UNIVERSITY OF CALIFORNIA, SAN DIEGO

# The role of caspase-8 in regulating dendritic cell activation during both homeostasis and chronic viral infection

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

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by

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The Dissertation of Jennifer S. Tsau is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chapter II, in full, is a reprint of the material as it appears in Ch'en, I. L., Tsau, J. S., Molkentin, J. D., Komatsu, M., & Hedrick, S. M. (2011). Mechanisms of necroptosis in T cells. *Journal of Experimental Medicine*, *208*(4), 633–641. The dissertation author was the second author of this paper.

Chapter III, in large part, is under preparation for submission of the material to the *Journal of Immunology*. The dissertation author was the primary author of this paper. Stephen Hedrick was the corresponding author of this paper.

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# PUBLICATIONS

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# ABSTRACT OF THE DISSERTATION

**The role of caspase-8 in regulating dendritic cell activation during both homeostasis and chronic viral infection**

by

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Doctor of Philosophy in Biology

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Professor Stephen Hedrick, Chair

Chapter I introduces the innate and adaptive immune systems, which serve as the context for my dissertation, as well as the protein of interest in both systems.

Chapter II is a reprint of a study on which I was the second author. In this study, we investigated the role of caspase-8 in T cell survival. Caspase-8 deficient T cells were found to accumulate defectively in response to antigenic stimulation *in vitro*, and mice with caspase-8 deficient T cells (*tCasp8*$^{-/-}$ mice) were unable to mount a response to an acute viral infection. Inhibition of Ripk1

kinase was shown to rescue this accumulation defect *in vitro*, and this form of death was termed necroptosis due to its dependence upon Ripk1 and lack of shared morphological characteristics with apoptosis. However, it was unclear whether other proteins were also required for necroptosis. Both programmed necrosis (as defined by a requirement for cyclophilin D) and autophagy (as defined by a requirement for Atg7) had been proposed to play a role in T cell necroptosis. Ripk3 had also been shown to interact with Ripk1 and trigger programmed necrosis. We used genetic studies to show that while neither cyclophilin D nor Atg7 could rescue the accumulation defect of caspase-8 deficient T cells, Ripk3 did. Only mice with caspase-8 deficient T cells and an additional loss of Ripk3 (DKO mice) were able to mount a response to an acute viral infection. We also found that DKO mice developed a lymphoproliferative disease with similarities to *lpr* mice, which have a lupus-like autoimmune disease.

Chapter III examines the role of caspase-8 in another type of immune cell, dendritic cells (DCs). Caspase-8 was shown to negatively regulate the Rig-I innate immune signaling pathway, which produces type I interferons in response to RNA viruses. I found that mice with caspase-8 deficient DCs (*dcCasp8*$^{-/-}$ mice) develop an age-dependent autoimmunity characterized by hyperactivated DCs and T cells, organ immunopathology, and helper T cells that skew towards a Th1 phenotype. Since DC activation is considered required for a robust T cell response to viral infection, we next infected *dcCasp8*$^{-/-}$ mice

with a chronic virus and found that they had an enhanced virus-specific T cell response characterized by less exhausted T cells and lower viral loads. Caspase-8 deficient DCs appeared to hyperactivate in response to Rig-I stimulation, which was likely dependent on endogenous activation of both IRF3 and NF-κB in the absence of caspase-8.

# CHAPTER I:

# Diverse roles of caspase-8 in innate and adaptive immunity

## Innate vs. adaptive immunity

Upon infection with a pathogen, all organisms mount an innate immune response. The innate response is generally thought of as non-specific and is characterized by the activation of pathogen recognition receptors (PRRs) by pathogen-associated molecular patterns (PAMPs). Depending on the organism, PRR signaling leads to a variety of downstream effects, such as an influx of innate immune cells to sites of pathogen invasion or a release of antimicrobial peptides (1, 2). In humans and mice, the most common innate immune cells are neutrophils, first responders that phagocytose bacteria; followed by macrophages, which also phagocytose foreign particles; then dendritic cells (DCs), which uptake microbial antigens and present them to T cells, a type of adaptive immune cell. When a DC presents a pathogenic antigen to a T cell which recognizes the antigen, that T cell clonally expands to produce many copies of itself. Those expanded T cells then go on to combat the pathogen - either directly, through CD8 cytotoxic T cells, or indirectly, through CD4 helper T cells. The early adaptive immune response is thus characterized by T cell proliferation.

While all organisms possess some form of innate immunity, which is not targeted towards specific pathogens, only vertebrates (generally speaking) possess adaptive immunity, which is. Innate immune PRR genes are germline-encoded, detect broad classes of PAMPs and are fixed for the lifespan of the individual. On the other hand, in adaptive immunity, also known as acquired

immunity, the individual acquires specific PRRs due to various pathogens it encounters throughout its lifetime.

## Role of DCs as the bridge between innate and adaptive immunity

DCs were first identified in 1973 as a "large stellate cell with distinct properties" from previously identified cells in the mouse spleen, such as lymphocytes, granulocytes and mononuclear phagocytes (3). In a series of seminal papers beginning in the 1970's, Steinman and colleagues described DCs as morphologically and functionally distinct from other immune cells. Unlike lymphocytes and macrophages, DCs do not directly kill cells, but rather act as sentries that detect invading pathogens. Once DCs detect a pathogen, they stimulate and activate lymphocytes. Thus, DCs bridge innate and adaptive immunity, but are generally considered innate immune cells because they are activated early in infection. For his pioneering work on DC biology, Steinman was awarded the Nobel Prize in Physiology or Medicine in 2011 (4).

DCs are hematopoietic cells that, like all other immune cells, originate from the bone marrow. Fully differentiated but immature DCs constantly circulate through the body via both the lymphatic and blood vessels in the steady state and monitor for signs of infection. DCs are found in most tissues and are especially prevalent in areas that directly contact the external environment, such as the skin, lung and gut (5). Upon pathogen invasion, DCs detect microbial

antigens they encounter in the tissues or vessels with PRRs, and ingest those antigens via continuous receptor-mediated endocytosis and phagocytosis. As DCs migrate to the lymph nodes and spleen, the ingested antigens are processed and presented on the cell surface, and antigen uptake is arrested. Antigen presentation on DCs is mediated by either major histocompatibility (MHC) I or II molecules, with MHCI being recognized by CD8 T cells, and MHCII molecules by CD4 T cells. Under the pro-inflammatory conditions of pathogen infection, DCs also upregulate two cell surface co-stimulatory molecules, CD80 and CD86, which are so named for their role in stimulating T cells in conjunction with MHCs. A DC that expresses both MHC (signal one) and CD80/CD86 (signal two) on its cell surface is considered activated, and when it encounters a T cell with the specific T cell receptor (TCR) that recognizes the presented antigen, clonal proliferation of the cognate T cell occurs (6). DCs are considered professional antigen-presenting cells (APCs) for their proficiency in presenting antigen and stimulating T cell responses. B cells and macrophages are also professional APCs and possess some antigen presentation capability, but DCs are considered the most important and versatile APCs—at least in terms of a primary immune response (7).

DCs can detect a wide range of PAMPs, which are pathogen-associated molecules that are never found naturally in animals. Some examples are lipopolysaccharide (LPS), a component of bacterial cell walls, and molecules such as single-stranded or double-stranded RNA. DCs detect PAMPs with a

variety of cell surface, cytosolic and endosomal PRRs. Toll-like receptors (TLRs) are a major class of PRRs that are found both on the cell surface and in endosomes, and Nod-like (NLR) and Rig-I-like (RLR) receptors are PRRs found in the cytosol. Each PRR specializes in recognizing a specific type of PAMP (8, 9). It is well established that DC detection of PAMPs leads to the initiation of T cell-mediated adaptive immunity. Another group of molecules has been demonstrated to have similar adjuvant properties as PAMPs. Danger-associated molecular patterns (DAMPs) are pro-inflammatory molecules that are released from cells dying in a non-physiological manner (10). Physiological modes of death include programmed cell death by apoptosis, while non-physiological death occurs when cells die from non-programmed necrosis. Death by necrosis causes cellular membrane rupture and releases DAMPs such as HMGB1, uric acid and heat shock proteins, while cells dying by apoptosis retain membrane integrity and thus are able to conceal DAMPs within themselves (11). While many studies have shown that either PAMPs or DAMPs have adjuvant and pro-inflammatory properties, it is unclear whether both are required and/or may be acting synergistically to initiate adaptive immunity.

## Detection of RNA viruses by Rig-I

One subset of PAMPs recognized by DCs is viral RNA. The life cycle of RNA viruses includes a stage in which its RNA is double-stranded. Since dsRNA is not normally found in human cells, the immune system has evolved to

distinguish dsRNA as a PAMP. Humans and mice possess at least two PRRs to detect viral dsRNA: Toll-like receptor (TLR) 3 and retinoic-acid inducible gene-I (Rig-I). Detection of viral dsRNA by either PRR results in activation of the transcription factor IRF3 and production of type I interferon (IFN-I), an important anti-viral cytokine (12, 13). However, different cell types often rely on one PRR or the other. Conventional DCs (cDCs) specialize in antigen presentation to T cells and are more common than plasmacytoid DCs (pDCs), which produce large amounts of IFN-I but are not thought to be involved in antigen presentation. Although both cDCs and pDCs detect viral dsRNA, cDCs require Rig-I for detection, while pDCs require TLR3 (13).

Upon detection of viral dsRNA by Rig-I, a signaling complex consisting of the kinase RIP1 (RIPK1), TRADD, FADD, TANK and MAVS forms at the mitochondrial membrane. The formation of this mitochondrial signaling complex (MSC) leads to enhanced expression of the transcription factor IRF3 in the nucleus. Activation of IRF3 in turn leads to production of anti-viral IFN-I. The formation of the RIPK1-containing complex downstream of Rig-I activation is held in check by the cysteine-aspartic protease caspase-8 (Figure 1; (14).

## The many functions of caspase-8

Caspases are a family of cysteine proteases with well-defined roles in programmed cell death (PCD) and inflammation, and have recently been found to regulate aspects of immunity. Apoptotic caspases, which control PCD, fall

into either the intrinsic or extrinsic category. Intrinsic apoptosis is triggered by environmental cues such as growth factor withdrawal, while extrinsic apoptosis is triggered by death receptor signaling. The apoptotic caspase family consists of both initiator caspases (-8, -9) which unleash a caspase cascade, and executioner caspases (-3, -7) which cleave substrates and effect death in apoptotic cells (15).

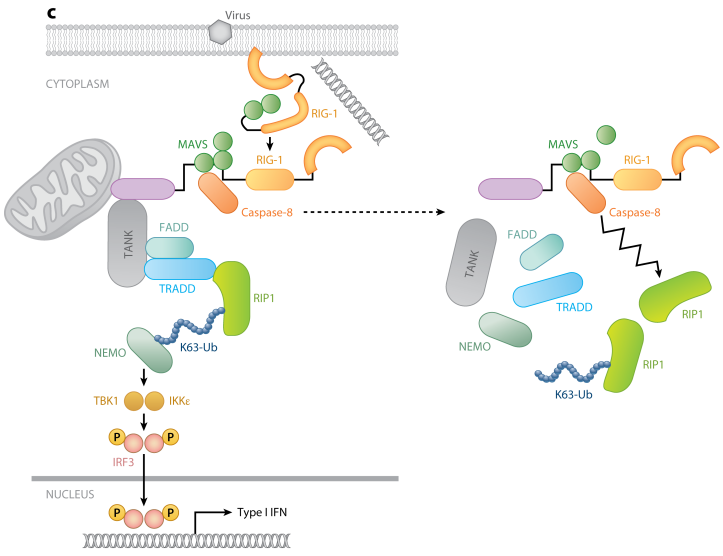


**Figure 1. Recognition of viral RNA by RIG-I leads to the formation of a mitochondria-associated signaling complex (MSC).**

The MSC includes MAVS, TANK, RIP1, FADD, and TRADD. Cleavage of RIP1 by caspase-8 destabilizes the RIG-I complex and generates a short IRF3-inhibiting RIP1 fragment (14).

Caspase-8 is considered the key initiator of extrinsic apoptosis, but ablation of caspase-8 in different tissue types led to surprising phenotypes. Mice completely deficient in caspase-8 are embryonic lethal due to irregular heart development and accumulation of erythrocytes. Fibroblasts from caspase-8-

deficient mice did not die upon stimulation of TNF receptors, which leads to cell death in normal cells (16). Loss of caspase-8 in epidermal keratinocytes or intestinal epithelium leads to chronic skin inflammation or susceptibility to colitis, respectively (17, 18). Caspase-8 and its adaptor, FADD, was also found to play an enigmatic role in T cell homeostasis. DCs initiate adaptive immunity by presenting pathogenic antigens to cognate T cells, which then clonally expand to combat the infection. After the pathogen is cleared, the large pool of T cells contract possibly as a means of limiting the risk of autoimmunity. This apoptotic process is known as activation-induced cell death (AICD) (19). Although an expectation from this was that T cells lacking caspase-8 would not contract following infection-induced expansion, T cells deficient for either caspase-8 or FADD were instead found to be unable to accumulate in response to any form of of T cell stimulation (20-24). Furthermore, mice with a T cell-specific deletion of *Casp8* ("*tCasp8*$^{-/-}$ mice") were unable to respond to an acute lymphocytic choriomeningitis (LCMV) infection (Salmena et al., 2003).

Programmed necrosis, or necroptosis, was identified to be responsible for the accumulation defect in caspase-8 deficient T cells. Necroptosis is an alternative form of PCD that is also triggered by death receptor ligation, but is mediated by the kinases Ripk1 and Ripk3, and requires inhibition of caspase-8 or FADD (25-32). Thus, cells lacking caspase-8 undergo necroptosis when death receptor signaling is triggered. When T cells lacking caspase-8 were treated with a Ripk1 inhibitor, or Ripk1 was knocked down, the accumulation

defect in response to TCR (anti-CD3 and anti-CD28) stimulation was rescued, and *tCasp8*$^{-/-}$ mice with a germline deletion of Ripk3 recovered a robust response to acute LCMV infection (28, 33). Significantly, deletion of germline Ripk3 in caspase-8 deficient mice rescued their embryonic lethality, showing the universality of necroptosis as an alternative mode of PCD in caspase-8 deficient conditions (34, 35).

Recently, caspase-8 was also found to regulate innate immune signaling. A number of studies have provided evidence for the mechanism of this regulation. Ablation of caspase-8 in murine cell lines was shown to increase IRF3 expression upon polyI:C stimulation (polyI:C is a surrogate for dsRNA) (36). This was a result of caspase-8 cleavage of Ripk1 and subsequent inhibition of MSC formation. Another study showed that caspase-8 directly cleaves IRF3, leading to its degradation (37). Moving into animal models, one group found that mice with caspase-8 deficient DCs ("*dcCasp8*$^{-/-}$ mice") produce increased amounts of the pro-inflammatory cytokine IL-1β upon LPS injection. The increased IL-1β levels were linked to enhanced NLRP3 inflammasome activation in caspase-8 deficient DCs, and dependent upon Ripk1 and Ripk3 (38). Additional phenotyping of *dcCasp8*$^{-/-}$ mice revealed that these mice develop age-dependent autoimmune disease, characterized by activated DCs and T cells, increased serum levels of pro-inflammatory cytokines, and kidney immunopathology. Uncontrolled TLR activation was found to be the source of

autoimmunity in these mice, as deleting the key TLR-pathway adaptor protein, MyD88, rescued the systemic autoimmunity of aged *dcCasp8*$^{-/-}$ mice (39).

## Role of DCs in autoimmunity

Historically, the immune system has been thought to distinguish between harmless "self" and harmful "non-self" molecules using PRRs. The "danger model" is an alternative theory which posits that the immune system has evolved to detect danger signals (danger associated molecular patterns, or DAMPs) such as inflammation and necrosis (10). In either model, an immune response against self-molecules (whether a result of misidentifying "self" as "non-self" or because of the presence of DAMPs) can lead to autoimmune disease. In addition to the crucial role DCs play in initiating the adaptive immune response, they are also important for maintaining immune tolerance. Immune tolerance is the process by which the adaptive immune system is either passively or actively prevented from responding to harmless antigens. These include molecules found both inside and outside of the body, such as food particles, gut microbes and benign environmental molecules (40). There is also emerging evidence that the immune system becomes tolerant of certain persistent viral infections for which a sustained immune response may be counter-productive (41-43).

The study of immune tolerance has a long history. In 1945, Ray Owen observed that cattle dizygotic twins share blood circulation, leading to a chimeric

population of red blood cells in each adult twin (44). In crucial later studies that experimentally demonstrated the existence of immune tolerance, a mouse fetus injected with donor allogeneic cells prevented the rejection of a skin graft from the same donor after the mice were born (45).

Immune tolerance comes in two forms. In central tolerance, developing B and T cells that are reactive against self-antigens are deleted, become anergic, or have their antigen receptor edited so as to be non-reactive to self. In peripheral tolerance, DCs play a crucial role in tolerizing self-reactive T cells that have escaped the thymic negative selection of central tolerance (40). DCs mediate peripheral tolerance using the same mechanism by which they initiate an adaptive immune response. Early studies demonstrated that DCs pulsed with antigen can stimulate antigen-specific T cells in the absence of infection or adjuvants (46, 47). Several groups then showed that when bone marrow (BM)-derived APCs presented self-antigen to peripheral T cells, the T cells were either deleted or became anergic. However, self-antigen presentation by non-BM cells did not lead to either T cell deletion or anergy, suggesting the importance of a hematopoietic cell for maintaining peripheral tolerance (48, 49). Crucial experiments that followed identified DCs as the cell type with these tolerogenic properties. Antigen delivered to steady-state DCs via DEC-205, a surface membrane protein important for antigen presentation, was found to rapidly expand the pool of antigen-specific T cells initially. However, a week later,

numbers of antigen-specific T cells were greatly reduced and the remaining cells did not respond to re-challenge with the original antigen (50, 51).

Based on these findings, a model for DC-mediated peripheral tolerance was developed. In the absence of infection, DCs continuously uptake self-antigen as they circulate through the vasculature or reside in tissues. The DCs then migrate to the lymphoid organs and present self-antigen/MHC complexes to T cells. The pro-inflammatory and pro-maturation signals that occur during infection are absent under homeostatic conditions. The DCs thus do not upregulate the co-stimulatory molecules CD80/CD86 (52-54). When antigen presentation to T cells occurs, the presence of the self-antigen/MHC complex (signal one) in the absence of co-stimulation (signal two) does not lead to T cell clonal proliferation. Instead, the T cells become "educated" that this antigen is self-derived, and are repressed through several different mechanisms, such as being deleted or becoming anergic (55, 56). This process of immune tolerance prevents T cells that recognize self-antigens from initiating a response when they encounter those self-antigens later.

Given the importance of DCs in maintaining immune tolerance, it is not surprising that dysregulation of DCs have been linked to a variety of autoimmune diseases, from multiple sclerosis and type 1 diabetes (T1D) to systemic lupus erythematosus (SLE) (57). In support of this connection, uninfected and partially mature DCs from *Leishmania*-infected mice can stimulate the proliferation of naïve OT-I T cells in the absence of cognate

antigen (58). In patients with SLE, excess IFN-I production is thought to lead to increased DC differentiation and maturation as IFN-I is a major activating source for DCs (59). This in turn leads to the expansion of autoreactive T cells commonly seen with the disease (60). A role for DCs in SLE pathogenesis is supported by the finding that DCs from SLE patients have been shown to upregulate CD86 expression independent of activating stimuli (61, 62).

The constitutive production of IFN-I seen in SLE could occur in a number of ways. Although most cells can produce some IFN-I upon microbial infection, DCs produce up to 1000-fold more and are considered specialized IFN-I producers. Within different DC subsets, plasmacytoid DCs (pDCs), which largely do not participate in antigen presentation, produce the most IFN-I. However, non-pDC conventional DCs (cDCs), whose main function is antigen presentation, also produce large amounts of IFN-I during certain types of viral infection (63). IFN-I production is driven by the family of transcription factors known as interferon regulatory factors (IRFs). IRFs are activated when certain PRRs on or within the cell detect specific PAMPs. While the PAMP-PRR combinations that lead to IFN-I production are diverse, many of them converge on activation of the same IRF, with TLR3, TLR4 and Rig-I signaling all activating IRF3 as just one example (9).

PRR signaling is activated during infection, but it can be activated in the steady state as well. Loss of negative regulation of RLR signaling pathways can mimic RLR detection of PAMPs, leading to IRF activation and IFN-I production

(9). Rig-I is one such RLR that is subject to negative regulation. Upon detection of viral RNA by Rig-I, a signaling complex which includes RIPK1 forms at the mitochondrial membrane and results in enhanced expression of IRF3 in the nucleus. Activation of IRF3 in turn leads to production of IFN-I. Caspase-8 negatively regulates this signaling pathway by inhibiting RIPK1, possibly by cleavage. In the steady state, spontaneous assembly of the RIPK1 mitochondrial complex does not occur, presumably due to caspase-8 inhibition of RIPK1. However, a loss of caspase-8 in fibroblasts was shown to increase IRF3 activation upon Rig-I stimulation (36). It is unclear whether caspase-8 deficient fibroblasts produce increased IFN-I as a result of IRF3 activation. It is also unknown whether a loss of caspase-8 in DCs, which are specialized IFN-I producers, would result in higher levels of IFN-I and a subsequent loss of immune tolerance.

## Programmed cell death (PCD) maintains immune homeostasis

Caspase-8, a negative regulator of Rig-I signaling, plays a key role in maintaining immune homeostasis. Caspase-8 is required in T cells to prevent autoimmunity, as demonstrated by the age-dependent lymphoinfiltration and lymproliferation seen in *tCasp8*$^{-/-}$ mice (64). While mice completely lacking caspase-8 are embryonic lethal, children with a homozygous caspase-8 deficiency survive but have defective lymphocyte apoptosis, as well as defective

activation of T cells, B cells and NK cells. As a result, these patients suffer from immunodeficiency (24). Adults with the same caspase-8 mutation also suffered from immunodeficiency, but additionally suffered from pulmonary hypertension and neurological disease (65). However, in both cases the patients retained a residual amount of caspase-8 expression, suggesting some caspase-8 activity is required for survival.

Since caspase-8 deficient mice are embryonic lethal, other mouse models have been developed to shed light on the importance of PCD in preventing autoimmunity. Several groups have generated mice lacking Fas or Fas ligand (FasL) in various tissues. Fas (CD95) is a death receptor upstream of caspase-8 that initiates apoptosis upon ligation with FasL (CD95L). MRL.*Fas*$^{lpr}$ mice are germline deficient in Fas and develop an SLE-like systemic autoimmunity (66). Specifically, MRL.*Fas*$^{lpr}$ mice develop peripheral T cell repertoires that are predominated by double negative CD4$^{-}$CD8$^{-}$B220$^{+}$ (DN) T cells, which are normally eliminated via Fas-dependent apoptosis (67). If mice lack both caspase-8 and Ripk3 on T cells, they develop a similar population of peripheral DN T cells, suggesting that when DN T cells are unable to be eliminated by either extrinsic apoptosis or necroptosis, they proliferate and accumulate in the periphery (33). Mice with a T cell-specific deletion of Fas also exhibit lymphoproliferative disease, but it is attenuated compared to MRL.*Fas*$^{lpr}$ mice, suggesting the importance of Fas on non-T cells to regulate immune homeostasis (68). To investigate this possibility, Stranges et al. created mice

with a DC-specific Fas deletion and found that these mice also developed systemic autoimmunity (69).

Significantly, in humans deficiencies in Fas, FasL, caspase-8 and caspase-10 mostly affect immune function. Other organ systems do not appear to be affected by a lack of these proteins, further emphasizing the importance of PCD in maintaining immune homeostasis (70).

## RLR components linked to autoimmune diseases

Mutations in RLR signaling components have also been implicated in various autoimmune diseases, especially ones that are driven by excess IFN-I production. MDA5 is a Rig-I like receptor (RLR) that is structurally similar to Rig-I and, like Rig-I, detects RNA viruses and induces IFN-I production. Single nucleotide polymorphisms (SNPs) in *IFIH1*, the gene encoding MDA5, have been linked to susceptibility to SLE (71). Consistent with these findings, mice with gain-of-function mutations in *IFIH1* developed spontaneous lupus-like symptoms, which were dependent upon both the adaptor protein MAVS and IFN-I receptor signaling (72). Additionally, loss-of-function SNPs in *IFIH1* were shown to be protective against development of T1D (73, 74). Although the mechanism for protection against T1D is unclear, Nejentsev et al. hypothesize that loss of MDA5 function prevents an innate immune response to enteroviruses, which are detected by MDA5 and have been linked to T1D development (75). While dysregulation of MDA5 has been implicated in

autoimmunity in both humans and mice, the role of Rig-I in these diseases is still unclear.

For my graduate work, I examined aspects of both innate and adaptive immunity as they pertained to loss of caspase-8 in specific immune cells. Chapter II addresses our investigations into the role of caspase-8 in T cells, while Chapter III looks at the role of caspase-8 in DCs.

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# CHAPTER II:

# The role of caspase-8 in regulating T cell survival

# Abstract

Caspases are a family of cysteine proteases with well-defined roles in programmed cell death (PCD) and inflammation, and have recently been found to regulate aspects of immunity. Caspase-8 is considered the key initiator of extrinsic apoptosis, but was also found to play an enigmatic role in T cell homeostasis. T cells clonally expand to combat pathogens. After pathogen clearance, the large pool of T cells contract, possibly as a means of limiting the risk of autoimmunity. Although an expectation from this was that T cells lacking caspase-8 would not contract following infection-induced expansion, these cells were instead found to be unable to accumulate in response to pro-apoptotic TCR stimulation, and mice with caspase-8 deficient T cells ("*tCasp8*$^{-/-}$ mice") had an impaired T cell response to an acute lymphocytic choriomeningitis (LCMV) infection. When T cells lacking caspase-8 were treated with a Ripk1 inhibitor, their accumulation defect in response to TCR stimulation was restored. Here we demonstrate that programmed necrosis, or necroptosis, is responsible for the accumulation defect in caspase-8 deficient T cells. While we found that necroptosis in caspase-8 deficient T cells is not mediated by cyclophilin D-dependent programmed necrosis or autophagy-related protein 7-dependent autophagy, a loss of Ripk3 completely rescued the survival of caspase-8 deficient T cells. *tCasp8*$^{-/-}$ mice with a germline deletion of Ripk3 also recovered a robust response to acute LCMV infection. Additionally, a loss of both T cell-specific caspase-8 and Ripk3 led to the development of a lymphoproliferative

disease with similarities to *lpr* or *gld* mice. Taken together, we conclude that necroptosis an alternative form of PCD triggered by antigen stimulation in caspase-8 deficient T cells, and is mediated by the kinase Ripk3.

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Brief Definitive Report

# Mechanisms of necroptosis in T cells

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**Cell populations are regulated in size by at least two forms of apoptosis. More recently, necroptosis, a parallel, nonapoptotic pathway of cell death, has been described, and this pathway is invoked in the absence of caspase 8. In caspase 8–deficient T cells, necroptosis occurs as the result of antigen receptor–mediated activation. Here, through a genetic analysis, we show that necroptosis in caspase 8–deficient T cells is related neither to the programmed necrosis as defined by the requirement for mitochondrial cyclophilin D nor to autophagy as defined by the requirement for autophagy-related protein 7. Rather, survival of caspase 8–defective T cells can be completely rescued by loss of receptor-interacting serine-threonine kinase (Ripk) 3. Additionally, complementation of a T cell–specific caspase 8 deficiency with a loss of Ripk3 gives rise to lymphoproliferative disease reminiscent of *lpr* or *gld* mice. In conjunction with previous work, we conclude that necroptosis in antigen-stimulated caspase 8–deficient T cells is the result of a novel Ripk1- and Ripk3-mediated pathway of cell death.**

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Abbreviations used: 7AAD, aminoactinomycin D; Atg7, autophagy-related protein 7; DKO, double KO; Fadd, Fas-associated protein with death domain; mPTP, mitochondrial permeability transition pore; Ripk, receptor-interacting serine-threonine-protein kinase; LCMV, lymphocytic choriomeningitis virus; TUNEL, TdT-mediated dUTP-biotin nick end labeling.

The maintenance of T cell population size is controlled by two forms of apoptosis, one that is initiated by permeabilization of the mitochondrial outer membrane and propagated by the release of cytochrome *c* and another that is initiated by death receptor ligation (Green, 2005). Engaged death receptors in turn bind Fas-associated protein with death domain (Fadd) and activate the initiator cysteine protease caspase 8. These interactions unleash the cascade of proteolytic events performed by executioner caspases. The manner in which these two forms of apoptosis regulate various aspects of T cell development and homeostasis is still being studied.

In the course of exploring a role for death receptor–mediated apoptosis in T cell population dynamics, another form of cell death emerged. T cells deficient for Fadd or caspase 8 might have been expected to expand to abnormally high levels in response to T cell antigen receptor (TCR)-mediated stimulation, and yet, such T cells proliferate poorly in culture and exhibit little expansion in vivo in response to viral infection (Hedrick et al., 2010). The cause of this defect has been controversial. One study characterized human and mouse T cells deficient for caspase 8 and concluded that they do not activate the prosurvival NF-κB pathway (Su et al., 2005), although this has been contested for mouse T cells and B cells deficient in either Fadd or caspase 8 (Salmena et al., 2003; Arechiga et al., 2005; Beisner et al., 2005; Imtiyaz et al., 2006; Ch'en et al., 2008). For example, TCR-stimulated mouse T cells with an inactivated *Casp8* gene exhibit normal degradation of IκB, nuclear localization of RelA, normal induction of active NF-κB dimers as measured by electrophoretic mobility shift assay, and no differences in the induction of NF-κB target genes. Other studies have suggested that there is a cell cycle progression defect in Fadd- or caspase 8–deficient T cells (Zhang et al., 2001; Arechiga et al., 2007), and yet, by several criteria, caspase 8-deficient and wild-type T cells divide at the same rate, both in culture and in vivo (Salmena et al., 2003; Ch'en et al., 2008).

Experiments measuring the viability of stimulated T cells showed that the deficit in T cell expansion caused by a loss of caspase 8 was

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clearly explained by a continuous loss in cell viability; however, the death was not apoptotic. No DNA fragmentation was evident, as measured by DNA laddering or TdT-mediated dUTP-biotin nick end labeling (TUNEL; Ch'en et al., 2008). Other studies have suggested that this death occurred as a result of overexuberant autophagy (Yu et al., 2004; Bell et al., 2008), although an RNA interference screen for suppression of nonapoptotic death did not uncover autophagy genes (Hitomi et al., 2008). Instead of acting to preserve cell viability under conditions of starvation, this form of autophagy was proposed to give rise to the accumulation of reactive oxygen species (Yu et al., 2006).

Other investigations suggested that this death was related to that of cells signaled to die through TNFRI, but defective for either Fadd or caspase 8 (Schulze-Osthoff et al., 1994). This death has been termed necroptosis, and it can be blocked by the receptor-interacting serine/threonine-protein kinase (Ripk) 1 kinase inhibitor necrostatin-1 (Degterev et al., 2005, 2008). Consistent with these results, the expansion defect in caspase 8–deficient T cells was rescued by necrostatin-1 or a knockdown of Ripk1 (Ch'en et al., 2008). As such, it would appear that caspase 8 can function as both an initiator of apoptosis and an inhibitor of necroptosis; in its absence, perhaps a consequence of viral infection, T cells die via necroptosis.

Recent work has suggested that Ripk1 and Ripk3 function as a complex to induce programmed necrotic cell death through the synthesis of reactive oxygen species (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). This suggests that in TCR-stimulated caspase 8–deficient T cells, necroptotic death is similarly mediated, although a recent work could find no evidence for the participation of Ripk3 in the death associated with the loss of Fadd in T cells (Osborn et al., 2010).

In this report, we have investigated T cell death associated with a loss of caspase 8 with respect to the role of programmed necrosis as defined by the requirement for cyclophilin D, the role of autophagy as defined by the requirement for autophagy-related protein 7 (Atg7), and Ripk3-dependent necroptosis. Genetic complementation experiments show that only a loss of Ripk3 is able to rescue the expansion of caspase 8–deficient T cells and reveal an abnormal $CD3^+CD4^-CD8^-B220^+$ population of T cells characteristic of human beings and mice with mutations in *TNFRSF6* (*Fas*).

## RESULTS AND DISCUSSION

### Caspase 8–deficient T cells do not die by classical necrosis

The process of necroptosis, mediated by Ripk1, is thought to function through programmed necrosis involving the formation of a mitochondrial permeability transition pore (mPTP; Vandenabeele et al., 2010). For example, both necroptosis and ischemia reperfusion death can be blocked by necrostatin-1 (Degterev et al., 2005, 2008; Ch'en et al., 2008). Separate studies showed that mice lacking the *Ppif* gene encoding cyclophilin D, an essential component of the mPTP, were also protected from ischemia reperfusion (Baines et al., 2005; Nakagawa et al., 2005). We thus reasoned that Ripk1 and cyclophilin D might be important for necroptosis associated with the loss of caspase 8 (Vandenabeele et al., 2010).

To test this possibility, we crossed *Casp8*$^{f/f}$ *Cd4Cre* (*tCasp8*$^{-/-}$) mice with mice harboring a *Ppif*-null allele and generated four genotypes for analysis: *Casp8*$^{f/f}$ (*WT*), *tCasp8*$^{-/-}$, *Ppif*$^{-/-}$, and *tCasp8*$^{-/-}$ *Ppif*$^{-/-}$ (*double KO* [*DKO*]) mice. To confirm the deletion appropriate to each genotype, purified T cells were immunoblotted for caspase 8 and cyclophilin D (Fig. S1). As previously shown, *tCasp8*$^{-/-}$ mice have a reduced percentage of LN T cells (Salmena et al., 2003), whereas *Ppif*$^{-/-}$ mice showed no apparent changes in the proportion or number of T and B cells (Fig. 1 A and not depicted). The four genotypes showed no differences in the proportion of $CD44^+$ memory-effector cells (unpublished data).

To analyze the dynamics of T cell expansion, CFSE-labeled cells were cultured for 72 h, and collected such that the area under each curve is representative of the total accumulation of cells (Fig. 1 B). In accord with previous results (Ch'en et al., 2008), there was a diminished accumulation of *tCasp8*$^{-/-}$ $CD4^+$ T cells, although the number of cell divisions was unchanged from wild-type. *Ppif*$^{-/-}$ T cells showed no difference from wild-type, but contrary to the prediction described above, the loss of *Ppif* did not rescue the decreased viability found in *tCasp8*$^{-/-}$ T cells. Similar results were found for $CD8^+$ T cells (unpublished data).

To test this further, mice were infected with lymphocytic choriomeningitis virus (LCMV) Armstrong, and the number of LCMV-specific T cells measured at the peak of the response, day 9, and at day 14 after a marked contraction of the population. As depicted in Fig. 1 C and previously reported, there was little expansion in *tCasp8*$^{-/-}$ mice when compared with wild-type mice (Salmena et al., 2003; Ch'en et al., 2008). Again, the loss of *Ppif* did not rescue this diminished proliferation (Fig. 1 C). We have also found no defects in LCMV-mediated expansion in *Ppif*$^{-/-}$ mice (unpublished data).

Although the mPTP complex has been characterized as consisting of the adenosine nucleotide translocator, the voltage-dependent anion channel, and cyclophilin D among other molecules, only a mutation in *Ppif* is sufficient to protect against ischemia reperfusion injury in vivo (Kokoszka et al., 2004; Baines et al., 2007). Such programmed necrosis was thought to be synonymous with necroptosis, especially given the finding that TNF-induced adenosine nucleotide translocator inhibition leading to cell death was shown to be Ripk1 dependent (Temkin et al., 2006). Notwithstanding these strong connections, we conclude that Ripk1-dependent necroptosis induced in activated T cells does not rely on the activity of cyclophilin D. Either this is a form of necroptosis that is distinct from that initiated by TNF, or in general, necroptosis does not use the pathway involving mPTP formation.

### Autophagy and cell death in T cells

Experiments have shown that monocytic cell lines or primary macrophages treated with irreversible caspase inhibitors or with diminished caspase 8 expression spontaneously died.

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An analysis indicated that there was a corresponding increase in autophagic vacuoles and that death could be inhibited by RNA interference–mediated diminution of the autophagy pathway components Atg7 and Beclin-1 (Yu et al., 2004). Similar results were found for Fadd- or caspase 8–deficient T cells using pharmacologic inhibition of phospoinositide 3 kinase or an short hairpin RNA knockdown of *Atg*7 (Bell et al., 2008).

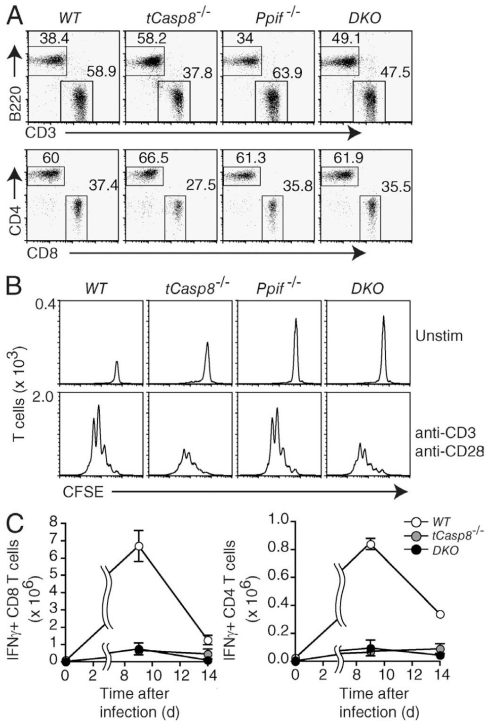


**Figure 1. Caspase 8–deficient T cells do not die by classical necrosis.** (A) The percentages of live-gated T and B cells from the lymph nodes of *WT* (*Casp8*$^{f/f}$), *tCasp8*$^{-/-}$ (*Casp8*$^{f/f}$ *Cd4Cre*), *Ppif*$^{-/-}$ (*Casp8*$^{f/f}$ *Ppif*$^{-/-}$), and *DKO* (*Casp8*$^{f/f}$ *Ppif*$^{-/-}$ *Cd4Cre*) mice were determined by flow cytometry. Data are representative of seven independent experiments. (B) Purified T cells were labeled with CFSE, and then cultured in media alone or stimulated with anti-CD3 and anti-CD28 for 72 h. All cells were resuspended in an equal volume and collected for the same amount of time on the flow cytometer. The numbers on the ordinate indicate the number of T cells per interval of intensity, where the area under the curve equals the total number of T cells collected. Data are representative of seven independent experiments. (C) Cohorts of mice were infected with LCMV Armstrong. On days 9 and 14 after infection, mice were sacrificed, and splenocytes were stimulated with LCMV peptides for 5 h in vitro. Intracellular IFN-γ in gated CD4$^+$ and CD8$^+$ T cells was measured by flow cytometry. Error bars represent the SEM. Data are representative of two independent experiments.

As such, we sought to determine whether a targeted deletion of *Atg*7 could likewise rescue the death associated with the loss of caspase 8 in T cell activation.

Mice with a conditional *Atg*7 deletion (Komatsu et al., 2005) were crossed with mice bearing *Casp8*$^{f/f}$. We generated the following four genotypes for analysis: *Casp8*$^{f/f}$ (*WT*), *Casp8*$^{f/f}$ *Cd4Cre* (*tCasp8*$^{-/-}$), *Atg7*$^{f/f}$ *Cd4Cre* (*tAtg7*$^{-/-}$), and *Casp8*$^{f/f}$ *Atg7*$^{f/f}$ *Cd4Cre* (*DKO*). To confirm the deletion appropriate to each genotype, purified T cells were immunoblotted for caspase 8 and Atg7 (Fig. S2 A). Analysis of the total T cells and B cells (Fig. S2 B), along with the number and proportion of memory–effector T cells (Fig. S2 C) verified previously published data showing a loss in T cell viability in *tAtg7*$^{-/-}$ mice because of abnormally high numbers of mitochondria. We found that the addition of a *Casp8*-null allele accentuated the loss of T cells (Fig. S2 B); at present we do not have an explanation for this genetic interaction.

To determine if the proliferation defect in the absence of *Casp8* is caused by an increase in autophagic death, we characterized proliferation and accumulation of T cells with or without the addition of necrostatin-1. Upon stimulation, a T cell–specific deficiency in either *Casp8* or *Atg*7 caused reduced recovery (Fig. 2 A). The double-mutant T cells exhibited an additional decrease in accumulation, whereas there was no defect in the number of cell divisions. With the addition of necrostatin-1, the accumulation of *tCasp8*$^{-/-}$ T cells was rescued as expected, whereas the addition of necrostatin-1 to *tAtg7*$^{-/-}$ T cells had no effect. The addition of necrostatin-1 to double-mutant T cells partially restored accumulation, presumably overcoming the loss of *Casp8* but not *Atg*7 (Fig. 2, A and B). These results show that the decrease in T cell accumulation caused by an *Atg*7 loss of function is not dependent on the kinase activity of Ripk1. More importantly, the presence of an Atg7 mutant allele did not rescue the necrostatin-sensitive cell death associated with a loss of caspase 8.

The loss of viability in T cells deficient for *Atg*7 was shown to originate from a build-up of mitochondria and overproduction of reactive oxygen species (Pua et al., 2009). Compared with *tAtg7*$^{-/-}$ T cells, DKO T cells were rescued at a substantially greater level with the addition of necrostatin-1, and one possible implication of this result is that a caspase 8 deficiency partially attenuates the loss of Atg7 and the build-up of mitochondria. To test this, we measured mitochondrial mass in T cells using MitoTracker (Fig. 2 C). Indeed, there was a decrease in the fluorescence corresponding to the mitochondrial mass in T cells with mutations in both *Casp8* and *Atg*7 when compared with *Atg*7-defective T cells.

We next determined if the loss of *Atg*7 could complement the loss of *Casp8* in the proliferation associated with LCMV infection. Mice were infected with LCMV, and spleens were collected and analyzed 8 and 14 d after infection. As shown in Fig. 2 D, there was little accumulation of LCMV-specific CD8$^+$ or CD4$^+$ T cells from either *tCasp8*$^{-/-}$ or *tAtg7*$^{-/-}$ mice. In addition, T cells from DKO mice also did not expand. We note that a hemizygous loss of *Atg*7 also did not complement the *Casp8* defect (unpublished data).

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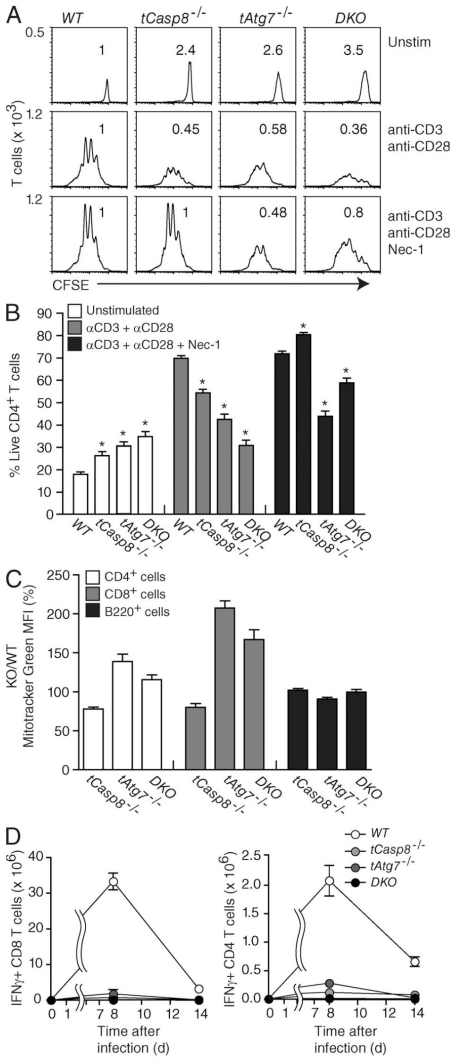


**Figure 2. Loss of Atg7 does not rescue caspase 8–deficient T cell proliferation.** (A) Purified *WT* (*Casp8*$^{f/f}$), *tCasp8*$^{-/-}$ (*Casp8*$^{f/f}$ *Cd4Cre*), *tAtg7*$^{-/-}$ (*Atg7*$^{f/f}$ *Cd4Cre*), and *DKO* (*Casp8*$^{f/f}$ *Atg7*$^{f/f}$ *Cd4Cre*) T cells were labeled with CFSE, and then cultured in media alone or stimulated with anti-CD3 and anti-CD28 in the absence or presence of necrostatin-1 (Nec-1) for 72 h. Flow cytometry analysis was performed as described in the legend to Fig. 1 B. Numbers above each curve represent the proportion of recovered cells relative to *WT*. Data are representative of eight independent experiments. (B) Percentage of live T cells was determined from proliferation described in A. Data are cumulative from eight independent experiments, and error bars indicate the SEM. Asterisks indicate a significant difference from the *WT* control; $P < 0.01$. (C) Mitochondrial mass was measured with MitoTracker Green and percent positive in *tCasp8*$^{-/-}$, *tAtg7*$^{-/-}$, and *DKO* T cells versus *WT* T cells was calculated. Data are cumulative from three independent experiments, and the bars indicate SEM. (D) Cohorts of mice were infected with LCMV Armstrong. On days 8 and 14 after infection, mice were sacrificed and splenocytes were stimulated with LCMV peptides for 5 h in vitro. Intracellular IFN-γ in gated CD4+ and CD8+ T cells was measured by flow cytometry. Data are representative of three independent experiments.

### Increased apoptotic death in the absence of Atg7

The analysis of the *Atg7* mutation as a means of complementing a loss of caspase 8 is complicated by the diminished viability of T cells associated with a defect in autophagy. We therefore sought to characterize the form of death occurring as a consequence of a loss of caspase 8, Atg7, or both. As a first analysis, we measured the proportion of viable versus dead or dying T cells by double staining for Annexin V and 7-aminoactinomycin D (7AAD). Annexin V detects a loss of membrane asymmetry associated with apoptosis, and 7AAD fluoresces after DNA intercalation in cells with a compromised plasma membrane characteristic of the early phases of necrosis. Both of these measurements are time-dependent, and all dying cells eventually become positive for both Annexin V and 7AAD. Cells that do not stain with either dye are viable.

T cells cultured in the absence of stimulation undergo Bim-dependent apoptosis at a high rate (Marrack and Kappler, 2004). This apoptosis is greatly diminished in cultures optimally stimulated with agonistic antibodies specific for CD3 and CD28, and this is illustrated by the analysis of wild-type T cells from *Casp8*$^{f/f}$ mice (Fig. 3 A and Fig. S3 A, *WT*). As previously shown, similarly stimulated caspase 8–deficient T cells undergo cell death at a higher rate (Fig. 3 A and Fig. S3 A, *tCasp8*$^{-/-}$ vs. *WT*). A previous study showed that T cells directly explanted from *Atg7*$^{f/f}$ *LckCre* mice showed diminished viability (Pua et al., 2009); however, in this study we found that after 72 h of culture, *tAtg7*$^{-/-}$ T cells displayed a similar or increased viability compared with wild-type T cells. With stimulation through CD3 and CD28, the viability was substantially reduced compared with *WT* T cells (Fig. 2 A and Fig. 3 A, *tAtg7*$^{-/-}$), and interestingly, the viability of stimulated *DKO* T cells was further reduced such that they did not display increased viability over the unstimulated controls (Fig. 3 A).

To directly compare the form of death for T cells from each genotype, a similar analysis was performed with the addition of necrostatin-1, or qVD, which is a pan-caspase inhibitor highly effective in blocking apoptosis. The addition of necrostatin-1 diminished death in *tCasp8*$^{-/-}$ T cells, as previously described, but had no effect on *tAtg7*$^{-/-}$ T cells (Fig. 3 A). In contrast, the addition of qVD rescued *tAtg7*$^{-/-}$ T cells, but had no effect on *tCasp8*$^{-/-}$ T cells. Double-mutant T cells exhibited the highest death of all, and this death was partially diminished with necrostatin-1, but not with qVD. These results indicate that the loss of the autophagy pathway did not affect

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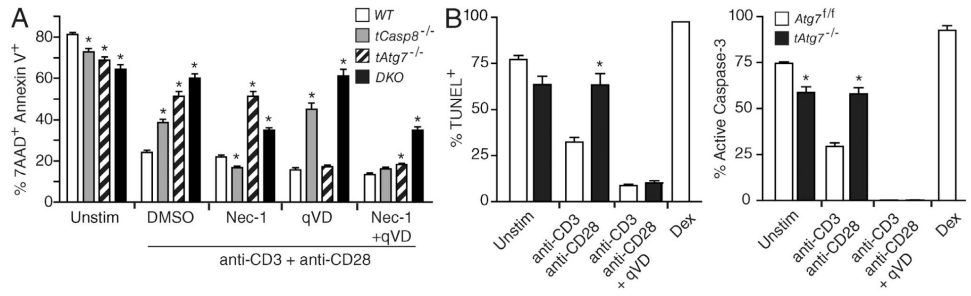


**Figure 3. Increased apoptotic death in the absence of Atg7.** Purified T cells were cultured in media alone (Unstim) or stimulated with anti-CD3 and anti-CD28 in the absence or presence of necrostatin-1 (Nec-1), qVD, or both for 72 h. Dead cells were detected by staining for Annexin V and 7AAD (A) or TUNEL and active caspase 3 (B). Genotypes are abbreviated as listed in legend to Figure 2. Data are cumulative from eight (A) or five (B) independent experiments, bars indicate the SEM, and asterisks indicate a significant difference from the *WT* control; $P < 0.01$.

the necroptosis associated with a loss of caspase 8, and the DKO mice maintained a component of death that was eliminated with the addition of necrostatin-1.

To further characterize the form of death displayed by *Atg7* mutant T cells, we measured DNA fragmentation, a defining characteristic of apoptosis. The presence of DNA fragmentation, as measured by TUNEL, is accompanied by the activation of caspases, most notably, caspase 3. All of the death associated with a loss of *Atg7* showed hallmarks of apoptosis—the cells were positive for TUNEL staining and active caspase 3 and the death was completely inhibited with the addition of qVD (Fig. 3 B and Fig. S3, A and B). These experiments reveal that in the absence of autophagy, stimulated T cells are highly sensitive to death by apoptosis, and we presume this sensitivity corresponds with earlier studies showing an abnormal increase in mitochondrial mass with corresponding increases in oxidative stress (Pua et al., 2009). Because we previously showed that *tCasp8*$^{-/-}$ T cells do not exhibit hallmarks of apoptosis, mutations in *Atg7* or *Casp8* are clearly affecting different pathways.

Previous studies on TNF signaling in the absence of Fadd or caspase 8 revealed the induction of autophagy, although inhibition of the autophagic signaling components did not prevent necroptotic cell death (Degterev et al., 2005). These results contrasted with other studies on caspase 8–deficient cells that demonstrated a form of cell death dependent on components of autophagy (Yu et al., 2004; Bell et al., 2008); however, the experiments presented here show that the loss of *Atg7* does not diminish the necroptotic death associated with a loss of caspase 8 in T cells.

A recent work showed that there is an etoposide-generated autophagy that is Atg5 and Atg7 independent (Nishida et al., 2009). It does not include lipidation of LC3 (the mammalian Atg8 orthologue), but does require Beclin-1 and a Rab9-dependent fusion of the phagophore with vesicles derived from the trans-Golgi and late endosomes. Although it was shown to be operative in the clearance of mitochondria from developing erythrocytes, it may be rare under physiological conditions, and it is not redundant with the many examples of defective autophagy found in *Atg7*-null cells (Komatsu and Ichimura, 2010). Whether this alternative pathway is involved in cell death is presently unknown. We think it is unlikely that this process is involved in the cell death studied here, and conclude that caspase 8–deficient T cells die by a process that does not require autophagy.

### Targeted deletion of Ripk3 rescues caspase 8–dependent necroptosis

The mechanism by which Ripk1 mediates cell death is not understood. The process includes neither DNA fragmentation nor caspase activation, but as described in the introduction, in cells signaled through TNF, a programmed necrosis occurs that requires Ripk1 complexed with Ripk3. The proximal cause of death may involve the overproduction of reactive oxygen species (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). To determine whether this applies to T cells stimulated to divide through the antigen receptor, we crossed a targeted mutation in the *Ripk3* gene into the *Casp8*$^{f/f}$ *Cd4Cre* strain to generate *Casp8*$^{f/f}$ (*WT*), *Casp8*$^{f/f}$ *Cd4Cre* (*tCasp8*$^{-/-}$), *Ripk3*$^{-/-}$, and *Casp8*$^{f/f}$ *Ripk3*$^{-/-}$ *Cd4Cre* (*DKO*) mice (Newton et al., 2004). To confirm the deletion appropriate to each genotype, purified T cells were immunoblotted for caspase 8 and Ripk3 (Fig. S4). The first indication that the *Ripk3* loss of function allele complemented *tCasp8*$^{-/-}$ was that *DKO* mice exhibited lymphadenopathy caused by the expansion of a population of abnormal CD3$^+$CD4$^-$CD8$^-$ T cells that also expressed B220—a cell type found in mice with a Fas or FasL deficiency (Cohen and Eisenberg, 1991). This population accrued in mice with a double mutation, but not in either single KO alone (Fig. 4, A and B), implicating that Ripk3-mediated cell death can replace Fas-mediated clearance of naturally occurring B220$^+$ T cells (Mohamood et al., 2008). In addition, the presence of the CD3$^+$CD4$^-$CD8$^-$B220$^+$ T cell population was not observed until mice were 8 wk of age.

To determine directly whether the *Ripk3* KO can complement a caspase 8 deficiency, T cells were stimulated through

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CD3 and CD28, and the division and accumulation in culture was recorded as described in Fig. 1. As shown, the paucity of T cell accumulation of T cells deficient in caspase 8 was rescued by the additional genetic deletion of *Ripk3* (Fig. 4 C). No changes in T cell expansion were noted in T cells with a mutant version of *Ripk3* alone. Finally, we wished to confirm the rescue by determining the expansion of T cells in response to LCMV infection. As shown, there was little T cell accumulation in the absence of caspase 8, but this phenotype was rescued at the peak of the immune response by the additional deletion of *Ripk3*. By 14 d after infection, the contraction of the T cell population with defects in either *Rip3*$^{-/-}$ or both *tCasp8*$^{-/-}$ and *Rip3*$^{-/-}$ was similar to *WT*.

The cellular pathways of necroptosis, autophagy, and apoptosis appear to be highly interconnected, and thus identifying a specific pathway can be complicated in different experimental models in which cell death occurs (Vandenabeele et al., 2010). We have shown that neither programmed necrosis, as defined by a requirement for cyclophilin D, nor autophagy, as defined by the requirement for Atg7, is operative in the necroptosis observed in caspase 8–deficient T cells. Rather, necroptosis is a unique death pathway mediated by Ripk1 and Ripk3 (Cho et al., 2009; He et al., 2009; Zhang et al., 2009), and here we show that this is the unique cell death pathway initiated by TCR-mediated T cell activation in the absence of caspase 8. We can deduce that caspase 8 prevents

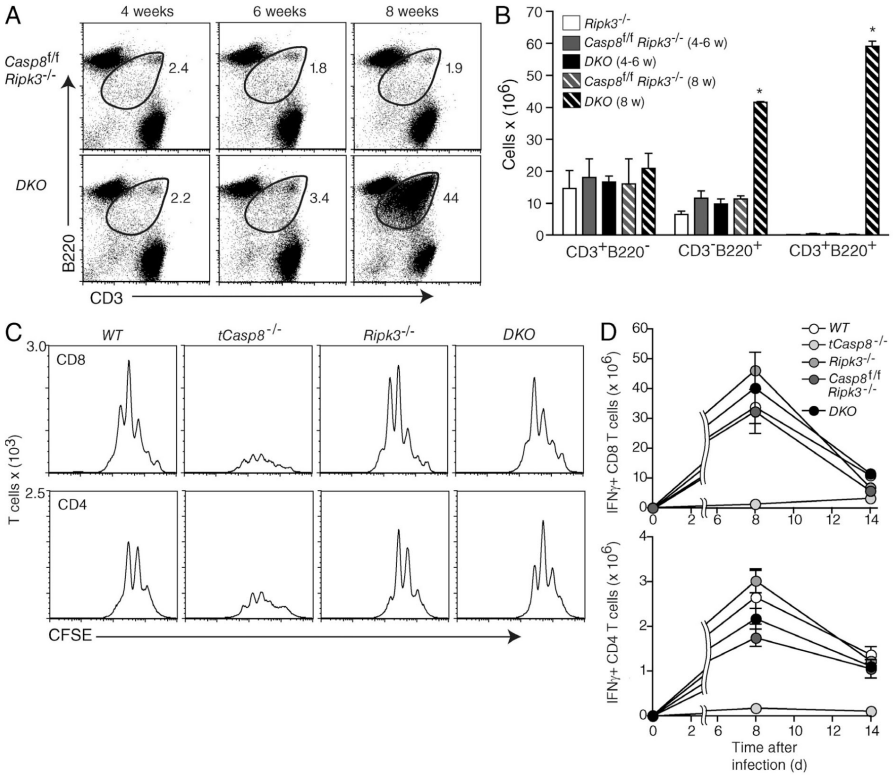


**Figure 4. Targeted deletion of Ripk3 rescues caspase 8–dependent necroptosis.** (A) Live-gated *Casp8*$^{f/f}$ *Ripk3*$^{-/-}$ and *DKO* (*Casp8*$^{f/f}$ *Ripk3*$^{-/-}$ *Cd4Cre*) lymphocytes from mice of the indicated ages were stained with CD3 and B220. Data are representative of three independent experiments. (B) The number of live-gated CD3$^+$B220$^-$, CD3$^-$B220$^+$, and CD3$^+$B220$^+$ cells from the lymph nodes of *Ripk3*$^{-/-}$, *Casp8*$^{f/f}$ *Ripk3*$^{-/-}$, and *DKO* mice were determined by flow cytometry. Data are cumulative from three independent experiments. Bars indicate the SEM, and asterisks indicate a significant difference from the *WT* control; $P < 0.01$. (C) Purified T cells were labeled with CFSE and stimulated with anti-CD3 and anti-CD28 for 72 h. Flow cytometry analysis was performed as described in the legend to Fig. 1 B. Data are representative of three independent experiments. (D) Cohorts of mice were infected with LCMV Armstrong. On days 8 and 14 after infection, mice were sacrificed and splenocytes were stimulated with LCMV peptides for 5 h in vitro. Intracellular IFN-γ in gated CD4$^+$ and CD8$^+$ T cells was measured by flow cytometry. Data for days 8 and 14 after infection are representative of three and one independent experiments, respectively.

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the activation of the Ripk1–Ripk3 axis, and in its absence, necroptosis results. As previously discussed, the physiological significance of such signaling circuitry may be that the absence of caspase 8 activity serves as a pathogen-associated molecular pattern indicating the presence of a virally expressed caspase inhibitor (Ch'en et al., 2008).

The lymphoproliferative disease found in mice or human beings with a germline Fas mutation, *lpr* disease, is characterized by an accumulation of $CD3^+CD4^-CD8^-B220^+$ cells in the peripheral lymphoid organs (Cohen and Eisenberg, 1991; Bidère et al., 2006). In a direct comparison with a germline deletion of *Fas*, this disease was not found in C57BL/6 mice with a T cell–specific deletion of Fas (*Fas*$^{f/f}$ *Cd4Cre*), at least up to 8 mo of age (Hao et al., 2004); rather, there was a gradual decline in T and B cell numbers in secondary lymphoid organs eventually resulting in complete lymphopenia. The genetic background can be determinative because (C57BL/6 x MRL)$F_1$ hybrid mice with a T cell–specific deletion of *Fas* did manifest *lpr*-like lymphoproliferation. We have found that the *tCasp8*$^{-/-}$ C57BL/6 mice also do not manifest *lpr* disease, or any type of lymphoproliferation, up to at least 1 yr of age (unpublished data); however, Salmena and Hakem (2005) found an obvious, generalized lymphoproliferation in aged *Casp8*$^{f/f}$ *Lck Cre* mice from a mixed 129 and C57BL/6 genetic background. In contrast, *tCasp8*$^{-/-}$ *Ripk3*$^{-/-}$ C57BL/6 mice displayed a typical *lpr*-like lymphoproliferative disease, which was consistently manifested at 8 wk of age and included the expansion of $CD3^+CD4^-CD8^-B220^+$ cells. We interpret this to indicate that, at least in C57BL/6 mice, a loss of the extrinsic apoptosis death pathway confined to T cells, along with a loss of the necroptosis pathway, is sufficient to invoke *lpr* disease.

Experiments with MRL.*Fas*$^{lpr}$ mice appear to reveal a requirement for dendritic cells in the expansion of T cells leading to lymphoproliferation (Teichmann et al., 2010), although there are substantial complexities to these studies (Platt and Randolph, 2010). Clearly, the role of other cell types and strain-specific background genes in lymphoproliferation require further investigation, including the role of necroptosis in lymphocyte homeostasis.

In mice, there is a single caspase that is activated via Fas, caspase 8, whereas in human beings there is a second downstream caspase, caspase 10. Interestingly, human beings deficient in caspase 10, but not caspase 8, exhibit lymphoproliferative disease (Bidère et al., 2006). This correlation implies that caspase 8 and caspase 10 are not entirely redundant, and specifically, caspase 8, but not caspase 10, exerts negative control on the necroptosis pathway.

## MATERIALS AND METHODS

**Mice.** *Ppif*$^{-/-}$ (encoding cyclophilin D), *Casp8*$^{f/f}$, *Atg7*$^{f/f}$, and *Ripk3*$^{-/-}$ mice were previously described (Newton et al., 2004; Baines et al., 2005; Beisner et al., 2005; Komatsu et al., 2005). *Ripk3*$^{-/-}$ mice were provided by V. Dixit (Genentech, Inc., South San Francisco, CA). Strains were crossed and backcrossed to obtain mice with single and double mutations with or without *Cd4Cre* (Taconic). *Ppif*$^{-/-}$, *Casp8*$^{f/f}$, and *Atg7*$^{f/f}$ strains were at least 5, 10, and 5 generations backcrossed to C57BL/6, respectively, whereas *Ripk3*$^{-/-}$ mice were generated on a C57BL/6 background. All mice were analyzed between 4 and 12 wk of age. Mice were bred and maintained in the animal care facilities at the University of California San Diego. Animal protocols were approved by the Institutional Animal Care and Use Committee.

**Isolation of T cells.** Single-cell suspensions were prepared from lymph nodes and spleen. Resting T cells were purified by magnetic separation with an autoMACS (Miltenyi Biotec). Biotinylated antibodies specific for B220, MHCII, CD11b, and DX5 (eBioscience) were added to the cells, followed by the addition of streptavidin microbeads (Miltenyi Biotec). The purity of these negatively selected cells was determined to be >95% $CD3^+$ as verified by flow cytometry.

**In vitro proliferation.** Purified T cells were labeled with 0.5 µM CFSE (Invitrogen) for 10 min in PBS/0.1% BSA at 37°C and stimulated with 100 ng plate-bound anti-CD3 (145-2C11) and 1 µg/ml purified anti-CD28 (eBioscience). All cells were resuspended at $10^6$ cells/ml in a 24-well plate. Where indicated, 30 µM 7-Cl-O-necrostatin (provided by A. Degterev, Tufts University, Medford, MA) or 20 µM Q-VD-OPH (SM Biochemicals LLC) was added to the culture.

**Flow cytometry.** Cells were incubated with the appropriate antibodies for 15–20 min in FACS buffer (1x PBS, 1% FBS, 10 mM EDTA, and 0.1% Azide). FITC-, PE-, and allophycocyanin-conjugated antibodies specific for B220, CD3, CD4, CD8, CD44, CD62L, and IFN-γ were purchased from eBioscience. CD8 conjugated to PerCP was purchased from BioLegend. Cultured CFSE-labeled cells were resuspended in an equal volume of FACS buffer and collected for a constant length of time on the FACSCalibur (BD). Flow cytometric analysis was performed using FlowJo software (Tree Star). To stain for mitochondrial mass, lymphocytes were incubated for 30 min at 37°C with MitoTracker Green (Invitrogen) in RPMI 1640 complete media.

**LCMV infection.** 4–12-wk-old mice were infected by intraperitoneal injection with $2 \times 10^5$ plaque-forming units of LCMV Armstrong in 0.5 ml of PBS. At the indicated time points, spleens were harvested from infected and noninfected mice. Splenocytes were stimulated for 5 h in vitro with gp33 and NP396 peptides, MHC class I–restricted LCMV epitopes or gp61 and NP309 peptides, MHC class II–restricted LCMV epitopes, and Brefeldin A (GolgiStop; BD). Cells were labeled with CD4, CD8, and CD44 surface markers, fixed and permeabilized with the Cytofix/Cytoperm kit (BD), and then stained for intracellular IFN-γ. Standard error was calculated for each time point.

**Cell death assays.** Cells were incubated with Annexin V (Invitrogen) in Annexin V–binding buffer (140 mM NaCl, 2.5 mM $CaCl_2$, and 10 mM Hepes) for 15 min at room temperature. Staining with 7AAD was done on unpermeabilized cells in FACS buffer. TUNEL was performed using the In situ Cell Death Detection kit TMR red from Roche. To measure intracellular active caspase-3, cells were fixed, permeabilized, and stained according to manufacturer's protocol and measured by flow cytometry (BD).

**Western blotting.** Purified T cells were lysed on ice with Cell Lysis Buffer (Cell Signaling Technology) supplemented with protease and phosphatase inhibitors, and spun for 10 min at 4°C. Protein concentrations were determined using a Bio-Rad protein assay (Bio-Rad Laboratories). 10-15 µg of protein was separated on 4–12% or 12% Bis-tris NuPage Gels (Invitrogen), transferred to Immobilon-P PVDF membranes (Millipore), and blotted with antibodies specific for caspase 8, clone 1G12 (Enzo Life Sciences); cyclophilin D, clone E11AE12BD4 (MitoSciences Inc.); ATG7 (Cell Signaling Technology); Ripk3 (ProSci Inc.); and PLCγ (Santa Cruz Biotechnology). Membranes were incubated with a horseradish peroxidase–conjugated antibody to the appropriate species and visualized on Hyblot CL Autoradiography Film (Denville Scientific Inc.) using the ECL system.

**Statistics.** Statistical analyses were performed using Prism 4 for Mac (GraphPad Software, Inc.). Error bars represent SEM, and p-values were calculated with a two-tailed Student's *t* test.

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**Online supplemental material.** Fig. S1 shows the efficiency of deletion in *WT*, *tCasp8*$^{-/-}$, *Ppif*$^{-/-}$, and DKO (*tCasp8*$^{-/-}$ *Ppif*$^{-/-}$) purified T cells. Fig. S2 shows the efficiency of deletion in *WT*, *tCasp8*$^{-/-}$, *tAtg7*$^{-/-}$, and DKO (*tCasp8*$^{-/-}$ *tAtg7*$^{-/-}$) purified T cells, and the accumulated phenotypic data describing peripheral lymphocytes and splenocytes. Fig. S3 shows example profiles measuring the increased apoptotic death observed in *tAtg7*$^{-/-}$ and DKO (*tCasp8*$^{-/-}$ *tAtg7*$^{-/-}$) stimulated cell cultures when compared with *WT* and *tCasp8*$^{-/-}$ (accumulated data shown in Fig. 3). Fig. S4 shows the efficiency of deletion in *WT*, *tCasp8*$^{-/-}$, *Ripk3*$^{-/-}$, and DKO (*tCasp8*$^{-/-}$ *Ripk3*$^{-/-}$) purified T cells. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20110251/DC1.

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# CHAPTER III:

# The role of caspase-8 in regulating dendritic cell activation during both homeostasis and chronic viral infection

## Abstract

Caspase-8 is canonically known as an executioner of apoptotic programmed cell death (PCD), and has also been found to inhibit alternative necroptotic PCD. Recently, caspase-8 has been suggested to negatively regulate Rig-I signaling. Here, we examined the effect of a loss of caspase-8 in dendritic cells (DCs). We found that mice lacking DC-specific caspase-8 (*dcCasp8*$^{-/-}$ mice) develop age-dependent symptoms of autoimmune disease in the steady state, characterized by hyperactive DCs and T cells, spleen and liver immunopathology, and polarization of $CD4^+$ T cells towards the Th1 subset. *dcCasp8*$^{-/-}$ mice infected with chronic lymphocytic choriomeningitis virus (LCMV), an RNA virus detected by Rig-I, mounted an enhanced LCMV-specific immune response as measured by increased proportions of antigen-specific $CD4^+$ T cells and effector cytokine producing $CD4^+$ and $CD8^+$ T cells. When the Rig-I pathway was stimulated, *Casp8*$^{-/-}$ DCs upregulated CD86 and produced pro-inflammatory cytokines to a greater degree than WT DCs. Both IRF3 and NF-kB were endogenously hyperactive in *Casp8*$^{-/-}$ DCs, consistent with a role for caspase-8 as a negative regulator of Rig-I signaling.

## Introduction

Dendritic cells (DCs) bridge innate and adaptive immunity by acting as sentries that detect invading pathogens. In addition, they act as professional antigen-presenting cells (APCs) capable of potently activating antigen-specific T cells (4, 76). As a consequence of pathogen recognition, DCs undergo a program of maturation that includes antigen presentation by cell surface MHC molecules and enhanced expression of essential co-stimulatory molecules including CD80 and CD86 (54, 77). In the steady state, immature DCs instead contribute to immune tolerance by presenting self antigens to autoreactive lymphocytes, leading to their anergy, deletion, or conversion to iTreg cells (50, 51, 78). DCs that become mature in the absence of infection can thus provoke an immune response to self antigens, as seen in autoimmune diseases from multiple sclerosis and type 1 diabetes (T1D) to systemic lupus erythematosus (SLE) (57). A role for DCs in human SLE pathogenesis is supported by the finding that DCs from SLE patients have been shown to upregulate CD86 expression independent of activating stimuli (61, 62).

Immature DCs utilize endocytic pathogen recognition receptors (PRRs) to detect and ingest infectious agents, or their constituents, that bear pathogen associated molecular patterns (PAMPs), and upon processing, present associated oligopeptides to T cells (79). For example, DCs can detect viral double-stranded RNA using Rig-I-like receptors (RLRs) such as retinoic-acid

inducible gene-I (Rig-I) (13, 80). Initiation of Rig-I signaling leads to the formation of a mitochondrial signaling complex (MSC) consisting of MAVS, FADD, TRADD, TANK and Ripk1, activation of interferon regulatory factors (IRFs) 3 and 7, and production of type I interferons (IFN-I) (80-86), a cytokine with pleiotropic effects in immunity and disease (87). IFN-I is an important anti-viral cytokine, but is also considered a key driver of disease development in SLE (55, 60).

The cysteine–aspartic acid protease, caspase-8, canonically known for its role in executing death receptor-mediated apoptosis, also inhibits necroptosis, an alternative form of programmed cell death (PCD) (88). Necroptosis can be triggered by the same stimuli that initiate apoptosis, such as death receptor ligation or T cell receptor stimulation, and it is mediated by the kinases Ripk1 and Ripk3 (26, 29-32, 34, 35). Loss of caspase-8 in a variety of tissues led to Ripk1 or Ripk3-dependent necroptotic death (16, 18, 28, 33, 89). A small proportion of DCs have been shown to undergo necroptosis upon stimulation with poly(I:C), and this appears to depend on the dissociation of caspase-8 from the MSC and subsequent Ripk1 ubiquitination and activation (90). However, *Casp8*$^{-/-}$ DCs are not more prone to dying upon treatment with the death receptor ligand TNF (38).

In addition to its inhibitory effect on necroptosis, caspase-8 is also a negative regulator of Rig-I signaling. Caspase-8 inhibits the formation of the MSC by cleaving Ripk1, and a loss of caspase-8 in non-hematopoietic cells resulted in enhanced phosphorylation of IRF3 (36). Caspase-8 was also shown to directly cleave IRF3, leading to its degradation (37). Subsequent studies showed that caspase-8 ablation in DCs led to increased production of pro-inflammatory cytokines, due to a loss of inhibition of either Ripk3-mediated NLRP3 inflammasome activation, or Ripk1- and MyD88-mediated DC activation (38, 39). Mice with *Casp8*$^{-/-}$ DCs were also shown to develop an age-dependent autoimmune disease (39). However, the role of Rig-I signaling in disease development of these mice is unclear, as is the overall contribution of the Rig-I pathway to the function of *Casp8*$^{-/-}$ DCs.

Here, we show that mice with *Casp8*$^{-/-}$ DCs develop an age-dependent hyperactivation of DCs and T cells, immunopathology, and predominance of IFN-$\gamma$ producing Th1 cells. Loss of caspase-8 in DCs also allowed mice to mount an enhanced response to chronic viral infection, leading to less exhausted antigen-specific T cells and lower viral loads. Finally, *Casp8*$^{-/-}$ DCs were more sensitive to Rig-I stimulation *in vitro*, highlighting the importance of caspase-8 in regulating both the development of autoimmune-like disease and enhanced viral response *in vivo*. Thus, caspase-8 inhibits spontaneous DC activation, but limits the immunogenicity of DCs during viral infection.

# Results

### Mice with *Casp8*$^{-/-}$ DCs develop an age-dependent autoimmune phenotype

To investigate the contribution of caspase-8 in the regulation of DCs, mice with a *Casp8*$^{f/f}$ allele were crossed with Cd11cCre mice to generate *Casp8*$^{flox/flox}$ *Cd11cCre* conditional knockout (cKO) mice (hereafter referred to as "*dcCasp8*$^{-/-}$ mice"). These mice displayed higher proportions of hyperactive CD11b$^{+}$ and CD8$^{+}$ conventional DCs (hereafter referred to as “DCs”) at three months of age, as assessed by the expression of CD86 (Fig. 6A), and DCs from *dcCasp8*$^{-/-}$ mice remained hyperactive through 14-plus months of age. To determine whether the hyperactivation seen in DCs was reflected in the population of T cells, we assessed the status of T cells by enumerating previously activated (CD44$^{hi}$CD62$^{lo}$) vs. naïve (CD44$^{lo}$CD62L$^{hi}$) T cells found in the spleen and blood. As shown, CD4$^{+}$ T cells from *dcCasp8*$^{-/-}$ mice generated increased proportions of activated T cells at three months of age, while increased in activated CD8$^{+}$ T cells were not apparent until six months of age (Fig. 6B). The proportion of previously activated T cells in WT mice increased with age; however, this was substantially accelerated in *dcCasp8*$^{-/-}$ mice for both CD4$^{+}$ and CD8$^{+}$ subsets.

We next determined whether the hyperactive DCs and T cells in aged *dcCasp8*$^{-/-}$ mice had an effect on organ immunopathology. Spleens from aged *dcCasp8*$^{-/-}$ mice displayed a disrupted splenic architecture, as demonstrated by an expansion of white pulp (Fig. 6C). Livers from aged *dcCasp8*$^{-/-}$ mice showed an increase in polymorphic infiltrates similar to the infiltrating leukocytes seen in autoimmune mice with Fas-deficient DCs (69) (Fig. 6C).

Since aged *dcCasp8*$^{-/-}$ mice appeared to display signs of autoimmunity, we next examined whether the number of FOXP3$^{+}$ regulatory T (Treg) cells was diminished. On the contrary, we found increased percentages and numbers of Foxp3$^{+}$ Tregs in ten month old *dcCasp8*$^{-/-}$ mice (Fig. 6D), consistent with a loss of DC-specific CD86/80 leading to a reduction in peripheral Tregs (91).

**CD4$^{+}$ T cells from aged *dcCasp8*$^{-/-}$ mice skew towards a Th1 phenotype**

Many autoimmune diseases are characterized by the emergence of a paarticular CD4$^{+}$ T helper (Th) subset characterized by its associated hallmark cytokine. For example, IFN-$\gamma$-producing Th1 cells play a key role in driving disease in mouse models of systemic lupus erythematosus (SLE) (92, 93) and these T cells are more prevalent in the peripheral blood of human SLE patients (94). To determine whether CD4$^{+}$ T cells in aged *dcCasp8*$^{-/-}$ mice were polarized for a specific Th subset, we stimulated splenocytes *in vitro* with PMA and ionomycin for 4 hr and analyzed the appearance of diagnostic cytokines.

The results showed an increased subset of $CD4^+$ T cells from aged *dcCasp8*$^{-/-}$ mice that produced IFN-γ, when compared to WT, whereas the numbers of $CD4^+$ T cells producing IL-4 and IL-17 were similar (Fig. 7A).

**Young adult *dcCasp8*$^{-/-}$ mice mount an enhanced antigen-specific T cell response to chronic viral infection**

Since specific pathogen free *dcCasp8*$^{-/-}$ mice develop hyperactive DCs and T cells over time, we wanted to determine how *dcCasp8*$^{-/-}$ mice would respond to a viral infection. Taking into account our observation that *dcCasp8*$^{-/-}$ mice developed a detectable increase in activated DCs and $CD4^+$ T cells by 3 months of age, we decided to infect young adult (6-10 week old) mice before the appearance of phenotypic manifestations of the *Casp8* deletion. Specifically, we wished to determine whether *dcCasp8*$^{-/-}$ mice would mount an exaggerated response to viral infection as a result of enhanced DC activation and antigen presentation to T cells.

Lymphocytic choriomeningitis virus (LCMV) is an RNA virus that is detected by Rig-I, and its containment is dependent upon antigen-specific CD8 T cells (95-97). Inoculation of C57BL/6 mice with $10^6$ PFU LCMV clone 13 (LCMV Cl13), the chronic form of LCMV, results in a long and persistent viral infection (98). Virus is cleared from the blood and peripheral lymphoid organs over a period of 2-3 months, but it is never cleared from the kidneys (99). A

characteristic of the $CD4^+$ and $CD8^+$ T cell response is the induction of multiple negative feedback pathways starting day 12 post infection, and these mechanisms appear to prolong the viral infection while lessening the associated immunopathology (100, 101). Previous work has shown that removal of negative regulation can be readily detected (102-104). In addition, LCMV Cl13 is able to establish a chronic infection partly because it selectively infects and suppresses DCs (105), and thus, enhanced DC function might be expected to mitigate this form of LCMV virulence.

Upon infection of 6-10 week old *dcCasp8*$^{-/-}$ mice, the expansion of antigen-specific T cell responses was examined using tetramers specific for the major immunodominant $CD4^+$ and $CD8^+$ T cell epitopes. We found that *dcCasp8*$^{-/-}$ mice had higher proportions of I-$A^b$ $GP_{66}^+$ $CD4^+$ T cells at days 8 and 15 p.i. (Fig. 8A). *dcCasp8*$^{-/-}$ mice had similar proportions of H2-$D^b$ $GP_{33}^+$ $CD8^+$ and H2-$D^b$ $GP_{276}^+$ $CD8^+$ T cells from days 8 to 30 p.i., but interestingly, *dcCasp8*$^{-/-}$ mice had larger percentages of H2-$D^b$ $NP_{396}^+$ $CD8^+$ T cells at days 8 and 30 (but not 15) p.i. (Fig. 8B). Overall, $GP_{66}$-specific $CD4^+$ T cells from *dcCasp8*$^{-/-}$ mice exhibited a substantially stronger expansion compared to wildtype early in the infection, and this would be predicted to have an important effect on the expansion of $CD8^+$ T cells and the clearance of virus (106).

**T cells from chronically infected *dcCasp8*$^{-/-}$ mice retain effector function**

T cells achieve their antiviral effector function, in part, by producing cytokines such as interferon-γ (IFN-γ) and tumor necrosis alpha (TNF-α). T cells that simultaneously produce both cytokines are considered the most functional and effective in clearing a viral infection (107). We assessed the ability of T cells from LCMV Cl13-infected *dcCasp8*$^{-/-}$ mice to produce IFN-γ and TNF-α upon re-stimulation with either CD4$^{+}$ or CD8$^{+}$ T cell LCMV-specific peptides ($GP_{61-80}$ and $GP_{33-41}$, respectively). We found that, consistent with the expansion of LCMV-specific T cells, a higher proportion of CD4$^{+}$ T cells from *dcCasp8*$^{-/-}$ mice produced both IFN-γ and TNF-α at days 8 and 15 p.i. (Fig. 8C), and a higher proportion of CD8$^{+}$ T cells from *dcCasp8*$^{-/-}$ mice produced both IFN-γ and TNF-α at days 15 and 30 p.i. (Fig. 8D). Taken together, these results suggest that chronically infected *dcCasp8*$^{-/-}$ mice have an early (days 8-15 p.i.) enhancement in the CD4$^{+}$ T cell response, and a late (days 15-30 p.i.) enhancement in the CD8$^{+}$ T cell response.

**The effect of DC-specific *Casp8* deletion on T cell exhaustion and viral clearance**

T cell exhaustion is defined by a loss of effector function, including antiviral cytokine production, and the upregulation of inhibitory surface molecules such as PD-1, LAG-3 and TIM-3 (107). Since we observed that there

were more effector $CD4^+$ and $CD8^+$ T cells from *dcCasp8*$^{-/-}$ mice compared with WT, we next investigated whether T cells from *dcCasp8*$^{-/-}$ mice expressed differential levels of the inhibitory receptor PD-1. Analysis showed that H2-$A^b$ $GP_{66}^+$ $CD4^+$ T cells from *dcCasp8*$^{-/-}$ mice expressed lower levels of PD-1 by both MFI and the percentage of PD-$1^{hi}$ expressing cells at day 30 p.i. (Fig. 9A). Similar results were found analyzing H2-$D^b$ $GP_{33}^+$ $CD8^+$ T cells from *dcCasp8*$^{-/-}$ mice.

Since *dcCasp8*$^{-/-}$ mice possessed T cells that retained the ability to produce antiviral cytokines and were less exhausted at d30 after infection, there was the possibility that virus load would be correspondingly reduced. Consistent with the time-course of T cell expansion and effector function *dcCasp8*$^{-/-}$ mice displayed lower viral loads in serum, liver and kidney at day 30 p.i. as measured by plaque assay (Fig. 9B-C).

### *dcCasp8*$^{-/-}$ mice have more antigen-specific Tfh cells and higher levels of IgG2a antibody a month after chronic infection

While WT mice clear an LCMV Cl13 infection (in lymphoid tissues) in two to three months, mice lacking $CD4^+$ T cells do not clear the virus for over four months (106), and this appears to be due to the importance of $CD4^+$ T cells both in providing help to $CD8^+$ T cells and in promoting LCMV-specific antibody responses (108-111). Since we saw an enhanced LCMV-specific $CD4^+$ T

response in *dcCasp8*$^{-/-}$ mice, we examined whether the antibody response was also playing a role in virus clearance. Analyses showed that *dcCasp8*$^{-/-}$ mice had greater proportions of H2-A$^{b}$ GP$_{66}^{+}$ CD4$^{+}$ follicular helper T (Tfh) cells at day 30 p.i. (Fig. 9D). Unexpectedly, we found that *dcCasp8*$^{-/-}$ mice had substantially fewer germinal center (GC) B cells as measured by the expression of GL-7 and Fas (112) (Fig. 9E). In keeping with this result, within the class switched (IgM$^{-}$IgD$^{-}$) B cell population from *dcCasp8*$^{-/-}$ mice there were very few cells expressing high amounts of CD86. Yet, measuring levels of LCMV-specific antibody levels at day 30 p.i. showed that *dcCasp8*$^{-/-}$ mice had higher levels of LCMV-specific IgG2a, not only the dominant isotype produced during LCMV Cl13 infection but also the isotype preferentially driven by increased IFN-$\gamma$ (113, 114). No difference in levels of LCMV-specific total IgG or IgG1 were found (Fig. 9F).

The discrepancy between increased numbers of Tfh cells, increased antibodies, but reduced GC B cells has not been resolved. GC B cells can differentiate into plasmablasts, which then form antibody-producing plasma cells (115). As plasmablasts and plasma cells are not characterized by expression of high levels of GL-7 and Fas (116), we cannot rule out the possibility that there are higher proportions of these B cell subsets in infected *dcCasp8*$^{-/-}$ mice. Another possibility is that a chronic viral infection accelerates the disruption of splenic architecture observed in uninfected aged *dcCasp8*$^{-/-}$

mice (Fig. 6C), leading to a loss of splenic structure required for GC B cell formation (117). Lupus-prone MRL.*Fas*$^{lpr}$ mice spontaneously develop GCs, which are lost over time and are not detectable in 6 month old mice, perhaps due to the collapse of splenic architecture (1, 2).

**LCMV-specific CD4 T cells from chronically infected *dcCasp8*$^{-/-}$ mice have enhanced expansion and effector function**

To address the possibility that pre-existing hyperactivated CD4$^+$ T cells in *dcCasp8*$^{-/-}$ mice contribute to the larger proportion of either H2-A$^b$ GP$_{66}$$^+$ CD4$^+$ T cells or IFN-$\gamma$/TNF-$\alpha$ producing CD4$^+$ T cells in these mice (Fig. 8A and B), we adoptively transferred CD4$^+$ T cells from SMARTA transgenic mice into WT and *dcCasp8*$^{-/-}$ recipients. SMARTA mice have CD4$^+$ T cells specific for the gp67-77 epitope of LCMV (118). We found that CD45.1$^+$CD45.2$^+$ SMARTA CD4$^+$ T cells transferred into *dcCasp8*$^{-/-}$ hosts expanded to a greater degree in the blood at day 5 p.i. (Fig. 10A), and in the spleen at day 8 p.i. (Fig. 10B). When we examined the ability of SMARTA CD4$^+$ T cells from *dcCasp8*$^{-/-}$ hosts to produce effector cytokines upon re-stimulation with the CD4$^+$ T cell LCMV-specific peptide (GP$_{61-80}$), we found that a higher proportion of SMARTA CD4$^+$ T cells from *dcCasp8*$^{-/-}$ mice produced either IFN-$\gamma$ alone, both IFN-$\gamma$ and TNF-$\alpha$, or both IFN-$\gamma$ and IL-2, consistent with the enhanced expansion of these cells in *dcCasp8*$^{-/-}$ hosts (Fig. 10C).

**DCs lacking caspase-8 hyperactivate upon Rig-I stimulation**

We hypothesized that, in *dcCasp8*$^{-/-}$ mice, both the age-dependent autoimmune phenotype and the enhanced response to chronic infection resulted from the loss of negative regulation of Rig-I signaling by caspase-8 in DCs. To directly test this, we activated Rig-I in *Casp8*$^{-/-}$ DCs by transfecting them with short-length poly(I:C), which is a surrogate for dsRNA and is specifically detected by Rig-I (119, 120). We isolated splenic conventional DCs from WT and *dcCasp8*$^{-/-}$ mice and transfected them with increasing doses of short-length poly(I:C), then assessed the upregulation of the co-stimulation molecule CD86. We found that both CD11b$^{+}$ and CD8$^{+}$ DCs upregulated CD86 to a greater degree than WT DCs in a poly(I:C) dose-dependent manner (Fig. 11A).

Loss of caspase-8 in a variety of cell types leads to necroptosis, or programmed necrosis, an alternative mode of programmed cell death that occurs when a cell receives death stimuli and apoptosis is inhibited (33-35). Immature DCs become mature upon co-culture with cells undergoing necrosis or exposure to necrotic cell supernatant, likely due to the release of danger-associated molecular patterns (DAMPs) during necrosis, but do not mature when cultured with apoptotic cells or apoptotic cell supernatant (120, 121). Likewise, DCs incubated with apoptotic cells cannot upregulate CD86 and do

not produce IL-12 upon stimulation with LPS, indicating that exposure to apoptotic signals suppresses DC maturation (122, 123).

To ensure that the amount of cell death was consistent between poly(I:C)-transfected WT and *Casp8*$^{-/-}$ DCs, we used a membrane permeable dye to compare levels of cell death in transfected DCs. We found that levels of cell death in poly(I:C)-transfected WT and *Casp8*$^{-/-}$ DCs were comparable (Fig. 11B).

**DCs lacking caspase-8 have endogenously hyperactive IRF3 and NF-κB**

To directly assess whether *Casp8*$^{-/-}$ DCs produce more type I interferon upon Rig-I stimulation, as our hypothesis predicted, we measured levels of IFN-β in the supernatant of poly(I:C)-transfected DCs. To our surprise, we found that the supernatant of *Casp8*$^{-/-}$ DCs contained significantly higher levels of IFN-β at only one concentration of transfected poly(I:C) (Fig. 11C). To investigate further, we measured whether levels of phosphorylated IRF3 (P-IRF3) in poly(I:C)-transfected *Casp8*$^{-/-}$ DCs were higher, also as our hypothesis predicted. We found that levels of P-IRF3 were indeed higher in unstimulated CD11b$^{+}$CD4$^{+}$ *Casp8*$^{-/-}$ DCs, and remained higher than in WT CD11b$^{+}$CD4$^{+}$ DCs regardless of the duration of poly(I:C) transfection (Fig. 12A).

Since we did not observe a dramatic increase in either the level of IFN-β in the supernatant of poly(I:C)-transfected *Casp8*$^{-/-}$ DCs, or in the activation of

IRF3 in the poly(I:C)-transfected *Casp8*$^{-/-}$ DCs themselves, we examined whether a non-IRF3/type I interferon mechanism could be contributing to the hyperactive phenotype of *Casp8*$^{-/-}$ DCs. Thus, we assessed the levels of several other pro-inflammatory cytokines (IFN-$\gamma$, TNF-$\alpha$, IL-6, MCP-1) in the supernatant of poly(I:C)-transfected DCs. We found that all four of these cytokines were all upregulated in the supernatant of *Casp8*$^{-/-}$ DCs in response to increasing doses of transfected poly(I:C) (Fig. 11C). This led us to examine whether NF-$\kappa$B signaling was responsible for the hyperactivation phenotype of the *Casp8*$^{-/-}$ DCs, as NF-$\kappa$B can also be activated by Rig-I signaling (in addition to IRF3/7) and its target genes include IFN-$\gamma$, TNF-$\alpha$, IL-6 and MCP-1 (80, 85).

I$\kappa$B is an inhibitor of NF-$\kappa$B. In unstimulated cells, I$\kappa$B interacts with and sequesters NF-$\kappa$B in the cytoplasm; upon activation by a number of different stimuli (i.e. TCR, BCR, TLR, RLR or TNFR agonists), I$\kappa$B is targeted for degradation by the ubiquitin-proteasome pathway (124, 125). To assess NF-$\kappa$B activation in Rig-I stimulated *Casp8*$^{-/-}$ DCs, we measured the degradation of I$\kappa$B upon poly(I:C) transfection. Similar to our observations with P-IRF3, we found that NF-$\kappa$B appeared to be endogenously hyperactive in CD11b$^{+}$CD4$^{+}$ *Casp8*$^{-/-}$ DCs. Levels of I$\kappa$B were lower in unstimulated CD11b$^{+}$CD4$^{+}$ *Casp8*$^{-/-}$ DCs, and remained lower than in WT CD11b$^{+}$CD4$^{+}$ DCs regardless of the duration of poly(I:C) transfection (Fig. 12B). Of note, WT and *Casp8*$^{-/-}$ CD8$^{+}$

DCs expressed similar levels of phospho-IRF3 and IkB, regardless of whether they were transfected with poly(I:C) or not (Fig. 12A&B).

## Discussion

Although dendritic cells (DCs) represent a small proportion of total immune cells, they are essential for both initiating and preventing adaptive immune responses. These seemingly paradoxical outcomes depend in large part on whether the DCs are activated. Immature, unactivated DCs help prevent autoimmunity by presenting self-antigens to cognate T cells in the peripheral lymphoid organs, which then undergo deletion or anergy in a process contributing to self-tolerance (40). If mature DCs present oligopeptides to T cells, however, T cell activation and proliferation result. In principle, presentation of an self-peptide by a mature DC could result in an autoimmune response (57). The fact that T cells do not become activated in response to antigen presentation by DCs alone, but also require the DCs to be stimulated to mature (such as through exposure to adjuvant or by pathogen-recognition receptor signaling) is the basis for "the immunologist's dirty little secret" first proposed by Charles Janeway, Jr. (126).

The importance of DCs in mediating immune responses is illustrated in mouse models of constitutive DC ablation. CD11c-DTA mice, which primarily lack DCs (but also certain macrophages, NK cells, and activated T cells), display impaired $CD4^+$ and $CD8^+$ T cell responses, and in one study they developed steady-state autoimmunity characterized by CD4+ T cell activation (127, 128). When DCs are constitutively deleted from lupus-prone MRL.$Fas^{lpr}$ mice, age-

dependent T cell expansion and differentiation into IFN-g producing effector cells is diminished, leading to disease reduction (93).

However, when activated DCs are unable to be eliminated via Fas-mediated killing, systemic autoimmunity develops (69). Several genes have been identified as negative regulators of DC activation; for example, mice with DC-specific ablations of A20 or SHP1 developed spontaneously mature DCs that produced more inflammatory cytokines and drove the increased activation and differentiation of T cells (57). Vaccination of mice with A20-silenced DCs boosted the tumor-infiltrating T cell response in a mouse melanoma model, and improved T cell cytokine production and expression of gut-homing receptors in response to HIV infection (129, 130).

The effect of deleting a DC negative regulator on the viral immune response *in vivo* is less clear. Caspase-8 is a downstream negative regulator of Rig-I signaling, a key pathway for detection of RNA viruses in DCs (13, 36). Mice with a DC-specific ablation of caspase-8 have previously been shown to develop an age-dependent autoimmunity that depends on TLR signaling (39). We show here that stimulated $CD4^+$ T cells from aged *dcCasp8*$^{-/-}$ mice skew towards a Th1 phenotype, indicating a possible role for IFN-g in disease pathogenesis. We also explore the consequences of hyperactive DCs in the context of a chronic viral infection. Our study reveals an adjuvant-like effect of losing caspase-8 in DCs, as *dcCasp8*$^{-/-}$ mice have an enhanced immune

response to LCMV Cl13, an RNA virus detected by Rig-I. The observation that hyperactive DCs can aid in the resolution of chronic viral infection, despite their damaging role in driving steady-state autoimmunity, is consistent with the finding that although mice with Fas-deficient DCs develop systemic autoimmunity, they also clear a chronic LCMV infection more rapidly (69, 131).

Over time, *dcCasp8*$^{-/-}$ mice develop systemic autoimmunity in the steady-state, defined by the absence of any external pathogen challenge. This suggests that peripheral tolerance is lost when hyperactivated caspase-8 deficient DCs present self-antigen, possibly derived from commensal bacteria, to autoreactive T cells. RNA species from intestinal microbiota can activate Rig-I signaling and lead to inflammatory cytokine production (132). Peptide-pulsed caspase-8 deficient DCs were shown to have a greater capacity to stimulate proliferation of cognate T cells *in vitro* (39), and we would predict that *dcCasp8*$^{-/-}$ mice injected with peptide would manifest an enhanced proliferation of cognate T cells *in vivo*, in the absence of any adjuvant.

*DcCasp8*$^{-/-}$ mice had higher proportions of activated DCs and CD4$^{+}$ T cells as early as three months of age and was seen to have increased by six months of age (Fig. 6A&B). Since we infected 6-8 week old mice with LCMV Cl13, and examined one cohort as late as 30 days post infection, this leaves open the possibility that the enhanced T cell functionality seen in *dcCasp8*$^{-/-}$ mice may have been derived from either pre-existing activated T cells in the 6-

8 week old mice, or T cells that became activated by a non-LCMV, endogenous mechanism during the course of infection. To address the first possibility, we transferred SMARTA (LCMV-specific $CD4^+$) T cells into *dcCasp8*$^{-/-}$ hosts, infected them with LCMV Cl13, and found that SMARTA T cells in *dcCasp8*$^{-/-}$ mice underwent a larger early (days 5 and 8 p.i.) expansion, and had greater anti-viral cytokine production at day 8 p.i. (Fig. 10). This finding supports the idea that the enhanced antigen-specific $CD4^+$ T cell response (by both proportion and functionality) at day 8 p.i. in LCMV Cl13 infected *dcCasp8*$^{-/-}$ mice (Fig. 8A&B) is likely directly due to the DCs and not any pre-existing hyperactive T cells.

We predicted that a loss of caspase-8 in DCs would lead to hyperactive IRF3 and increased type I interferon production. While we did not find a dramatic increase in IFN-$\beta$ secretion from poly(I:C)-transfected *Casp8*$^{-/-}$ DCs (Fig. 11C), levels of phospho-IRF3 were elevated in both unstimulated and Rig-I stimulated *Casp8*$^{-/-}$ DCs (Fig 12A). To determine whether a non-IRF3 signaling pathway could be contributing to the hyperactive *Casp8*$^{-/-}$ DC phenotype, we examined the supernatant of poly(I:C)-transfected DCs and found that *Casp8*$^{-/-}$ DCs produced higher levels of four pro-inflammatory cytokines (IFN-$\gamma$, TNF-$\alpha$, IL-2, MCP-1) (Fig. 11C). As production of all of these cytokines are downstream of NF-$\kappa$B activation, we assessed the degradation of I$\kappa$B in *Casp8*$^{-/-}$ DCs as a readout of NF-$\kappa$B activation. Similar to expression of phospho-IRF3, we also

found endogenously upregulated levels of activated NF-κB in *Casp8*$^{-/-}$ DCs as determined by lower levels of IκB (Fig. 12B). CD8$^{+}$ DCs express very low levels of Rig-I (133), and consistent with this, there was no difference in activation of IRF3 or NF-κB between WT and *Casp8*$^{-/-}$ DCs, regardless of Rig-I stimulation. We conclude that our findings are consistent with studies that have shown that, in DCs, removal of a negative regulator leads to hyperactivation of downstream elements (134-137).

Canonically known as an initiator of apoptosis, caspase-8 was recently shown to be required to prevent RIP kinase-dependent necroptosis in a variety of cell types (138). Caspase-8 is needed for both preventing necroptosis of proliferating T cells during the initiation of an adaptive immune response, as well as triggering apoptosis of T cells during the contraction of the response (28, 33, 139). Although a small percentage of DCs have been shown to undergo necroptosis upon treatment with poly(I:C) (90), we did not observe any differences in cell death in poly(I:C)-transfected wild-type versus caspase-8 deficient DCs (Fig. 11C). Our study adds to the growing body of work on the non-canonical functions of caspase-8 (140) by describing a non-programmed cell death role for caspase-8 in regulating DC activation during both homeostasis and chronic viral infection.

# Materials and Methods

## Mice

Mice in which exon 3 of *Casp8* is flanked by *loxP* sites (B6.129-*Casp8*$^{tm1Hed}$) (backcrossed to C57BL/6J, n > 10) (141) were crossed to *Cd11cCre* mice (B6.Cg-Tg(Itgax-cre)1-1Reiz/J) (142) to generate *Casp8*$^{f/f}$ *Cd11cCre* mice. These mice are referred to as *dcCasp8*$^{-/-}$. SMARTA transgenic mice (TCR transgenes specific for H2A$^{b}$ bound with LCMV glycopeptide 61–80) (118) were a gift from A. Goldrath, University of California, San Diego (UCSD). Animal work was performed according to UCSD guidelines.

## Viral Infections

6- to 10-week old *Casp8*$^{f/f}$ *Cd11cCre* mice were infected intravenously (i.v.) via the retroorbital sinus with 2 x $10^{6}$ pfu LCMV Cl13. Virus was grown, identified, quantified, and titered by plaque assay as previously described (98).

## Flow Cytometry

Spleens were harvested from mice and treated with Collagenase D (Roche) at 37°C for 20 min. Splenic single cell suspensions were made and incubated with the indicated fluorochrome-conjugated antibodies. For intracellular staining of Foxp3, cytokines (IFN-$\gamma$, TNF-$\alpha$ and IL-2) and I$\kappa$B, FoxP3 Fix/Perm kit (eBioscience) was used. For intracellular staining of phosphorylated IRF3 (P-IRF3), Cytofix Fixation Buffer and Perm Buffer III (BD) were used. LCMV-

specific T cells were examined with MHC class II (H2-$A^b$ $GP_{66}$) and MHC class I (H2-$D^b$ $GP_{33}$, H2-$D^b$ $GP_{276}$, H2-$D^b$ $NP_{396}$) tetramers obtained from the NIH Tetramer Core Facility (Emory University). All antibodies for surface and intracellular cytokine staining were purchased from eBioscience, BioLegend or BD. Antibodies for IκB and P-IRF3 were purchased from Cell Signaling Technology. Data were collected on an LSR Fortessa (BD) and analyzed with FlowJo software (Tree Star).

### *In Vitro* Stimulations

$10^6$ splenocytes were isolated and stimulated for one hour with PMA (10 ng/ml) and Ionomycin (1 μM) to assess CD4 T cell skewing, or LCMV peptides $GP_{33-41}$ (0.5 μM) or $GP_{61-80}$ (2 μg/ml) to assess cytokine production, after which monensin (eBioscience) was added for an additional 3.5 hours, for a total stimulation of 4.5 hours.

### Dendritic Cell Transfections

4 spleens per genotype were pooled to isolate CD11c+ cells to at least 90% purity according to EasySep CD11c Positive Selection Kit (Stemcell). For CD86 upregulation and pro-inflammatory cytokine measurements, isolated DCs were transfected with short-length poly(I:C)-LyoVec (Invivogen) for 20 hours at the indicated concentrations. Lipopolysaccharide was added at 0.1 ug/well. Cytokines were assessed in cell culture supernatants by cytometric bead assay

(BioLegend). For IκB and P-IRF3 expression studies, isolated DCs were transfected with short-length poly(I:C)-LyoVec (5 ug/ml) for the indicated lengths of time. Poly(I:C) preparations and media used were endotoxin free.

**Histopathology**

Paraffin-embedded spleen and liver sections were stained with hematoxylin & eosin (H&E). Splenic white and red pulp areas were quantified by ImageJ software (NIH).

**LCMV-Specific Antibody ELISAs**

LCMV-specific ELISAs were performed as previously described (143).

**SMARTA Adoptive Transfer**

LCMV-specific CD45.1$^+$ CD45.2$^+$ transgenic SMARTA CD4$^+$ T cells were isolated by negative selection to at least 85% purity (Miltenyi Biotec). 5,000 SMARTAs were then transferred i.v. into recipient *Casp8*$^{f/f}$ *Cd11cCre* mice 1 day prior to LCMV Cl13 infection.

## Acknowledgements

I thank Brittney Wellisch for her assistance with this project, and Laura Shaw and Kyla Omilusik for providing the SMARTA mice. I also thank the Goldrath, Murre and Zuniga labs at UC San Diego for sharing equipment, reagents and technical advice.

Chapter III, in large part, is under preparation for submission to the *Journal of Immunology*. The dissertation author is the primary author of this paper. Stephen Hedrick is the corresponding author of this paper.

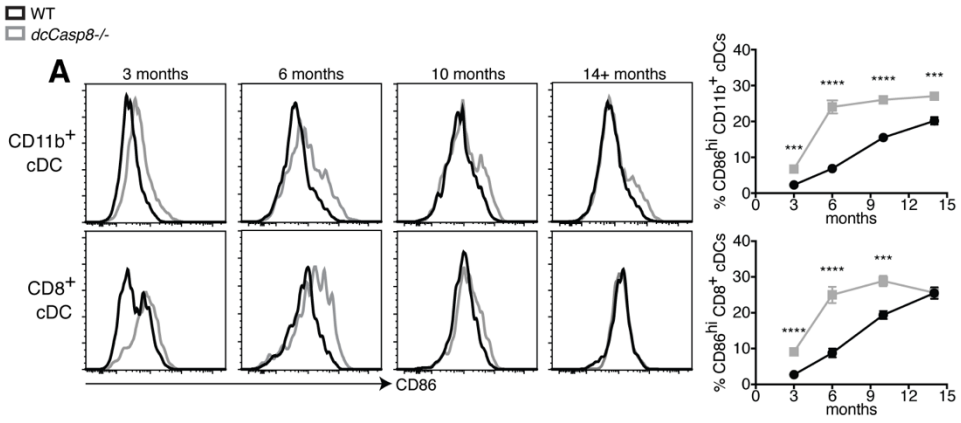


**Figure 6. Phenotype of aged *dcCasp8*$^{-/-}$ mice.**

**A.** The expression of CD86 in splenic DCs from WT and *dcCasp8*$^{-/-}$ mice was determined at 3, 6, 10 and 14+ months (left), and the percentage of DCs expressing high levels of CD86 is depicted (right). Error bars represent S.E.M. Data are representative of 3 independent experiments for each time point, with $n = 4$.

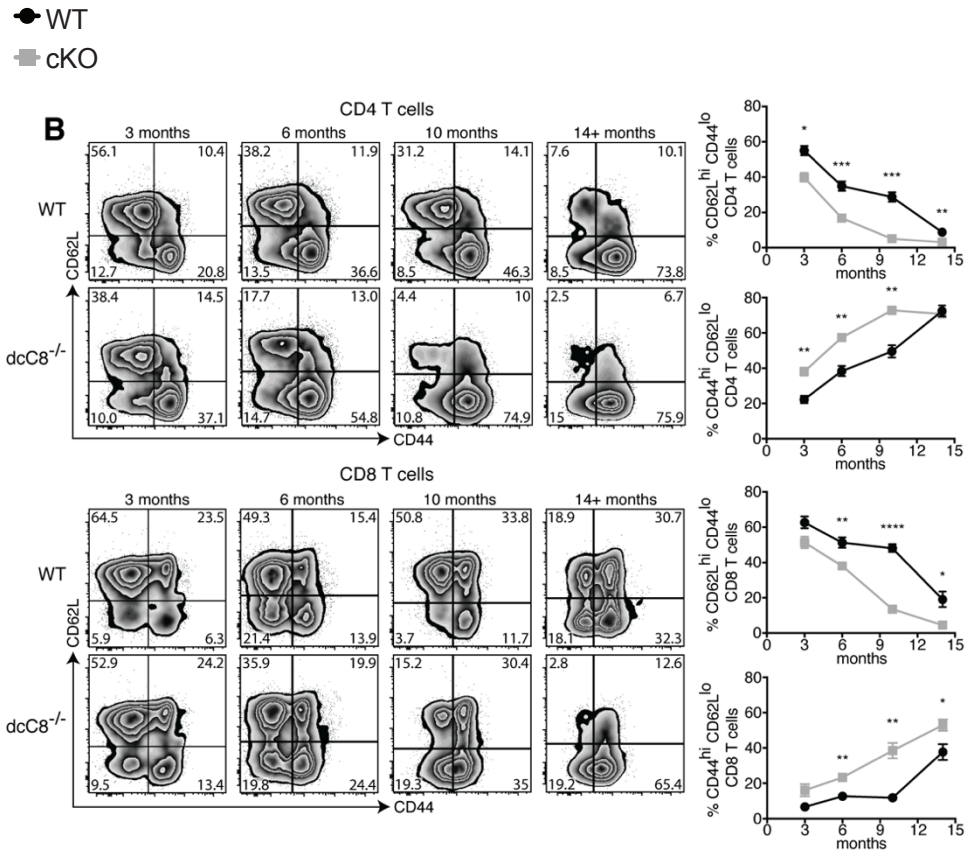


**Figure 6. Phenotype of aged *dcCasp8*$^{-/-}$ mice (continued).**

**B.** The expression of CD62L and CD44 in splenic and blood T cells from WT and *dcCasp8*$^{-/-}$ mice was determined at 3, 6, 10 and 14+ months (left), and the percentage of T cells that were naive (CD62L$^{hi}$CD44$^{lo}$) or activated (CD62L$^{lo}$CD44$^{hi}$) is depicted (right). Error bars represent S.E.M. Data are representative of 3 independent experiments for each time point, with $n$ = 4.

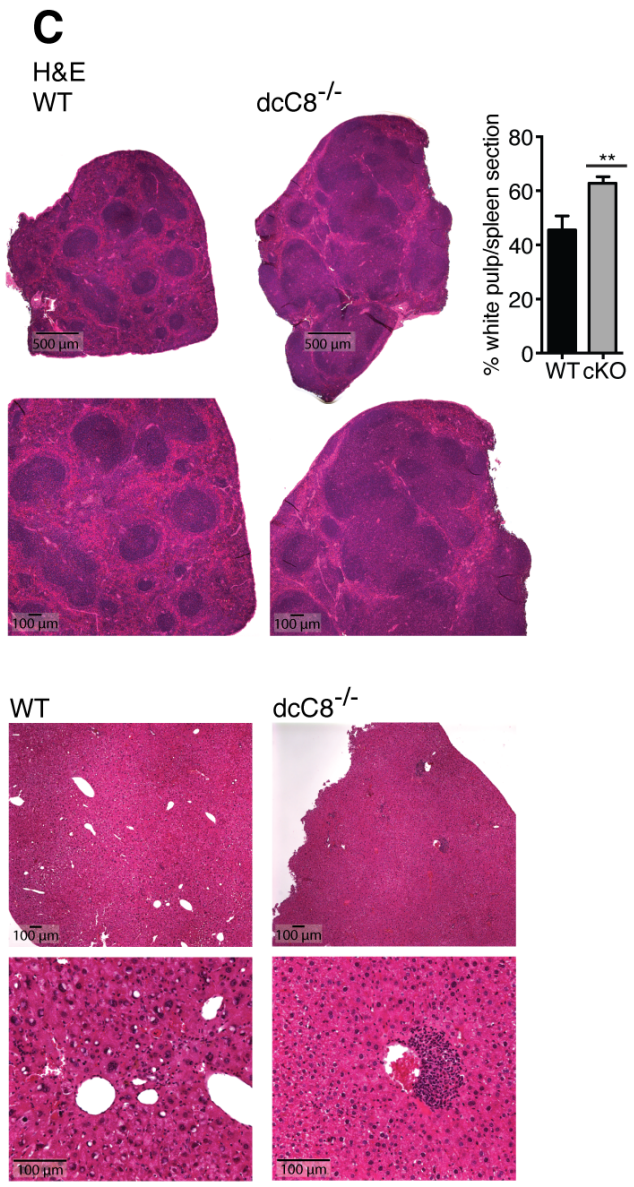


**Figure 6. Phenotype of aged *dcCasp8*$^{-/-}$ mice (continued).**

**C.** Formalin-fixed and paraffin-embedded spleens and livers from 10-month old WT and *dcCasp8*$^{-/-}$ mice were sectioned and stained with hematoxylin & eosin (H&E). The percentage of white pulp area per spleen section in WT vs. *dcCasp8*$^{-/-}$ mice is depicted (center). Error bars represent S.E.M. Data are representative of 16 mice.

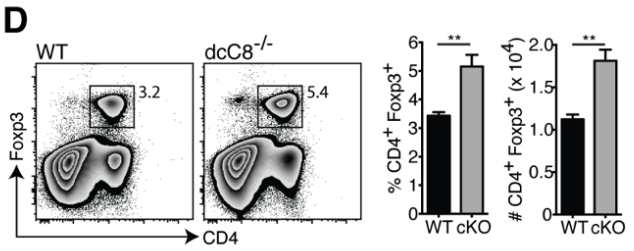


**Figure 6. Phenotype of aged *dcCasp8*$^{-/-}$ mice (continued).**

**D.** The expression of Foxp3 in splenic CD4 T cells from 10-month old WT and *dcCasp8*$^{-/-}$ mice was determined by percentage (right) and absolute number per spleen (far right). Error bars represent S.E.M. Data are representative of 3 independent experiments for each time point, with $n$ = 4.

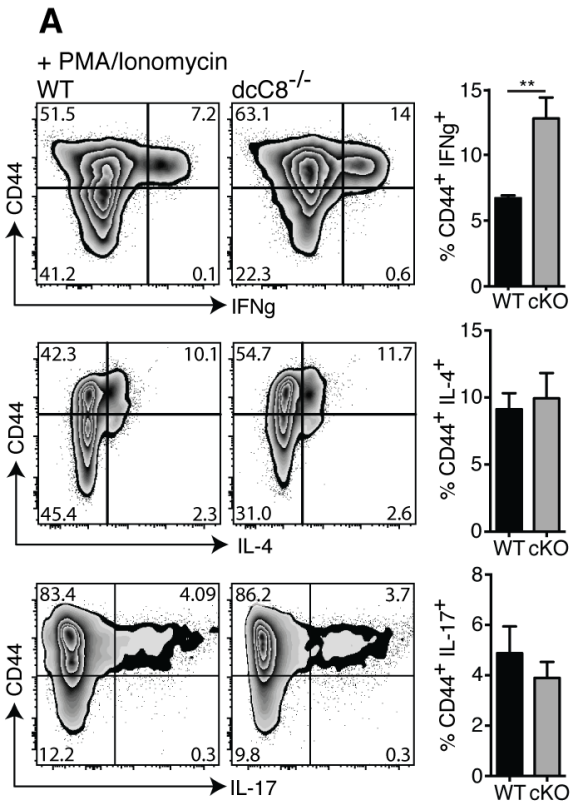


**Figure 7. CD4 T cells from aged *dcCasp8*$^{-/-}$ mice skew towards a Th1 phenotype.**

**A.** Splenocytes from 10-month old WT and *dcCasp8*$^{-/-}$ mice were stimulated *in vitro* for one hour with PMA/Ionomycin, then monensin was added for an additional 3.5 hours, for a total stimulation of 4.5 hours. The expression of IFN-γ, IL-4 and IL-17 was assessed by intracellular staining. The percentage of CD44$^{+}$ CD4 T cells expressing IFN-γ, IL-4 or IL-4 is depicted (right). Error bars represent S.E.M. Data for A are representative of 3 independent experiments, with $n$ = 4.

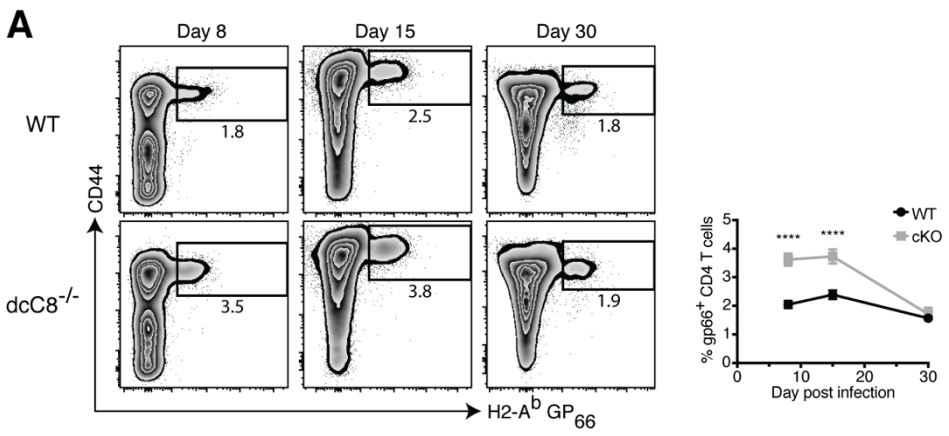


**Figure 8. Young adult *dcCasp8*$^{-/-}$ mice mount an enhanced T cell response to chronic LCMV.**

6- to 10-week old WT and *dcCasp8*$^{-/-}$ mice were infected intravenously with LCMV-cl13. After 8, 15 and 30 days, spleen cells were harvested and analyzed.

**A.** The expression of $GP_{66}$ in $CD44^+$ CD4 T cells was assessed by tetramer staining. The percentage of $GP_{66}^+$ $CD44^+$ CD4 T cells is depicted (right). The expression of $GP_{33}$, $GP_{276}$ or $NP_{396}$ in $CD44^+$ CD8 T cells was also assessed by tetramer staining. The percentage of $GP_{33}^+$, $GP_{276}^+$ or $NP_{396}^+$ $CD44^+$ CD8 T cells is depicted (right). Error bars represent S.E.M. Data depict at least 12 pooled mice.

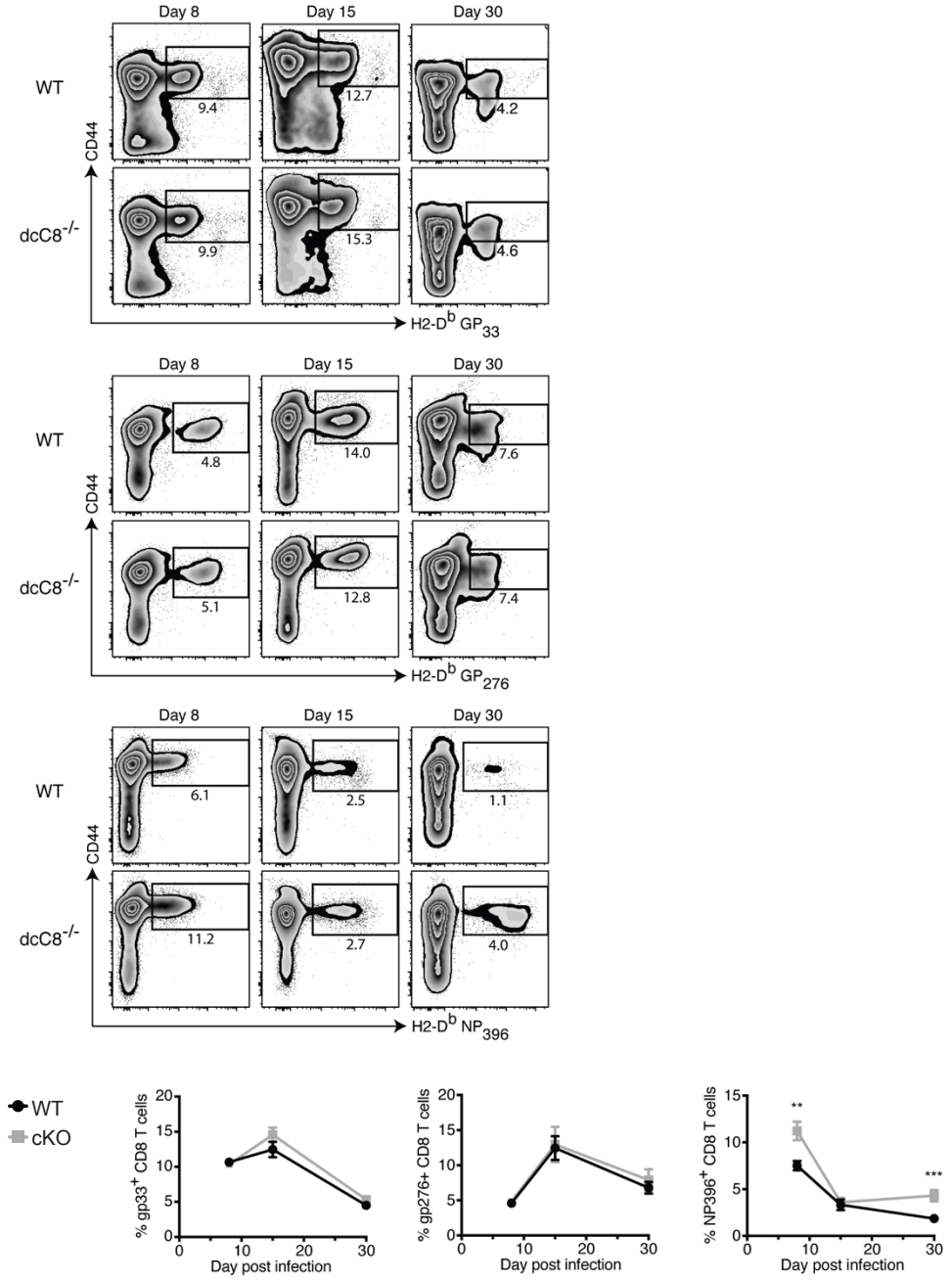


**Figure 8. Young adult *dcCasp8*$^{-/-}$ mice mount an enhanced T cell response to chronic LCMV (continued).**

**B.** The expression of $GP_{33}$, $GP_{276}$ or $NP_{396}$ in $CD44^+$ CD8 T cells was assessed by tetramer staining. The percentage of $GP_{33}^+$, $GP_{276}^+$ or $NP_{396}^+$ $CD44^+$ CD8 T cells is depicted (right). Error bars represent S.E.M. Data depict at least 12 pooled mice.

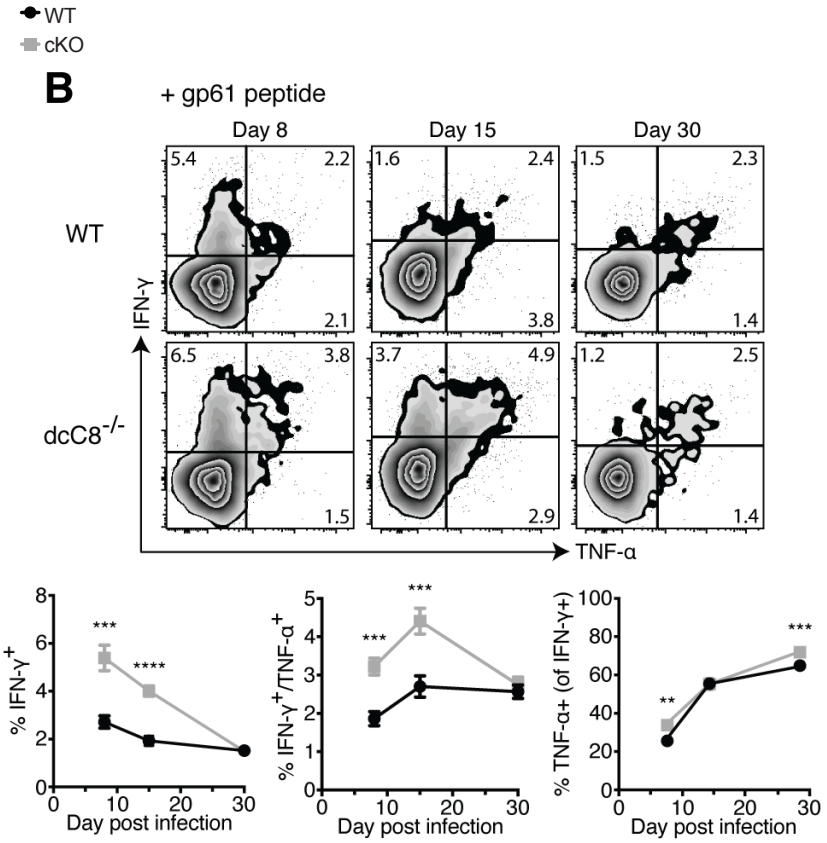


**Figure 8. Young adult *dcCasp8*$^{-/-}$ mice mount an enhanced T cell response to chronic LCMV (continued).**

**C.** Splenocytes were stimulated *in vitro* for one hour with $GP_{61-80}$ (CD4) peptide, then monensin was added for an additional 3.5 hours, for a total peptide stimulation of 4.5 hours. The expression of IFN-γ and TNF-α was assessed by intracellular staining (top). The percentage of $CD44^+$ CD4 T cells that produce either IFN-γ alone, both IFN-γ and TNF-α, or TNF-α in addition to IFN-γ is depicted (bottom). Error bars represent S.E.M. Data for depict at least 10 pooled mice.

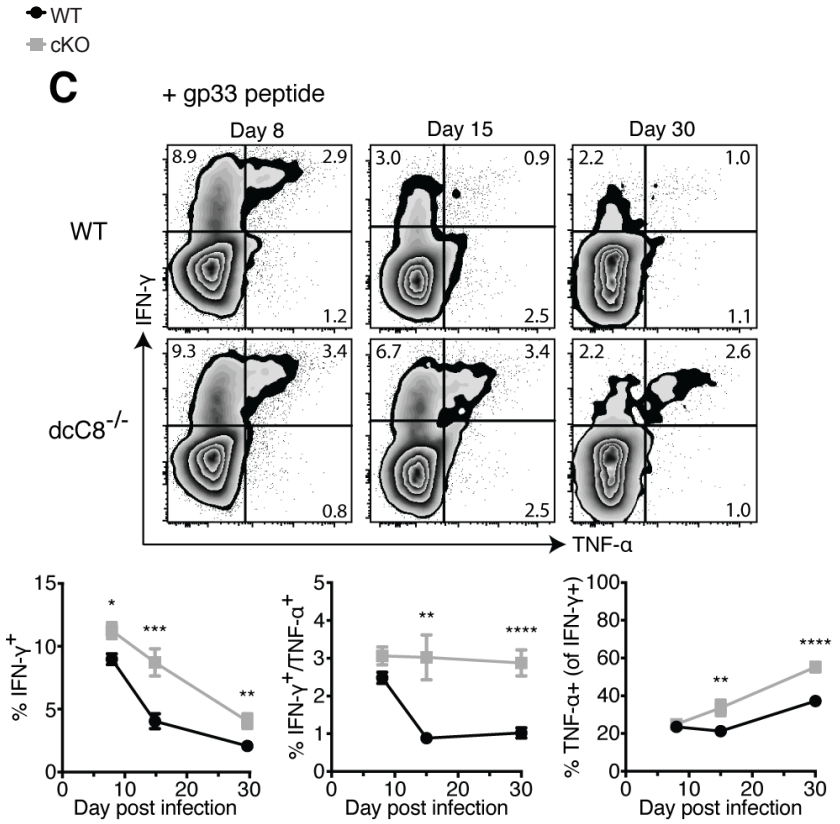


**Figure 8. Young adult *dcCasp8*$^{-/-}$ mice mount an enhanced T cell response to chronic LCMV (continued).**

**D.** Splenocytes were stimulated *in vitro* for one hour with either B) $GP_{61-80}$ (CD4) or C) $GP_{33-41}$ (CD8) peptide, then monensin was added for an additional 3.5 hours, for a total peptide stimulation of 4.5 hours. The expression of IFN-γ and TNF-α was assessed by intracellular staining (top). The percentage of $CD44^+$ CD4 and CD8 T cells that produce either IFN-γ alone, both IFN-γ and TNF-α, or TNF-α in addition to IFN-γ is depicted (bottom). Error bars represent S.E.M. Data for depict at least 10 pooled mice.

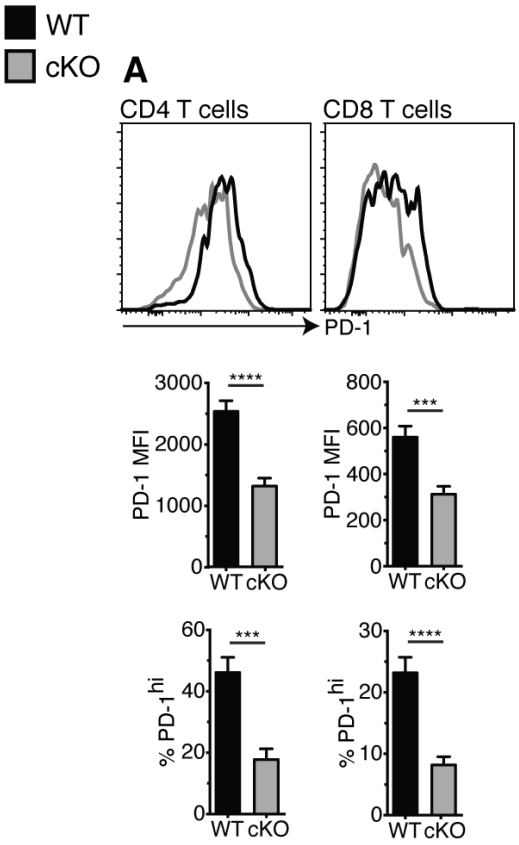


**Figure 9. Phenotype of chronically infected young adult *dcCasp8*$^{-/-}$ mice.**

6- to 10-week old WT and *dcCasp8*$^{-/-}$ mice were infected intravenously with LCMV Cl13. After 30 days, various organs and blood were harvested and analyzed.

**A.** The expression of PD-1 in $GP_{66}^{+}$ $CD4^{+}$ or $GP_{33}^{+}$ $CD8^{+}$ T cells was assessed. The PD-1 median fluorescence intensity (MFI) and percentage of cells expressing high levels of PD-1 is depicted (bottom). Error bars represent S.E.M. Data depict at least 12 pooled mice.

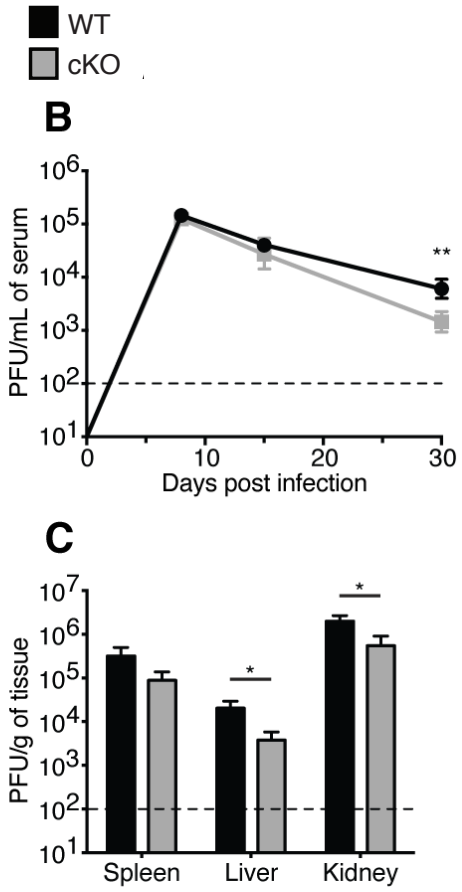


**Figure 9. Phenotype of chronically infected young adult *dcCasp8*$^{-/-}$ mice (continued).**

**B.** Plaque assays were performed using Vero cells to determine virus titers in serum. Error bars represent S.E.M. Data depict 8 pooled mice (days 8 & 15 p.i.) and 20 pooled mice (day 30 p.i.).

**C.** Plaque assays were performed using Vero cells to determine virus titers in spleen, liver and kidney. Error bars represent S.E.M. Data depict 8 pooled mice.

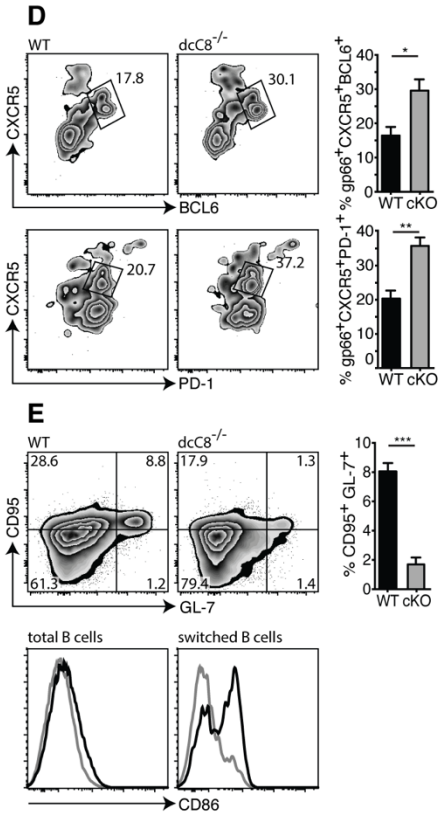


**Figure 9. Phenotype of chronically infected young adult *dcCasp8*$^{-/-}$ mice (continued).**

**D.** The expression of CXCR5 and BCL6 or PD-1 in $GP_{66}^{+}$ $CD44^{+}$ CD4 T cells was assessed, and the percentage of $CXCR5^{hi}BCL6^{hi}$ and $CXCR5^{hi}PD\text{-}1^{hi}$ cells is depicted (right). Error bars represent S.E.M. Data depict at least 12 pooled mice.

**E.** The expression of CD95 and GL-7 in B cells was assessed, and the percentage of $CD95^{hi}GL\text{-}7^{hi}$ cells is depicted (bottom). The expression of CD86 on total vs. switched B cells was also examined. Error bars represent S.E.M. Data depict at least 12 pooled mice.

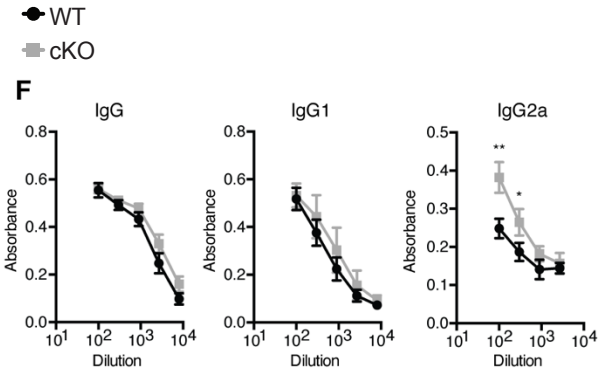


**Figure 9. Phenotype of chronically infected young adult *dcCasp8*$^{-/-}$ mice (continued).**

**F.** ELISAs were performed to determine the amount of LCMV-specific IgG, IgG1 and IgG2a antibodies in serum. Error bars represent S.D. Data are representative of 2 independent experiments, with $n$ = 4.

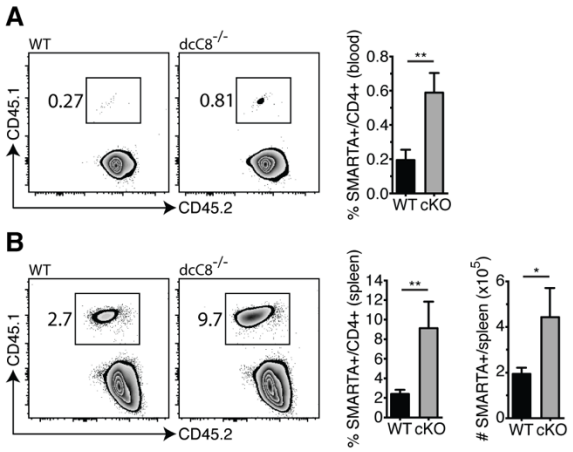


**Figure 10. SMARTA T cells adoptively transferred into *dcCasp8*$^{-/-}$ mice undergo greater expansion and are more polyfunctional upon chronic LCMV infection.**

LCMV-specific CD45.1$^{+}$ CD45.2$^{+}$ transgenic CD4$^{+}$ T cells (SMARTA) were transferred i.v. 1 day prior to LCMV Cl13 infection.

**A.** The frequency of blood CD4$^{+}$ T cells that were SMARTA was assessed at day 5 p.i. Error bars represent S.E.M. Data depict at least 10 pooled mice.

**B.** The frequency and number of spleen CD4$^{+}$ T cells that were SMARTA was assessed at day 8 p.i. Error bars represent S.E.M. Data depict at least 10 pooled mice.

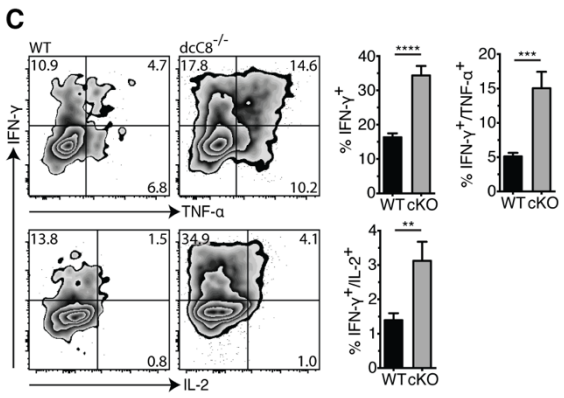


**Figure 10. SMARTA T cells adoptively transferred into *dcCasp8*$^{-/-}$ mice undergo greater expansion and are more polyfunctional upon chronic LCMV infection (continued).**

**C.** Splenocytes isolated at day 8 p.i. were stimulated *in vitro* for one hour with $GP_{61-80}$ (CD4) peptide, then monensin was added for an additional 3.5 hours, for a total peptide stimulation of 4.5 hours. The expression of IFN-γ, TNF-α and IL-2 by SMARTA $CD4^+$ T cells was assessed by intracellular staining (left). The percentage of SMARTA $CD4^+$ T cells that produce either IFN-γ alone, IFN-γ and TNF-α, or IFN-γ and IL-2 is depicted (right). Error bars represent S.E.M. Data depict at least 8 pooled mice.

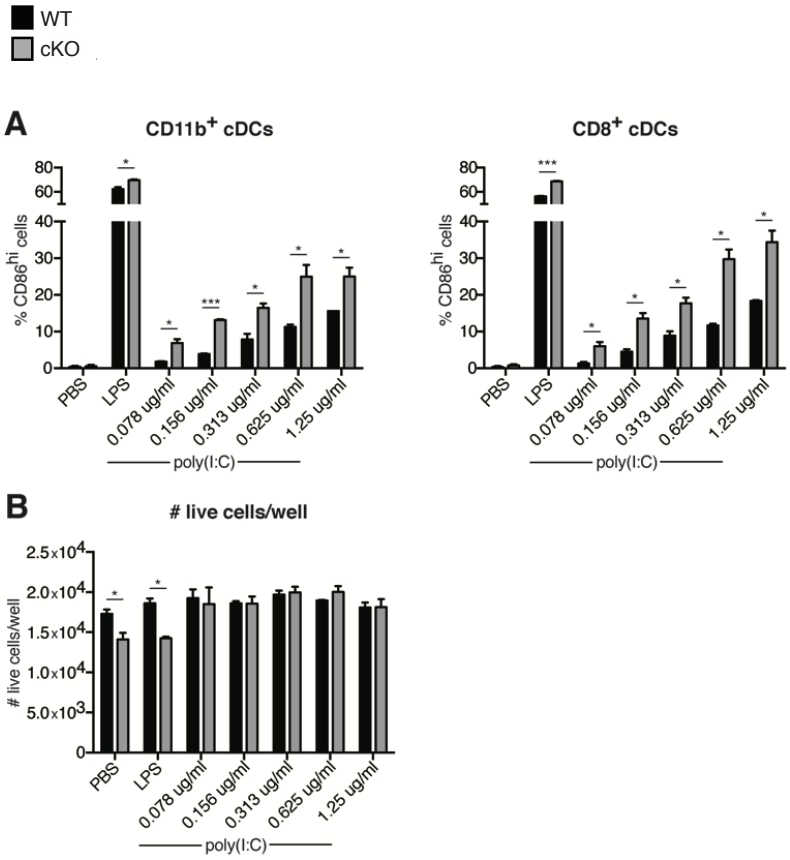


**Figure 11. Caspase-8 deficient DCs hyperactivate in response to Rig-I stimulation.**

DCs were isolated from the spleen and transfected with short-length poly(I:C) for 20 hours.

**A.** The expression of CD86 was assessed in $CD11b^+$ and $CD8^+$ DCs. Error bars represent S.D. Data are representative of 3 independent experiments, with three replicates per genotype.

**B.** Cell death was assessed by staining DCs with a membrane permeable dye. Error bars represent S.D. Data are representative of 3 independent experiments, with three replicates per genotype.

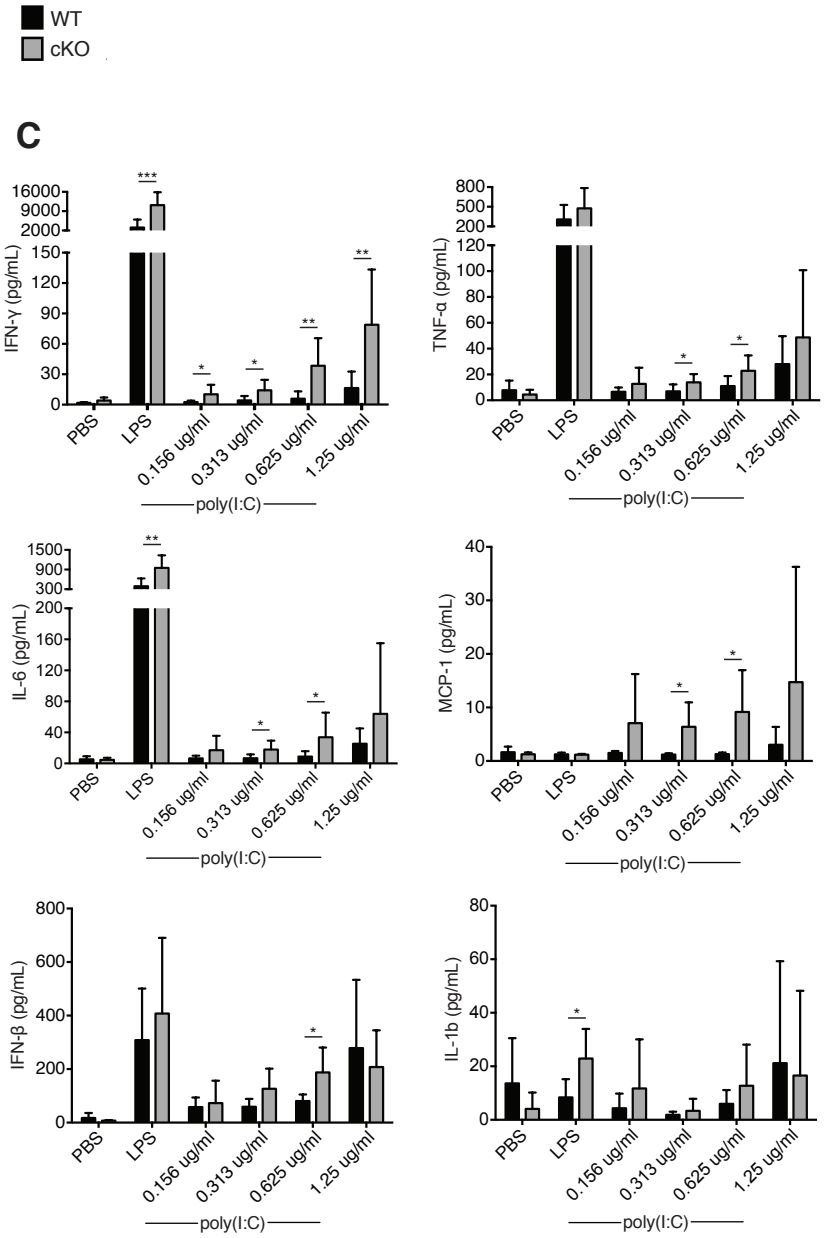


**Figure 11. Caspase-8 deficient DCs hyperactivate in response to Rig-I stimulation (continued).**

**C.** Cell culture supernatants were analyzed for the presence of inflammatory cytokines using a cytometric bead array. Error bars represent S.D. Data are representative of 2 independent experiments, with two replicates per genotype.

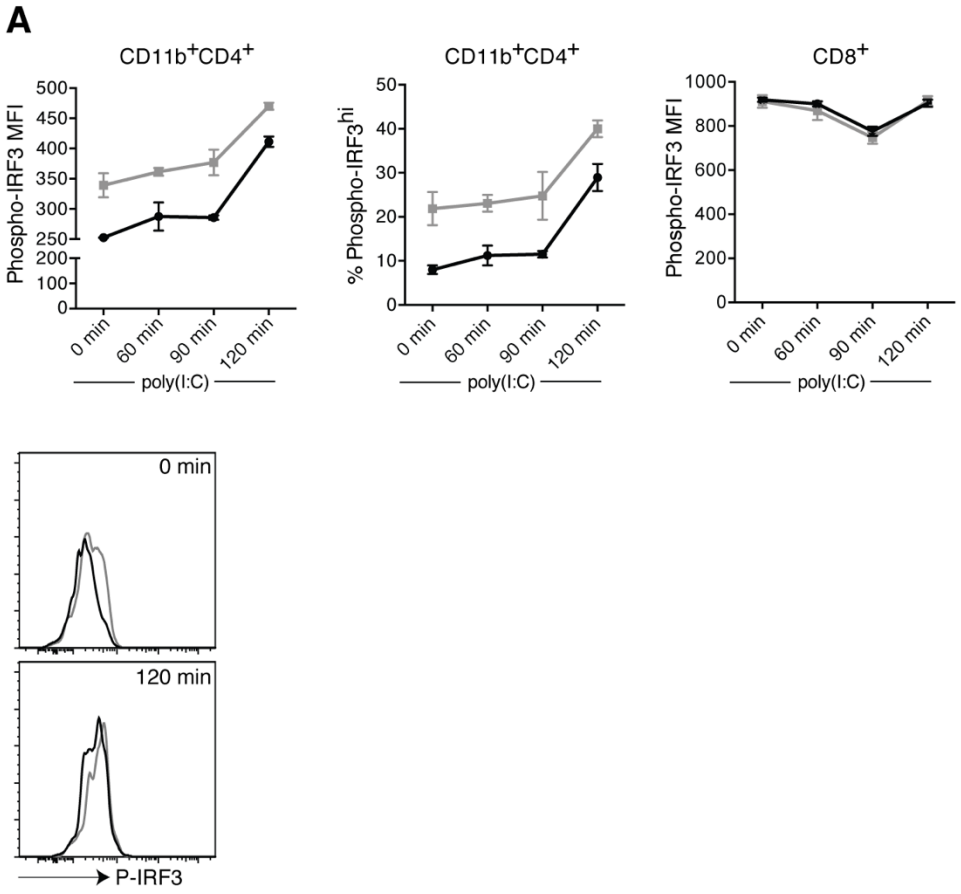


**Figure 12. IRF3 and NF-κB are endogenously activated in caspase-8 deficient DCs.**

**A.** DCs were isolated from the spleen and transfected with short-length poly(I:C) for the indicated periods. The expression of phosphorylated IRF3 was assessed by intracellular staining in $CD11b^+CD4^+$ and $CD8^+$ DCs (top). A representative histogram depicts expression of phosphorylated IRF3 in $CD11b^+CD4^+$ DCs (bottom). Error bars depict S.D. Data are representative of 2 independent experiments, with three replicates per genotype.

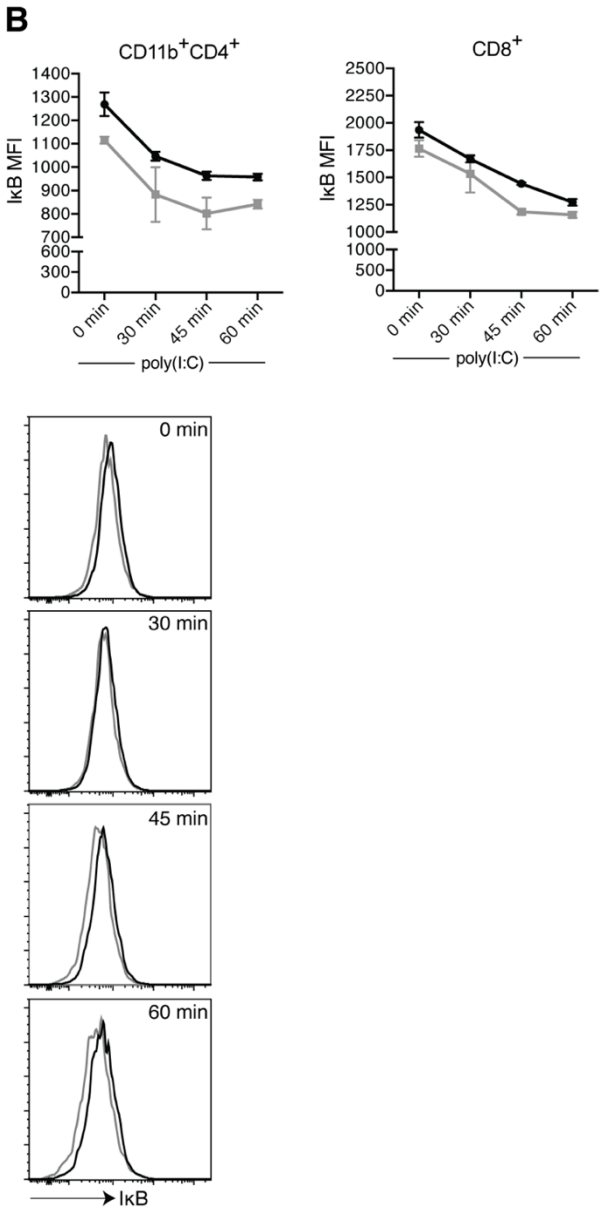


**Figure 12. IRF3 and NF-κB are endogenously activated in caspase-8 deficient DCs (continued).**

**B.** DCs were isolated from the spleen and transfected with short-length poly(I:C) for the indicated periods. The expression of IκB was assessed by intracellular staining in $CD11b^+CD4^+$ and $CD8^+$ DCs (top). A representative histogram depicts expression of IκB in $CD11b^+CD4^+$ DCs (bottom). Error bars depict S.D. Data are representative of 2 independent experiments, with three replicates per genotype.

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# CHAPTER IV:

# Future Directions

# Mechanisms driving the steady state autoimmunity of *dcCasp8*$^{-/-}$ mice

### What is activating *Casp8*$^{-/-}$ DCs?

We found that caspase-8 negatively regulates DC activation, as DCs lacking caspase-8 become hyperactivated in the steady state. However, it remains unclear what stimuli may be triggering this hyperactivation. Caspase-8 has been shown to negatively regulate Rig-I signaling (36), suggesting that the stimulus may be an endogenous Rig-I ligand. RNA species derived from the intestinal commensal microbiota have been found to activate MAVS, which is part of the Rig-I pathway, and drive production of IFN-β (132). By altering the intestinal microbiota of *dcCasp8*$^{-/-}$ mice, we could test whether microbiota-derived RNA ligands are responsible for activating *Casp8*$^{-/-}$ DCs.

Recently described “pet store” mice are thought to have immune systems that are much more similar to adult humans than standard laboratory mice, which are raised under sterile and specific pathogen free (SPF) conditions (144, 145). Pet store mice are infected with a number of mouse pathogens, and presumably have a significantly different gut microbiota than SPF mice. Co-housing mice has been shown to lead to horizontal transfer of gut microbiota between cagemates (146). Thus, co-housing *dcCasp8*$^{-/-}$ mice with pet store mice might accelerate the steady-state autoimmunity of *dcCasp8*$^{-/-}$ mice, if an

intestinal microbiota-derived RNA ligand is indeed triggering the hyperactivation of *Casp8*$^{-/-}$ DCs.

Likewise, eliminating the gut microbiota of *dcCasp8*$^{-/-}$ mice could inhibit the development of autoimmunity. Germ-free (GF) mice are reared in an ultra-sterile environment devoid of any exposure to live microbes and thus lack commensal gut bacteria (147). Rederivation of *dcCasp8*$^{-/-}$ mice (via hysterectomy of pups and transfer into GF foster mothers) into a GF facility would remove the gut microbiota from these mice, allowing us to analyze the contribution of commensal bacteria to the develop of autoimmunity in *dcCasp8*$^{-/-}$ mice.

**Do *dcCasp8*$^{-/-}$ mice undergo a loss of immune tolerance?**

Our studies, as well as those of Cuda et al. 2014, suggest that *dcCasp8*$^{-/-}$ mice lose immune tolerance as a result of hyperactive DCs that stimulate and activate $CD4^+$ and $CD8^+$ T cells in the steady state. However, this has been yet to be directly shown. An established method of inducing immune tolerance involves adoptively transferring OT-I and/or OT-II cells (T cells with OVA peptide-specific TCRs) into host mice, then intravenously injecting the mice with OVA peptide (148). If the DCs in host mice become activated, such as through exposure to an adjuvant like LPS, they will upregulate co-stimulation molecules like CD86/CD80. These activated DCs will then present antigen derived from OVA peptide to, and stimulate the proliferation of, OVA-specific OT-I/OT-II T

cells. If the DCs are not activated, they will present OVA-derived antigen to OT-I/OT-II T cells in the absence of co-stimulation, which will result in the anergy and eventual deletion of those T cells.

To test whether *dcCasp8*$^{-/-}$ mice undergo a loss of immune tolerance, we would adoptively transfer OT-I or OT-II T cells into WT or *dcCasp8*$^{-/-}$ hosts, then intravenously inject the mice with OVA peptide without the addition of adjuvant. We would predict that OT-I/OT-II T cells will proliferate in *dcCasp8*$^{-/-}$, but not WT, hosts, due to the constitutively upregulated levels of CD86 on DCs from *dcCasp8*$^{-/-}$ mice. We would also predict that OT-I/OT-II T cell proliferation will be greater in 10 month old versus 3 month old *dcCasp8*$^{-/-}$ mice, based on the much higher expression of CD86 in 10 month old mice.

## Contribution of *Casp8*$^{-/-}$ DCs towards clearing chronic LCMV infection

### A possible role for CD4$^{+}$ T cell help to CD8$^{+}$ T cells

We found that the enhancement in the antigen-specific CD4$^{+}$ T cell response in *dcCasp8*$^{-/-}$ mice is likely due to the *Casp8*$^{-/-}$ DCs themselves and not any pre-existing hyperactive CD4$^{+}$ T cells (Fig. 10). However, it is less clear whether the middle to late (days 15 & 30 p.i.) enhancement in antigen-specific CD8$^{+}$ T cell function is a direct result of caspase-8 deficient DCs. One possibility is that the CD8$^{+}$ T cells in infected *dcCasp8*$^{-/-}$ mice are receiving more "help"

from $CD4^+$ T cells. $CD4^+$ T cell help to $CD8^+$ T cells can take many forms, such as by $CD4^+$ T cell licensing and activation of APCs via CD40L-CD40 interactions, or by $CD4^+$ T cells producing cytokines like IL-2 (149). In LCMV Cl13 infected mice, IL-21 production by $CD4^+$ T cells was shown to be essential for maintaining virus-specific $CD8^+$ T cells and for permitting viral clearance (109-111). Given that a greater proportion of antigen-specific $CD4^+$ T cells are polyfunctional (in terms of cytokine production) in *dcCasp8*$^{-/-}$ mice at days 8 & 15 p.i., we predict that the production of IL-2 and IL-21 by $CD4^+$ T cells is also enhanced at those timepoints.

## Signaling pathways responsible for the hyper-activation of *Casp8*$^{-/-}$ DCs

### Signals upstream of IRF3 and NF-κB

Activation of Ripk1 can lead to subsequent activation of either IRF3 or NF-κB. We found that unstimulated *Casp8*$^{-/-}$ DCs expressed higher levels of phosphorylated IRF3 and lower levels of IκB (Fig. 12), suggesting an endogenous activation of IRF3 and NF-κB independent of stimulation through Rig-I. One possibility is that Ripk1 is constitutively active in *Casp8*$^{-/-}$ DCs, since caspase-8 cleaves and inhibits Ripk1 (14). Active Ripk1 could then continuously activate IRF3 and NF-κB. To test whether enhanced Ripk1 activity is responsible for the endogenous activation of IRF3 and NF-κB, Ripk1 could be

inhibited either pharmacologically or genetically in *Casp8*$^{-/-}$ DCs. Ripk1 can be inhibited with a small molecule inhibitor, necrostatin-1 (Nec-1), so addition of Nec-1 to *Casp8*$^{-/-}$ DCs may inhibit the increased activation of IRF3 and NF-κB (29, 88). Alternatively, the effect of Ripk1 on expression of IRF3 and NF-κB could be examined by isolating DCs from mice deficient in caspase-8, Ripk1 and Ripk3 (150).

## Therapeutic applications of *Casp8*$^{-/-}$ DCs

### Hyperactive *Casp8*$^{-/-}$ DCs in cancer immunotherapy

Our study found that mice with hyperactive DCs lacking caspase-8 have delayed T cell exhaustion during chronic LCMV infection, indicating a possible therapeutic application for these DCs. T cell exhaustion is characterized by a loss of effector cytokine production and upregulation of inhibitory receptors such as PD-1 and CTLA-4, and plays a major role in disease progression of many chronic infections and cancer (107). Clinical trials with antibodies against PD-1 and CTLA-4 showed improved overall survival rates of human patients with various forms of cancer, demonstrating the importance of these signaling pathways in T cell dysfunction and leading to anti-PD-1 and anti-CTLA-4 being approved for human use within the last five years (with anti-PD-L1, the ligand for PD-1 expressed on DCs, currently in testing) (151). However, these immune checkpoint therapies still need much improvement. It would be interesting to see whether combining the hyperactive caspase-8 deficient DCs with an antibody

blockade of PDL-1 on DCs would prolong T cell function and accelerate viral clearance in an LCMV Cl13 infection (Fig. 9A-C).

Dendritic cells themselves are also a relatively new and promising target of cancer immunotherapy. DCs can either be generated *ex vivo*, loaded with relevant cancer antigens, then injected into patients, or they can be targeted with antigen *in vivo*. In both cases, the DCs need to be activated to prime T cells, which requires exposure to or co-injection with an adjuvant such as poly(I:C) (152). If caspase-8 can be specifically inhibited in DCs, such as via CRISPR-Cas9 mediated deletion of caspase-8 in DCs prior to vaccination of patients (153), or by delivery of the caspase-8 inhibitor Z-IETD-FMK (such as by fusion with a DEC-205 antibody) to DCs *in vivo* (51), the resulting constitutively activated DCs could supplant the need for adjuvants in current DC targeted immunotherapies.

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