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Conditional Fas-Associated Death Domain Protein (FADD): GFP Knockout Mice Reveal FADD Is Dispensable in Thymic Development but Essential in Peripheral T Cell Homeostasis

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Fas-associated death domain protein (FADD)/mediator of receptor-induced toxicity-1 is required for signaling induced by death receptors such as Fas. In earlier studies, FADD-deficient mice died in utero, and a FADD deficiency in embryonic stem cells inhibited T cell production in viable FADD−/−→RAG-1−/− chimeras. To analyze the temporal requirement of FADD in the development and function in the T lineage, it is necessary to establish viable mutant mice producing detectable FADD-deficient T cells. We generated mice that express a functional FADD:GFP fusion gene reconstituting normal embryogenesis and lymphopoiesis in the absence of the endogenous FADD. Efficient T cell-specific deletion of FADD:GFP was achieved, as indicated by the presence of a high percentage of GFP-negative thymocytes and peripheral T cells in mice expressing Lck-Cre or CD4-Cre. Sorted GFP-negative thymocytes and peripheral T cells contained undetectable levels of FADD and were resistant to apoptosis induced by Fas, TNF, and TCR restimulation. These T cell-specific FADD-deficient mice contain normal thymocyte numbers, but fewer peripheral T cells. Purified peripheral FADD-deficient T cells failed to undergo extensive homeostatic expansion after adoptive transfer into lymphocyte-deficient hosts, and responded poorly to proliferation induced by ex vivo TCR stimulation. Furthermore, deletion of FADD in preactivated mature T cells using retrovirus-Cre resulted in no proliferation. These results demonstrate that FADD plays a dispensable role during thymocyte development, but is essential in maintaining peripheral T cell homeostasis and regulating both apoptotic and proliferation signals. The Journal of Immunology, 2005, 175: 3033–3044.

Proliferation and apoptosis (or programmed cell death) are induced at various stages during lymphocyte development and immune responses (1–3). For example, signal transduction induced by the pre-TCR results in proliferation of CD4−CD8− double-negative (DN) T cells and subsequent differentiation into CD4+CD8+ double-positive (DP) T cells in the thymus (4). At the DP stage, autoreactive T cells are eliminated by apoptosis (5, 6), and those that evade this thymic-negative selection process are deleted in the periphery primarily by activation-induced cell death (AICD) (7, 8). In normal and healthy mice, the peripheral naive T cell pool is maintained at a steady state by homeostatic mechanisms, which in part involve TCR and self peptide/MHC interaction (9, 10). During an immune response, Ag-specific T cells are induced to proliferate and differentiate into effector cells, and AICD is required in the subsequent contraction phase to eliminate activated T cells (2, 3, 5). One of the major players involved in negative selection and AICD is Bim, the proapoptotic member of the Bcl-2 family proteins (11, 12). The death receptor (DR) Fas (Apo-1 or CD95) also plays an important role in lymphocyte apoptosis required for maintaining homeostasis in the immune system (13, 14). Mutations in the Fas gene result in a lymphoproliferative syndrome characterized by lymphadenopathy as well as autoimmune diseases (15–17).

The Fas-associated death domain-containing protein (FADD) was initially identified as an adaptor required for cell death signal transduction initiated by Fas (18–20). Subsequent studies indicated that FADD is also involved in signaling induced by other DRs such as TNFR-I, TRAIL receptors (or DR4/5), and DR3 (21–27). FADD contains two protein-protein interaction structures: the death domain (DD) at the carboxy terminus and the death effector domain (DED) at the amino terminus. The DD of FADD binds to a similar DD located within the intracellular tail of Fas, whereas the DED of FADD associates with the DED present in procaspase-8 (28, 29). Cell death signaling is initiated by clustering of Fas induced by engagement of the trimeric Fas ligand (FasL). As a result, a death-inducing signaling complex containing FasL, Fas, FADD, and procaspase-8 is assembled (30), and aggregation of procaspase-8 in the death-inducing signaling complex facilitates its autoproteolysis. After assembly of the resulting subunits to become a fully active cysteine protease, caspase-8 processes and activates downstream caspases, leading to apoptotic cell death.

The in vivo function of FADD has been previously investigated by gene targeting in germ cells (31, 32). The resulting FADD-deficient (FADD−/−) mice died in utero, an unexpected phenotype given the normal development of mice lacking Fas, TNFR-I,
TRAIL receptor, or DR3 (33–37). In an alternative approach, homozygous FADD−/− embryonic stem (ES) cells were generated and injected into blastocysts of lymphocyte-deficient RAG−/− mice (31). The resulting viable FADD−/−→Rag−/−/− mice produced few thymocytes and an undetectable level of B cells. In other studies, a truncated FADD (FADD-DD) containing the DBD but lacking the DED was shown to block evivo apoptotic responses (20, 22), and expression of FADD-DD specifically in T cells was shown to block ex vivo apoptotic responses (20, 22), and expression of FADD-DD specifically in T cells was shown to block ex vivo apoptotic responses. The data obtained from analysis of FADD-deficient T cells were readily detected as the very few FADD-deficient T cells recovered in FADD−/−→Rag−/−/− mice (31). The resulting viable FADD−/−→Rag−/−/− mice were backcrossed with wild-type C57BL/6 (B6) mice for five to six generations. Heterozygous FADD knockout (FADD+/−) mice, generated in a previous study (31), were crossed to B6 mice for >12 generations. The FADD-GFP transgene was then crossed into FADD−/−/− mice, and the resulting FADD−/−/− FADD-GFP mice were backcrossed with FADD−/−/− mice to produce viable FADD−/−/−FADD-GFP+ mice. To generate T cell-specific FADD-deficient mice, Lck-Cre and CD4-Cre transgenes were crossed with Taconic, and were crossed with FADD−/−/− mice. The resulting FADD−/−/− Lck-Cre+ or FADD−/−/−CD4-Cre+ mice were crossed with FADD−/−/−FADD-GFP+ mice to generate FADD−/−/−FADD-GFP+ Lck-Cre+ or FADD−/−/−FADD-GFP+ CD4-Cre+ mice. For genotyping, Southern blot analysis was performed using mouse tail DNA and the 0.4-kb probe, which detects the endogenous and knockout alleles as well as FADD-GFP as EcoRI fragments of different sizes. The Lck-Cre and CD4-Cre transgenes were detected by PCR-based genotyping using mouse ear tissue lysates and Cre-specific primers (5′-CCACGTCAAACATGCTATCATGC-3′ and 5′-CCTGATCCGTGGCAATTTCGG-3′). All of the animal studies were approved by the Institutional Review Board at Thomas Jefferson University. Flow cytometry Lymphocytes were isolated from the thymus, spleen, and lymph nodes of 2- to 7-mo-old mice. To determine GFP expression, single-cell suspensions were directly subjected to flow cytometric analysis using a Coulter Epics XL analyzer (Beckman Coulter). To detect the expression of CD4 and CD8, lymphocytes were stained on ice for 20–30 min with fluorescein-conjugated Abs (Caltag Laboratories) in PBS containing FBS (1%) and sodium azide (0.05%). The WinMDI software (U. Trotter, The Scripps Institute, La Jolla, CA) was used for generating histograms and dot-plots. MoFlo high-speed cell sorters (DakoCytomation) at Kimmel Cancer Center at Thomas Jefferson University and the Wistar Institute were used to isolate GFP+ and GFP− T cells. Cell purity was typically >90%. Western blot analysis Total thymocytes, splenocytes, and lymph node cells were isolated from mice of various genotypes. GFP+ and GFP− cells were purified from the spleen and lymph nodes by high-speed cell sorting. Activated GFP+ and GFP− T cells were prepared by stimulation with anti-CD3 and anti-CD28 Abs for 2 days and incubation for another 2 days in the presence of IL-2 (see below). Cells were washed with ice-cold PBS and resuspended in a lysis buffer containing Tris-HCl (50 mM; pH 8.0), NaCl (150 mM), EDTA (1 mM), Nonidet P-40 (1%; Calbiochem), PMSF (1 mM; Sigma-Aldrich), pepstatin A (0.7 μg/ml; Roche Biochemical Laboratories), and a protease inhibitor mixture (Roche Biochemical Laboratories). After incubation on ice for 30 min, cell lysates were collected after a 5-min centrifugation (14,000 × g) at 4°C, and protein concentrations were determined using a Bio-Rad kit (Bio-Rad). Proteins (20 μg) were resolved by a 10% SDS-PAGE and blotted to Protran nitrocellulose membranes (Schleicher & Schuell Microscience), followed by staining with Ponceau S solution (Sigma-Aldrich) to assure equal loading and transfer. Rabbit polyclonal anti-FADD Abs and reaction conditions have been described elsewhere (20). The Western Lighting Chemiluminescence Reagent Plus (PerkinElmer) was used to detect signals on x-ray films (Kodak). Cell culture T cells were grown in RPMI 1640 medium (Mediatech) supplemented with FBS (10%), penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (2 mM), and 2-ME (0.25 mM). All of the media supplements were purchased from Mediatech. Cells were incubated at 37°C in the presence of CO2 (5%). The IL-2-secreting cells were provided by Dr. F. Melchers (Max Planck Institute for Infection Biology, Berlin, Germany) and grown
in RPMI 1640 medium with the above-mentioned supplements. After the culture reaches saturation, the IL-2-containing supernatant was collected and used at a 1/50 dilution in T cell cultures.

**Cell death assay**

Thymocyte killing was performed as described previously (48). Various amounts of anti-Fas Abs (Jo2; BD Pharmingen) and cycloheximide (30 μg/ml; Sigma-Aldrich) were added into triplicate wells (10^6 of thymocytes/well) in a 96-well plate. Sixteen hours after treatment, cell death was determined by propidium iodide (PI; Sigma-Aldrich) uptake assays as described previously (20). Resting GFP+/FADD− T cells were purified from the spleen and lymph nodes by sorting and seeded to 96-well plates (10^5/well). sFasL (Alexis) was added at various concentrations from the spleen and lymph nodes by sorting and used at a 1/50 dilution in RPMI 1640 medium containing supplements (see above) at a concentration of 6 × 10^6/ml. This cell suspension was added to a 6-well plate (4 ml/well) and stimulated with soluble anti-CD3 (1/1000 dilution) and anti-CD28 (1/1000 dilution) Abs. After 18-h incubation at 37°C, these activated cells were washed with and resuspended in RPMI 1640 medium (6 × 10^6/ml). This cell suspension was added into wells in triplicate. Cell death was determined by PI uptake and analyzed by flow cytometry.

**Adaptive transfer**

B6.RAG-1−/− mice were obtained from The Jackson Laboratory and were subject to a 500-rad irradiation. GFP+ donor T cells were isolated from the spleen and lymph nodes of FADD−/− FADD:FADD-GFP+/− Lck-Cre+ and FADD−/− FADD:FADD-GFP+/− Lck-Cre+ mice, which were crossed to B6 mice for at least five generations. Cells were resuspended at a concentration of 4 × 10^6/ml in PBS + FBS (5%), and mixed with equal volumes of CFSE (20 μM; Molecular Probes) in PBS + FBS. After incubation for 25°C for 5 min, the labeled cells were washed three times with PBS + FBS, once with PBS, and were injected i.v. (4–10 × 10^6 cell/mice) into irradiated B6.RAG-1−/− and nonirradiated B6 mice. Three days after injection, cells were recovered from the spleen and lymph nodes and analyzed using a flow cytometer.

**T cell proliferation assays**

Ninety-six-well plates were coated with anti-CD3 ascites (1/1000 dilution; clone 500A2). GFP+ and GFP− cells were purified from the spleen and lymph nodes by high-speed sorting, and 10^5 cells were seeded into each well in 100 μl of RPMI 1640 medium. Anti-CD28 ascites (1/1000 dilution; clone 37.51) were then added into wells when necessary. Forty hours post-stimulation, 1 μCi of [3H]thymidine (ICN Biochemicals) was added into each well, followed by additional 8–10-h incubation. Incorporated [3H]thymidine in each well was determined using a Wallac beta counter (PerkinElmer). Data were obtained from triplicate samples for each treatment. To ensure that the GFP tag would not affect the function of FADD, we performed analyses using FADD-deficient (FADD−/−) mouse embryonic fibroblasts (MEFs) (26). The GFP gene was cloned at the 3′ end of the FADD cDNA, and the resulting FADD-GFP fusion cDNA was introduced into FADD−/− MEFs by retrovirus-mediated gene transfer. These FADD-GFP-reconstituted FADD−/− MEFs were killed by FasL as effectively as those reconstituted with an untagged FADD (data not shown), indicating that the FADD-GFP fusion protein functions similarly to the wild-type FADD protein in transducing the death signal initiated by Fas.

To express FADD-GFP in mice, we used a 12-kb genomic DNA fragment isolated from the MIEG3-Cre plasmid (provided by D. Williams, University of California, Berkeley, CA). A nuclear localization signal peptide was linked to the NH2 terminus of Cre, and the resulting fusion gene was then cloned into a murine stem cell virus (MSCV) vector (provided by W. Pear, University of Pennsylvania, Philadelphia, PA). For virus packaging, the resulting MSCV-Cre plasmid was cotransfected along with the helper plasmid pCL-Eco (Invitrogen) into HEK 293T cells. Two to 3 days after transfection, the virus-containing supernatant was collected and frozen at −80°C. Total splenic and lymph node cells were isolated from FADD−/− FADD-GFP+ mice and suspended in RPMI 1640 medium containing supplements (see above) at a concentration of 6 × 10^6/ml. This cell suspension was added to a 6-well plate (4 ml/well) and stimulated with soluble anti-CD3 (1/1000 dilution) and anti-CD28 (1/1000 dilution) Abs. After 18-h incubation at 37°C, these activated cells were washed with and resuspended in RPMI 1640 medium (6 × 10^6/ml). This cell suspension was added into wells in triplicate. Cell death was determined by PI uptake and analyzed by flow cytometry.

**Results**

**FADD-GFP functions indistinguishably from FADD in vivo**

To ensure that the GFP tag would not affect the function of FADD, we performed analyses using FADD-deficient (FADD−/−) mouse embryonic fibroblasts (MEFs) (26). The GFP gene was cloned at the 3′ end of the FADD cDNA, and the resulting FADD-GFP fusion cDNA was introduced into FADD−/− MEFs by retrovirus-mediated gene transfer. These FADD-GFP-reconstituted FADD−/− MEFs were killed by FasL as effectively as those reconstituted with an untagged FADD (data not shown), indicating that the FADD-GFP fusion protein functions similarly to the wild-type FADD protein in transducing the death signal initiated by Fas.

To express FADD-GFP in mice, we used a 12-kb genomic DNA fragment isolated from the mouse FADD locus containing two coding exons (Fig. 1A). This FADD minigene, when present as a transgene, can restore normal development in FADD−/− mice lacking the endogenous alleles of FADD (20, 45). We cloned GFP

![FIGURE 1. FADD-GFP reconstitutes normal development in FADD−/− mice. A. Diagrams of the wild-type (WT) FADD gene consisting of two exons (boxes 1 and 2); the knockout allele (K/O); and the FADD-GFP construct. LoxP sites and the 0.4-kb probe used for genotyping by Southern blots are indicated. RI, EcoRI; RV, EcoRV. B. Genotyping by Southern blot analysis using mouse tail DNA identified various genotypes present in the offspring of crosses between FADD−/− and FADD−/− FADD-GFP+ mice. FADD−/− mice can become viable and develop normally only when FADD: GFP is present. C. Anti-FADD Abs detected the FADD and FADD-GFP proteins in thymocytes, splenocytes, and lymph node cells from mice of various genotypes by Western blot analysis. FADD-GFP differs in size from the endogenous FADD, which is absent in FADD−/− FADD-GFP+ mice.](http://www.jimmunol.org/)
at the 3' end of the FADD coding region within exon 2 in the 12-kb FADD minigene and inserted two LoxP sites flanking the coding region of the FADD:GFP fusion (Fig. 1A). The resulting DNA construct was injected into mouse embryos, and 15 transgenic lines were generated. Genotyping by Southern blot analysis revealed the FADD:GFP fusion gene as a 5.4-kb EcoRI fragment, distinguishable from the 4.7-kb fragment representing the endogenous FADD alleles (Fig. 1B, lane 1). Western blot analysis was performed on mice carrying FADD:GFP to detect expression of the FADD:GFP fusion protein. As shown in Fig. 1C, anti-FADD Abs detected a band of 56 kDa in cells isolated from the thymus, spleen, and lymph nodes, and this protein is absent in control heterozygous FADD+/− mice. This is the expected size of the full-length FADD:GFP fusion protein and is distinct from the size of the endogenous FADD (27 kDa; Fig. 1C). A monoclonal anti-GFP Ab detected only the 56-kDa protein band (data not shown). Expression of FADD:GFP was also detectable by flow cytometric analyses, as indicated by the single GFP+ peak in histograms of thymocytes, spleen, and lymph node cells in mice carrying FADD:GFP (Fig. 2).

To determine whether expression of FADD:GFP would complement a FADD deficiency, we introduced FADD:GFP into FADD−/− mice in which the endogenous FADD gene was inactivated in germ cells by deletion of the promoter region and exon 1 (Fig. 1A) (31). Because FADD−/− mice die at around day 10 of gestation, we first crossed FADD:GFP into viable heterozygous FADD+/− mice. The resulting FADD−/−/FADD:GFP+ mice were backcrossed with FADD+/− mice. Genotypes of the offspring were determined by Southern blotting (Fig. 1B), and viable homozygous knockout FADD−/− mice containing FADD:GFP were detected at expected Mendelian genetic frequencies. These FADD−/− FADD: GFP+ mice showed no obvious abnormalities at various stages during development, indicating that FADD:GFP functions similarly to endogenous FADD and can restore normal embryogenesis in FADD−/− mice. To analyze the immune system of FADD−/− FADD:GFP+ mice, the thymus, spleen, and lymph nodes were dissected and found to be similar in size and cellularity to those of wild-type FADD+/+ and heterozygous FADD+/− FADD:GFP+ mice (data not shown). Flow cytometric analysis revealed uniform expression of FADD:GFP, indicated by a single GFP+ peak in thymocytes, spleen, and lymph node cells (Fig. 2, top). Additionally, Western blot analysis confirmed expression of the FADD:GFP protein (56 kDa) and absence of the endogenous FADD protein (27 kDa; Fig. 1C). Flow cytometric analysis revealed that FADD−/− FADD:GFP+ mice contain DN, DP, and SP subpopulations of thymocytes similar to those in FADD+/+ mice (data not shown). Therefore, the GFP tag does not appear to affect the function of FADD in lymphocyte development, and FADD−/− FADD:GFP+ mice were used frequently as controls in subsequent experiments.

**T cell-specific deletion of FADD:GFP**

To induce deletion of the FADD:GFP fusion gene in DN immature thymocytes, we crossed the Lck-Cre transgene (47) into FADD−/− mice. The resulting FADD+/− Lck-Cre mice were then crossed with FADD−/− FADD:GFP+ mice, and litters in the offspring were analyzed by flow cytometry. A major GFP+ cell population (80–95%) was detected in the thymus of FADD−/− FADD: GFP+ Lck-Cre+ mice, which was not present in mice lacking

![FIGURE 2](http://www.jimmunol.org/) Detection of the expression and deletion of FADD:GFP by flow cytometric analysis. Gene deletion efficiency in various lymphoid organs was indicated by the percentage of GFP+ cells detected in FADD−/− FADD:GFP+ mice containing either Lck-Cre or CD4-Cre. Cells from FADD+/− FADD:GFP+ mice (top) were used as GFP+ controls, which uniformly express FADD:GFP as indicated by a single right histogram peak (shaded). Cells from wild-type FADD+/+ mice were used as GFP− controls (bottom). Using these two different controls, GFP+ cells in mice expressing either Lck-Cre or CD4-Cre were determined to be those represented by areas shaded in histograms (two middle rows).
Lck-Cre (Fig. 2). Western blot analyses were then performed with total thymocytes, showing dramatically reduced levels of the FADD:GFP fusion protein in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> and FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice in comparison with control mice lacking Lck-Cre (Fig. 3A). The remaining FADD:GFP protein may be that expressed in the 10–20% of GFP cells in the thymus (Fig. 2). GFP<sup>−</sup> cells were also detected in the spleen (16%) and lymph nodes (44%) in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice (Fig. 2), and these cells express the T cell marker CD3 (data not shown). When total peripheral lymphocytes were analyzed by Western blots, reduction of the FADD:GFP protein in FADD<sup>+/+</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> or FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice was not as obvious as that detected in thymocytes (Fig. 3A), probably due to the presence of higher percentages of GFP<sup>−</sup> cells in the spleen (84%) and lymph nodes (56%) (Fig. 2). These peripheral GFP<sup>+</sup> populations are mostly B220-expressing B cells (data not shown). GFP<sup>−</sup> T cells were isolated from the thymus, spleen, and lymph nodes by FACS, and analyzed by Western blotting. As shown in Fig. 3B, these purified GFP<sup>−</sup> T cells contain undetectable levels of the FADD:GFP protein in comparison with control GFP<sup>+</sup> T cells isolated from FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice. After activation and growth in culture for several days, the FADD:GFP protein remains undetectable in mature GFP<sup>−</sup> T cells (Fig. 3B). Thus, the GFP<sup>−</sup> population in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice represents FADD-deficient (FADD<sup>−/−</sup>) T cells.

We also introduced into FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice the CD4-Cre transgene, which is expressed at the DP stage in the thymus (47). Unlike FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice, which contain a discrete GFP<sup>−</sup> population in the thymus, FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> CD4-Cre<sup>−</sup> mice contained thymocytes expressing various levels of FADD:GFP, indicated by a GFP-peak shift in flow cytometric histograms (Fig. 2). In the periphery, however, GFP<sup>−</sup> cells were detected as a distinct population in the spleen (43%) and lymph nodes (23%) in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> CD4-Cre<sup>−</sup> mice (Fig. 2). The FADD:GFP protein was undetectable in sorted peripheral GFP<sup>−</sup> cells by Western blot analysis (data not shown). These results indicate that T cells are not completely FADD-deficient until the FADD:GFP protein, synthesized before CD4-Cre-mediated gene deletion at the DP stage, is degraded during the process of maturation and subsequent migration to the periphery.

FADD deficiency abrogates cell death in T cells

To determine Fas-induced cell death responses in FADD-deficient immature T cells, total thymocytes were prepared from FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice and found to contain 80–97% of GFP<sup>−</sup> cells. These thymocytes were cultured in the presence of anti-Fas Abs, which induce cell death preferentially in the DP population (48). Although control thymocytes from FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice were killed in a dose-dependent manner, FADD-deficient thymocytes are highly resistant to stimulation with various concentrations of anti-Fas Abs (Fig. 4A). Although resting mature T cells are resistant to stimulation with anti-Fas Abs, they are readily killed by soluble FasL (sFasL) (49). To analyze cell death response in resting mature T cells, GFP<sup>−</sup> T cells were isolated from the spleen and lymph nodes in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice, and these cells were killed in a dose-dependent manner by sFasL (FADD:GFP<sup>+</sup>; Fig. 4B). In contrast, GFP<sup>−</sup> (FADD<sup>−/−</sup>) T cells isolated from the spleen and lymph nodes in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>−</sup> mice were resistant to stimulation by sFasL at various concentrations (Fig. 4B).

Repeated stimulation of the TCR results in death in activated T cells, a process called AICD involving the action of FasL and TNF (50–52). To determine cell death responses in activated T cells, GFP<sup>−</sup> and GFP<sup>+</sup> T cells were isolated by FACS from the spleen and lymph nodes in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice with or without the presence of the Lck-Cre transgene, respectively. These mature T cells were activated by stimulation with anti-CD3 and anti-CD28 Abs for 2 days and cultured for an additional 2 days in the presence of IL-2 to sensitize cells for Fas-induced death (53). These activated T cells were then treated with increasing concentrations of anti-Fas Abs. This treatment induced a dose-dependent cell death in activated T cells (50, 54). We prepared activated FADD-deficient and control mature T cells as described above by stimulation using anti-CD3 and anti-CD28 Abs. At a concentration of 50 ng/ml, TNF induced 20% cell death in control-activated T cells (FADD:GFP<sup>+</sup>) and <5% cell death in activated FADD-deficient T cells (FADD:GFP<sup>−</sup>), as indicated by the dramatic reduction of the FADD:GFP protein in activated FADD-deficient thymocytes and mature resting and activated T cells was confirmed by anti-FADD Western blots. GFP<sup>−</sup> T cells isolated from FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice were used as controls. Ponceau S staining shown at the bottom in panels A and B indicates equal loading and transfer of proteins.
death in mutant-activated T cells (FADD$^{-/-}$; Fig. 4D). To examine AICD responses, activated splenic and lymph node T cells were prepared as in Fas-induced cell death assays by stimulation with anti-CD3 and anti-CD28 Abs and a 2-day incubation in the presence of IL-2. After restimulation with anti-CD3 Abs, >40% death was induced in FADD:GFP$^+$ control T cells, whereas <5% death was detected in FADD-deficient T cells (Fig. 4D).

T cell-specific FADD-deficient mice contain normal thymocyte populations but have a homeostatic defect in peripheral T cells

We analyzed the thymus, spleen, and lymph nodes in 1- to 7-month-old FADD$^{-/-}$ FADD:GFP$^+$ mice containing either the Lck-Cre or CD4-Cre transgenes, and these lymphoid organs appear to be normal in size and total cellularity in comparison with littermate control mice containing one endogenous FADD allele (FADD$^{+/+}$ FADD:GFP$^+$ Lck-Cre$^+$ or CD4-Cre$^+$) (data not shown). T cell subpopulations were further analyzed by flow cytometry after staining for the CD4 and CD8 coreceptors. The DN, DP, and SP thymocytes were present in FADD$^{-/-}$ FADD:GFP$^+$ Lck-Cre$^+$ mice in a pattern similar to that detected in littermate control FADD$^{+/+}$ FADD:GFP$^+$ Lck-Cre$^+$ mice (Fig. 5). Normal thymocyte subsets were also detected in FADD$^{-/-}$ FADD:GFP$^+$ CD4-Cre$^+$ mice (Fig. 5). When peripheral T cells were analyzed by flow cytometry, a reduction in the number of both CD4$^+$ and CD8$^+$ T cells was detected in the spleen and lymph nodes from FADD$^{-/-}$ FADD:GFP$^+$ mice containing either Lck-Cre or CD4-Cre (Fig. 5). Among the FADD$^{-/-}$ FADD:GFP$^+$ Lck-Cre$^+$ mice analyzed, there is an average 46% reduction in the number of peripheral T

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**FIGURE 4.** FADD-deficient T cells are resistant to cell death induction. FADD$^{-/-}$ cells were the GFP$^+$ population isolated from FADD$^{+/+}$ FADD:GFP$^+$ Lck-Cre$^+$ mice. Littermate FADD$^{+/+}$ FADD:GFP$^+$ mice were used to isolate the control GFP$^+$ T cell population (FADD:GFP$^+$). Error bars represent ±SD of the means from three to five mice of each genotype in each treatment. A, Thymocytes were stimulated with various concentrations of Fas Abs to induce cell death. B, Cell death was induced in resting mature T cells by treatment with various concentrations of sFasL. Proliferating T cells, activated by stimulation with anti-CD3 and anti-CD28 Abs, were incubated with various concentrations of anti-Fas Abs (C), TNF (50 ng/ml), or anti-CD3 Abs (D) to induce cell death. Percentage cell death is indicated on y-axis.
cells in comparison to FADD+/− FADD:GFP+ Lck-Cre+ littermate controls (Tables I and II). In the FADD−/− FADD:GFP+ CD4-Cre+ mice analyzed, the peripheral T cell number is ~70% of that in littermate control FADD+/− FADD:GFP+ CD4-Cre+ mice. Concomitant with the reduction in the peripheral T cell number, there was an increase in the number of B cells in the periphery (Fig. 5 and data not shown). This is most likely a result of homeostatic proliferation of FADD-expressing B cells when there is a decrease in the T cell population.

The overall size and composition of the naive T cell pool are controlled by homeostasis mechanisms (9, 10). The reduced peripheral T cell number phenotype detected in the T cell-specific FADD-deficient mice prompted us to examine whether FADD-deficient T cells have a defect in homeostatic responses. In a lymphopenic environment, wild-type T cells tend to fill the “space” by expansion or homeostatic proliferation. We performed adoptive transfer experiments using RAG-1−/− mutant mice that lack B and T cells. Donor FADD-deficient (FADD−−) T cells were isolated by sorting for the GFP+ population from the spleen and lymph nodes of FADD−/− FADD:GFP+ Lck-Cre+ mice. For controls, FADD+/− T cells containing a single allele of the endogenous FADD gene were isolated by sorting for the GFP+ population from littermate FADD+/− FADD:GFP+ Lck-Cre+ mice. Purified mature T cells were labeled with CFSE, and the fluorescent intensity of CFSE is progressively reduced due to partitioning of labeled intracellular molecules from the mother cell into daughter cells during cell divisions. Three days after adoptive transfer into RAG-1−/− mice, cells were isolated from the spleen and lymph nodes and analyzed by flow cytometry. As indicated in Fig. 6, 29% of FADD-deficient T cells had divided once or twice in the lymph nodes of RAG-1−/− mice, less than that detected in control FADD+/− T cells (55.4%; Fig. 6). The third cell division was detectable in FADD+/− T cells, but not in FADD-deficient T cells. There were more undivided FADD-deficient T cells (68.3%) than undivided FADD+/− T cells (37.5%) in the lymph nodes. Similar homeostatic proliferation defects were also detected in the spleen in RAG-1−/− hosts (Fig. 6). Therefore, FADD deficiency appears to inhibit homeostatic expansion of peripheral T cells. As controls, CFSE-labeled cells were also injected into nonirradiated wild-type B6 mice, in which there was an undetectable level of division of transferred FADD−/− and FADD+/− T cells due to the presence of the wild-type lymphocyte pool in the periphery (Fig. 6).

FADD is required for ex vivo TCR-induced proliferation

To analyze TCR-induced proliferation responses, FADD-deficient T cells were isolated by sorting for the GFP+ population from the spleen and lymph nodes in FADD−/− FADD:GFP+ Lck-Cre+ mice, and stimulated with anti-CD3 Abs. FADD+/− T cells were isolated from age- and sex-matched wild-type mice and used as controls. Two days after stimulation, [3H]thymidine was added into each culture, followed by an additional 8-h incubation. The relative numbers of cells accumulated in each culture were indicated by the amount of [3H]thymidine incorporation. In comparison with FADD+/− T cells, proliferation of FADD-deficient T cells was reduced by 56% (Fig. 7A). When T cells were stimulated with both anti-CD3 and anti-CD28 Abs, FADD-deficient T cells only reached 40% of the wild-type T cell proliferation capacity, indicating that costimulation provided by CD28 signaling could not overcome the TCR-induced proliferation defect. A direct assessment of proliferation was performed by determining the total

Table I. Reduced peripheral T cell numbers (means of nine mice of each genotype ±SD) in Lck-Cre-induced FADD-deficient mice

<table>
<thead>
<tr>
<th></th>
<th>FADD+/+ (×10^6)</th>
<th>FADD−− (×10^6)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>Thymus</td>
<td>4.6 ± 1.1</td>
<td>0.9 ± 0.4</td>
</tr>
<tr>
<td>SPLN</td>
<td>14.4 ± 3.2</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>LN</td>
<td>7.9 ± 1.9</td>
<td>3.1 ± 0.9</td>
</tr>
</tbody>
</table>

* SPLN, Spleen; LN, lymph node.

Table II. Reduced peripheral T cell numbers (means of three mice of each genotype ±SD) in CD4-Cre-induced FADD-deficient mice

<table>
<thead>
<tr>
<th></th>
<th>FADD+/+ (×10^6)</th>
<th>FADD−− (×10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD4+</td>
<td>CD8+</td>
</tr>
<tr>
<td>Thymus</td>
<td>14.1 ± 2.1</td>
<td>3.8 ± 1.8</td>
</tr>
<tr>
<td>SPLN</td>
<td>15.5 ± 2.4</td>
<td>6.9 ± 1.5</td>
</tr>
<tr>
<td>LN</td>
<td>15.3 ± 3.8</td>
<td>9.1 ± 2.3</td>
</tr>
</tbody>
</table>

* SPLN, Spleen; LN, lymph node.
node T cells were isolated from cause MSCV only infects dividing cells, resting spleen and lymph based vector was used to clone the bacterial phage Cre much higher growth rate was observed in control proliferation in FADD-deficient T cells than stimulations with Con A, a mentions with anti-CD3 and anti-CD28 Abs induced a better prolif-ation in FADD-deficient T cells than stimulations with Con A, a much higher growth rate was observed in control FADD:GFP T cells treated with anti-CD3 and CD28 Abs (Fig. 6B).

Defective proliferation may be an indirect effect of abnormal T cell maturation and differentiation when FADD is deleted at earlier DN and DP stages using Lck-Cre and CD4-Cre. To resolve this issue, we induced deletion of FADD:GFP specifically in peripheral T cells, using a retroviral Cre delivery system. A MSCV-based vector was used to clone the bacterial phage Cre gene. Because MSCV only infects dividing cells, resting spleen and lymph node T cells were isolated from FADD−/− FADD:GFP+ mice and were preactivated by stimulation with anti-CD3 and anti-CD28 Abs. The resulting dividing T cells were infected with MSCV-Cre or control MSCV viruses. Two days after infection, GFP+ T cells were isolated by sorting and confirmed to lack the FADD:GFP population isolated from FADD−/− FADD:GFP+ Lck-Cre+ mice, which divided at largely reduced rates. Control FADD−/− T cells were isolated from FADD−/− FADD:GFP+ Lck-Cre+ litters. The percentage of cells that underwent various divisions is indicated in brackets on the graph. CFSE-labeled T cells, adoptively transferred into wild-type B6 hosts, were used as nonproliferation controls (top).

FIGURE 6. Defective homeostatic responses in FADD-deficient T cells. Adoptively transferred FADD-deficient (FADD−/−) T cells were the GFP+ population isolated from FADD−/− FADD:GFP+ Lck-Cre+ mice, which divided at largely reduced rates. Control FADD−/− T cells were isolated from FADD−/− FADD:GFP+ Lck-Cre+ litters. The percentage of cells that underwent various divisions is indicated in brackets on the graph. CFSE-labeled T cells, adoptively transferred into wild-type B6 hosts, were used as nonproliferation controls (top).

cell number in each culture for an extended period of time. Splenic and lymph node T cells were activated for 2 days by stimulation with either Con A or anti-CD3 plus anti-CD28 Abs. During an additional 5-day growth in IL-2-containing media, cell numbers were counted each day, and growth curves of each culture were generated. During the first 24 h, the number of Con A-stimulated FADD-deficient T cells decreased slightly, and then remained at a steady state during the rest of culturing (Fig. 7B). In the control GFP+ T cells (FADD:GFP+) isolated from littermate FADD−/− FADD:GFP+ mice, little growth was detected in the first 24 h, and an exponential growth was induced subsequently. Although treatments with anti-CD3 and anti-CD28 Abs induced a better proliferation in FADD-deficient T cells than stimulations with Con A, a much higher growth rate was observed in control FADD:GFP+ T cells treated with anti-CD3 and CD28 Abs (Fig. 6B).

During a 5-day period, whereas FADD:GFP+ T cells infected with control viruses grew exponentially before a decline at day 4. These results indicate that the proliferation defect is not due to abnormal development, rather it is an indication of a direct involvement of FADD in peripheral T cell function.

To further analyze the defect in FADD-deficient T cells, we determined cell division potential after activation is induced by TCR stimulation. FADD-deficient and FADD+/− T cells were iso-lated from the spleen and lymph nodes in FADD−/− FADD:GFP+ CD4-Cre+ or control littermate FADD+/− FADD:GFP+ CD4-Cre+ mice, respectively, and were uniformly labeled with CFSE. To induce proliferation, CFSE-labeled T cells were stimulated with anti-CD3 and anti-CD28 Abs and analyzed by flow cytometry. Three days after stimulation, <18% of FADD-deficient T cells had divided once or twice, in comparison to >40% in control FADD+/− T cells (Fig. 8, left). Approximately 72% of control FADD+/− T cells and 38% of FADD-deficient T cells had divided on the fourth day after activation (Fig. 8, center). Dividing FADD+/− T cells continued to accumulate on day 5 (88.3%), whereas a high percentage of undivided cells (39%) remained in the FADD-deficient T cell culture. These results indicate that the first division in T cells is dramatically inhibited by a FADD deficiency, and the lower numbers of dividing cells is due to a reduced division potential.

**Discussion**

Several reports have attempted to examine the function of FADD in the immune system. Studies using FADD-deficient ES cells in the RAG-deficient blastocyst complementation model have indicated that FADD is necessary for T cell development and apoptosis (31). However, it is not clear whether FADD is required at specific stages or throughout T cell development. There is a possibility that aberrant proliferation and apoptotic responses in T
cells are indirect effects caused by abnormal development because FADD is not expressed in hemopoietic stem cells in FADD<sup>−/−</sup>→RAG-1<sup>−/−</sup> chimeras. An alternative <i>tFADD</i> mice model was generated in an attempt to disrupt FADD specifically in the T cell lineage (45). In these <i>tFADD</i> mice, there was an accumulation of the DN population and reduction of total cellularity in the thymus. In contrast, T cell-specific deletion of caspase-8, which is immediately downstream of FADD in DR-induced signaling, had no impact on thymocyte development (46), leading to the suggestion of a caspase-8-independent function of FADD in regulating early thymocyte development. Although ES cell-derived peripheral FADD-deficient T cells in FADD<sup>−/−</sup>→RAG-1<sup>−/−</sup> chimeras and FADD-DD transgenic T cells are impaired in TCR-induced proliferation, this defect was not detected in peripheral T cells in <i>tFADD</i> mice (45). It is possible that T cell-specific deletion of <i>tFADD</i> was ineffective, because a reduction of FADD protein expression in T cells was not detectable in <i>tFADD</i> mice expressing Lck-Cre (45). By expressing a functional GFP-tagged FADD in mice lacking the endogenous FADD and then achieving efficient stage-specific deletion of the resulting FADD:GFP fusion gene in T cells, we have been able to identify processes where FADD activity is required in T cells.

Deletion of FADD in >90% of the cells had no obvious effect on total thymic cellularity or subset representations (Fig. 5), indicating that FADD plays a dispensable role during development from DN to DP and SP stages in the thymus. Because a FADD deficiency in ES cells inhibits T cell production in FADD<sup>−/−</sup>→RAG-1<sup>−/−</sup> chimeras (31), FADD must play a more important role at earlier prethymic stages during hematopoiesis. This can be further investigated by induction of FADD:GFP deletion in hemopoietic stem cells using additional Cre-expression systems. Recent studies showed caspase-8 deletion in bone marrow cells resulted in arrest of hemopoietic progenitor functioning (55). Although FADD-deficient thymocytes develop normally, these cells are defective in Fas-induced cell death.

In contrast to the undetectable effect in the thymus, induction of FADD:GFP deletion at the DN stage using Lck-Cre led to a reduction in the size of the peripheral T cell pool (Fig. 5). Deletion of FADD:GFP at later stages using CD4-Cre resulted in a similar defect in the periphery. These results indicate that FADD plays a more important role in mature peripheral T cells than in immature thymocytes. The nature of this FADD function is not clear. It is likely that the reduced peripheral T cell number in FADD-deficient mutant mice is a result of defective homeostatic proliferation, as indicated by results from adoptive transfer experiments using RAG-1-deficient hosts (Fig. 6). Homeostatic responses in naive T cells are regulated in part by signals from TCR in contact with self-MHC/peptide complexes (9, 10). The homeostatic proliferation defect of FADD-deficient T cells could be an indication of abnormal signaling induced by the TCR. In ex vivo TCR stimulation assays, a FADD deficiency induced by the action of Lck-Cre or CD4-Cre dramatically inhibited proliferation responses in mature T cells (Fig. 7). Thus, FADD deficiency may block a key step shared by both the homeostatic and Ag-specific proliferation signaling processes downstream of the TCR. Deletion of FADD using retrovirus-mediated delivery of Cre into mature T cells that have been previously activated also resulted in a reduced growth rate (Fig. 7C), suggesting that proliferation defects are not due to defective development and differentiation of FADD-deficient T cell and that FADD is involved in not only initiation but also maintenance of mitogenic signals induced by TCR.

Fas-induced cell death plays a more important role in AICD in the periphery than in thymic negative selection (56, 57). The absence of lymphoproliferative diseases in FADD-deficient mice has raised the possibility that alternative Fas-induced pathways exist in peripheral T cells. This issue was not resolved previously using FADD<sup>−/−</sup>→RAG-1<sup>−/−</sup> chimeras, which contains few peripheral T cells (31) or in <i>tFADD</i> mice, which appears to contain undeleteable peripheral FADD-deficient T cells (45). Using FADD:

**FIGURE 7.** FADD is required for TCR-induced proliferation responses. 

A. Peripheral T cells were stimulated with anti-CD3 Abs in the presence (+) or absence (−) of anti-CD28 Abs for 2 days and then pulsed with [3H]thymidine for 12 h. Levels of proliferation were indicated by the amount of radioactivity incorporated into T cells. Mature FADD<sup>−/−</sup>/T cells were the peripheral GFP<sup>−</sup> population isolated from FADD<sup>−/−</sup>/FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> mice. Peripheral T cells from wild-type mice (FADD<sup>+/+</sup>) were used as control. B. Peripheral T cells were stimulated for 2 days with anti-CD3 and anti-CD28 Abs (Δ and □) or with Con A (○ and △). The resulting activated T cells were cultured in IL-2-containing media. Growth curves were generated by counting the cell number in T cell cultures at various times after activation. Peripheral FADD<sup>−/−</sup>/T cells were the GFP<sup>+</sup> population isolated from FADD<sup>−/−</sup>/FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> mice. The control peripheral FADD<sup>+/−</sup>/FADD:GFP<sup>+</sup> T cells were isolated from FADD<sup>−/−</sup>/FADD:GFP<sup>+</sup> littermates. C. Deletion of FADD:GFP in activated mature T cells also resulted in a reduced growth rate. FADD:GFP<sup>+</sup> T cells were activated by stimulation with anti-CD3 and anti-CD28 Abs for 16 h to induce cell cycle entry. The resulting activated, dividing T cells were infected with MSCV-Cre or control MSCV viruses. Two days after infection, cells were cultured in fresh IL-2-containing media, and cell number is determined at indicated times. Error bars represent ±SD of the arithmetic means from three to five mice of each genotype in each treatment.
GFP mice, we demonstrated that Fas-induced death was inhibited by >97% in FADD-deficient mature resting and activated T cells (Fig. 4), indicating that alternative FADD-independent Fas-induced pathway is unlikely present in these cells. Although FADD-DN could inhibit cell death induced by TNF in tumor cells (22), whether FADD is involved in TNFR-I signaling in primary T cells had not been determined in previous studies. In this report, activated FADD-deficient T cells were shown to be highly resistant to TNF treatment (Fig. 4D). Although TCR stimulation of resting T cells induces exit from the G0-G1 phase of the cell cycle and subsequent proliferation, the same treatment in proliferating T cells leads to apoptosis. This so-called AICD process is believed to be mediated in part by Fas, and is required in vivo to eliminate self-reactive lymphocytes and Ag-specific effectors during the contraction phase following an immune response. As expected, activated FADD-deficient T cells are highly resistant to AICD induced by a second challenge with anti-CD3 Abs (Fig. 4D). In Fas mutant mice, there is an age-dependent development of lymphadenopathy, obvious at 6 mo of age. We have analyzed FADD−/− FADD:GFP−/− Lck-Cre or CD4-Cre mice of 6–7 mo of age, and none of these mice develop lymphoproliferative diseases similar to those in Fas mutant mice. It is possible that the lymphoproliferation disease, which normally develops in the absence of cell death, was inhibited by the compromised homeostatic and proliferative responses in FADD-deficient T cells. Recently, conditional Fas knockout mice were generated (58). Interestingly, inactivation of Fas in lymphocytes using the Lck-Cre and CD19-Cre transgenes did not lead to lymphoproliferation diseases. However, deletion of Fas in not only lymphocytes but also other cell types by using the IFN-responsive MX1-Cre transgene resulted in the lpr phenotype. It remains to be determined whether deletion of FADD in both lymphocytes and nonlymphoid cells would cause similar lymphoproliferation diseases.

Fas is only one of the many proteins that participate in regulating apoptosis in lymphocytes. The Bcl-2 family members also play a critical role in either inhibiting or activating apoptosis in lymphocytes (59, 60). Bcl-2 can protect cells from apoptosis. However, previous studies have shown that Bcl-2 mediates death pathways distinct from that regulated by Fas. For example, transgenic overexpression of Bcl-2 failed to protect death of activated T cells induced by Fas signaling (61). The BH3-only member of the Bcl-2 family, Bim, has a proapoptotic activity. In Bim-deficient mice, thymocyte apoptosis induced by TCR stimulation is dramatically inhibited, suggesting a role for Bim in thymic negative selection (11). Other studies showed that endotoxin-induced death of activated T cells is largely dependent on the function of Bim (12). In addition, reactive oxygen species apparently are also essential for AICD in T cells (62). Therefore, multiple mechanisms are used to ensure effective deletion of autoreactive lymphocytes and to maintain homeostasis in the immune system.

It is clear that FADD interacts with caspase-8 in signal transduction induced by DR. This and previous studies showed that FADD-caspase-8-deficient mice exhibit overall phenotypic similarity to T cell-specific caspase-8-deficient mice (46), indicating that FADD and caspase-8 also interact in nonapoptotic signaling required for peripheral T cell homeostatic and proliferative responses. At least two additional proteins, FLIP and TRADD, were shown to interact with FADD. Similar to FADD or caspase-8 deficiency, inactivation of FLIP in germ cells also leads to early embryonic lethality in mice (63). Therefore FADD, caspase-8, and FLIP may be part of a novel signaling mechanism independent of
References


