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The Long Isoform of Cellular FLIP Is Essential for T Lymphocyte Proliferation through an NF-κB-Independent Pathway

Nu Zhang, Kaycie Hopkins, and You-Wen He

Although the long isoform of cellular FLIP (c-FLIP_L) has been implicated in TCR-mediated signaling, its role in T cell proliferation remains controversial. Some studies have demonstrated that overexpression of c-FLIP_L promotes T cell proliferation and NF-κB activation, whereas others have reported that c-FLIP_L overexpression has no effect or even inhibits T cell proliferation. To establish the role of c-FLIP_L in T lymphocyte proliferation, we have generated a conditional knockout mouse strain specifically lacking c-FLIP_L in T lymphocytes. c-FLIP_L−/− mice exhibit severely impaired effector T cell development after Listeria monocytogenes infection in vivo and c-FLIP_L-deficient T cells display defective TCR-mediated proliferation in vitro. However, c-FLIP_L−/− T cells exhibit normal NF-κB activity upon TCR stimulation. These results demonstrate that c-FLIP_L is essential for T lymphocyte proliferation through an NF-κB-independent pathway. The Journal of Immunology, 2008, 180: 5506–5511.

Materials and Methods

Generation of c-FLIP_L bacterial artificial chromosome (BAC) tg mice

A c-FLIP wild-type (WT) BAC clone was modified using a system developed by Eberl G. et al. (18). In short, c-FLIP_L cDNA, including its own stop codon and poly(A) signal, was fused to the start codon in exon 1 of c-FLIP gene (Fig. 1A). A 60 kb BAC fragment that is 15 kb downstream of the nearest 3′ gene (Als2c12) and 10 kb upstream of the nearest 3′ gene (caspase-8) was used for injection. Three founders were obtained for each line and crossed to c-FLIP_L-Lck-cre mice (5), and all mice displayed a similar phenotype. Animal usage was conducted according to protocols approved by the Duke University Institutional Animal Care and Use Committee.

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PCR analysis

PCR to assess the presence of the c-FLIP\textsubscript{L} BAC transgene was performed with 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 120 s. The primers are: forward, 5'-CAAGTGGAGGAGTGGCATG-3'; and reverse, 5'-TCAGAGACCTCTTAATACG-3'.

Bacterial infection, intracellular IFN-γ staining, and CTL assay

The recombinant Listeria monocytogenes strain engineered to secrete chicken OVA and pMHC/peptide tetramers were kindly provided by M. Bevan (University of Washington, Seattle, WA) (19). The recombinant Listeria monocytogenes strain engineered to secrete chicken OVA was grown in brain-heart infusion broth supplemented with 5 μg/ml erythromycin. Bacteria were diluted in PBS and injected i.v. at a dose of 2 × 10\textsuperscript{3} CFU for primary infection. To examine IFN-γ producing T cells, total splenocytes were incubated with 10\textsuperscript{-7} M OVA\textsubscript{256–264} peptide in the presence of 3 μM monensin for 6 h. Alternatively, total splenocytes were incubated with Listeria-infected bone marrow macrophages for 20 h with the last 6 h in the presence of monensin. After ex vivo stimulation, splenocytes were surface stained, fixed, permeabilized with 0.1% saponin, and stained for IFN-γ. To examine the CTL activity of infected mice, splenocytes were surface stained, fixed, and stained with FITC-, PE/Cy5-, or allophycocyanin-labeled mAb or biotinylated mAb followed by PE-streptavidin, and washed with FBS containing 2% FCS. A total of 1–5 × 10\textsuperscript{5} events were collected on a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software. All fluorescence-labeled Abs, including anti-CD3, -CD4, -CD8, -CD24, -CD25, -CD69, -CD44, -CD62L, -TCRβ, -Qa-2, -IL-4, and -IFN-γ, were obtained from eBioscience, BioLegend, or BD Biosciences. Apoptotic cells were defined by Annexin V and 7-AAD staining using an Annexin V-PE kit (BD Biosciences).

Flow cytometric analysis

Single-cell suspensions of the thymus, spleen, and lymph nodes were incubated with an FcR blocker (2.4G2; eBioscience) after RBC lysis stained on ice for 30 min with FITC-, PE/Cy5-, or allophycocyanin-labeled mAb or biotinylated mAb followed by PE-streptavidin, and washed with PBS containing 2% FCS. A FACS analysis of CD4\textsuperscript{+} T cells was performed using a FACS-Scan flow cytometer (BD Biosciences) and analyzed using CellQuest software. All fluorescence-labeled Abs, including anti-CD3, -CD4, -CD8, -CD24, -CD25, -CD69, -CD44, -CD62L, -TCRβ, -Qa-2, -IL-4, and -IFN-γ, were obtained from eBioscience, BioLegend, or BD Biosciences. Apoptotic cells were defined by Annexin V and 7-AAD staining using an Annexin V-PE kit (BD Biosciences).

Lymphocyte activation and Western blot assays

Peripheral T cells were purified from the spleen and lymph nodes using an EasyStep mouse T cell enrichment kit from StemCell Technologies according to the manufacturer's instructions. T cells were incubated with 10 μg/ml anti-CD3 (2C11) on ice for 30 min, washed with ice-cold RPMI 1640 containing 10% FBS, and cross-linked with 75 μg/ml rabbit anti-hamster IgG (Sigma-Aldrich) at 37°C for the amount of time indicated in the figures. Total cell lysates were generated after TCR stimulation and subjected to Western blot analysis. Abs used for Western blots were anti-c-FLIP (clone D2-2; Alexis Biochemical), anti-γ-tubulin, and -ErbK (Santa Cruz Biotechnology), and anti-pJNK, -pp38, -p-I-kBα, and -IκBα (Cell Signaling Technology).

EMSAs

A total of 10\textsuperscript{7} purified T cells were incubated in complete medium or stimulated with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 for 16 h. Nuclear extracts were prepared using a nuclear extraction kit from Active Motive according to the manufacturer's instructions. A total of 10 μl of each extract was subjected to bandshift analysis as described (20). In short, EMASAs were performed using the following oligonucleotides and binding buffers: AP-1 oligonucleotide, 5′-CGCTGTAGGACTCGAGCCGAA-3′; AP-1 5X binding buffer, 50 mM Tris-HCl (pH 7.5), 500 mM KCl, 2.5 mM MgCl\textsubscript{2}, 0.5 mM EDTA, 50% glycerol, 250 μg/ml poly(dI:dC), 5 mM DTT, and 1 mg/ml BSA; NF-κB oligonucleotide, 5′-ACCAAGAGGAGGATCTCAATAAC-3′; and NF-κB 5X binding buffer, 25 mM Tris-HCl (pH 7.5), 5 mM EDTA, 250 μg/ml poly(dI:dC), 5 μM DTT, and 1 mg/ml BSA. A total of 2 × 10\textsuperscript{4} cpm of labeled probe was used in each reaction, and bandshifts were resolved on 5% polyacrylamide gels in 1× TBE running buffer.

Cell proliferation assays

CFSE-labeled (Molecular Probes) splenocytes were stimulated with plate-bound anti-CD3 (2C11; eBioscience) and/or anti-CD28 (clone 37.51; BioLegend) for 3 days, and proliferation was assessed by measuring CFSE dilution in CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells by FACS analysis.

Semiquantitative RT-PCR analysis

A total of 5 × 10\textsuperscript{6} purified peripheral T cells were stimulated with 10 μg/ml plate-bound anti-CD3 Ab for 3 h. Total RNA was extracted and subjected to RT-PCR analysis. The PCR reaction used to measure 16S rRNA expression consisted of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The primers are: forward, 5′-AGGATGACTCTGGCTTTATGTA-3′; and reverse, 5′-GCCACTTTTCAATTTGTCAGA-3′.

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**FIGURE 2.** Impaired development of effector CD8\(^+\) T cells in c-FLIP\(_L^{-/-}\) mice. A and B, FACS analysis of IFN-γ-producing Ag-specific CD8\(^+\) effector T cells in c-FLIP\(_L^{-/-}\) mice. Seven days after *Listeria* infection, total splenocytes were incubated with Ova257–264 for 6 h (A) or with *Listeria*-infected bone marrow-derived macrophages for 20 h (B) and stained with anti-IFN-γ and anti-CD8. Shown are frequencies of Ag-specific CD8\(^+\) T cells. C, FACS profile of splenic CD8\(^+\) T cells before and after *Listeria* infection. Numbers represent frequency of CD44\(^{high}\)CD62L\(^{low}\) activated/memory T cells. D, Lack of ex vivo CTL activity in c-FLIP\(_L^{-/-}\) mice. Data are representative of killing activity from seven individual mice. E, Number of IFN-γ-producing cells in c-FLIP\(^{−/−}\), c-FLIP\(_L^{-/-}\), and c-FLIP\(^{−/−}\)c-FLIP\(_L^{−/−}\) BAC tg mice. Mice were infected with *Listeria* and the number of Ova-specific CD8\(^+\) T cells was determined by FACS as in A.

**Results**

**Generation of c-FLIP\(_L\) conditional knockout mice**

Conventional c-FLIP-deficient mice die during embryogenesis (21). To examine the role of c-FLIP in T lymphocytes, we generated mice conditionally lacking both c-FLIP\(_L\) and c-FLIP\(_S\) isoforms in T lymphocytes (c-FLIP\(^{−/−}\)Lck-cre, referred to here as c-FLIP\(_L^{-/-}\)) and demonstrated that loss of c-FLIP expression results in a severe defect in the mature T cell compartment due to massive apoptosis at the SP thymocyte stage (5). This lack of mature T cells in c-FLIP\(_L^{-/-}\) mice prevented further analysis of c-FLIP function in mature T cell proliferation and signaling.

As both c-FLIP\(_L\) and c-FLIP\(_S\) are expressed in thymocytes and the major function of c-FLIPS is to inhibit apoptosis (1), we reasoned that conditional deletion of c-FLIP\(_L\) but not c-FLIP\(_S\) in thymocytes would allow development of mature T cells. To achieve this, we have generated a BAC tg mouse line expressing c-FLIP\(_S\) (Fig. 1A) and crossed this tg line to the c-FLIP\(_L^{-/-}\) line. The resulting c-FLIP\(^{−/−}\)c-FLIP\(_S\) BAC tg mice lack the expression of c-FLIPS in T lymphocytes but express c-FLIP\(_S\) under the control of endogenous regulatory elements and, therefore, are referred to as c-FLIP\(_L^{-/-}\) mice. As expected, c-FLIP\(_L\) was only detected in WT thymocytes, while c-FLIP\(_S\) was detected in both WT and c-FLIP\(_L^{-/-}\) thymocytes (Fig. 1B). As a control, neither isoform was detected in c-FLIP\(_L^{-/-}\)tg thymocytes.

Expression of the c-FLIP\(_S\) BAC tg rescued mature T cell development in c-FLIP\(_L^{-/-}\) mice, as indicated by the comparable frequency of mature HSA\(^{low}\)/TCR\(^{hi}\) CD8\(^+\) SP and HSA\(^{low}\)/Qa-2\(^+\)/CD4\(^+\) SP thymocytes in c-FLIP\(_L^{-/-}\) and c-FLIP\(_L^{-/-}\)control mice (Fig. 1C). Furthermore, the absolute numbers of mature CD4\(^+\) and CD8\(^+\) T cells in the spleen, lymph node, and peripheral blood of c-FLIP\(_L^{-/-}\) mice were comparable to those in control mice (Fig. 1, D and E; data not shown). Importantly, the apoptotic rates of mature T cells in c-FLIP\(_L^{-/-}\) mice with or without TCR stimulation were similar to those in control (c-FLIP\(^{−/−}\) or c-FLIP\(^{−/−}\)BAC tg) mice (Fig. 1F), indicating that the lack of c-FLIP\(_L\) expression in mature T cells does not result in enhanced apoptosis, and further supporting the notion that the major function of c-FLIP\(_S\) is to inhibit T cells from apoptosis.

**Impaired effector T cell development in c-FLIP\(_L^{-/-}\) mice**

We first examined the role of c-FLIP\(_L\) in the development of CD8\(^+\) T effector cells using a *L. monocytogenes* infection model (22). c-FLIP\(_L^{-/-}\) and control mice were infected i.v. with 2 × 10\(^7\) *L. monocytogenes* expressing chicken OVA (Ova). Seven days later, the primary CD8\(^+\) T cell response was examined by intracellular staining of IFN-γ after restimulation with Ova257–264 peptide or *L. monocytogenes*-infected macrophages. Under both conditions, IFN-γ-producing CD8\(^+\) effector T cells were readily detected in control mice (Fig. 2, A and B). Strikingly, only background levels of IFN-γ CD8\(^+\) T cells were detected in c-FLIP\(_L^{-/-}\) mice (Fig. 2, A and B). The absolute number of Ova-specific CD8\(^+\) effector T cells in c-FLIP\(_L^{-/-}\) mice was dramatically lower than that in littermate controls (Fig. 2E). Similar results were observed using MHC class I/Ova tetramer staining (data not shown). Furthermore, the frequency of activated/memory CD8\(^+\) T cells (CD62L^hi/Cd44^hi) in c-FLIP\(_L^{-/-}\) mice was significantly lower than that in control mice 7 days after infection (Fig. 2C).

A previous study demonstrated that overexpression of c-FLIP\(_S\) in T cells inhibited T cell proliferation (23). To rule out the possibility that the impaired CD8\(^+\) effector T cell differentiation observed in c-FLIP\(_L^{-/-}\) mice was due to inhibition by the c-FLIP\(_S\) BAC tg we compared CD8\(^+\) effector T cell development in c-FLIP\(_L^{-/-}\) and c-FLIP\(_S^{−/−}\)BAC tg mice. The presence of the c-FLIP\(_S\) BAC tg did not affect CD8\(^+\) effector T cell development upon *L. monocytogenes* infection, indicating that the defect observed in c-FLIP\(_L^{-/-}\) mice is due to loss of c-FLIP\(_S\) expression (Fig. 2E). Consistent with this lack of CD8\(^+\) effector T cells, no CTL-mediated killing was detected in c-FLIP\(_L^{-/-}\) mice (Fig. 2D). These results demonstrate that c-FLIP\(_L\) is essential for CD8\(^+\) effector T cell development in vivo.
The defective effector T cell development observed in c-FLIPL-/- mice was examined in c-FLIPL-/- mice (24). FLIPL promotes Th2 differentiation in overexpression studies (24), and CD4+ T cell proliferation in c-FLIPL-/- mice. Eight days after immunization with 100 μg/mouse DNP-KLH, splenocytes were labeled with CFSE and stimulated with DNP-KLH for 3 days in vitro. Cell proliferation was assessed by FACS analysis. Histograms were gated on 7-AAD+CD4 cells. These data demonstrate that the development of effector T cells in vivo, a fraction of c-FLIPL-/- CD8+ T cells responded to TCR stimulation in vitro (Fig. 4), suggesting that strong stimulation of TCR with anti-CD3 Ab may partially overcome the lack of c-FLIPL.

Defective TCR-induced proliferation in c-FLIPL-/- T cells

The defective effector T cell development observed in c-FLIPL-/- mice may be due to defective T cell activation and/or proliferation because apoptosis was not enhanced after TCR activation in c-FLIPL-/- T cells (Fig. 1F). We examined T cell proliferation after stimulation with plate-bound anti-CD3 with or without anti-CD28. The frequencies of proliferating c-FLIPL-/- CD4+ and CD8+ T cells after TCR stimulation were greatly reduced when compared with those of control T cells (Fig. 4). Engagement of costimulatory molecules with mAb against CD28, OX40, 4-1BB, and CD27 did not rescue the proliferative defects in c-FLIPL-/- T cells (Fig. 4; data not shown). Interestingly, in contrast to the complete lack of CD8+ effector T cells in vivo, a fraction of c-FLIPL-/- CD8+ T cells responded to TCR stimulation in vitro (Fig. 4), suggesting that strong stimulation of TCR with anti-CD3 Ab may partially overcome the lack of c-FLIPL.

Next, we examined CD4+ effector T cell development in c-FLIPL-/- mice using L. monocytogenes infection and DNP-keyhole limpet hemocyanin (DNP-KLH) immunization. Seven days after Listeria infection, CD4+ effector T cells were determined by intracellular IFN-γ staining. The percent of CD4+ effector T cells was reduced by ~70% in c-FLIPL-/- mice when compared with that in control mice (Fig. 3A). Furthermore, in vitro Ag restimulation-induced CD4+ effector T cell proliferation was decreased in c-FLIPL-/- mice immunized with DNP-KLH (Fig. 3B). Since c-FLIPL-/- promotes Th2 differentiation in overexpression studies (24), IL-4 production was examined in c-FLIPL-/- CD4+ T cells. As shown in Fig. 3C, both IL-4 and IFN-γ production was impaired in vitro differentiated c-FLIPL-/- effector CD4+ T cells under neutral condition. These data demonstrate that the development of effector CD4+ T cells also depends on c-FLIPL.

Defective CD4+ effector T cell development in c-FLIPL-/- mice. A, FACS analysis of IFN-γ-producing CD4+ effector T cells in c-FLIPL-/- mice 7 days after Listeria infection. Total splenocytes were stimulated with Listeria-infected bone marrow-derived macrophages for 20 h before intracellular cytokine staining. B, Decreased effector CD4+ T cell proliferation in c-FLIPL-/- mice. Eight days after immunization with 100 μg/mouse DNP-KLH, splenocytes were labeled with CFSE and stimulated with DNP-KLH for 3 days in vitro. Cell proliferation was assessed by FACS analysis. Histograms were gated on 7-AAD+CD4 cells. These data demonstrate that the development of effector T cells in vivo, a fraction of c-FLIPL-/- CD8+ T cells responded to TCR stimulation in vitro (Fig. 4), suggesting that strong stimulation of TCR with anti-CD3 Ab may partially overcome the lack of c-FLIPL.

Defective TCR-induced proliferation in c-FLIPL-/- T cells

The defective proliferation of c-FLIPL-/- T cells may reflect impaired activation. We thus examined the expression of activation markers CD25 and CD69 on c-FLIPL-/- T cells. At 6 h after anti-CD3 stimulation, the expression of CD69 and CD25 on c-FLIPL-/- T cells was slightly lower than that on control T cells (Fig. 5A; data not shown). However, at 24 h after TCR activation, c-FLIPL-/- T cells expressed slightly higher levels of CD69 and CD25 than control cells (Fig. 5B). These results suggest that while
c-FLIPL in T cell proliferation

C. These results indicate that c-FLIPL is not essential for T cell proliferation in a loss-of-function model without the complication of cell death. Our conditional c-FLIPL knockout mice are ideal for this purpose.

Discussion

Most data supporting a role for c-FLIPL in T cell proliferation and NF-κB activation were generated in overexpression studies using tg mice or cell lines (8, 9, 11). Although our previous study and those of others have used loss-of-function models to examine the role of c-FLIP in SP thymocyte and mature T cell proliferation (4, 5), these results are likely complicated by the fact that T cells lacking both c-FLIPL and c-FLIPS die rapidly after activation. Thus, it is essential to assess the function of c-FLIPL in T cell proliferation in a loss-of-function model without the complication of cell death.

IL-2 production in c-FLIPL–/– T cells

Another possible cause for the impaired proliferation of c-FLIPL–/– T cells is that these cells exhibit defective IL-2 production. To test this, we measured the amount of IL-2 in the supernatants of anti-CD3-stimulated T cells by ELISA. c-FLIPL–/– T cells produced 40–50% less IL-2 than control T cells 16 h after stimulation, but produced comparable levels of IL-2 48 h after stimulation (Fig. 5C). Furthermore, exogenous IL-2 cannot rescue the proliferative defects in c-FLIPL–/– T cells (data not shown). These data indicate that IL-2 production is defective in the early phase but normal in the late phase of activation of c-FLIPL–/– T cells and suggest that the defective proliferation of c-FLIPL–/– T cells is not likely due to defective IL-2 production.

MAPK pathways and NF-κB activity in c-FLIPL–/– T cells

To examine the mechanisms of c-FLIPL-dependent T cell proliferation, we examined the MAPK pathway in c-FLIPL–/– T cells. Phosphorylation of ERK, JNK, and p38 in c-FLIPL–/– T cells was comparable to that in WT T cells upon anti-CD3 stimulation (Fig. 6A). Furthermore, c-FLIPL–/– T cells exhibit nuclear AP-1 DNA binding activity similar to that in WT T cells upon TCR stimulation (Fig. 6C). These results indicate that c-FLIPL is not essential for activation of the MAPK/AP-1 pathways.

Next, we examined NF-κB activity in c-FLIPL–/– T cells. It has been shown that overexpression of c-FLIPL activates NF-κB through interaction with TRAF2 and receptor interacting protein (9). However, c-FLIPL–/– T cells exhibited normal l-κBα degradation, phosphorylation (Fig. 6A), and nuclear NF-κB DNA binding activity (Fig. 6C) after TCR stimulation. In addition, l-κBα mRNA induction after TCR stimulation, which is another well-accepted indicator of NF-κB function (25), was similar in both c-FLIPL–/– and control T cells (Fig. 6B). Collectively, these results demonstrate that c-FLIPL is not required for TCR-induced NF-κB activation.

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Disclosures

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References


