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c-FLIP Protects T Lymphocytes from Apoptosis in the Intrinsic Pathway

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Apoptosis can be induced by either death receptors on the plasma membrane (extrinsic pathway) or the damage of the genome and/or cellular organelles (intrinsic pathway). Previous studies suggest that cellular caspase 8 (FLICE)-like inhibitory protein (c-FLIP) promotes cell survival in death receptor–induced apoptosis pathway in T lymphocytes. Independent of death receptor signaling, mitochondria sense apoptotic stimuli and mediate the activation of effector caspases. Whether c-FLIP regulates mitochondrion-dependent apoptotic signals remains unknown. In this study, c-FLIP gene was deleted in mature T lymphocytes in vitro, and the role of c-FLIP protein in intrinsic apoptosis pathway was studied. In resting T cells treated with the intrinsic apoptosis inducer, c-FLIP suppressed cytochrome c release from mitochondria. Bim-deletion rescued the enhanced apoptosis in c-FLIP-deficient T cells, whereas inhibition of caspase 8 did not. Different from activated T cells, there was no necroptosis or increase in reactive oxygen species in c-FLIP-deficient resting T cells. These data suggest that c-FLIP is a negative regulator of intrinsic apoptosis pathway in T lymphocytes.

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The online version of this article contains supplemental material.

Materials and Methods

Animals

c-FLIP<sup>−/−</sup> mice were generated previously (17). c-FLIP conditional knockout mice were generated by crossing c-FLIP<sup>−/−</sup> to Er-Cre<sup>+</sup> mice (18). Mice deficient for Bim, Bak, and/or Bak were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were genotyped as described previously (17, 19) and used at 6–8 wk of age. Animal usage was conducted according to protocols approved by the Duke University Institutional Animal Care and Use Committee.
In vitro deletion and cell culture

Single-cell suspensions prepared from spleens and peripheral lymph nodes are resuspended in ACK lysis buffer (0.15 M NH4Cl, 10 mM KHCO3, 0.1 mM EDTA pH 7.4) for up to 3 min for RBC lysis. For induced depletion, total splenocytes were cultured in complete RPMI 1640 medium containing 10% FBS, 200 nM 4-hydroxytamoxifen (4-OHT; Sigma-Aldrich) and 1 nM/ml IL-7 (PeproTech) at 37°C in the presence of 5% CO2 for 3 d. Live cells were purified after deletion using Ficoll. T lymphocytes were enriched using an EasySep mouse T cell–negative enrichment kit from Stemcell Technologies according to the manufacturer’s instructions. T lymphocytes were cultured in complete RPMI 1640 medium containing 10% FBS at 37°C in the presence of 5% CO2 for indicated time. A total of 10 μM zVAD-fmk (Sigma-Aldrich), 10 μM zETD-fmk (BD Pharmaningen), 10 μM zLEHD-fmk (BD Pharmaningen), 100 nM necrostatin-1 (Nec-1; Enzo Life Sciences), and 10 mM acetylcysteine (NAC) were added in lymphocyte cultures as indicated.

Cell death analysis

T lymphocytes were incubated with an FcR-blocking Ab (2.4G2), stained with FITC–, PE–, PE/Cy5–, allophycocyanin–, allophycocyanin-Cy7–, or Pacific blue–labeled mAbs on ice for 20 min, and washed with FACS buffer (2% FBS, 0.02% NaN3 in PBS). Then cells were resuspended in Annexin V–binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) and incubated with Annexin V–PE (BD Biosciences) and 7-AAD and then washed with FACS buffer and analyzed by FACS immediately. All fluorescence-labeled Abs, including anti-CD3, anti-CD4, anti-CD8, and anti-CD19 were obtained from Biolegend.

Cytochrome c release

Cytochrome c release was tested based on previously published protocol (20). In brief, T lymphocytes were enriched using an EasySep mouse T cell enrichment kit from StemCell Technologies according to the manufacturer’s instructions. Cell purity was determined by flow cytometry to be >95%. Cytosol was released by 200 mg/ml digitonin in 80 mM KCl buffer. The mitochondria/nuclear fraction was lysed in cell lysis buffer (50 mM Tris-Cl [pH 6.8], 50 mM 2-ME, 2% SDS, 0.2% bromophenol blue, and 10% glycerol). Abs used for Western blots were achieved with SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). For fluorescent Western blot, the secondary Abs were anti-rabbit IgG-HRP, anti-mouse IgG-HRP, anti-hamster IgG-HRP, and anti-goat IgG-HRP (Jackson Immunoresearch).

Western blot

T cells with a >95% purity were purified using an EasySep mouse T cell enrichment kit (Stemcell Technologies). 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). Abs were used for Western blots were rabbit anti–LC3 (polyclonal; MBL International), hamster anti–Bcl-2 (polyclonal; BD Pharmaningen), rabbit anti–Bcl-xL (polyclonal; Novus), rabbit anti–c-Flipf/f (polyclonal; Stemcell Technologies), rabbit anti–c-Flipf/f (polyclonal; Sigma-Aldrich), mouse anti–cytochrome c (7H8.2C12; Biolegend), mouse anti–c-FLIP (7H8.2C12; Biolegend), mouse anti–α-Tubulin (B-5-1-2; Sigma-Aldrich), and goat anti–β-actin (polyclonal, Santa Cruz Biotech). For HRP-labeled Western blot, the secondary Abs were anti-rabbit IgG–HRP, anti-mouse IgG–HRP, anti-hamster IgG–HRP, and anti-goat IgG–HRP (Jackson Immunoresearch). The development of the Western blot was achieved with SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific). For fluorescent Western blot, the secondary Abs were anti-rabbit IgG–Alexa Fluor 680, anti-mouse IgG–Alexa Fluor 680, and anti-goat IgG–Alexa Fluor 790 (Molecular Probes, Invitrogen).

LC3 fluorescence microscopy

Enriched primary T lymphocytes were first intracellularly stained with anti–LC3 (P015; MBL International), then stained with Cy3–, FITC– and Pacific blue–labeled, anti-rabbit IgG, anti-CD4, and anti-CD8. All images were captured with a custom-built Zeiss Observer D1 using a Zeiss 3100 objective lens and a 1.4 numerical aperture. Images were captured using a Photometrics Cool SNAP HQ2 and analyzed using MetaMorph software. Images were deconvoluted and thresholded. Deconvolution was done blind at 40 iterations. LC3+ staining is defined as >180% of background signal. LC3 puncta is defined as any enclosed LC3+ staining area <10 pixels.

Reactive oxygen species analysis

Single-cell suspensions were incubated with 2.5 mM dihydroethidium (DHE; Sigma-Aldrich) in RPMI 1640 medium at 37°C for 30 min or with 2.5 mM CM-H2DCFDA (Invitrogen) in Dulbecco’s PBS at 37°C for 15 min. The cells were then washed with RPMI 1640 medium; stained with anti–CD4-allophycocyanin, anti–CD8-allophycocyanin/Cy7, and 7-AAD; and then washed with FACS buffer and analyzed by FACS immediately.

Statistics

Unpaired two-tailed Student t tests were used to compare the means of different samples.

FIGURE 1. Impaired survival of c-FLIP–deficient T lymphocytes upon STS treatment. (A) The rate of live cells in c-FLIP–deficient T cells upon intrinsic apoptosis induction. Lymphocytes pooled from spleen and lymph nodes from c-FLIP−/− and c-FLIP−/− ER-Cre+ mice were cultured for 3 d with 4-OHT for in vitro deletion. Live cells were enriched with Ficoll and then cultured in the presence or absence of STS or ETP for 16 h. Cell death was measured by Annexin V/7-AAD staining before and after 16 h treatment. (B) Statistics of (A). Live cells were analyzed by gating on the Annexin V−/7-AAD− population within the total CD4+ or CD8+ populations (n = 6). All error bars represent the SEM. *p < 0.05, **p < 0.01, ***p < 0.001, n.s., nonsignificant.
Results

Impaired survival of c-FLIP–deficient T lymphocytes upon intrinsic apoptosis induction

Conditional deletion of c-FLIP in the thymocytes leads to impaired survival and severe periphery T cell reduction (17). The low cell number of c-FLIP–deficient T cells, along with the accumulative effect of the deletion, makes this model unsuitable for studying the role of c-FLIP in mature T lymphocytes. Therefore, we crossed c-Flipf/f mice with ER-Cre+ mice to generate inducible c-FLIP knockout mice. Because in vivo deletion of c-FLIP leads to lethality of the animals caused by liver failure (21, 22), c-FLIP was deleted in vitro in T lymphocytes by 4-OHT (4). In brief, T cells were cultured in the presence of 0.2 μM 4-OHT and 1 ng/ml IL-7 for 72 h. This culture generally maintained the viability of unstimulated T cells, and no difference in the survival rates was observed between c-FLIPf/f and c-FLIPf/f ER-Cre+ T cells during the culture (Supplemental Fig. 1). After deletion, live cells were purified and the cell viability was confirmed by flow cytometry–based apoptosis assay (Fig. 1A, 1B). Although short-term IL-7 stimulation downregulated surface IL-7Rα, T cells restored their IL-7Rα expression on the cell membrane at the end of 3-d culture (Supplemental Fig. 2A). After in vitro–induced deletion, IL-7 stimulation effectively induced phosphorylation of Stat5 in purified wild type (WT) and c-FLIP–deficient T cells (Supplemental Fig. 2B). These data demonstrate that in vitro–induced deletion system did not alter T cell viability or IL-7R–mediated cell signaling.

After the 3-d induced deletion, live cells were enriched by Ficoll and cultured in the presence of 1 ng/ml IL-7. More than 95% of T cells were Annexin V−7-AAD− after the enrichment (Fig. 1B). Apoptosis was induced in the cultured cells by the addition of staurosporine (STS) or etoposide (ETP). STS inhibits Protein kinase C and induces caspase 3–dependent apoptosis (23). ETP causes irreversible double-strand or single-strand breaks in DNA (24). Cell death was analyzed by Annexin V/7-AAD staining in CD4+ and CD8+ T cells 16 h after the addition of STS or ETP. As previously reported (4), c-FLIP–deficient T lymphocytes showed increased spontaneous death (Fig. 1A). The survival of c-FLIP–deficient T cells was dramatically impaired compared with that of WT T cells when treated with STS (Fig. 1B). In contrast, genomic toxin ETP did not trigger more cell death in c-FLIP–deficient T cells than in WT T cells (Fig. 1B).

c-FLIPL has been shown to regulate necroptosis in activated T lymphocytes (16). Therefore, we first determined whether STS treatment induces necroptosis in c-FLIP–deficient T cells. The induction of apoptosis involves the activation of a series of caspases, which can be inhibited by pan-caspase inhibitor z-VAD-fmk. Necroptosis induction requires the activity of RIP-1 kinase, which can be repressed by Nec-1 (25). The impaired survival of c-FLIP−/− T cells was partially rescued by z-VAD-fmk, but not by Nec-1 (Fig. 2A). Addition of Nec-1 to z-VAD-fmk–treated T cells did not further increase cell survival. Another biochemical marker of apoptosis is the caspase-dependent cleavage of PARP-1.
FIGURE 3. Role of c-FLIP in the intrinsic apoptosis pathway in T lymphocytes. (A) Effect of z-VAD-fmk, z-LEHD-fmk, and z-IETD-fmk on the cell death of c-FLIP$^{-/-}$ T cells. Inducible deletion was conducted as in Fig. 1. Live T cells were then cultured in the presence or absence of STS, z-IETD-fmk, z-LEHD-fmk, or z-VAD-fmk for 16 h. Live cells were analyzed by gating on the Annexin V$^+$ 7-AAD$^+$ population within the total CD4$^+$ or CD8$^+$ populations ($n = 4$). All error bars represent the SEM. *$p < 0.05$. (B) Cytochrome $c$ release upon STS treatment. Live T cells were enriched by Ficoll after in vitro deletion of c-FLIP (viability > 95%) and then cultured in the presence of STS for indicated time. Cytosol was separated by digitonin solution as described in Materials and Methods. The level of cytochrome $c$ was quantified and normalized to the expression of COX IV and GAPDH, in the mitochondria and cytosol fraction, respectively.

Protective role of c-FLIP in the intrinsic apoptosis pathway in T lymphocytes

Caspase 1 and caspase 3 activation are involved in the apoptosis induction by STS (27, 28). We used inhibitors of different caspases to test which apoptotic pathway is activated. Extrinsic pathway of apoptosis is triggered by death receptor ligation, followed by activation of caspases 8. Intrinsic pathway is characterized by cytochrome $c$ release from mitochondria to cytosol and the activation of caspases 9 by apoptosome (29). The impaired survival of c-FLIP-deficient T lymphocytes was partially rescued by both caspase 9 inhibitor z-LEHD-fmk and pan-caspase inhibitor z-VAD-fmk (Fig. 3A). Caspase 9 inhibitor z-LEHD-fmk showed better rescue, especially in CD4$^+$ T cells. Caspase 8 inhibitor z-IETD-fmk, however, failed to rescue the enhanced apoptosis in c-FLIP-deficient T lymphocytes (Fig. 3A). We did not detect active form of caspase 8 in either group of T cells (data not shown). The requirement of caspases 9 in STS-induced apoptosis suggested the involvement of the intrinsic apoptotic pathway in c-FLIP-deficient T cells. We measured cytochrome $c$ release from mitochondria to cytosol in both WT and c-FLIP-deficient T cells, isolating cytosolic fraction by digitonin (20). The kinetic study showed that cytochrome $c$ release was faster in c-FLIP-deficient T cells than in WT controls (Fig. 3B). These data indicate that c-FLIP protects T cells from the intrinsic pathway-induced apoptosis.

c-FLIP suppresses Bim-dependent apoptosis in T lymphocytes

To further establish the role of c-FLIP in protecting T cells from the intrinsic apoptotic pathway, we determined whether deletion of Bcl-2 family proapoptotic proteins rescues their impaired survival upon STS treatment. c-Flip$^{f/f}$ ER-Cre$^+$ mice were crossed with Bak$^{-/-}$, Bax$^{-/-}$, and Bim$^{-/-}$ mice to generate double knockouts. During apoptosis induction in the intrinsic pathway, Bak and Bax form oligomeric complex in the outer membrane of the mitochondria to mediate the release of cytochrome $c$ (30, 31). Bak and Bax compensate for each other in the process of mitochondrial outer membrane permeabilization. Bak$^{-/-}$/Bax$^{-/-}$ cells show resistance to multiple death stimuli that mediates apoptosis through the intrinsic pathway, whereas Bax- and Bak-deficient cells are susceptible to them (32). Deletion of Bak or Bax did not rescue the apoptotic phenotype of c-FLIP$^{-/-}$ T cells (Fig. 4A, 4B). Proapoptotic protein Bim responds to several stimuli and regulates the homeostasis of hematopoietic cells (33). Loss of Bim increased the survival of c-FLIP$^{-/-}$ CD4$^+$ T cells and fully rescued the impaired survival in c-FLIP$^{-/-}$ CD8$^+$ T cells after STS treatment (Fig. 4C). These results suggest that c-FLIP protects STS-induced apoptosis in T cells in a Bim-dependent pathway.

We next examined the expression level of Bcl-2 family member in c-FLIP-deficient T cells. The expression levels of proapoptotic protein Bcl-2, Mcl-1, and Bcl-xL were comparable between WT and c-FLIP$^{-/-}$ T cells (Fig. 4D). After STS treatment, Bcl-2 and Mcl-1 were downregulated in WT cells, whereas the expression of Bcl-xL was increased (Fig. 4D). c-FLIP-deficient T cells failed to upregulate Bcl-xL but retained the expression of Mcl-1. Previous data showed that c-FLIP interacts with Bcl-xL (34). How this interaction regulates the function of Bcl-xL remains to be studied. Interestingly, the surviving
WT and c-FLIP–deficient T cell after apoptosis induction showed decreased levels of Bax and Bim, which may be the consequence that cells expressing a low level of proapoptotic proteins preferentially survived. These data suggest the cross talk between c-FLIP and Bcl-2 family members in the regulation of apoptosis in T lymphocytes.

Autophagy is not involved in the enhanced apoptosis in c-FLIP–deficient T lymphocytes upon intrinsic apoptosis induction

Because c-FLIP, modulates autophagy in T cells upon TCR stimulation (16), we tested whether c-FLIP–modulated autophagy in T lymphocytes was involved in the impaired survival in c-FLIP–deficient T cells upon intrinsic apoptosis induction. The conversion of LC3-I to LC3-II isoform and the consequent insertion of LC3-II in the isolation membrane are essential for autophagosome formation. Monitoring LC3 puncta formation inside the T cells is used in autophagy detection (35). We first determined the level of LC3-II in c-FLIP–deficient and WT T cells by Western blot. LC3-II level was very low in both WT and c-FLIP–deficient T cells in the absence of apoptosis stimuli. After STS treatment, LC3-II was increased, especially in c-FLIP–deficient T cells (Fig. 5A). We then intracellularly stained c-FLIP–deficient T cells and WT T cells with anti-LC3 to examine the LC3 puncta for autophagosome quantitation. The number of LC3+ puncta in c-FLIP−/− T cells was not significantly higher than that in WT T cells (Fig. 5B). However, c-FLIP–deficient T cells showed larger LC3 puncta and stronger fluorescent intensity, indicating larger autophagosome formed or higher LC3 protein level in the absence of c-FLIP (Fig. 5B). We further investigated whether blocking autophagy altered the cell survival in c-FLIP−/− T lymphocytes upon STS treatment. Loss of autophagy-related gene Atg3 in T lymphocyte leads to inhibition of autophagy (11). Short-term deletion of Atg3 does not cause increased apoptosis in resting T cells (16). Therefore, c-FLIP−/− ER-Cre mice were crossed to Atg3−/− mice to generate Atg3−/− c-FLIP−/− mice. Atg3−/− mice were used as controls. Inducible deletion of c-FLIP and Atg3 was conducted in vitro by 4-OHT treatment as previously described (16). Deletion of Atg3 in c-FLIP–deficient T cells that were treated with STS had no effect on their cell survival after STS treatment (Fig. 5C). These data suggest that autophagy is not the cause of the enhanced apoptosis of c-FLIP–deficient T lymphocytes after STS treatment.
Role of reactive oxygen species in the enhanced apoptosis in c-FLIP–deficient T lymphocytes upon intrinsic apoptosis induction

STS has been shown to rapidly increase intracellular reactive oxygen species (ROS) in HeLa cells, and the high level of ROS contributes to caspase 3 activation and apoptosis induction (23). Notably, the long isoform of c-FLIP has been shown to promote cell survival by controlling ROS in activated T lymphocytes (16). Therefore, it was important to test whether ROS caused the increased apoptosis in STS-treated c-FLIP–deficient T lymphocytes. We examined the intracellular ROS by analyzing DHE and DCFDA staining using flow cytometry and found that loss of c-FLIP did not alter ROS production in T cells, in the presence or absence of STS (Fig. 6A, 6B). Addition of antioxidant NAC did not alter ROS production in c-FLIP–deficient T cells. In this report, we show that c-FLIP protects T cells against intrinsic apoptosis induction in an ROS-independent pathway.

Discussion

Although the role of c-FLIP in the extrinsic apoptosis pathway in T cells has been well established, it is not clear whether c-FLIP also plays a critical role in the intrinsic apoptosis pathway in T cells. In this report, we show that c-FLIP–deficient T cells have impaired cell survival upon intrinsic apoptosis induction by STS. The enhanced apoptosis in c-FLIP–deficient T cells upon STS treatment is likely caused by a more rapid cytochrome c release and caspase 9 activation. The autonomous activation of procaspase 8 was not the cause of the apoptotic phenotype in resting c-FLIP–deficient T lymphocytes upon STS treatment. Furthermore, deletion of Bim significantly improved the survival of c-FLIP–deficient T cells in the presence of STS. Although autophagy is enhanced in c-FLIP–deficient T cells, inhibition of autophagy fails to rescue their impaired survival upon intrinsic apoptosis induction.

Interestingly, c-FLIP–deficient T cells were susceptible to STS-induced, but not ETP-induced, apoptosis. Although both drugs are widely used as intrinsic apoptosis inducers, they provoke apoptosis in distinct manners. ETP induces apoptosis through generating double-strand or single-strand break in the genomic DNA (24), whereas STS inhibits Protein kinase C activity (25). ETP fails to rescue their impaired survival upon intrinsic apoptosis induction.

The enhanced intrinsic pathway-mediated apoptosis in resting c-FLIP–deficient T cells after STS treatment is not due to the secondary effect of spontaneous caspase 8 activation. In the Fas-induced extrinsic apoptosis pathway, active caspase 8 cleaves Bid, a BH3-only proapoptotic protein, to generate the truncated form of Bid (tBid). tBid efficiently inserts into mitochondrial outer membrane and augments cytochrome c release (39). It has been shown that c-FLIP–deficient T cells have higher caspase 8 activity.
deficient T lymphocytes. Antioxidant NAC slightly enhanced the survival of all STS-treated T cells. Thus, the minimum cytoprotective effect of NAC was not specific to c-FLIP–deficient T cells. Furthermore, the loss of c-FLIP (both isoforms) did not lead to an increase of ROS production in resting T cells. In contrast, the loss of c-FLIPp120 leads to increased ROS production and ROS-dependent cell death in activated T cells (16). These results suggest that c-FLIP proteins function differently in resting and activated T lymphocytes.

Necroptosis was not detected in resting c-FLIP−/− T cells. This result was consistent with previous publications showing necroptosis induction only in activated T lymphocytes (13, 15, 42, 43). Why activated T cells are susceptible to necroptosis has yet to be investigated. RIP-1 kinase plays an important role in T lymphocytes for proper TCR signaling. RIP-1–deficient T cells show impaired TCR-induced phosphorylation of p65 NF-κB (44). Activated T cells may upregulate RIP-1 for the benefit of cell signaling, whereas loss of adequate inhibition in RIP-1’s kinase activity ends up killing the cells.

Together, we show that c-FLIP regulates apoptosis signaling in the Bim-dependent intrinsic pathway. The mechanism of the cross talk of c-FLIP and Bim remains to be determined in future investigation. Our results suggest that c-FLIP protects T lymphocyte survival through multiple pathways.

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Disclosures

The authors have no financial conflicts of interest.

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