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A role for c-FLIP$_L$ in the regulation of apoptosis, autophagy, and necroptosis in T lymphocytes

M-X He*[1] and Y-W He*[1]

Caspase 8 plays a dual role in the survival of T lymphocytes. Although active caspase 8 mediates apoptosis upon death receptor signaling, the loss of caspase 8 activity leads to receptor-interacting protein (RIP)-1/RIP-3-dependent necrotic cell death (necroptosis) upon TCR activation. The anti-apoptotic protein c-FLIP (cellular caspase 8 (FLICE)-like inhibitory protein) suppresses death receptor-induced caspase 8 activation. Moreover, recent findings suggest that c-FLIP is also involved in inhibiting necroptosis and autophagy. It remains unclear whether c-FLIP protects primary T lymphocytes from necroptosis or regulates the threshold at which autophagy occurs. Here, we used a c-FLIP isoform-specific conditional deletion model to show that c-FLIP$_L$-deficient T cells underwent RIP-1-dependent necroptosis upon TCR stimulation. Interestingly, although previous studies have only described necroptosis in the absence of caspase 8 activity, we found that pro-apoptotic caspase 8 activity and apoptosis were also enhanced in c-FLIP$_L$-deficient T lymphocytes. Furthermore, c-FLIP$_L$-deficient T cells exhibited enhanced autophagy, which served a cytoprotective function. Together, these findings indicate that c-FLIP$_L$ plays an important antinecroptotic role and is a key regulator of apoptosis, autophagy, and necroptosis in T lymphocytes.
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Cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) is a potential regulator for apoptosis, autophagy, and necroptosis in primary T lymphocytes. When death receptor Fas (CD95) interacts with its ligand FasL, the cytoplasmic tail of Fas recruits several proteins, including c-FLIP, the adaptor protein Fas-associated via death domain (FADD), and pro-caspase 8, which form a membrane-bound receptor complex referred to as the CD95 death-inducing signaling complex (DISC).[1] The homodimerization of pro-caspase 8 in the DISC leads to autocatalytic cleavage and the generation of active caspase 8, which in turn triggers the downstream activation of caspase 3 and eventually leads to apoptosis.[2] Two isoforms of c-FLIP protein have been identified in mice: the 24-kD c-FLIP$_R$ and the 55-kD c-FLIP$_L$. Both isoforms form heterodimers with pro-caspase 8 to inhibit its activation.[2] However, when dimerized with c-FLIP$_L$, pro-caspase 8 is partially activated and cleaves c-FLIP$_L$ to create molecules with signaling capacity, such as p43FLIP.[3] In contrast, because of lack of pro-caspase 8-like C-terminal domains, c-FLIP$_R$ solely functions to inhibit caspase 8 activation, and the formation of c-FLIP$_R$-pro-caspase 8 heterodimers does not result in pro-caspase 8 cleavage.[2] c-FLIP$_L$ and c-FLIP$_R$ are both expressed in mature T lymphocytes, and the expression of either isoform is sufficient to protect resting T cells from apoptosis.[4]

Necroptosis is a form of receptor-interacting protein-1/receptor-interacting protein-3 (RIP-1/RIP-3)-dependent programmed necrotic cell death that is induced when caspase 8 activation is suppressed in embryonic and hematopoietic cells.[5] Previous reports suggest that c-FLIP$_L$ is involved in the regulation of necroptosis. c-FLIP$_L$ protects cIAP antagonist-treated cells from Fas-induced cell death, which involves both apoptosis and necroptosis.[6] c-FLIP$_L$ inhibits the formation of the cell death-inducing 'Ripoptosome', which functions in TLR3-induced apoptosis and necroptosis.[7] Furthermore, siRNA-mediated silencing of c-FLIP$_L$ sensitizes cells to TNF-induced RIP1/RIP3-dependent necroptosis.[8] However, the role of c-FLIP$_L$ in necroptosis has yet to be studied in primary T cells in a genetically deficient model.

Autophagy plays a critical role in regulating T-cell homeostasis.[9,10] Overexpression of c-FLIP suppresses autophagy, as c-FLIP competes with LC3 for Atg3 binding.[11] Moreover, recent studies suggest a correlation between caspase 8 inhibition and excessive autophagy.[12–14] Whether c-FLIP directly regulates autophagy or contributes to autophagy regulation by controlling caspase 8 activity in primary cells has not yet been addressed. More importantly, whether enhanced autophagy leads to necroptosis in T lymphocytes remains controversial.[14–16] Thus, the role of c-FLIP in regulating autophagy and autophagy-related cell death in primary T cells has yet to be determined.

We previously reported that c-FLIP is essential for T-cell development and survival,[4,17] and c-FLIP$_L$-deficient T cells failed to respond to *Listeria monocytogenes* infection.[18] Here, we show that c-FLIP$_L^{-/-}$ T cells fail to proliferate upon TCR stimulation due to extensive Fas-dependent cell death. Both apoptosis and RIP-1-dependent necroptosis contribute to

[1]Department of Immunology, Duke University Medical Center, Durham, NC 27710, USA
*Corresponding author: Y-W He, Department of Immunology, Duke University Medical Center, Jones Building 335, Box 3010, Research Drive, Durham, NC 27710, USA. Tel: +919 613 7870; Fax: +9196848982; E-mail: youwen.he@duke.edu
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Abbreviations: c-FLIP, cellular caspase 8 (FLICE)-like inhibitory protein; FADD, Fas-associated via death domain; DISC, CD95 death-inducing signaling complex; RIP-1, receptor-interacting protein-1; RIP-3, receptor-interacting protein-3; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; BAC, bacterial artificial chromosome; MOMP, mitochondrial outer membrane permeabilization
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the loss of cellularity after T-cell activation. Interestingly, c-FLIP$_L^{-/-}$ T cells generate more active caspase 8 (p18) than wild-type T cells, indicating that the apoptotic and antinecrotic activities of caspase 8 may be independent. In addition, c-FLIP$_L^{-/-}$ T cells display increased induction of autophagy, which promotes T-cell survival. Together, our results identify c-FLIP$_L$ as a key regulator of apoptosis, necroptosis, and autophagy in primary T lymphocytes.

Results

c-FLIP$_L$-deficient T lymphocytes display enhanced cell death upon TCR stimulation. We previously generated conditional knockout mice lacking only the c-FLIP$_L$ isoform in T lymphocytes (referred to as c-FLIP$_L^{-/-}$) and found that c-FLIP$_L^{-/-}$ mice failed to generate effector T-cell responses against *Listeria monocytogenes* infection *in vivo*.[18] The impaired effector response in c-FLIP$_L^{-/-}$ mice was initially attributed to defective T-cell proliferation (Figure 1a). Because impaired proliferation could be caused by enhanced cell death, we analyzed cell survival and found that the death rate of c-FLIP$_L^{-/-}$ T cells upon TCR stimulation was dramatically increased when compared with that of wild-type T cells (Figures 1b and c and Supplementary Figure S1). Regardless of the strength of the TCR signal, a majority of c-FLIP$_L^{-/-}$ T cells (both CD4$^+$ and CD8$^+$) died within 36 h of simulation (Figure 1b and Supplementary Figure S1). The decreased numbers of live c-FLIP$_L^{-/-}$ T cells were apparent at <24 h after TCR stimulation, suggesting that this phenomenon was not attributable to impaired T-cell proliferation, as T lymphocytes do not divide in the first 24 h following stimulation (Supplementary Figure S2). In contrast to these results, c-FLIP$_L^{-/-}$ T cells displayed no survival defects in the absence of TCR stimulation (Figure 1b and Supplementary Figure S1B), likely because of the protective effect of c-FLIP$_R$. Interestingly, we observed that c-FLIP$_L^{-/-}$ CD4$^+$ T cells consistently exhibited slightly better survival than wild-type T cells in the absence of TCR stimulation (Figures 1b and c). Although mature T lymphocytes expressed both c-FLIP isoforms (Supplementary Figure S3B), this survival defect was not observed in c-FLIP$_R$-deficient T cells (Supplementary Figure S3A).

c-FLIP$_L$ functions downstream of death receptor signals.[2] Blocking Fas signaling by anti-FasL mAb treatment completely rescued the survival defect in c-FLIP$_L^{-/-}$ CD4$^+$ T cells (Figure 2a) and partially rescued the survival

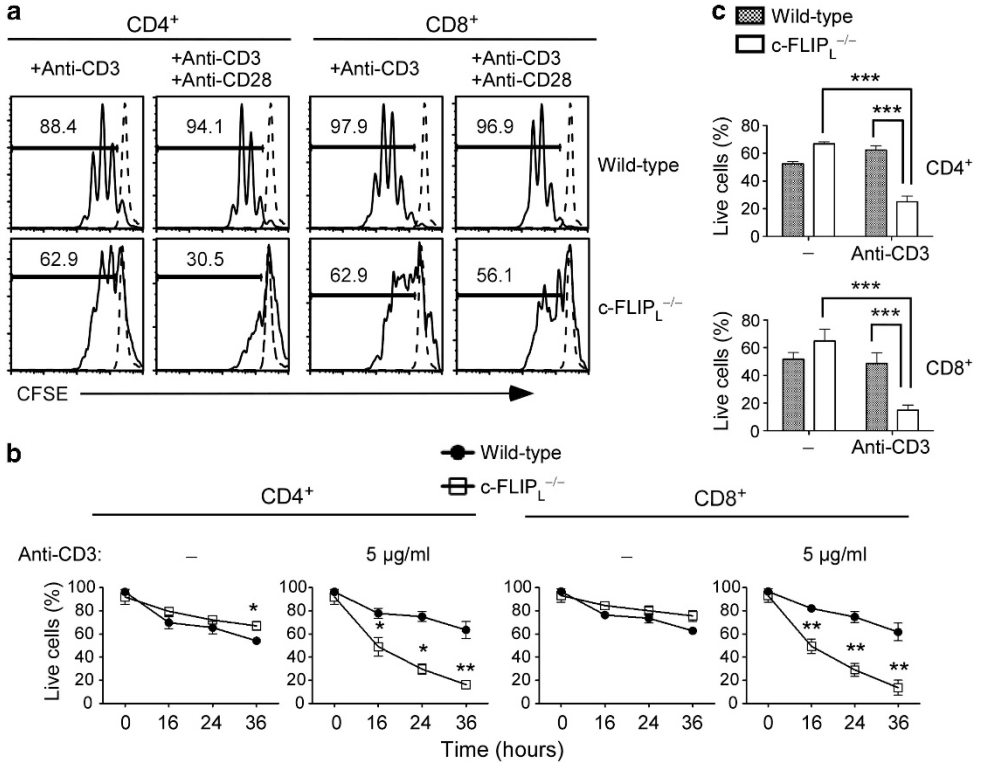

Figure 1 Defective proliferation and enhanced cell death after TCR stimulation in c-FLIP$_L$-deficient T lymphocytes. (**a**) Proliferation of c-FLIP$_L^{-/-}$ T cells. Total splenocytes from c-FLIP$_L^{-/-}$ and wild-type (c-FLIP$^{f/f}$) mice were labeled with CFSE and stimulated with anti-CD3 in the presence or absence of anti-CD28 for 3 days. Cell proliferation was analyzed by flow cytometry. Histograms were gated on 7-AAD$^-$ CD4$^+$ or CD8$^+$ populations. Numbers represent the frequency of T cells that have undergone cell proliferation. Dashed lines represent unstimulated T cells. The data shown are representative of >5 experiments. (**b**) Time course analysis of the survival rate of naive and activated c-FLIP$_L^{-/-}$ T cells. Total splenocytes were cultured in the presence or absence of anti-CD3. The cell survival rates of CD4$^+$ and CD8$^+$ T cells were analyzed by flow cytometry at different time points after TCR activation. Live cells were analyzed by gating on the Annexin V$^-$ 7AAD$^-$ population within the total CD4$^+$ or CD8$^+$ populations ($n=4$). (**c**) Cell death rate of TCR-stimulated c-FLIP$_L^{-/-}$ T cells after 40 h of culture. Total splenocytes were cultured in the presence or absence of anti-CD3 for 40 h. Cell death rates were analyzed as described in (**b**; $n=6$). All error bars represent S.E.M. *$P<0.05$, **$P<0.01$, ***$P<0.001$

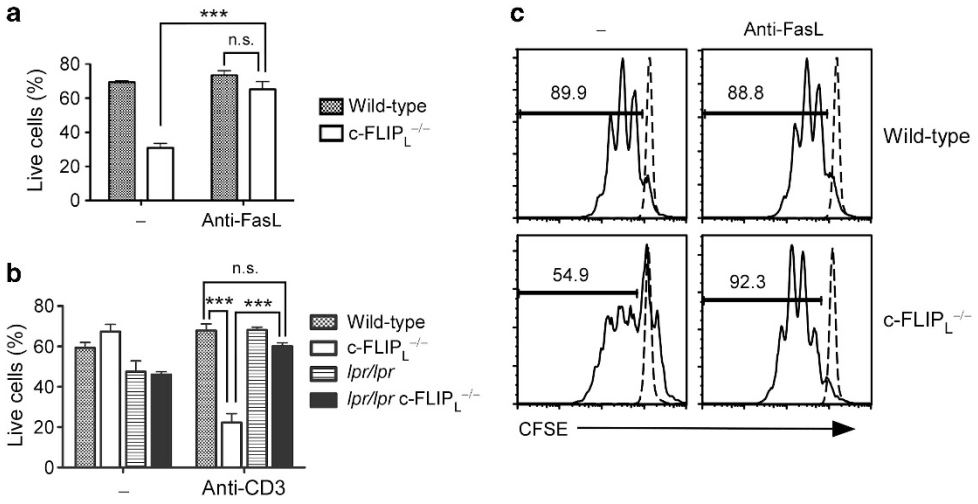

Figure 2 The increased cell death observed in c-FLIP$_L^{-/-}$ T lymphocytes is Fas dependent. (**a**) The effect of blocking FasL on cell death in TCR-stimulated c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 and cultured for 40 h in the presence or absence of anti-FasL mAb as indicated. Cell death of CD4$^+$ T cells was analyzed as described in Figure 1 ($n=5$). (**b**) Cell death of T cells from c-FLIP$_L^{-/-}$ mice crossed to *lpr/lpr* mice. Total splenocytes from different mice were stimulated with anti-CD3 and cultured for 40 h, and apoptosis was measured in CD4$^+$ T cells as described in Figure 1 ($n=4$). All error bars represent S.E.M. ***$P<0.001$. (**c**) Effect of anti-FasL mAb on the proliferation of c-FLIP$_L^{-/-}$ T cells. Proliferation assays were performed as shown in Figure 1a in the presence or absence of anti-FasL mAb. Histograms were gated on 7-AAD$^-$ CD4$^+$ populations. The data shown were obtained from three independent experiments

defect in c-FLIP$_L^{-/-}$ CD8$^+$ T cells (Supplementary Figure S4A). In contrast, anti-FasL mAb treatment did not further enhance cell survival in wild-type T lymphocytes, as these cells are fully protected by endogenous c-FLIP. We further confirmed the role of Fas in the death of activated c-FLIP$_L^{-/-}$ T cells by crossing c-FLIP$_L^{-/-}$ mice to *lpr/lpr* mice, which do not express functional Fas on the cell surface.[19] The survival of T cells from c-FLIP$_L^{-/-}$ *lpr/lpr* mice was comparable to that of T cells from wild-type or *lpr/lpr* mice (Figure 2b and Supplementary Figure S4B). Importantly, rescuing c-FLIP$_L^{-/-}$ T-cell survival restored their ability to proliferate (Figure 2c and Supplementary Figure S4C), suggesting that the impaired effector T-cell expansion observed upon TCR stimulation in c-FLIP$_L^{-/-}$ mice is a secondary effect of enhanced cell death. Interestingly, when the Fas/FasL interaction was blocked, c-FLIP$_L^{-/-}$ T cells consistently proliferated slightly faster than wild-type T cells, whereas anti-FasL mAb treatment had no effect on wild-type T-cell proliferation (Figure 2c). These data are consistent with the fact that NF-κB signaling is increased in c-FLIP$_L^{-/-}$ T lymphocytes.[18] Taken together, these findings indicate that the enhanced cell death observed in activated c-FLIP$_L^{-/-}$ T cells was dependent on the Fas/FasL interaction.

We next tested whether c-FLIP$_L^{-/-}$ T cells displayed altered expression of death receptors or death receptor ligands. Both untreated and activated c-FLIP$_L$-deficient T lymphocytes expressed Fas at levels comparable to those in wild-type T cells (Supplementary Figure S5A). As FasL expression is undetectable in naive T cells, we analyzed cell surface FasL 24 h after TCR stimulation. Similar levels of FasL were observed in wild-type and c-FLIP$_L^{-/-}$ T lymphocytes (Supplementary Figure S5B). These results show that the enhanced Fas-dependent cell death observed in c-FLIP$_L^{-/-}$ T cells was not due to changes in the expression of Fas or FasL.

c-FLIP$_L$-deficient T-cell death is partially mediated through caspase-dependent apoptosis. As c-FLIP$_L$ inhibits death receptor-mediated caspase 8 activation,[2] we next investigated whether the loss of c-FLIP$_L$ led to increased caspase activity in T lymphocytes. Active caspase 8 (p18) was not detected in naive T cells regardless of c-FLIP$_L$ expression (Figure 3a). However, after TCR stimulation, c-FLIP$_L^{-/-}$ T cells generated high levels of active caspase 8 (p18), and blocking the Fas/FasL interaction with an anti-FasL blocking antibody completely prevented active caspase 8 generation (Figure 3a). The accumulation of pro-caspase 8 upon blockade of FasL in both wild-type and c-FLIP$_L^{-/-}$ T cells indicated that the cleavage of pro-caspase 8 in activated T cells occurred through a Fas-dependent mechanism. In contrast, wild-type T cells do not generate significant amounts of active caspase 8 (p18), likely because c-FLIP$_L$ forms heterodimers with caspase 8 and inhibits its complete cleavage. Pro-caspase 8 levels was increased in activated T cells, likely because the catalytic activity of pro-caspase 8 is essential for the survival of activated T cells.[20]

We further tested whether suppressing active caspase 8 generation and apoptosis could rescue the cell survival defect in c-FLIP$_L^{-/-}$ T cells. Surprisingly, the caspase 8 inhibitor zIETD only partially rescued the survival defect observed in c-FLIP$_L^{-/-}$ CD4$^+$ T cells and failed to rescue that observed in c-FLIP$_L^{-/-}$ CD8$^+$ T cells (Figure 3b). Similar results were obtained using the pan-caspase inhibitor zVAD to block apoptosis (Figure 3b). We next analyzed the kinetics of apoptosis in c-FLIP$_L^{-/-}$ T cells. During early stages of apoptosis, phosphatidylserine can be detected on the cell membrane by Annexin V staining. We used Annexin V to detect cells undergoing apoptosis and 7-AAD to label late apoptotic or necrotic cells. Upon TCR stimulation, the frequencies of Annexin V$^+$ cells among 7-AAD$^-$ cells were initially higher in c-FLIP$_L^{-/-}$ T-cell samples (Figure 3c).

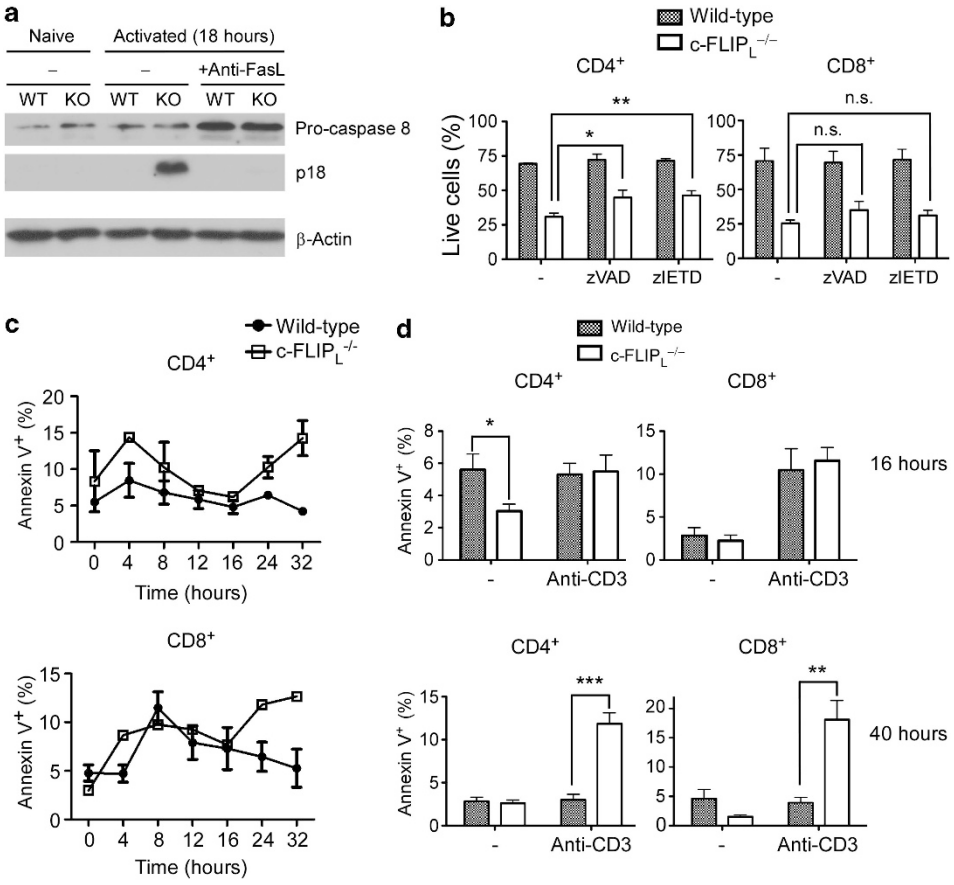

Figure 3 Role of caspase in the death of c-FLIP$_L^{-/-}$ T cells. (**a**) Western blot analysis of caspase 8 activation in c-FLIP$_L^{-/-}$ T cells. Total splenocytes from c-FLIP$_L^{-/-}$ and wild-type mice were activated with anti-CD3 for 18 h. T lymphocytes were enriched (>95%) and lysed for blotting. The data shown are representative of three independent experiments. (**b**) The effect of caspase inhibitors on cell death in c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 and cultured for 40 h in the presence of zVAD or zIETD, and cell survival was measured ($n=6$). (**c**) The kinetics of apoptotic rates in c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 and analyzed by flow cytometry at different time points ($n=2$). Annexin V$^+$% was calculated as follows: the number of Annexin V$^+$ 7-AAD$^-$ CD4$^+$ (or CD8$^+$) cells divided by the total number of 7AAD$^-$ CD4$^+$ (or CD8$^+$) cells. (**d**) Apoptotic rates at 16 h ($n=6$) and 40 h ($n=4$) after TCR stimulation. All error bars represent S.E.M. *$P<0.05$, **$P<0.01$, ***$P<0.001$

At time points between 12 and 16 h following TCR stimulation, the wild-type and c-FLIP$_L^{-/-}$ T cells displayed comparable apoptotic rates (Figures 3c and d). At later time points (32 or 40 h), more apoptotic cells were detected in the c-FLIP$_L^{-/-}$ CD4$^+$ and CD8$^+$ T-cell samples (Figures 3c and d). Meanwhile, the death rate (7-AAD$^+$) steadily increased in the c-FLIP$_L^{-/-}$ samples (Supplementary Figure S6). Although cell death was enhanced in c-FLIP$_L^{-/-}$ T cells, inhibiting caspases failed to rescue these cells from death, suggesting that a nonapoptotic cell death pathway may contribute to the enhanced death observed in c-FLIP$_L^{-/-}$ T cells.

c-FLIP$_L$-deficient T cells undergo necroptosis upon TCR stimulation. We then analyzed whether activated c-FLIP$_L^{-/-}$ T cells died through necrosis. Apoptosis is characterized by the generation of mononucleosomal and oligonucleosomal DNA fragments, which are released first to the cytosol during early stages of apoptosis and then to the culture medium outside the cells at the late stage of apoptosis.[21] In contrast, necrotic cells directly release randomly digested DNA into the culture medium as they lose membrane integrity.[21] Necropoptosis and apoptosis can be induced in L929 cells by zVAD and etoposide, respectively.[12,22] The enzyme-linked immunosorbent assay (ELISA) results confirmed that zVAD-treated L929 cells immediately released fragmented DNA to the supernatant, whereas etoposide-treated L929 cells showed a pattern of DNA release typical of apoptosis: the fragmented DNA was first detected in the cytosol before being released to the supernatant (Supplementary Figure S7). We thus assessed the release of fragmented DNA in wild-type and c-FLIP$_L^{-/-}$ T cells upon TCR stimulation using the same methods. The wild-type T cells displayed a fragmented DNA release pattern similar to that observed upon induction of apoptosis in L929 cells. In contrast, c-FLIP$_L^{-/-}$ T cells quickly released fragmented DNA to the supernatant, suggesting that they were undergoing necrosis. Fragmented DNA was also detected in the cytosol of activated c-FLIP$_L^{-/-}$ T cells, suggesting that some of the cells died from apoptosis (Figure 4a).

Previous data suggested that c-FLIP$_L$ might recruit RIP-1 to the DISC, leading to caspase 8-dependent RIP-1 cleavage.[23,24] Therefore, we investigated whether RIP-1 was involved in the death of c-FLIP$_L$-deficient T lymphocytes.

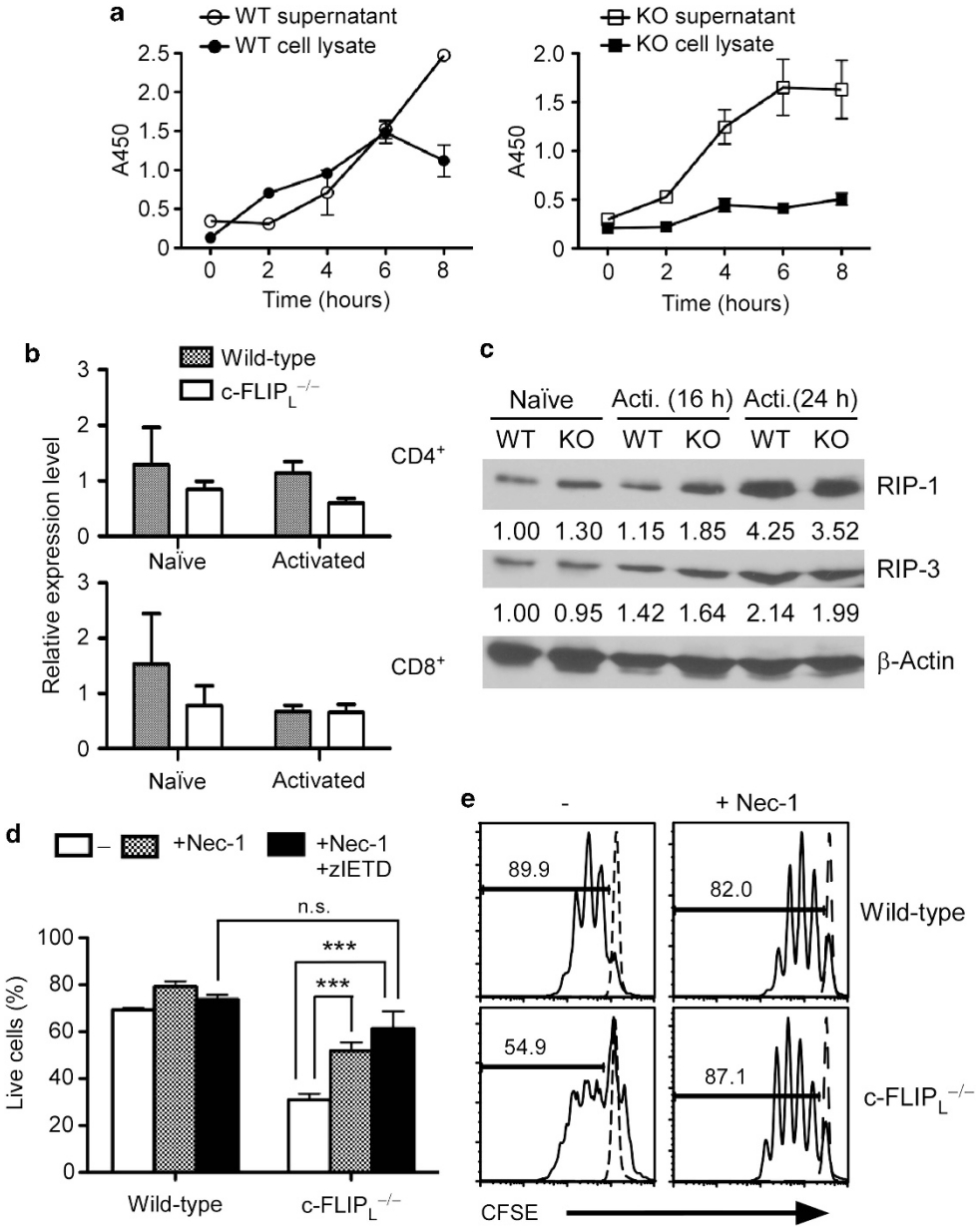

Figure 4 RIP-1-dependent necroptosis in c-FLIP$_L^{-/-}$ T cells. (**a**) Release of fragmented DNA by T cells upon activation. T cells in c-FLIP$_L^{-/-}$ and control mice were labeled *in vivo* by i.p. BrdU injection and stimulated *in vitro* with plate-bound anti-CD3 for the indicated periods of time. BrdU-labeled fragmented DNA was measured in cell lysates and supernatants by ELISA. (**b**) RIP-1 mRNA levels in resting or activated c-FLIP$_L^{-/-}$ T cells. For naive T cells, live CD44^{low} CD4$^+$ or CD8$^+$ T cells were sorted from freshly harvested splenocytes. For activated T cells, total splenocytes were activated with anti-CD3 for 18 h, and live CD4$^+$ or CD8$^+$ T cells were sorted. (**c**) RIP-1 and RIP-3 protein expression in resting or activated c-FLIP$_L^{-/-}$ T cells. Live T lymphocytes were enriched (>95%) for blotting. The numbers underneath each band represent the relative expression level after normalization to β-actin. (**d**) Effect of necrostatin-1 (Nec-1) and zIETD on the cell death of c-FLIP$_L^{-/-}$ T cells. Total splenocytes were stimulated with anti-CD3 (5 μg/ml) and cultured in the presence or absence of Nec-1 and zIETD for 40 h. All error bars represent S.E.M. ***$P<0.001$. (**e**) Effect of Nec-1 on the proliferation of c-FLIP$_L^{-/-}$ T cells. Proliferation assays were performed as shown in Figure 1a, and Nec-1 was added at the beginning of the culture period. Histograms were gated on 7-AAD$^-$ CD4$^+$ populations. The data shown were obtained in three independent experiments

RIP-1 mRNA levels were not increased in c-FLIP$_L^{-/-}$ T cells before or after TCR stimulation (Figure 4b). However, the protein level of RIP-1 was higher in naive c-FLIP$_L^{-/-}$ T cells than in controls. At 16 h after TCR stimulation, the difference in RIP-1 expression was more obvious (Figure 4c). c-FLIP$_L^{-/-}$ and wild-type T cells displayed comparable levels of RIP-1 expression 24 h after TCR stimulation, likely because of the death of cells expressing high levels of RIP-1. The expression of RIP-3, another necroptosis-related protein, was slightly enhanced in the absence of c-FLIP$_L$ in T lymphocytes (Figure 4c). Moreover, the RIP-1 inhibitor necrostatin-1 (referred to as Nec-1) partially rescued the enhanced cell death (Figure 4d) and completely restored the defective proliferation capacity observed in c-FLIP$_L^{-/-}$ T cells (Figure 4e and Supplementary Figure S4C). More importantly, combined treatment with the caspase 8 inhibitor zIETD

and Nec-1 completely rescued the enhanced cell death observed in c-FLIP$_L^{-/-}$ T cells (Figure 4d). Taken together, these results indicate that in addition to apoptosis, c-FLIP$_L$-deficient T cells also undergo RIP-1-dependent necroptosis upon TCR activation.

Autophagy is enhanced in c-FLIP$_L^{-/-}$ T lymphocytes. A correlation between necroptosis and excessive autophagy has been observed in several previous studies.[12,14,25] Hence, we investigated whether the c-FLIP$_L$-deficient T lymphocytes displayed altered levels of autophagy. During autophagy induction, LC3-I is conjugated to phosphatidylethanolamine (PE) to form LC3-II. LC3-I/LC3-II conversion in mammalian cells is a reliable marker for autophagy induction.[26] After TCR stimulation (18 h), the LC3-II/LC3-I ratio increased in both wild-type and c-FLIP$_L^{-/-}$ T cells; however, LC3-II levels were much higher in activated c-FLIP$_L^{-/-}$ T cells than in wild-type T cells (Figure 5a). We next counted LC3 puncta as a measure of the number of autophagosomes per cell[26,27] (Figure 5b, upper panel). Naive and activated c-FLIP$_L^{-/-}$ T cells displayed significantly more LC3 puncta than did wild-type T cells (Figure 5b). We further analyzed the intracellular structures in wild-type and c-FLIP$_L^{-/-}$ T cells by transmission electron microscopy (TEM). Activated c-FLIP$_L^{-/-}$ T cells contained more intracellular double-membrane vacuoles (autophagosomes) than wild-type T cells (Figures 5c and d), and the percentage of cells containing autophagosomes was much higher in activated c-FLIP$_L^{-/-}$ T cells than in wild-type T cells (Figure 5e). Moreover, autophagy levels were also higher in naive c-FLIP$_L^{-/-}$ T cells than in wild-type T cells (Figures 5f and g). Interestingly, we observed an accumulation of membrane-containing autophagosomes in naive c-FLIP$_L$-deficient T lymphocytes that were entirely absent in naive wild-type cells (Figures 5f and h). Together, these data demonstrate that c-FLIP$_L^{-/-}$ T cells display enhanced autophagy induction, especially after T-cell activation.

Overexpression of c-FLIP protein inhibits autophagy in several cell lines.[11] However, whether c-FLIP in primary T cells has a similar effect remains unclear. We thus assessed autophagy levels in activated T cells expressing c-FLIP isoform-specific BAC transgenes (referred to as c-FLIP$_R$-Tg and c-FLIP$_L$-Tg). Both c-FLIP$_R$-Tg and c-FLIP$_L$-Tg inhibited LC3 punctum formation upon TCR stimulation; however, c-FLIP$_R$-Tg inhibited punctum formation more efficiently than c-FLIP$_L$ (Supplementary Figure S8). These data suggest that c-FLIP can modulate autophagy in activated T lymphocytes.

Enhanced autophagy plays a cytoprotective role in c-FLIP$_L$-deficient T lymphocytes. Conflicting findings have been reported regarding the role of caspase inhibition-mediated autophagy in cell death. Previous studies have concluded that autophagy either causes[12,25,28] or protects cells from zVAD-induced necrotic cell death.[29] We therefore tested the role of autophagy in the death of c-FLIP$_L^{-/-}$ T lymphocytes. Atg3 plays an essential role in autophagosome formation by catalyzing LC3 processing.[30] Inducible short-term deletion of Atg3 in T lymphocytes resulted in reduced autophagy induction (Supplementary Figure S9A) but did not cause enhanced cell death under either resting or activated conditions[31] (Supplementary Figure S9B). Therefore, we crossed c-FLIP$_L^{-/-}$ (c-FLIP$^{f/f}$ x c-FLIP$_R$-BAC Tg) mice to Atg3$^{f/f}$ ER-Cre mice. In this model, c-FLIP and Atg3 are successfully deleted *in vitro* upon 4-hydroxytamoxifen (4-OHT) treatment.[4,31] We found that the loss of Atg3 further decreased cell survival in c-FLIP$_L^{-/-}$ T cells, especially upon TCR stimulation (Figure 6). These data suggest that autophagy plays a cytoprotective role in activated c-FLIP$_L^{-/-}$ T lymphocytes.

Necroptosis in c-FLIP$_L$-deficient T cells involves reactive oxygen species (ROS). CM-H$_2$DCFDA staining showed that ROS levels were slightly increased in c-FLIP$_L$-deficient T cells under both resting and activated conditions (Supplementary Figure S10A). The antioxidant *N*-acetylcysteine (NAC) partially inhibited TCR-induced cell death in both wild-type and c-FLIP$_L$-deficient T cells. Moreover, adding Nec-1 to NAC-treated cells did not improve their survival (Supplementary Figure S10B). Therefore, the production of ROS likely contributes to necroptosis in c-FLIP$_L$-deficient T cells. The major cellular reservoir of ROS production is the mitochondria.[32] In T lymphocytes, the numbers of mitochondria are tightly regulated by autophagy.[31] However, the enhanced autophagy observed in naive and activated c-FLIP$_L^{-/-}$ T cells did not appear to affect the mitochondrial numbers in these cells (Supplementary Figure S10C). In zVAD-treated L929 cells, excessive autophagy causes ROS accumulation by degrading catalase, the major ROS scavenger.[28] Whether the same mechanism applies to T cells remains to be determined. On the other side, altered RIP-1/RIP-3 activity may also lead to abnormal ROS production.[28,33]

Discussion

Here we reported that the loss of c-FLIP$_L$ in T cells resulted in dramatic cell death upon TCR stimulation. Although c-FLIP$_L$ has long been considered as an antiapoptotic protein, activated c-FLIP$_L$-deficient T cells showed extensive necroptosis. More interestingly, although previous studies showed the occurrence of necroptosis when caspase 8 activity was completely inhibited, we observed both necroptosis and enhanced caspase 8 cleavage in c-FLIP$_L^{-/-}$ T cells. Our results suggest that the antinecrotic and pro-apoptotic activities of caspase 8 might be independently regulated. The increased apoptosis and necroptosis observed in c-FLIP$_L^{-/-}$ T cells were both Fas dependent. In contrast, naive c-FLIP$_L^{-/-}$ T cells showed no defects in survival. Our findings demonstrate that c-FLIP$_L$ suppresses autophagy in both activated and resting T lymphocytes, and the enhanced autophagy observed in these cells is cytoprotective.

In c-FLIP$_L$-deficient T lymphocytes, necroptosis occurs downstream of Fas signaling following TCR stimulation. This finding is consistent with several recent publications showing that death receptor signaling induced necroptosis in *Cyld*$^{-/-}$ MEFs, caspase-8$^{-/-}$ hematopoietic cells, and FADD$^{-/-}$ keratinocytes.[34–36] Caspase 8- or FADD-deficient T lymphocytes also showed remarkable necroptosis upon TCR stimulation,[15,16] but whether Fas generates signals for

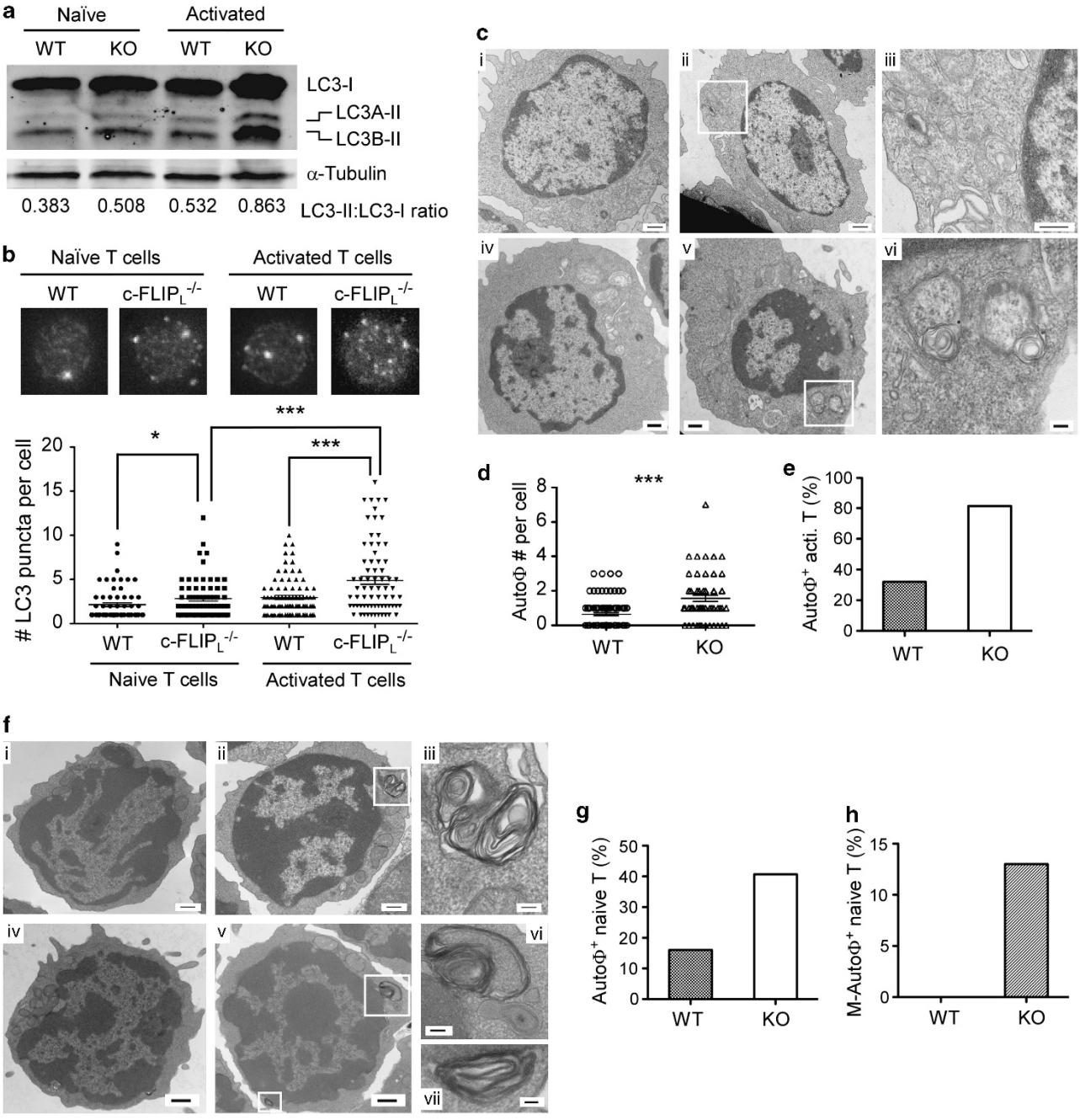

Figure 5 Enhanced autophagy in c-FLIP$_L^{-/-}$ T cells. (**a**) Western blot analysis of LC3 in resting and activated c-FLIP$_L^{-/-}$ T cells. T cells were activated with anti-CD3 for 18 h, and LC3-I/LC3-II conversion was measured by western blot as described in Figure 4c. (**b**) The numbers of LC3 puncta in c-FLIP$_L^{-/-}$ T cells. Activated T cells were intracellularly stained with anti-LC3 and analyzed for LC3 puncta by fluorescent microscopy. (**c**) TEM images of c-FLIP$_L^{-/-}$ T cells. Total splenocytes were activated with anti-CD3 for 18 h. Activated wild-type CD4$^+$ (I) or CD8$^+$ (IV) and c-FLIP$_L^{-/-}$ CD4$^+$ (II, III) or CD8$^+$ (V, VI) T cells were sorted and analyzed by TEM. III and VI are enlarged images of II and V, respectively. Bars represent 0.5 μm in I–V and 100 nm in VI. Shown are representative micrographs. (**d**) Numbers of autophagosomes per cell in activated T lymphocytes ($n \geq 60$). (**e**) Percentages of activated T cells containing autophagosomes among all activated T cells counted in (**d**). (**f**) Accumulated membrane inside autophagosomes in naive c-FLIP$_L^{-/-}$ T cells. Naive wild-type CD4$^+$ (I) or CD8$^+$ (IV) and c-FLIP$_L^{-/-}$ CD4$^+$ (II, III) or CD8$^+$ (V, VI, VII) T cells were sorted (CD44^{low}) and analyzed by TEM. III is an enlarged image of II. V and VII are enlarged images of IV. Bars represent 0.5 μm in I–II and IV–V, and 100 nm in III, VI, and VII. Shown are representative micrographs. (**g**) Percentages of naive T cells in which autophagosomes were observed among all naive T cells counted in (**f**; $n \geq 50$). (**h**) Percentages of naive T cells with membrane-containing autophagosomes among all naive cells. AutoΦ stands for autophagosomes; M-AutoΦ stands for membrane-containing autophagosomes. All error bars represent S.E.M. $^*P < 0.05$, $^{***}P < 0.001$

necroptosis in these knockout models remains to be determined. In addition to death receptor signaling, alternative necroptosis inducers may also exist. For example, TLR3 activation was shown to induce necroptosis in zVAD-treated HaCaT keratinocytes.[7] Therefore, future studies should focus on deciphering the signaling pathway of necroptosis induction

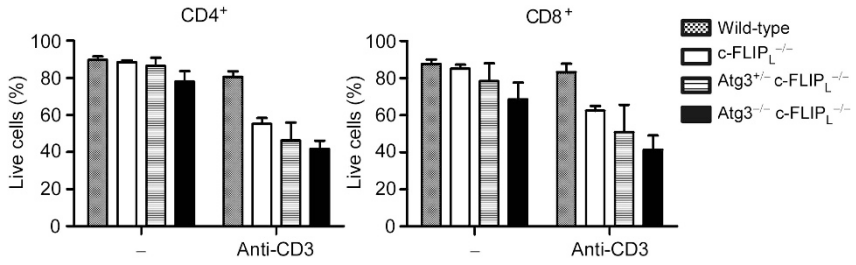

Figure 6 The cytoprotective role of autophagy in the cell death of c-FLIP$_L^{-/-}$ T cells. The effect of autophagy inhibition in the survival of c-FLIP$_L^{-/-}$ T cells. Total splenocytes from c-FLIP$^{f/f}$ Atg3$^{f/f}$, c-FLIP$^{f/f}$ c-FLIP$_R$-Tg$^+$ ER-Cre$^+$, c-FLIP$^{f/f}$ Atg3$^{f/+}$ c-FLIP$_R$-Tg$^+$ ER-Cre$^+$, and c-FLIP$^{f/f}$ c-FLIP$_R$-Tg$^+$ Atg3$^{f/f}$ ER-Cre$^+$ mice were cultured for 3 days in the presence of 4-OHT and IL-7 to induce deletion, resulting in wild-type, c-FLIP$_L^{-/-}$, cFLIP$_L^{-/-}$ Atg3$^{+/-}$, and cFLIP$_L^{-/-}$ Atg3$^{-/-}$ cells. Live cells were isolated using Ficoll, stimulated with plate-bound anti-CD3, and cultured for 40 h with IL-7 (1 ng/ml). Cell death was analyzed by flow cytometry analysis of 7-AAD staining. The data presented are a summary of data from five independent experiments

under physiological conditions and the biological implications of programmed necrosis.

Necroptosis usually occurs when caspase 8 activity and apoptosis are blocked. Here, we reported the existence of necroptosis in cells with enhanced apoptosis. Both enhanced apoptosis and necroptosis occur downstream of Fas/FasL ligation in c-FLIP$_L^{-/-}$ T cells. When Fas is activated, multiple proteins, including c-FLIP$_L$ and pro-caspase 8, are recruited to the DISC.[2] The fact that c-FLIP$_L$, caspase 8, and FADD are all involved in necroptosis regulation strongly suggests that the inhibition of RIP-1/RIP-3 occurs at the DISC.[15,16] The interaction between pro-caspase 8 and c-FLIP$_L$ at the DISC leads to the cleavage of c-FLIP$_L$ to generate p43FLIP.[2] p43FLIP and c-FLIP$_L$ recruit RIP-1 to the caspase 8/cFLIP/RIP-1 complex, and p43FLIP binds RIP-1 with higher affinity than do full-length c-FLIP$_L$ or caspase 8 in T lymphocytes.[24] Studies in human fibroblast cell lines showed that caspase 8 mediated the cleavage of RIP-1 to produce an inhibitory RIP-1 fragment.[23] It is possible that a similar inhibitory mechanism may occur in T lymphocytes: c-FLIP$_L$ may regulate RIP-1 activity by recruiting RIP-1 to the DISC for caspase 8-dependent degradation. In this model, without c-FLIP$_L$, the recruitment of RIP-1 is blocked. Therefore, RIP-1 cannot be regulated by caspase 8 cleavage, leading to increased RIP-1 activity and necroptosis. This hypothesis is supported by the accumulation of RIP-1 protein in c-FLIP$_L^{-/-}$ T cells without changes in RIP-1 mRNA levels. Furthermore, the loss of c-FLIP$_L$ protein causes enhanced caspase 8 activation and a subsequent increase in apoptosis in c-FLIP$_L^{-/-}$ T cells (Figure 7).

Autophagy is shown to be beneficial for cell survival in c-FLIP$_L$-deficient T lymphocytes. Autophagy supports cell survival in many circumstances; this cytoprotective role of autophagy is especially important upon nutrient withdrawal.[37] In T lymphocytes, autophagy is indispensible not only for surviving growth factor withdrawal, but also for regulating the mitochondria and endoplasmic reticulum.[31,38,39] How autophagy promotes cell survival in c-FLIP$_L^{-/-}$ T cells remains to be determined. In addition to clearing unwanted intracellular substances and providing energy, autophagy may benefit activated c-FLIP$_L^{-/-}$ T cells in a more direct way: studies in cells resistant to apoptosis revealed that autophagy actively removed active caspase 8.[40] Whether T lymphocytes employ a similar mechanism must be addressed in further studies. In addition, it will be interesting to investigate whether the enhanced autophagy observed in naive c-FLIP$_L^{-/-}$ T lymphocytes renders them more resistant to cell death.

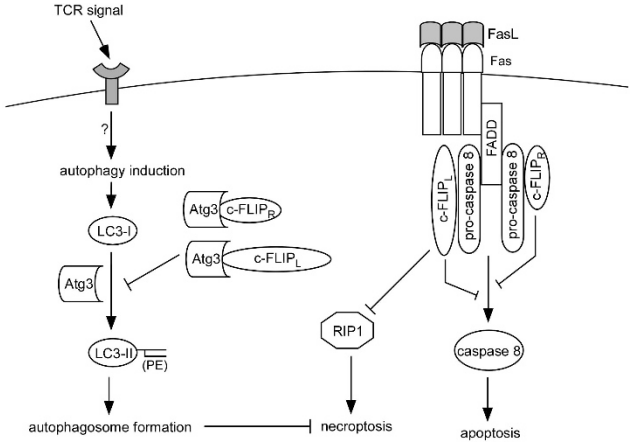

Figure 7 A proposed model for c-FLIP$_L$-mediated regulation of apoptosis, necroptosis, and autophagy. Upon TCR-mediated activation, T lymphocytes upregulate the expression of Fas and FasL. The binding of Fas to FasL on the cell surface induces the formation of DISC, where both c-FLIP$_L$ and c-FLIP$_R$ suppress the activation of pro-caspase 8 and downstream apoptosis. In addition, c-FLIP$_L$ and p43FLIP recruit RIP-1, inhibiting RIP-1 activity and necroptosis. Simultaneously, TCR activation induces autophagosome formation in T lymphocytes through an unknown mechanism. c-FLIP competes with LC3 for Atg3 binding, thereby reducing LC3 processing, and thus suppressing autophagy induction. In c-FLIP$_L$-deficient T cells, autophagy is enhanced and protects cells from RIP-1-dependent necrosis

Our study reveals that excessive ROS production contributes to cell death in c-FLIP$_L^{-/-}$ T lymphocytes upon TCR stimulation. This finding is consistent with previous reports showing that ROS accumulation leads to necroptosis in zVAD-treated L929 cells.[25,28] Mitochondria may serve as the site of ROS production because of increased Ca^{2+} flux and metabolic changes, in which RIP-1 activity is involved.[33] Studies in L929 cells suggested that increased ROS production occurs downstream of dysregulated autophagy. Whether enhanced autophagic flux in c-FLIP$_L$-deficient T cells leads to excessive ROS production remains to be determined. Nevertheless, it is possible that the downstream effects of autophagy are both beneficial and harmful for cell survival,

and the combination of these effects may determine the outcome.

c-FLIP$_L$ is a key pro-survival factor at the nexus of apoptosis, necroptosis, and autophagy. T-cell responses are tightly controlled to avoid the generation and activation of autoreactive cells and excessive tissue damage.[41] The presence of redundant death pathways guarantees that active lymphocytes are removed to avoid autoimmunity. Further studies will focus on developing c-FLIP as a therapeutic target to induce T-cell death or to enhance T-cell survival *in vivo* with the goal of improving adaptive responses to pathogens.

Materials and Methods

Animals. c-FLIP$_L^{-/-}$ mice were generated by crossing c-FLIP$_R$ bacterial artificial chromosome (BAC) transgenic mice[18] to c-FLIP$^{f/f}$ Lck-cre$^+$ mice.[17] c-FLIP$^{f/f}$ littermates were used as wild-type controls. The animals were genotyped as previously described and used at 6–8 weeks of age. Animal usage was conducted according to the protocols approved by the Duke University Institutional Animal Care and Use Committee.

Cell culture. Single-cell suspensions were prepared from spleens or peripheral lymph nodes after red blood cell lysis. T lymphocytes were enriched using an EasySep mouse T cell negative enrichment kit from StemCell Technologies (Vancouver, BC, Canada) according to the manufacturer's instructions. Total splenocytes or T lymphocytes were cultured in complete RPMI-1640 medium containing 10% FBS at 37 °C in the presence of 5% CO_2 for the indicated periods of time. For TCR stimulation, total splenocytes were stimulated with soluble anti-CD3 (2C11, 5 μg/ml, unless otherwise indicated in the figure legends). Anti-CD28 (clone 37.51, 2 μg/ml; BioLegend, San Deigo, CA, USA) was added to the cultures as indicated in the figure legends. Enriched T lymphocytes were activated with plate-bound anti-CD3 and anti-CD28. Some lymphocyte cultures were treated with 10 μM zVAD (Sigma, St Louis, MO, USA), 10 μM zIETD (BD Pharmingen, San Diego, CA, USA), 2 μg/ml anti-FasL (2MFL3, BioLegend), 100 nM Nec-1 (Enzo Life Science, Farmingdale, NY, USA) and 10 mM NAC as indicated in the figure legends.

Flow cytometry analysis. Single-cell suspensions were prepared from spleens and lymph nodes after red blood cell lysis, or from T lymphocytes that were cultured for the indicated periods of time. The cells were incubated with an FcR-blocking antibody (2.4G2), stained with FITC-, PE-, PE/Cy5-, APC-, APC-Cy7-, or Pacific Blue-labeled mAbs on ice for 20 min, and washed with FACS buffer (2% FBS, 0.02% NaN_3 in PBS). A total of 0.5–20 $\times$ 10^5 events were collected on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). All fluorescence-labeled Abs, including anti-CD3, anti-CD4, anti-CD8, anti-TCR-β, anti-CD19, anti-FasL, and anti-Fas, were obtained from BioLegend. To stain cell surface FasL, the cells were cultured with 1 $\times$ protease inhibitor (Aprotinin; Sigma) for 4 h immediately before staining.

Cell death assays. For Annexin V staining, the cells were first stained for surface markers following the methods described above. The cells were then incubated with Annexin V-PE (BD Biosciences) and 7-AAD (BD Biosciences) in Annexin V-binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) for 15 min at room temperature. The cells were then diluted in Annexin V-binding buffer and analyzed within 1 h. For mitochondrial outer membrane permeabilization (MOMP) staining, the cells were incubated with 40 nM TMRE at 37 °C for 20 min and then stained with surface marker and 7-AAD (Invitrogen, Grand Island, NY, USA) in FACS buffer on ice for 15 min. The cells were washed with FACS buffer, incubated on ice, and analyzed immediately.

Cell proliferation assays. CFSE-labeled (Molecular Probes, Grand Island, NY, USA) splenocytes were stimulated with anti-CD3 (2C11, 5 μg/ml) in the presence or absence of anti-CD28 (clone 37.51; BioLegend, 2 μg/ml) for 3 days. Proliferation was assessed by measuring CFSE dilution in 7-AAD$^-$ CD4$^+$ or CD8$^+$ T cells by flow cytometry.

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

Western blot. For activated T cells, total splenocytes were cultured for 16–18 h with anti-CD3 (2C11, 5 μg/ml) before purification. T cells were purified using an EasySep mouse T cell enrichment kit (StemCell Technologies) according to the manufacturer's instructions. Cell purity was determined by flow cytometry to be $>$95%. Purified T-cell lysates were prepared in sample buffer (50 mM Tris-Cl (pH 6.8), 50 mM 2-ME, 2% SDS, 0.2% bromophenol blue, and 10% glycerol). Antibodies used for western blots were anti-c-FLIP (clone Dave-2; Alexis Biochemical, Farmingdale, NY, USA), anti-full length caspase 8 (polyclonal; Cell Signaling), anti-active caspase 8 (polyclonal; Cell Signaling, Danvers, MA, USA), anti-RIP1 (38/RIP; BD Bioscience), anti-RIP3 (polyclonal, Axxora, Farmingdale, NY, USA), anti-LC3A/B (polyclonal; MBL International, Woburn, MA, USA), anti-α-tubulin (B-5-1-2; Sigma), and anti-β-Actin (polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

RT-PCR. Total RNA was extracted from sorted freshly isolated T cells and T cells cultured for 16–18 h with anti-CD3. The primers for RIP-1 were as follows: forward: 5′-GGAAGGATAATCGTGGAGGC-3′ (exon 4); reverse: 5′-AAGGAAGCC ACACCAAGATC-3′ (exon 5).

Fluorescent microscopy. Primary T lymphocytes were enriched using an EasySep mouse T cell enrichment kit (StemCell Technologies) according to the manufacturer's instructions. The cells were first stained with Cy3-, FITC-, and Pacific Blue-labeled anti-rabbit IgG, anti-CD4, and anti-CD8 and then stained with anti-LC3 (P015, MBL, Woburn, MA, USA). All images were captured with a custom-built Zeiss Observer D1 using a Zeiss 3100 objective lens (Zeiss, Thornwood, NY, USA) and a 1.4 numerical aperture. Images were captured using a Photometrics Cool SNAP HQ2 (Tucsan, AZ, USA) and analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA, USA). The images were blindly deconvoluted (40 iterations). Positive staining for LC3 was defined as $>$180% of background signal. LC3 puncta were defined as any enclosed LC3-positive area no smaller than 10 pixels.

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

Fragmented DNA release assay. Four-week-old mice were injected i.p. with 1–1.5 mg BrdU per animal per day for 2 weeks. Over 90% of the peripheral T lymphocytes were labeled with BrdU by the end of the injection period. The kinetics of fragmented DNA release were analyzed using a Cellular DNA Fragmentation ELISA kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instruction.

***In vitro* inducible deletion.** Splenocytes were cultured with 200 nM 4-OHT (Sigma-Aldrich, St Louis, MO, USA) for 3 days in the presence of IL-7 (1 ng/ml; PeproTech, Rocky Hill, NJ, USA). Live cells were purified after deletion using Ficoll (Sigma).

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

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)