeele, P., et al., *The role of the kinases RIP1 and RIP3 in TNF-induced necrosis*. Sci Signal, 2010. **3**(115): p. re4.
20. Lu, J.V., et al., *Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity*. Proc Natl Acad Sci U S A, 2011. **108**(37): p. 15312-7.
21. Kawadler, H., et al., *The paracaspase MALTI controls caspase-8 activation during lymphocyte proliferation*. Mol Cell, 2008. **31**(3): p. 415-21.
22. Misra, R.S., et al., *Caspase-8 and c-FLIPL associate in lipid rafts with NF-kappaB adaptors during T cell activation*. J Biol Chem, 2007. **282**(27): p. 19365-74.
23. Su, H., et al., *Requirement for caspase-8 in NF-kappaB activation by antigen receptor*. Science, 2005. **307**(5714): p. 1465-8.
24. Arechiga, A.F., et al., *Cutting edge: FADD is not required for antigen receptor-mediated NF-kappaB activation*. J Immunol, 2005. **175**(12): p. 7800-4.
25. Fredriksson, S., et al., *Protein detection using proximity-dependent DNA ligation assays*. Nat Biotechnol, 2002. **20**(5): p. 473-7.
26. Li, J., et al., *The RIP1/RIP3 necrosome forms a functional amyloid signaling complex required for programmed necrosis*. Cell, 2012. **150**(2): p. 339-50.
27. Leverrier, S., G.S. Salvesen, and C.M. Walsh, *Enzymatically active single chain caspase-8 maintains T-cell survival during clonal expansion*. Cell Death Differ, 2011. **18**(1): p. 90-8.
28. Alappat, E.C., et al., *Phosphorylation of FADD at serine 194 by CKIalpha regulates its nonapoptotic activities*. Mol Cell, 2005. **19**(3): p. 321-32.

CHAPTER 5

Conclusions

The investigations presented in this thesis aims to elucidate the crosstalk between necroptosis, apoptosis, and autophagy pathways in activated T lymphocytes. We have shown that FADD, caspase 8 and RIP kinases are all essential for clonal expansion, contraction, and antiviral responses. The interplay of these death pathways is also critical in immune tolerance, as inhibition of apoptosis and necroptosis leads to lymphoproliferative disease and accumulation of autoreactive T cells. We have established a role for autophagic signaling in T lymphocytes due to the observance of a significant hyperautophagic phenotype in activated FADD^Δ cells. Interestingly, deletion of autophagy genes led to impaired T cell activation and proliferation. Our data suggest dual roles for autophagy in controlling T cell survival and death. Autophagy also plays different roles in naïve and activated T cells. It appears to be essential for maintaining naïve T cell survival through regulation of mitochondrial levels, while deletion of autophagy proteins following TCR activation has no impact on T cell survival and proliferation. The balance between apoptosis, necroptosis, and autophagy are essential for maintaining immune homeostasis and for functional immune responses.

Although different stimuli contribute to the induction of necroptosis, these pathways converge at the formation of the RIPK1-RIPK3 necrosome. The main question regarding the regulation of death pathways is the regulation of caspase 8. Currently, the spatial and temporal kinetics that control the activation caspase 8 are still unknown. The necrosome is a ~2 megadalton complex [1,2] thus an ongoing goal is to identify the components of the necrosome and its targets. The events involved in the execution of necroptosis downstream of RIPK3 are

still uncharacterized. We expect to identify not only the known necrosome factors, RIPK1/3, caspase 8, CYLD, etc., but also novel components that may allow us to define the mechanism of necrosome assembly. It has been suggested that the complex is recruited directly to the Bcl-10/Carma-1/Malt-1 (BCM) complex following recruitment of RIPK1 and casp8, although our data would suggest the necrosome does not form in conjunction with initial TCR activation events, but as late as 24-36 hours post activation. Based on the molecules we identify we will further analyze their involvement in necroptosis induction by genetic knockout or retroviral-mediated shRNA. Mitochondrial proteins such as mitochondrial phosphatase, PGAM5 and Drp-1 (mitochondrial fission factor) [3], and mixed lineage kinase-like protein (MLKL) [4] have been recently identified as downstream components of the necroptotic pathway in HeLa cells. Recent findings [4] utilizing a chemical library screening approach identified a molecule, necrosulfonamide (NSA), which can block necroptosis downstream of RIPK3 activity. NSA blocks necrosis by modifying mixed lineage kinase like protein (MLKL1), which is a functional substrate of RIPK3. Interaction of MLKL1 and the necrosome is shown to be necessary for propagating the necroptotic signal in Jurkats, HT-29, and Hela cells. Wang et al. simultaneously identified mitochondrial protein phosphatase, phosphoglycerate mutase 5 (PGAM5), as a downstream effector that binds the necrosome on the mitochondrial membrane. PGAM5-MLKL1-RIPK1-3 interaction promotes Drp1 activation, a GTPase necessary for mitochondrial fission, and Drp1 inhibition blocks necrosis. The RIPK1-RIPK3 complex amplifies its necroptotic signal by recruiting mitochondrial fragmentation processes, and provides a potential mechanism of RIPK1-3 death regulation in the immune system. We suspect these proteins, in addition to other mitochondrial/cell cycle regulators will also be detected in the RIPK1-RIPK3 complex.

The mechanism by which caspase 8 is activated is a fundamental question that addresses all death pathways under its regulation. By developing probes that can detect the activated form of Caspase 8, we can utilize the PLA and Caspase 8 activity assays to address specific questions regarding the temporal and spatial mechanism of its activation. In addition, Caspase 8 activity is detectable at 24 hours of TCR stimulation, corresponding with the generation of necrosomes.

Only a small fraction of total RIPK1 in cells is recruited to the necrosome to promote necroptosis indicating a highly specific mechanism regulates its pro-death activity. The necroptotic pathway is under strict regulation and we have shown the inability to cleave RIPK1 is sufficient to dominantly promote necroptotic death. It has been shown that ubiquitination of RIPK1 determines whether it is pro- or anti-survival in cells, however, additional post-translational modifications of RIPK1 and RIPK3 in T cells and the mechanisms that control generation of the complex have not been clearly identified. We hypothesize that ubiquitination, phosphorylation, or proteolytic cleavage events may play a role in necrosome formation. Antibodies that recognize phosphorylation RIPK1 and RIPK3 are not currently available, thus we propose to employ two-dimensional gel electrophoresis (2DGE) and western blotting to analyze the phosphorylation events. As RIPK1-RIPK3 auto- or cross-phosphorylation has been detected in MEFS [5], we anticipate similar modifications to occur in T cells.

To further understand the interplay between autophagy and necroptosis, we utilize the Proximity Ligation Assay (PLA) in Atg5-deficient T cells. We propose that the autophagosomal membrane serves as a platform for recruitment of various complexes, such as the DISC or the necrosome etc. Atg5 harbors a death domain, and Atg5 and FADD have shown to interact and bind with one another [6]. Exploring the hyperautophagic hallmarks observed in FADD^{dd} and casp8^{-/-} T cells following activation will help to understand not only the needs for autophagy

during T cell activation, but more importantly, will help to establish the link between necroptosis and autophagy.

The FADD^Δ defect is primarily observed in CD8⁺ T cells, and not as profound in CD4⁺ T cells. We speculate the reason for the differing sensitivity to caspase 8 is because CD8⁺ T cells proliferate more rapidly than CD4⁺ T cells. Based on our PLA studies indicating the necrosome is detected at 24-36 hours post activation and the complex formation can be blocked with the addition of cell cycle inhibitors, we surmise necroptosis serves as a cell cycle checkpoint. Necroptosis is activated as a way to rapidly clear non-functional CD8⁺ T cells during their rapid proliferation and ensure optimal effector capabilities are intact to mount an effective antiviral response. FADD may interact with spindle fibers and we can validate this hypothesis using spindle fiber markers and inhibitors.

NF- κ B has long been known for its role in promoting activation and expansion in T cells by diminishing apoptotic sensitivity through influencing the expression of pro-and anti-apoptotic genes such as c-FLIP, Bcl-xL and IAP genes [7, 8]. Whether it is involved in the regulation of the necroptotic pathway is still unknown. RIPK1-deficient Jurkats reconstituted with a non-cleavable RIPK1 (D325A) predominantly undergo necroptosis in response to TNF. Blockade of NF- κ B signaling using an inhibitor of I κ B kinases (IKK) enhances the sensitivity of RIP1_D325A Jurkats to TNF α induced necroptosis. Addition of an IKK inhibitor led to a significant increase in the sensitivity of RIP1_D325A expressing Jurkats to diminishing doses of TNF α . The finding that this transcription factor is also involved in controlling the sensitivity of cells to necroptotic cell death will be important if we are to take advantage of necroptosis in overcoming the apoptotic insensitivity of undesired cells. Further, NF- κ B plays important roles in early T cell activation, and is directly activated via TCR proximal signaling pathways [9].

Should NF- κ B blockade enhance the sensitivity of FADD^{dd} T cells to necroptosis, we would anticipate a greater level of death in these cells following stimulation in the presence of the IKK inhibitor. Enhanced death that is blocked by Nec-1 in the wild type cultures would suggest that necroptosis signaling occurs in wild type T cells and plays an important physiological role in normal T cells [10]. Identifying the mechanism of inhibition by NF- κ B signaling will further delineate the elaboration of the downstream signaling induced during necroptosis.

As Nec-1 potently suppresses both RIPK1 catalytic activity as an allosteric kinase inhibitor, and also blocks necroptosis, it is necessary to determine if downstream mediators or RIPK1 itself is promoting autophagy. A novel necroptosis inhibitor, necrosulfonamide, which inhibits downstream of RIPK1 was recently developed. We use this inhibitor to evaluate the role of RIPK1 in autophagic signaling, and to delineate the sequence of events that precedes TNF-induced death. Future experimentation involves establishing the contribution of RIPK1 signaling in directly modulating autophagy by assessing the impact of the loss of RIPK3 on the hyper-autophagy FADD^{dd} phenotype in primary T cells derived from FADD^{dd}, RIPK3^{-/-} and FADD^{dd} x RIPK3^{-/-} mice.

Our data from Atg5-deficient mice implicates a necessary role for autophagy in naïve T cell homeostasis but not effector proliferation. The generation of memory cells following infection, and the transition from the effector population to the memory population as they revert back to the “naïve” stage is an important question. Using the ER-Cre mouse model, it would be highly applicable to determine the role of autophagy on memory cell generation. The development and maintenance of the memory population is still unclear, and autophagy may play a part. We would like to delete Atg5 prior to and post-infection with MHV, and assess the ability of these mice to mount an anti-viral response. Subsequently, we would also like to investigate

the ability to generate a recall response. These findings are highly applicable in effective vaccine development. As well, Atg serves as a potential therapeutic target in an autoimmune or tolerance model. We have demonstrated the importance of autophagy in survival of peripheral T cells as losing autophagy in a CD4-Cre mediated mouse model leads to significantly decreased peripheral T lymphocytes. Autophagy-deficient mice display lymphopenia, and we are interested in assessing the effects of deleting autophagy in a mouse that starts out with a compartment of wild type T cells that are capable of undergoing autophagy. Inducing the deletion after reconstituting a RAG^{-/-} mouse with Atg5^{fl/fl} x ER-Cre T cells led to defective homeostasis and a reduction in T cell numbers in the periphery after 30 days, further supporting the importance of autophagy in maintaining immune function. Since expansion capabilities of effector cells are intact in autophagy-deficient cells, it will be of interest to examine the development of the memory pool in the absence of autophagy. Atg5 provides a potential therapeutic target in an autoimmune or tolerance model for the deletion of autoreactive T cells.

References

1. Tenev, T., et al., *The Ripoptosome, a signaling platform that assembles in response to genotoxic stress and loss of IAPs*. Mol Cell, 2011. **43**(3): p. 432-48.
2. Feoktistova, M., et al., *cIAPs block Ripoptosome formation, a RIP1/caspase-8 containing intracellular cell death complex differentially regulated by cFLIP isoforms*. Mol Cell, 2011. **43**(3): p. 449-63.
3. Wang, Z., et al., *The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways*. Cell, 2012. **148**(1-2): p. 228-43.
4. Sun, L., et al., *Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase*. Cell, 2012. **148**(1-2): p. 213-27.
5. Cho, Y.S., et al., *Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation*. Cell, 2009. **137**(6): p. 1112-23.
6. Pyo, J.O., et al., *Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death*. J Biol Chem, 2005. **280**(21): p. 20722-9.
7. Sprent, J. and C.D. Surh, *Normal T cell homeostasis: the conversion of naive cells into memory-phenotype cells*. Nat Immunol, 2011. **12**(6): p. 478-84.
8. Bertrand, M.J. and P. Vandenabeele, *RIP1's function in NF-kappaB activation: from master actor to onlooker*. Cell Death Differ, 2010. **17**(3): p. 379-80.
9. van Oers, N.S. and Z.J. Chen, *Cell biology. Kinasing and clipping down the NF-kappa B trail*. Science, 2005. **308**(5718): p. 65-6.
10. Lu, J.V., et al., *Complementary roles of Fas-associated death domain (FADD) and receptor interacting protein kinase-3 (RIPK3) in T-cell homeostasis and antiviral immunity*. Proc Natl Acad Sci U S A, 2011. **108**(37): p. 15312-7.