

# 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- $\kappa$ B 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 I $\kappa$ B, nuclear localization of RelA, normal induction of active NF- $\kappa$ B dimers as measured by electrophoretic mobility shift assay, and no differences in the induction of NF- $\kappa$ B 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<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup>B220<sup>+</sup> 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<sup>fl/fl</sup> Cd4Cre* (*tCasp8<sup>-/-</sup>*) mice with mice harboring a *Ppif*-null allele and generated four genotypes for analysis: *Casp8<sup>fl/fl</sup> (WT)*, *tCasp8<sup>-/-</sup>*, *Ppif<sup>-/-</sup>*, and *tCasp8<sup>-/-</sup> Ppif<sup>-/-</sup>* (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<sup>-/-</sup>* mice have a reduced percentage of LN T cells (Salmena et al., 2003), whereas *Ppif<sup>-/-</sup>* 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<sup>+</sup> 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<sup>-/-</sup>* CD4<sup>+</sup> T cells, although the number of cell divisions was unchanged from wild-type. *Ppif<sup>-/-</sup>* 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<sup>-/-</sup>* T cells. Similar results were found for CD8<sup>+</sup> T cells (unpublished data).

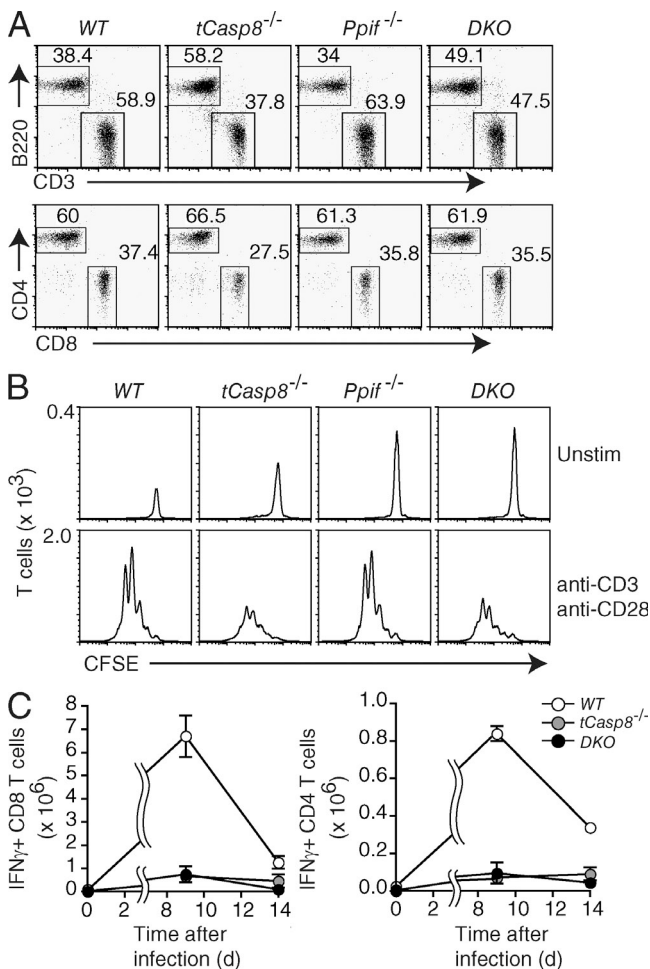
To test this further, mice were infected with lymphocytic choriomeningitis virus (LCMV) Armstrong, and the number of LCMV-specific T cells measured at the peak of the response, day 9, and at day 14 after a marked contraction of the population. As depicted in Fig. 1 C and previously reported, there was little expansion in *tCasp8<sup>-/-</sup>* 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<sup>-/-</sup>* 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.

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 phosphoinositide 3 kinase or an short hairpin RNA knockdown of *Atg7* (Bell et al., 2008).



**Figure 1. Caspase 8-deficient T cells do not die by classical necro-sis.** (A) The percentages of live-gated T and B cells from the lymph nodes of WT (*Casp8*<sup>fl/fl</sup>), *tCasp8*<sup>-/-</sup> (*Casp8*<sup>fl/fl</sup> Cd4Cre), *Ppif*<sup>-/-</sup> (*Casp8*<sup>fl/fl</sup> *Ppif*<sup>-/-</sup>), and DKO (*Casp8*<sup>fl/fl</sup> *Ppif*<sup>-/-</sup> 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<sup>+</sup> and CD8<sup>+</sup> T cells was measured by flow cytometry. Error bars represent the SEM. Data are representative of two independent experiments.

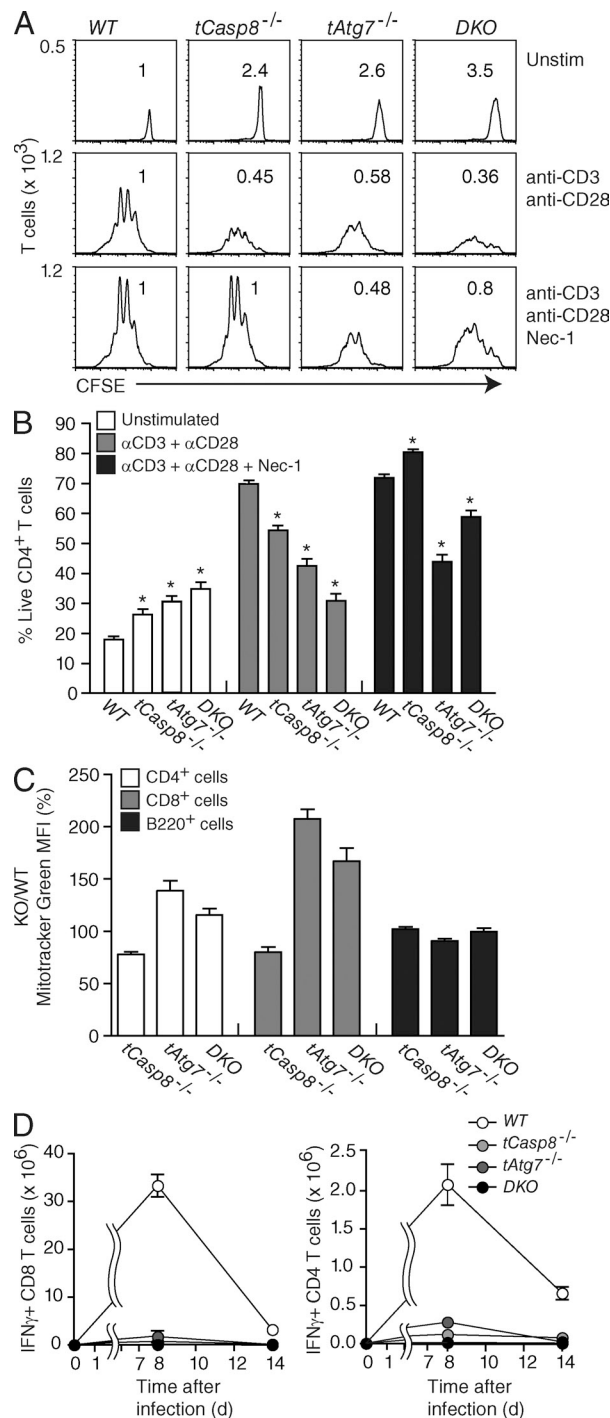
As such, we sought to determine whether a targeted deletion of *Atg7* could likewise rescue the death associated with the loss of caspase 8 in T cell activation.

Mice with a conditional *Atg7* deletion (Komatsu et al., 2005) were crossed with mice bearing *Casp8*<sup>fl/fl</sup>. We generated the following four genotypes for analysis: *Casp8*<sup>fl/fl</sup> (WT), *Casp8*<sup>fl/fl</sup> Cd4Cre (*tCasp8*<sup>-/-</sup>), *Atg7*<sup>fl/fl</sup> Cd4Cre (*tAtg7*<sup>-/-</sup>), and *Casp8*<sup>fl/fl</sup> *Atg7*<sup>fl/fl</sup> 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*<sup>-/-</sup> 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 *Atg7* 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*<sup>-/-</sup> T cells was rescued as expected, whereas the addition of necrostatin-1 to *tAtg7*<sup>-/-</sup> 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 *Atg7* (Fig. 2, A and B). These results show that the decrease in T cell accumulation caused by an *Atg7* 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 *Atg7* was shown to originate from a build-up of mitochondria and overproduction of reactive oxygen species (Pua et al., 2009). Compared with *tAtg7*<sup>-/-</sup> 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 *Atg7* when compared with *Atg7*-defective T cells.

We next determined if the loss of *Atg7* 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<sup>+</sup> or CD4<sup>+</sup> T cells from either *tCasp8*<sup>-/-</sup> or *tAtg7*<sup>-/-</sup> mice. In addition, T cells from DKO mice also did not expand. We note that a hemizygous loss of *Atg7* also did not complement the *Casp8* defect (unpublished data).



**Figure 2. Loss of Atg7 does not rescue caspase 8-deficient T cell proliferation.** (A) Purified WT (*Casp8*<sup>fl/fl</sup>), *tCasp8*<sup>-/-</sup> (*Casp8*<sup>fl/fl</sup> Cd4Cre), *tAtg7*<sup>-/-</sup> (*Atg7*<sup>fl/fl</sup> Cd4Cre), and DKO (*Casp8*<sup>fl/fl</sup> *Atg7*<sup>fl/fl</sup> 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

### Increased apoptotic death in the absence of Atg7

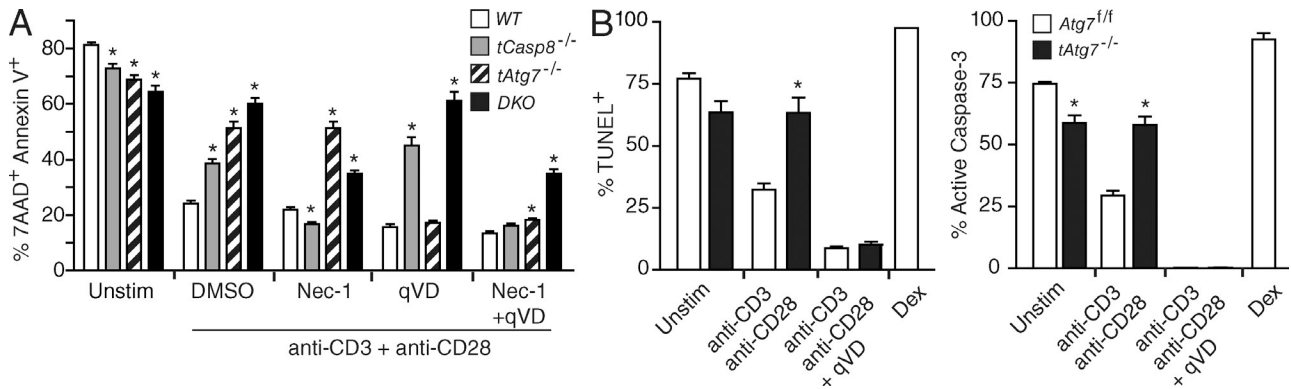
The analysis of the *Atg7* mutation as a means of complementing a loss of caspase 8 is complicated by the diminished viability of T cells associated with a defect in autophagy. We therefore sought to characterize the form of death occurring as a consequence of a loss of caspase 8, *Atg7*, or both. As 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*<sup>fl/fl</sup> 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*<sup>-/-</sup> vs. WT). A previous study showed that T cells directly explanted from *Atg7*<sup>fl/fl</sup> *LckCre* mice showed diminished viability (Pua et al., 2009); however, in this study we found that after 72 h of culture, *tAtg7*<sup>-/-</sup> 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*<sup>-/-</sup>), 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*<sup>-/-</sup> T cells, as previously described, but had no effect on *tAtg7*<sup>-/-</sup> T cells (Fig. 3 A). In contrast, the addition of qVD rescued *tAtg7*<sup>-/-</sup> T cells, but had no effect on *tCasp8*<sup>-/-</sup> 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

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*<sup>-/-</sup>, *tAtg7*<sup>-/-</sup>, and DKO T cells versus WT 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- $\gamma$  in gated CD4<sup>+</sup> and CD8<sup>+</sup> T cells was measured by flow cytometry. Data are representative of three independent experiments.





**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*<sup>-/-</sup> 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 physio-

logical 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.

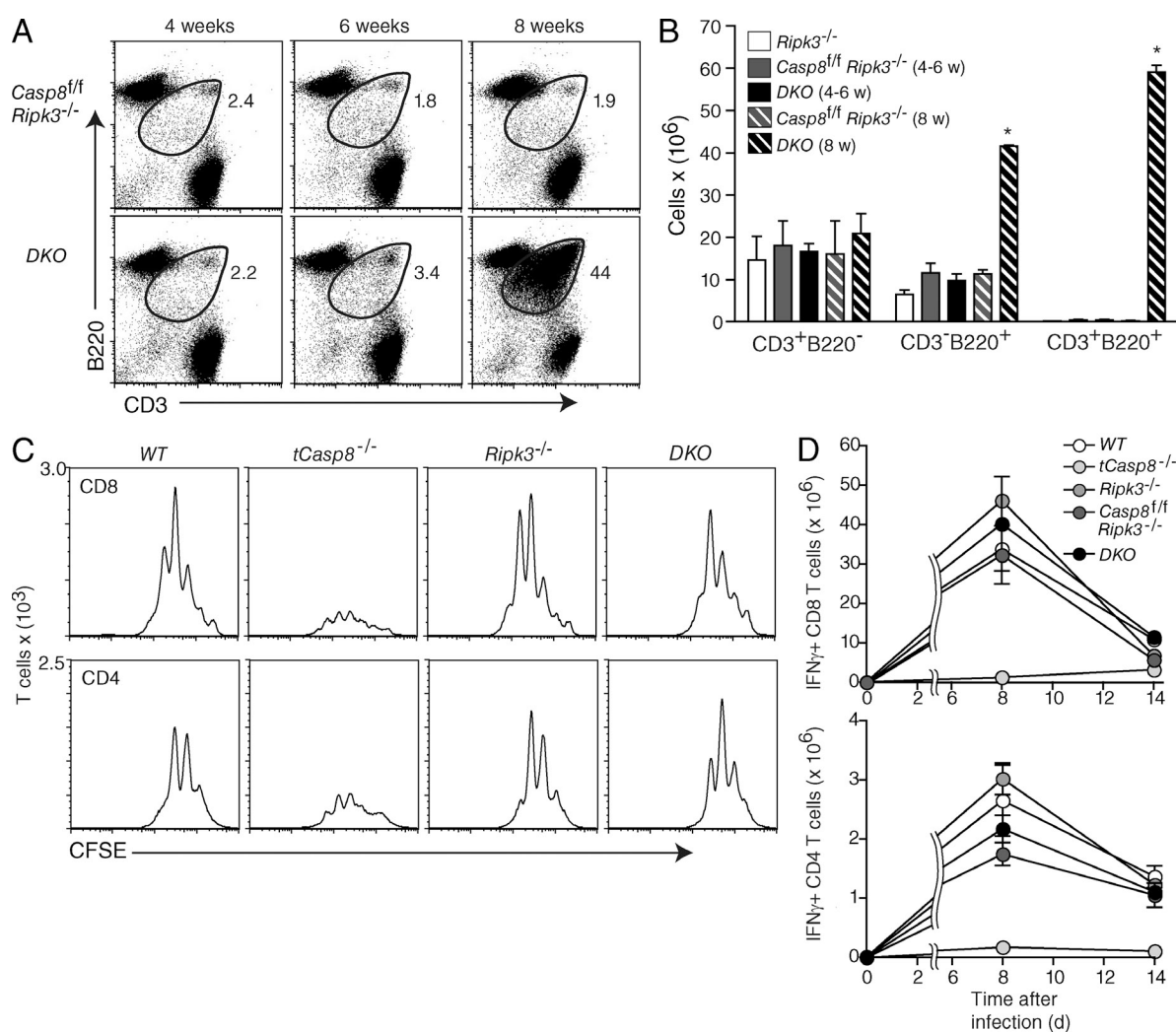
#### Targeted deletion of *Ripk3* rescues caspase 8-dependent necroptosis

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*<sup>f/f</sup> *Cd4Cre* strain to generate *Casp8*<sup>f/f</sup> (WT), *Casp8*<sup>f/f</sup> *Cd4Cre* (*tCasp8*<sup>-/-</sup>), *Ripk3*<sup>-/-</sup>, and *Casp8*<sup>f/f</sup> *Ripk3*<sup>-/-</sup> *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*<sup>-/-</sup> was that DKO mice exhibited lymphadenopathy caused by the expansion of a population of abnormal CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup> 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<sup>+</sup> T cells (Mohamood et al., 2008). In addition, the presence of the CD3<sup>+</sup>CD4<sup>+</sup>CD8<sup>-</sup>B220<sup>+</sup> 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

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*<sup>-/-</sup> or both *tCasp8*<sup>-/-</sup> and *Rip3*<sup>-/-</sup> 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*<sup>fl/fl</sup> *Ripk3*<sup>-/-</sup> and *DKO* (*Casp8*<sup>fl/fl</sup> *Ripk3*<sup>-/-</sup> *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<sup>+</sup>B220<sup>-</sup>, CD3<sup>-</sup>B220<sup>+</sup>, and CD3<sup>+</sup>B220<sup>+</sup> cells from the lymph nodes of *Ripk3*<sup>-/-</sup>, *Casp8*<sup>fl/fl</sup> *Ripk3*<sup>-/-</sup>, 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<sup>+</sup> and CD8<sup>+</sup> T cells was measured by flow cytometry. Data for days 8 and 14 after infection are representative of three and one independent experiments, respectively.

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<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>B220<sup>+</sup> 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*<sup>Δ<sub>ET</sub></sup> *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 × MRL)F<sub>1</sub> hybrid mice with a T cell–specific deletion of *Fas* did manifest *lpr*-like lymphoproliferation. We have found that the *tCasp8*<sup>−/−</sup> 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*<sup>Δ<sub>ET</sub></sup> *Lck Cre* mice from a mixed 129 and C57BL/6 genetic background. In contrast, *tCasp8*<sup>−/−</sup> *Ripk3*<sup>−/−</sup> 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<sup>+</sup>CD4<sup>+</sup>CD8<sup>−</sup>B220<sup>+</sup> 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*<sup>Δ<sub>ET</sub></sup> 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*<sup>−/−</sup> (encoding cyclophilin D), *Casp8*<sup>Δ<sub>ET</sub></sup>, *Atg7*<sup>Δ<sub>ET</sub></sup>, and *Ripk3*<sup>−/−</sup> mice were previously described (Newton et al., 2004; Baines et al., 2005; Beisner et al., 2005; Komatsu et al., 2005). *Ripk3*<sup>−/−</sup> 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*<sup>−/−</sup>, *Casp8*<sup>Δ<sub>ET</sub></sup>, and *Atg7*<sup>Δ<sub>ET</sub></sup> strains were at least 5, 10, and 5 generations backcrossed to C57BL/6, respectively, whereas

*Ripk3*<sup>−/−</sup> 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<sup>+</sup> 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<sup>6</sup> 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 (1× 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 × 10<sup>5</sup> 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<sub>2</sub>, 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.



**Online supplemental material.** Fig. S1 shows the efficiency of deletion in *WT*, *tCasp8<sup>-/-</sup>*, *Ppif<sup>-/-</sup>*, and DKO (*tCasp8<sup>-/-</sup> Ppif<sup>-/-</sup>*) purified T cells. Fig. S2 shows the efficiency of deletion in *WT*, *tCasp8<sup>-/-</sup>*, *tAtg7<sup>-/-</sup>*, and DKO (*tCasp8<sup>-/-</sup> tAtg7<sup>-/-</sup>*) 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<sup>-/-</sup>* and DKO (*tCasp8<sup>-/-</sup> tAtg7<sup>-/-</sup>*) stimulated cell cultures when compared with *WT* and *tCasp8<sup>-/-</sup>* (accumulated data shown in Fig. 3). Fig. S4 shows the efficiency of deletion in *WT*, *tCasp8<sup>-/-</sup>*, *Ripk3<sup>-/-</sup>*, and DKO (*tCasp8<sup>-/-</sup> Ripk3<sup>-/-</sup>*) purified T cells. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110251/DC1>.

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