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Cellular FLICE-inhibitory protein is required for T cell survival and cycling

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Fas-associated death domain (FADD) and caspase-8 are key signal transducers for death receptor–induced apoptosis, whereas cellular FLICE-inhibitory protein (cFLIP) antagonizes this process. Interestingly, FADD and caspase-8 also play a role in T cell development and T cell receptor (TCR)–mediated proliferative responses. To investigate the underlying mechanism, we generated cFLIP-deficient T cells by reconstituting Rag−/− blastocysts with cFLIP-deficient embryonic stem cells. These Rag chimeric mutant mice (rcFLIP−/−) had severely reduced numbers of T cells in the thymus, lymph nodes, and spleen, although mature T lymphocytes did develop. Similar to FADD− or caspase-8−deficient cells, rcFLIP−/− T cells were impaired in proliferation in response to TCR stimulation. Further investigation revealed that cFLIP is required for T cell survival, as well as T cell cycling in response to TCR stimulation. Interestingly, some signaling pathways from the TCR complex appeared competent, as CD3 plus CD28 cross-linking was capable of activating the ERK pathway in rcFLIP−/− T cells. We demonstrate an essential role for cFLIP in T cell function.

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 cFLIPK) 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 show 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 stimulated with suboptimal doses of anti-CD3 antibody showed enhanced proliferation and IL-2 production (21). In contrast, another group reported that in response to TCR cross-linking, cFLIP–overexpressing T cells were impaired in proliferation, as well as in activation of ERK, p38 mitogen-activated protein kinase, and NF-κB (22). A third group overexpressed the viral homologue of cFLIP (vFLIP) in T cells and showed that T cell survival is perturbed (23). Collectively, it appears that cFLIP is involved in T cell activation, but the exact function of cFLIP in this process remains very much in debate.

We examined cFLIP-deficient T cells in Rag2−/− chimeric (mFLIP−/−). mFLIP−/− mice had greatly reduced T cell counts in the thymus and periphery, and TCR-mediated proliferation was severely impeded in the mutant cells. The resemblance of mFLIP−/−, FADD−/−, and caspase-8−/− phenotypes to each other suggests that cFLIP may be a critical molecular link, at least for FADD and caspase-8, during the process of TCR signaling that affects T cell survival and proliferation.

RESULTS
cFLIP expression is constitutive in T cells and mildly induced after TCR stimulation

As we were interested in investigating the function of cFLIP in T cells and, potentially, the connection between cFLIP, FADD, and/or caspase-8 on TCR stimulation, we first examined the expression patterns of these proteins in T cells. Constitutive expression of cFLIPΔ in naive T lymphocytes was readily detectable, and the protein level was mildly enhanced on TCR activation. Interestingly, cFLIPp was undetectable in resting lymphocytes but was highly induced 6 h 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−/−/Rag2−/− chimeric mice

To evaluate the impact on T cells when cFLIP is absent, we generated cFLIP-deficient T cells by reconstituting Rag2−/− mice with cFLIP−/− 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−/− ES cell lines, along with one cFLIP−/− control line, were used for injections into Rag2−/− blastocysts to generate the chimeric mice (mFLIP−/− and mFLIP+/−).
The percentages of gated viable cells for each quadrant of the dot plots using antibodies against CD4 and CD8 and analyzed by flow cytometry. Positive and negative controls, respectively. Thymocytes were stained with a flow cytometric analysis of thymi from 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 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 reference control. Again, the total number of cFLIP+/− thymocytes (2.53 ± 1.40 × 10^6; n = 7) showed ~10-fold reduction compared with cFLIP+/+ thymocytes (36.7 ± 0.09 × 10^6; n = 4). As shown in Fig. 2D, cFLIP+/− thymus had a markedly reduced population of double-positive (DP) CD4+CD8+ cells when compared with cFLIP+/+ thymi. Nevertheless, cFLIP+/− thymocytes were capable of reaching the stage of single positive cell development, albeit at reduced numbers. Analysis of Ly9.1+ (a marker for the reconstituted ES cells) double-negative CD4−CD8− thymocytes by CD25/CD44 staining also revealed a reduction of early cFLIP+/− thymocytes and some accumulations of CD25+CD44− and CD25−CD44− cells when compared with the cFLIP+/+ counterparts (Fig. 2E). Interestingly, we found that the earlier we analyzed the chimerae, the more thymocytes and DP cells were present in cFLIP+/− mice (unpublished 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 are defective in TCR-stimulated proliferation and cell survival

One question of interest was whether cFLIP plays a role in T cell proliferation in response to TCR stimulation, as this process is defective in both FADD-deficient and caspase-8−/− deficient T cells (10, 11). Purified T cells from cFLIP−/− or cFLIP+/− mice were stimulated with a low (0.1 μg/ml) or high (1 μg/ml) concentration of anti-CD3 antibody, in the presence or absence of anti-CD28 antibody (1 μg/ml), for 48 h. As measured by [3H]thymidine incorporation, respectively. 4 wk after chimeric mice were born, the presence of reconstituted T lymphocytes in these mice was assessed by tail-blood staining for the presence of CD4 or CD8 (unpublished data). Because the caspase-8 and cflip gene loci are close to one another, we examined and confirmed that caspase-8 expression is equal in nFLIP−/− and control T cells (unpublished data).

Although peripheral mature T lymphocytes were present in nFLIP−/− mice, the numbers of total splenocytes and lymph node cells in these mutant mice were both severely reduced compared with nFLIP+/− mice. For example, nFLIP−/− mice (9.20 ± 3.85 × 10^6; n = 9) had approximately three- to fourfold fewer total splenocytes than nFLIP+/− mice (41.8 ± 29.6 × 10^6; n = 7). In lymph nodes, the cell numbers of nFLIP−/− mice were reduced by approximately ninefold. Reduction of lymphocyte numbers in nFLIP−/− 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. 2C).

To further characterize T cells in nFLIP−/− and nFLIP+/− 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 cFLIP+− thymocytes (2.53 ± 1.40 × 10^6; n = 7) showed ~10-fold reduction compared with cFLIP+/+ thymocytes (36.7 ± 0.09 × 10^6; n = 4). As shown in Fig. 2D, cFLIP+/− thymus had a markedly reduced population of double-positive (DP) CD4+CD8+ cells when compared with cFLIP+/+ thymi. Nevertheless, cFLIP+/− thymocytes were capable of reaching the stage of single positive cell development, albeit at reduced numbers. Analysis of Ly9.1+ (a marker for the reconstituted ES cells) double-negative CD4−CD8− thymocytes by CD25/CD44 staining also revealed a reduction of early cFLIP+/− thymocytes and some accumulations of CD25+CD44− and CD25−CD44− cells when compared with the cFLIP+/+ counterparts (Fig. 2E). Interestingly, we found that the earlier we analyzed the chimerae, the more thymocytes and DP cells were present in nFLIP−/− mice (unpublished 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. 3A). 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. 3A).

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. 3B, 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 nFLIP−/− and nFLIP+/− 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 before flow cytometry analysis. Results are shown in Fig. 5A. These data demonstrate that the viability of T cells is significantly reduced in nFLIP−/− cells compared with control T cells. In addition, CD3 + CD28 stimulation results in a further decrease in cell viability in nFLIP−/− T cells, indicating that the Fas signal plays a role in T cell proliferation in response to TCR activation. In contrast, stimulation with anti-CD3 alone does not significantly affect cell viability in either control or nFLIP−/− T cells.

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 anti-Thy1.2, Annexin V, and 7-AAD 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 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 grey 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 G0 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 nFLIP−/− Thy1.2+ T cells, compared with nFLIP+/− controls, was evident at both time points (Fig. 4, A and B). In fact, there was a mild increase of spontaneous cell death in unstimulated nFLIP−/− 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 nFLIP−/− or nFLIP+/− 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 nFLIP−/− 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.

**rcFLIP−/− 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 nFLIP−/− cells may be partially explained by impaired cell survival, at least two lines of evidence suggest that nFLIP−/− T cells may have additional defects in cell proliferation. First, when an excess number of nFLIP−/− T cells were plated such that after 48 h of TCR stimulation equal numbers of viable nFLIP−/− and nFLIP+/− cells were present, the proliferation of nFLIP−/− T cells, as measured by thymidine incorporation, was still lower compared with nFLIP+/− 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, nFLIP−/− 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 nFLIP−/− populations (3.94%) compared with resting nFLIP+/− cells (0.67%; Fig. 5 B). Collectively, the defective TCR response of nFLIP−/− 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 nFLIP−/− 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 nFLIP−/− 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 nFLIP−/− T cells, similar to what was observed in caspase-8−/− 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 signals. 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 with or without 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 nFLIP−/− 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 robust and independent assessment of cFLIP functions in T cell lineage, but the deletion of cFLIP is restricted to and after a certain developmental stage; e.g., lck-Cre deletes the targeted gene from the late double negative stage 2 of thymocyte development onward (29). In this regard, the Rag−/− complementation approach provides 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 through FasL/Fas, or TL1A/DR3 have been reported to be costimulatory for TCR activation (31, 32). However, a recent report suggested that the subcellular compartmentalization of FADD and caspase-8 induced by TCR stimulation is distinct from that triggered by DR ligation (33). In addition, T cells lacking competent Fas and TNFRI proliferation normally in response to TCR activation (34).

It is also possible that the three DED proteins play a role downstream of IL-2 signaling, secondary to TCR activation, although STAT5 phosphorylation appears normal in activated FADD-DN T cells (19). Many of the TCR signaling pathways leading to IL-2 production are reported to be normal in caspase-8−/− (11), FADD-DN (16), and cFLIP−/− T cells, except for a defect of calcium flux in FADD-DN cells (35). Furthermore, supplementation of TCR stimulation with IL-2 does not rescue proliferation in these mutant cells (11, 14, 17, 19). Alternatively, some DED proteins may somehow communicate with other TCR signaling complexes. One potential link came from a recent study showing that caspase-8 and FADD could interact with Bcl-10 and Malt-1, at least in a human T cell line, and play a role in the NF-κB signaling pathway (36). Whether or not cFLIP is involved in this association remains to be investigated.

It is possible that the DED proteins may collaborate downstream of TCR triggering. However, an alternative scenario of mutual regulation among these proteins, as in DR apoptotic signals, remains possible so that too much or too little of the signals mediated by these DED proteins would be detrimental to T cell proliferation. Indeed, the phenotypes of FADD−/−, caspase-8−/−, and cFLIP−/− T cells each exhibit its unique features. Further studies on the downstream impact of the DED protein signaling complex are important to sort out this issue.

One intriguing hint on the mechanism of the DED protein signaling is that during T cell proliferation, FADD appears to be regulated by phosphorylation at serine 191 (S191, murine FADD) (37). Consistently, an unknown kinase has been reported to phosphorylate FADD during the G2/M phase of cell cycling (37). T cells expressing FADD bearing constitutively phosphorylated S191 (FADD S191D) reproduced the cell cycle defect observed in FADD−/− T cells but had normal Fas-induced apoptosis (38). Studies of FADD phosphorylation may reveal the key to uncoupling the regulation of apoptosis and proliferation mediated by cFLIP, FADD, and caspase-8.

**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 leukaemia 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 × 10⁵ E14K ES cells (129J mouse background) were electroporated (0.34 kV, 250 μF) with 5 nM 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′-CTT ATG CAT AAA GGG CTG GCA AGA TG-3′) and to a portion of the endogenous 5′ piece of the short arm (primer sequence: 5′-CAA ATT ATT AAG GGC CAG CTC CCT CCT CC-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. fFLIP\textsuperscript{++} and fFLIP\textsuperscript{++} ES cell clones were injected into E3.5 Rag\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.** 20 \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 \textsuperscript{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\textsuperscript{+} only T cells, according to the manufacturer’s instructions (Miltenyi Biotec).

**[H]Thymidine proliferation assay.** 10\textsuperscript{5} 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 anti-CD28 antibodies for at least 3 h at 37\degree C. Wells 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 [\textsuperscript{3}H]thymidine per well. The amount of [\textsuperscript{3}H]thymidine incorporated by the cells was measured with a direct \beta-counter (Matrix 96; Canberra Packard).

**Cell death assay.** 10\textsuperscript{5} 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 phase (i.e., G0/G1, S, G2/M phase) by flow cytometry.

**CFSE labeling of T cells.** Purified CD4\textsuperscript{+} T cells were labeled with CFSE as previously described (24). In brief, cells were resuspended at a density of 20 \times 10\textsuperscript{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 fFLIP\textsuperscript{++} and fFLIP\textsuperscript{++} T cells were plated at 4 \times 10\textsuperscript{5} and 12 \times 10\textsuperscript{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\textsuperscript{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 \mu g/ml each of PMA (Sigma-Aldrich) plus calcium ionophore (A23187; Sigma-Aldrich). After incubation at 37\degree C for 5, 15, and 60 min (10 min for PMA plus ionophore), the cells were washed in PBS containing 10 mM Na\textsubscript{2}VO\textsubscript{4} and lysed in ice-cold lysis buffer (50 mM Tris, pH 8, 250 mM NaCl, 1% Triton X-100, 20 mM EDTA, 1 mM Na\textsubscript{2}VO\textsubscript{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.** 3 \times 10\textsuperscript{5} purified CD4\textsuperscript{+} 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\degree C for 3, 6, 12, 24, and 48 h, after which the cells were pelleted and lysed in radioimmunoprecipitation assay buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na\textsubscript{2}VO\textsubscript{4}, 10 mM NaF, and 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 (Apotex), FADD (a gift from D. Goeddel, Z. Cao, and G. Chen, Tulark/Amen, 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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