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Hien Chau,1,2 Veronica Wong,1,2 Nien-Jung Chen,1,2 Huey-Lan Huang,1,2 Wen-Jye Lin,1,2 Christine Mirtsos,1,2 Alisha R. Elford,1,2 Madeleine Bonnard,3 Andrew Wakeham,1,2 Annick Itie You-Ten,1,2 Bénédicte Lemmers,1 Leonardo Salmina,3 Marc Pellegrini,1,2 Razq Hakem,1 Tak W. Mak,1,2 Pamela Ohashi,1,2 and Wen-Chen Yeh1,2

Studies of apoptosis regulation and cell proliferation are critical to our understanding of various biological responses, including immune regulation. Proteins containing specialized domains, such as the death domain (DD) and the death effector domain (DED), have been found to play essential roles in regulating apoptotic signals, including signals mediated by death receptors such as the Fas antigen and TNFR1 (1–3). Death receptor–induced apoptosis requires two common signal transducers, Fas-associated DD (FADD) and caspase-8 (4). FADD is a bimodular adaptor (DED–DD) that interacts with death receptors or other adaptors through its DD while recruiting caspase-8 through its DED. Caspase-8 contains two DEDs that interact with FADD and a COOH-terminal caspase domain that can be activated to trigger downstream apoptotic cascades (5).

Cellular FLICE-inhibitory protein (cFLIP), on the other hand, contains two DEDs and a caspase-like domain similar to that in caspase-8, but it lacks the signature feature of proteolytic enzymes (6). Full-length cFLIP (cFLIPL) and several splice variants of cFLIP (including cFLIPΔ) containing truncated caspase-like domains have been described. All cFLIP variants are capable of binding to the DEDs of FADD and caspase-8, theoretically allowing them to physically interfere with caspase-8 recruitment to FADD (6). Consistent with this hypothesis, cFLIP−/− mouse embryonic fibroblasts are highly sensitive to apoptosis induced by TNF or FasL, indicating that cFLIP is an essential cell survival factor that counteracts FADD and caspase-8–mediated apoptosis (7). Interestingly, cFLIP−/− mice die during embryogenesis with a cardiac defect similar to that observed in FADD−/− and caspase-8−/− mice (7–9). Thus, this trio of DED–containing proteins may cooperate in certain signaling pathways such as that involved in heart development.

One outstanding question regarding the physiological functions of FADD and caspase-8 concerns their involvement in T cell development, survival, and proliferation. Although both molecules are required for the Fas-dependent arm of activation-induced cell death of T cells,
these proteins also promote proliferation induced by TCR engagement (10–12). Specifically, in FADD-deficient T cells, activation in response to TCR stimulation is impaired and cells do not proliferate (10). Further study of T cell–specific FADD-deficient mice revealed a role of FADD in early T cell development (13). Consistent with certain knockout phenotypes, mutant T cells from transgenic mice overexpressing a dominant negative mutant of FADD (FADD-DN) also showed impaired development in the thymus and fail to proliferate in response to TCR stimulation (14–19).

Recently, an essential role of caspase-8 in T cell proliferation was demonstrated in both human patients and mouse models (11, 12). Patients harboring homozygous caspase-8 mutations exhibited defective lymphocyte apoptosis and immunodeficiency, primarily caused by abnormal activation of T, B, and NK cells (12). Mouse peripheral T cells lacking caspase-8 showed impaired Fas-mediated apoptosis and activation-induced T cell expansion in vitro (11). More dramatically, deletion of caspase-8 in early hematopoietic precursors completely abolished T cell development (20). Collectively, both FADD and caspase-8 may play a role in TCR signals that affect T cell development and proliferation.

cFLIP is abundantly expressed in T cells (6). Recently, two laboratory groups have investigated the role of cFLIP in T lymphocytes using transgenic mice overexpressing cFLIP. One study demonstrated that cFLIP is important for promoting T cell proliferation. Mutant T cells derived from these transgenic mice exhibited proliferative responses similar to those in wild-type T cells, except that the levels of cFLIP after CD3 and CD28 cross-linking (Fig. 1). Expression of FADD and caspase-8, on the other hand, was more or less constant before and throughout TCR stimulation (Fig. 1).

We further examined the protein expression in a situation where proliferation was reduced because of the expression of FADD-DN (14). As shown in Fig. 1, the suboptimal proliferation of FADD-DN T cells after TCR stimulation appeared to correlate with the reduced level of c-Myc. Interestingly, expression levels of FADD, caspase-8, and cFLIP in FADD-DN T cells were comparable with those in wild-type littermate T cells, except that the levels of cFLIP after TCR stimulation appeared to be slightly accentuated in the mutant cells (Fig. 1). These data suggested that FADD-DN could antagonize TCR-mediated proliferative signals through an alternative mechanism instead of down-regulating any of the three DED proteins.

**Generation of cFLIP<sup>−/−</sup>/Rag<sup>−/−</sup> chimeric mice**

To evaluate the impact on T cells when cFLIP is absent, we generated cFLIP-deficient T cells by reconstituting Rag<sup>−/−</sup> mice with cFLIP<sup>−/−</sup> embryonic stem (ES) cells. To generate cFLIP-deficient ES cells, exon 1 of one cflip allele was replaced with a neomycin cassette, while exon 1 of the other allele was substituted by a hygromycin resistance gene (Fig. 2 A). Proper genomic recombination with the presence of both neomycin and hygromycin resistance alleles was confirmed using Southern blot analyses (not depicted), and the absence of cFLIP expression in a homozygous mutant cell line was demonstrated by Northern blotting (Fig. 2 B). Two independent cFLIP<sup>−/−</sup> ES cell lines, along with one cFLIP<sup>+/−</sup> control line, were used for injections into Rag<sup>−/−</sup> blastocysts to generate the chimeric mice (nFLIP<sup>−/−</sup> and nFLIP<sup>+/−</sup>).
positive and negative controls, respectively. Thymocytes were stained

tative flow cytometric analyses of thymi from

expression of CD25 and CD44. The percentage of gated cells in each

quadrant is shown.

Figure 2. Generation of cFLIP-deficient T cells. (A) The targeting
construct. Homologous recombination replaced a part of cFLIP exon 1,
including the ATG start codon, with the neomycin resistance cassette on
one of the wild-type alleles, resulting in a heterozygous ES cell line. A
second targeting construct containing a hygromycin resistance cassette
replaced the other allele, resulting in a cFLIP-null ES cell line. Primers
used for PCR screening are indicated by arrows. (B) Absence of cFLIP
expression confirmed by Northern blot analysis. 20 μg of total RNA
was isolated from homozygous wild-type (+/+=; homozygous mutant
(−/−), and heterozygous (+/−) ES cell clones and resolved on a form-
alddehyde-denaturing agarose gel. RNA from a wild-type embryonic
fibroblast cell line (EF) was also used as a control. The RNA was trans-
ferrred to nitrocellulose membrane and probed with a full-length cFLIP
cDNA probe (top) and a GADPH probe (bottom). (C) Expression of CD4
and CD8 in rcFLIP−/− peripheral T cells. Pooled lymph node cells from
wild-type and rcFLIP−/− mice were stained for the expression of T cell-
specific surface markers, CD4 and CD8, and analyzed by flow cytometry.
The percentage of gated viable cells in each quadrant of the dot plots is
shown. (D) Abnormal T cell development in rcFLIP−/− chimera. Representa-
tive flow cytometric analyses of thymi from rcFLIP−/−, rcFLIP+/−, wild-
type 129J, and Rag−/− mice are shown; the latter two were used as
positive and negative controls, respectively. Thymocytes were stained
using antibodies against CD4 and CD8 and analyzed by flow cytometry.
The percentages of gated viable cells for each quadrant of the dot plots
are shown. (E) Abnormal early thymocyte development in rcFLIP−/−
chimera. Lineage-negative (CD4−CD8−) and Ly9.1-positive thymocytes
from rcFLIP−/− and control mice (+/−) were gated and analyzed for
the expression of CD25 and CD44. The percentage of gated cells in each
quadrant is shown.

Although peripheral mature T lymphocytes were present in
rcFLIP−/− mice, the numbers of total splenocytes and lymph node cells in these mutant mice were both severely
reduced compared with rcFLIP+/− mice. For example, rcFLIP−/− mice (9.20 ± 3.85 × 10⁶; n = 9) had approximately three- to fourfold fewer total splenocytes than
rcFLIP+/− mice (41.8 ± 29.6 × 10⁶; n = 7). In lymph nodes, the cell numbers of rcFLIP−/− mice were reduced by approxi-
mately ninefold. Reduction of lymphocyte numbers in
rcFLIP−/− mice was partly caused by a deficiency of B cells,
as B cell development was also impaired in these mutant mice
(unpublished data). Flow cytometric analysis of the lymph
node cells revealed that the ratio of CD4+/CD8+ cells was comparable between mutant and control T cells (Fig. 2 C).

To further characterize T cells in rcFLIP−/− and rcFLIP+/−
mice, thymus cellular profiles were analyzed. Wild-type
129J mouse thymocytes were included in the analysis as a
reference control. Again, the total number of rcFLIP−/− thymo-
cytes (2.53 ± 1.40 × 10⁶; n = 7) showed ~10-fold redu-
cion compared with rcFLIP+/− thymocytes (36.7 ± 0.09 × 10⁶; n = 4). As shown in Fig. 2 D, rcFLIP−/− thymus had a markedly reduced population of double-positive (DP)
CD4+CD8+ cells when compared with rcFLIP+/− thymi.

rcFLIP−/− thymocytes were capable of reaching the stage of single positive cell development, albeit at re-
duced numbers. Analysis of Ly9.1+ (a marker for the recon-
stituted ES cells) double-negative CD4−CD8− thymocytes by CD25/CD44 staining also revealed a reduction of early
rcFLIP−/− thymocytes and some accumulations of CD25−
CD44+ and CD25−CD44− cells when compared with the
rcFLIP+/− counterparts (Fig. 2 E). Interestingly, we found
that the earlier we analyzed the chimerae, the more thy-
mocytes and DP cells were present in rcFLIP−/− mice (un-
published data), suggesting that thymocytes lacking cFLIP
may have an intrinsic survival defect. Similar defects of
thymocyte development and survival were reported in
FADD−/− mice (10).
rcFLIP−/− T cells failed to proliferate under all stimulatory conditions when compared with the rcFLIP+/− counterparts (Fig. 3 A). In addition, cFLIP-deficient T cells produced a reduced amount of IL-2 in response to TCR stimulation (not depicted), and a supplement of exogenous IL-2 failed to rescue the defect of proliferation (Fig. 3 A).

To further analyze the impact of cFLIP deficiency on T cells throughout TCR activation and cell division, the fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) was used to label T cells (24). As shown in Fig. 3 B, rcFLIP+/− T cells have undergone five rounds of cell division by 72 h after TCR activation using CD3.
cross-linking. In contrast, nFLIP−/− T cells remained undivided, appearing in the rightmost region where resting cells reside (Fig. 3 B). Similar results were observed with anti-CD3 and anti-CD28 costimulation of nFLIP−/− T cells (unpublished data). Interestingly, CD69 and CD25, surface markers for T cell activation, were equally induced in nFLIP−/− and the control T cells after CD3 alone and CD3 plus CD28 cross-linking (Fig. 3, C and D), indicating that some pathways downstream of TCR stimulation may operate normally in nFLIP−/− cells. It should be noted that measurement of these T cell activation markers was limited to live cell populations (Fig. 3 D).

Because cFLIP is a known antiapoptotic protein, we examined whether the deficiency of cFLIP affects the viability of T cells. Purified rcFLIP−/- and rcFLIP+/+ T cells were untreated or stimulated with anti-CD3 antibody for 24 or 48 h, and cells were harvested and stained with Annexin V and 7-AAD. Flow cytometry analysis showed that rcFLIP−/- T cells had a higher percentage of Annexin V-positive cells compared to the control T cells, indicating a survival defect of rcFLIP−/- T cells (Fig. 4).}

**Figure 4.** Survival defect of rcFLIP−/- T cells. (A) Enhanced spontaneous and TCR-induced cell death in T cells lacking cFLIP. Purified T cells from rcFLIP+/+ and rcFLIP−/- mice were stimulated with medium alone (untx) or 1 μg/ml of insoluble anti-CD3 antibody for 48 h. Cells were then stained with annexin-V, 7-AAD and Annexin V and examined by flow cytometry. Analysis was performed on Thy1.2-positive cells. The percentage of gated cells in each quadrant is shown. (B) Cell viability histogram of purified T cells from rcFLIP+/+ (gray bars) and rcFLIP−/- (black bars) mice stimulated with medium alone (untx), 1 μg/ml anti-CD3, or anti-CD3 plus 5 μg/ml anti-CD28 for 24 and 48 h. Cell viability was assessed by flow cytometric analysis of 7-AAD-negative cells, and the results shown were the average percentage of cell viability ± SD for duplicate samples. (C) The role of the Fas signal on rcFLIP−/- T cell proliferation. Purified T cells from rcFLIP+/+ and rcFLIP−/- mice were treated with medium alone (untx), 1 μg/ml of insoluble anti-CD3 antibody without or with 1 μg/ml anti-CD28 antibody in the absence or presence of 10 μg/ml of neutralizing anti-FasL antibody for 24 h. T cell proliferation was assessed by [3H]thymidine incorporation, and results shown are cpm ± SE from triplicate samples.

**Figure 5.** Impaired rcFLIP−/- T lymphocyte proliferation in response to TCR activation. (A, top) Viable cell counts by trypan blue exclusion from duplicate wells made of rcFLIP+/+ and rcFLIP−/- T cells after 48 h of culture without or with 1 μg/ml anti-CD3 treatment. Approximately twice as many rcFLIP−/- cells were seeded in each well as compared with rcFLIP+/+ cells. (bottom) Defective thymidine incorporation of rcFLIP−/- T cells in response to TCR stimulation. rcFLIP−/- (white bars) and rcFLIP+/+ (black and gray bars) T cells were cultured without or with 1 μg/ml anti-CD3 treatment as described in A (top), and proliferation was measured by [3H]thymidine incorporation with results shown as mean cpm ± SE. (B) Impairment of cell cycle entry in rcFLIP−/- T cells. purified T cells from rcFLIP−/- and rcFLIP+/+ mice were either untreated (untx) or treated with 1 μg/ml of plate-bound anti-CD3 antibody with or without 1 μg/ml anti-CD28 antibody. Cells were fixed and stained with FITC-labeled anti-BrdU antibody and 7-AAD. Cells in the different phases of the cell cycle (G0/G1, S, and G2/M) are indicated. The percentage of cells in the S and G2/M phases of the cell cycle is shown.
7-amino-actinomycin D (7-AAD), which detect early apoptotic and dead cells, respectively. A decrease in the viability of αFIP−/− Thy1.2+ T cells, compared with αFIP+/− controls, was evident at both time points examined (Fig. 4, A and B). In fact, there was a mild increase of spontaneous cell death in unstimulated αFIP−/− cells cultured over the period of 48 h (Fig. 4 A). This reduction of T cell viability was not caused by increased Fasl/Fas apoptotic signals (25) because neutralizing FasL (using an antibody) failed to prevent cell death in αFIP−/− or αFIP+/− T cells in the early stages of culturing and TCR stimulation (unpublished data). Consistently, the neutralizing antibody against FasL could not rescue the proliferation defect observed in αFIP−/− T cells (Fig. 4 C). This neutralizing antibody was capable of inhibiting Fasl/Fas-induced apoptosis in wild-type thymocytes (unpublished data), validating its ability to neutralize FasL.

αFIP−/− T cells are defective in cell cycling in response to TCR stimulation

Although the defects shown by thymidine incorporation and CFSE labeling of TCR-stimulated αFIP−/− cells may be partially explained by impaired cell survival, at least two lines of evidence suggest that αFIP−/− T cells may have additional defects in cell proliferation. First, when an excess number of αFIP−/− T cells were plated such that after 48 h of TCR stimulation equal numbers of viable αFIP−/− and αFIP+/− cells were present, the proliferation of αFIP−/− T cells, as measured by thymidine incorporation, was still lower compared with αFIP+/− T cells (Fig. 5 A). Second, when the cell cycle profile of TCR-stimulated T cells was analyzed by BrdU incorporation and 7-AAD staining, αFIP−/− T cells showed severe impairment of cell cycle entry, both in response to anti-CD3 alone and to anti-CD3 plus anti-CD28 stimulation (Fig. 5 B). The BrdU/7-AAD experiment also revealed a slight increase in G2/M phase cells in unstimulated αFIP−/− populations (3.94%) compared with resting αFIP+/− cells (0.67%; Fig. 5 B). Collectively, the defective TCR response of αFIP−/− cells is likely caused by a combination of proliferation defect and enhanced apoptosis.

Although FADD- and caspase-8–deficient T cells are both defective in TCR-activated responses, there are remarkable differences reported between these two knockout cells. Specifically, FADD−/− and caspase-8−/− T cells fail to proliferate in response to TCR stimulation because of defects in cell cycle regulation and survival, respectively (11, 26). Interestingly, the observed phenotypes of αFIP−/− T cells resemble caspase-8−/− T cells in their impaired survival, whereas caspase-8−/− cells show the defect only after TCR stimulation. The cell cycling defect of αFIP−/− T cells is reminiscent of FADD−/− T cells.

Stimulation of TCR triggers various intracellular signaling cascades, including activation of ERK, which contribute to cellular proliferation (27). FADD and caspase-8, however, do not appear to play a major role in promoting most known TCR signaling pathways, based on previous studies (11, 16). Consistently, activation of the ERK pathway in response to CD3 plus CD28 cross-linking did not appear to require cFLIP (Fig. 6). In fact, TCR–induced ERK phosphorylation appeared to be slightly enhanced in αFIP−/− T cells, similar to what was observed in caspase-8–deficient T cells (11).

DISCUSSION

The involvement of FADD and caspase-8 in TCR-mediated responses is intriguing, but the molecular mechanisms remain completely unknown. As cFLIP is capable of associating with FADD/caspase-8 in death receptor signaling (28) and of modulating their activities, we investigated whether cFLIP is also involved in TCR signaling. We demonstrated that cFLIP plays an important physiological role in T cell regulation. Importantly, cFLIP is required for the proper expansion of T cells in response to TCR stimulation. The presence of cFLIP not only ensures the survival of T cells without or with TCR activation, but also promotes proper cycling of T cells on stimulation. These findings support the possibility that this DED-containing protein may coordinate the death/survival decision, as well as the process of proliferation in the same cell (2).

We observed an impairment of thymocyte development and a reduction of T lymphocyte numbers in peripheral lymphoid organs in αFIP−/− mice, suggesting that ES cells lacking cFLIP are less capable of reconstituting or maintaining a T cell compartment. An alternative approach of studying the effect of cFLIP deficiency on T cells would be to generate a T cell-specific cFLIP knockout mouse (see Zhang and He on p. 395 of this issue). The tissue-specific knockout offers a complementary system in which deficiency of cFLIP is inherited from the beginning of T cell development. It may be worth pointing out that mice harboring FADD-deficient T cells, derived from RAG−/− complementation, also showed a similar reduction of thymocyte cellularity and a deficiency of DP cells (10, 30).
Transgenic mice expressing a dominant negative form of FADD also exhibited a T cell developmental defect (14, 17, 18). Recently, an induced deletion of caspase-8 using Mx-Cre and the interferon system, although not T cell specific, revealed a severe defect in thymocyte development (20).

Despite the defect in thymocyte development and cellularity, the numbers of peripheral FADD−/− T lymphocytes are comparable to the control counterparts in the Rag2−/− complementation study (10). Unlike FADD−/− T cells, nFLIP−/− T cells showed deficiencies in both thymocyte and peripheral T cell numbers. Both mutants may go through similar lymphopenic environments, at least at some stages, but FADD−/− peripheral T cells are perhaps capable of homeostatic proliferation and lack survival defects. Intriguingly, a fraction of the unstimulated FADD−/− T cells show characteristics of activated blasts, high levels of the T cell activation marker CD69, and early entry into the S phase. However, typical markers of homeostatic proliferation are not, or perhaps are no longer, present in FADD−/− cells (10). In contrast, we observed increased expression of homeostatic proliferation marker CD122 on nFLIP−/− T lymphocytes (unpublished data), suggesting that some degree of homeostatic proliferation does occur in nFLIP−/− mice. The fact that the homeostatic proliferation is unable to fill up the T cell compartment in the periphery of nFLIP−/− mice suggests an intrinsic defect in survival/proliferation in vivo.

The exact nature of the signals mediated by FADD, caspase-8, or cFLIP downstream of TCR triggering remains largely unknown. An involvement of death receptor (DR) signals in TCR signals remains a possibility, and signals largely unknown. An involvement of death receptor (DR) caspase-8, or cFLIP downstream of TCR triggering remains the pathway leading to IL-2 production are reported to be nor-

Geneticin (G418) and Hygromycin B were obtained from Gibco BRL. ES cells were cultured in DMEM supplemented with 15% FCS (Sigma-Aldrich), 2 mM L-glutamine (Gibco BRL), 2 mM sodium pyruvate (Gibco BRL), 50 μM β-mercaptoethanol (Gibco BRL), and leukemia inhibitory factor. Lymphocytes were cultured in RPMI 1640 with 10% FCS (Gibco BRL), 2 mM L-glutamine, 2 mM sodium pyruvate, and 50 μM β-mercaptoethanol. BM cells were harvested and kept in Iscove’s media supplemented with 2 mM L-glutamine and 50 μM β-mercaptoethanol until further use.

Materials and Methods

Reagents. DNA restriction enzymes were obtained from Boehringer and Gibco BRL. The phosphoglycerate kinase promoter–driven neomycin and hygromycin cassettes were cloned in pBluescript. Rag2−/− and 129J mice were purchased from the Jackson Laboratory. All antibodies used for flow cytometry and stimulation purposes were obtained from BD Biosciences or eBioscience. Geneticin (G418) and Hygromycin B were obtained from Gibco BRL. ES cells were cultured in DMEM supplemented with 15% FCS (Sigma-Aldrich), 2 mM L-glutamine (Gibco BRL), 2 mM sodium pyruvate (Gibco BRL), 50 μM β-mercaptoethanol (Gibco BRL), and leukemia inhibitory factor. Lymphocytes were cultured in RPMI 1640 with 10% FCS (Gibco BRL), 2 mM L-glutamine, 2 mM sodium pyruvate, and 50 μM β-mercaptoethanol. BM cells were harvested and kept in Iscove’s media supplemented with 2 mM L-glutamine and 50 μM β-mercaptoethanol until further use.

Generation of cFLIP heterozygous mutant ES cells by gene targeting. The cFLIP targeting construct consists of a 1.1-kb short arm and a 5.5-kb long arm, which are located at 5′ and 3′, respectively, outside of exon 1 of the targeted cflip locus and the neomycin resistance gene inserted in the reverse orientation. Homologous recombination will result in replacement of endogenous cflip exon 1, which contains the ATG starting codon, with the selection marker. 5′ and 3′-extension (primer sequence: 5′-CTT ATG CAT AAA GGG CTG GCA AGA TG-3′) with 5 mM of the linearized construct. 24 h later, cells were selected in 300 μg/ml G418 (neomycin) in complete ES media. Homologous recombinants were identified by PCR and further analyzed by genomic Southern blot hybridization with the short arm as a probe. Primers used for PCR screening bind to a portion of the neomycin marker gene (primer sequence: 5′-CGT GCA AGA TG-3′) and to a portion of the endogenous 5′ piece of the short arm (primer sequence: 5′-CAAT ATT AAG GGC CAG CTC ATT CCT CGT-3′).

Establishing cFLIP-null ES cells and blastocyst injection. The generation of cFLIP-null ES cells was established using two different methods. The first method subjected cFLIP−/− ES cells to high concentrations of G418 (3–12 mg/ml), forcing them to undergo mitotic recombination whereby the wild-type allele is replaced after duplication of the disrupted allele. Surviving clones were confirmed as homozygous mutants by genomic Southern blot analysis as described in the previous section. The second method used another targeting construct containing a hygromycin resistance cassette to disrupt the remaining wild-type allele in cFLIP−/− ES cells. After electroporation, colonies were selected in both G418 and hygromycin. The surviving clones were screened by PCR using primers 5′-AAG
CGC ATG CTC CAG ACT GCC TTG GGA A-3\' and 5\'-CAA ATT AAG GCC CAG CTC ATT CCT CC-3\' and confirmed by genomic Southern blot analysis as mentioned in the previous section. \textit{fFLIP}\textsuperscript{++} and \textit{fFLIP}\textsuperscript{++/−} ES cell clones were injected into E3.5 \textit{Rag2}\textsuperscript{−/−} (B6 background) blastocysts and reimplanted into pseudopregnant ICR foster mothers. The resulting chimeric offspring were subjected to tail bleeding to examine for the presence of lymphocytes. All mice were maintained under pathogen-free conditions in accordance with the guidelines of the Canadian Council for Animal Care, and all animal studies were approved by the University Health Network Animal Care Committee (protocol 00–155).

**Northern blot analysis.** Twenty \(\mu\)g of total RNA from ES cell clones was fractionated on a formaldehyde-denaturing agarose gel, and ethidium bromide was used to stain for the presence of ribosomal RNA (18S and 28S) to check for RNA integrity and equal loading. The RNA was then transferred to nitrocellulose membrane and probed sequentially with \(^{32}P\)-radiolabeled full-length cFLIP and GADPH cDNAs.

**Flow cytometry analysis.** Single cell suspensions from thymus, spleen, LN, BM, and Peyer’s patches were stained with a variety of mAbs conjugated to fluorescent moieties (BD Biosciences) to analyze the expression of cell surface markers. Antibodies conjugated with FITC, PE, biotinylated streptavidin-conjugated RED670, and allophycocyanin allowed the simultaneous assessment of multiple surface markers per cell sample using a flow cytometer (FACSCalibur; Becton Dickinson). Data was analyzed using CellQuest software (Becton Dickinson).

**T cell purification.** Single cell suspensions were obtained from spleen and LN (cervical, axillary, inguinal, and brachial) by running the tissues through a 70-\(\mu\)m mesh. RBCs were removed from spleens by incubating suspended cells in sterile hypertonic lysis buffer (0.155 mM ammonium chloride, 0.1 mM disodium EDTA, 0.01 M potassium bicarbonate) for 5 min on ice. One method used to enrich for T cells was by negative selection using magnetic Dynabeads (Dynal), depleting cells expressing B220 (B cells), Gr-1 (granulocytes), and Mac-1 (macrophages), according to the manufacturer’s instructions. An alternative method used to purify T cells used MACS magnetic beads conjugated to anti-CD4 and anti-CD8 antibodies (for positive selection) or biotinylated non-anti-CD4 antibody cocktail (for negative selection) to purify CD4\(^+\)-only T cells, according to the manufacturer’s instructions (Miltenyi Biotec).

**[\(^{3}H\)]Thymidine proliferation assay.** Ten cells/well were seeded in 96-well round-bottom plates in a total volume of 200 \(\mu\)l RPMI-supplemented media. T cells were stimulated with anti-CD3 antibodies (clones 145-2C11 or 17A2; BD Biosciences) with or without costimulatory anti-CD28 antibody (clone 37.51; BD Biosciences). To mimic ligation or aggregation of CD3 with or without costimulatory anti-CD28 antibodies for at least 3 h at 37\(^\circ\)C. Cells were then stained with antibodies for 24 or 48 h. Cells were then washed with PBS before adding the cells. Cells were stimulated for 48 h and pulsed for an additional 8 h with 1 \(\mu\)Ci [\(^{3}H\)]thymidine per well. The amount of [\(^{3}H\)]thymidine incorporated by the cells was measured with a direct \(\beta\)-counter (Matrix 96; Canberra Packard).

**Cell death assay.** Ten T cells/well (unpurified or purified) were seeded in a 96-well dish and left untreated or treated with anti-CD3 \(\pm\) anti-CD28 antibodies for 24 or 48 h. Cells were then stained for Thy1.2 or CD3 (T cell markers), Annexin V, and 7-AAD to evaluate the cell viability.

**BrdU proliferation assay.** The BrdU Flow Kit (BD Biosciences) was used for cell cycle analysis on peripheral T lymphocytes. After 48 h of TCR stimulation, cells were pulsed for 90 min with 10 \(\mu\)M BrdU, harvested, and stained with antibodies against TCR or CD3 to identify T cells. Cells were fixed and permeabilized to allow staining with anti-BrdU antibody conjugated to FITC. Cells were further stained with 7-AAD to assess cell cycle position (i.e., G0/G1, S, G2/M phase) by flow cytometry.

**CFSE labeling of T cells.** Purified CD4\(^+\) T cells were labeled with CFSE as previously described (24). In brief, cells were resuspended at a density of 20 \(\times\) \(10^5\) cells/ml in PBS. An equal volume of 5 \(\mu\)M CFSE (Molecular Probes) was added in PBS and the cells were incubated for 5–10 min at room temperature with intermittent mixing. An equal volume of PBS was added, and the labeled T cells were washed in PBS and resuspended in supplemented RPMI media. CFSE-labeled \textit{fFLIP}\textsuperscript{++/−} and \textit{fFLIP}\textsuperscript{++} T cells, were plated at 4 \(\times\) \(10^5\) and 12 \(\times\) \(10^5\) cells/well, respectively, in 48-well microtiter plates. CFSE-labeled T cells were harvested at 24, 48, and 72 h after no treatment or treatment with anti-CD3 \(\pm\) anti-CD28 antibodies (1 \(\mu\)g/ml each). All samples were analyzed by flow cytometry using 7-AAD and CD4 antibody.

**Phospho-ERK Western blotting.** Western blotting for phospho-ERK was performed as previously described (39). In brief, 3 \(\times\) \(10^5\) purified T cells were stimulated with soluble anti-CD3 plus anti-CD28 (10 \(\mu\)g/ml each) followed by cross-linking with 10 \(\mu\)g/ml of the appropriate secondary IgG antibody (Jackson ImmunoResearch Laboratories) or with 100 ng/ml each of PMA (Sigma-Aldrich) plus calcium ionophore (A23187; Sigma-Aldrich). After incubation at 37\(^\circ\)C for 5, 15, and 60 min (10 min for PMA plus ionophore), the cells were washed in PBS containing 10 mM Na\(_2\)VO\(_4\) and lysed in ice-cold lysis buffer (50 mM Tris, pH 8, 150 mM NaCl, 1% Triton X-100, 20 mM EDTA, 1 mM Na\(_2\)VO\(_4\), 1 mM NaF, and protease inhibitors), subjected to 10\% SDS-PAGE, and transferred to polyvinylidene difluoride membranes. Filters were incubated with antibodies against phospho-ERK1/2 (Cell Signaling) or actin (Sigma-Aldrich). The protein–antibody complexes were detected using the ECL detection system (GE Healthcare).

**Western blotting for DED protein expressions in T cells.** Three \(\times\) \(10^5\) purified CD4\(^+\) T cells from FADD-DN transgenic mice (a gift from A. Strasser, University of Melbourne, Parkville, Australia) and wild-type littermates were seeded on 48-well plates precoated with anti-CD3 (1 \(\mu\)g/ml) and anti-CD28 (5 \(\mu\)g/ml) antibodies. T cells were incubated at 37\(^\circ\)C for 3, 6, 12, 24, and 48 h, after which the cells were pelleted and lysed in radiolabelling precipitation assay buffer (10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na\(_2\)VO\(_4\), 10 mM NaF, 1 mM PMSF). 10 \(\mu\)g of the protein lysates were fractionated on 4–20% Tris-glycine polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were sequentially probed with antibodies against cFLIP (Apotech), FADD (a gift from D. Goeddel, Z. Cao, and G. Chen, Tulikir/Angen, San Francisco, CA), caspase-8 (Qbiogene), c-Myc (a gift from L. Penn, University Health Network, Toronto, Canada), and actin (Sigma-Aldrich). Protein–antibody complexes were detected using the ECL detection system (GE Healthcare).

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