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.

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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-1−/− mice (31). The resulting viable FADD−/−→Rag-1−/− mice produced few thymocytes and an undetectable level of B cells. In other studies, a truncated FADD (FADD-DD) containing the DBL domain but lacking the DED was shown to block ex vivo apoptotic responses (20, 22), and expression of FADD-DD specifically in T cells appears to perturb thymic development in some transgenic mouse lines (38) but not in others (39, 40). Although FADD-deficient thymocytes were resistant to Fas-induced apoptosis, there was no massive accumulation of T cells in the periphery of FADD−/−→Rag-1−/− chimeras (31), unlike that present in Fas-deficient mice. The very few FADD-deficient T cells recovered from the periphery of FADD−/−→Rag-1−/− chimeras proliferated poorly in response to TCR stimulation (31), and peripheral T cells expressing FADD-DD also have abnormal proliferation responses (38, 40–44).

Although there is a better understanding of the molecular process in FADD-mediated apoptosis signaling induced by DRs, little is known about the nature of the potential involvement of FADD in early T cell development and peripheral T cell proliferation responses. In FADD−/−→Rag-1−/− chimeras, FADD is absent in hemopoietic stem cells, and therefore the temporal requirement of FADD during T cell development could not readily be determined. In previously described tFADD mice expressing a LoxP-containing FADD transgene to reverse embryonic lethality caused by a lack of the endogenous FADD, thymic development appeared to be inhibited when the T cell-specific Lck-Cre transgene was expressed (45). Interestingly, T cell-specific deletion of caspase-8, which is known to be immediately downstream of FADD in DR signaling, did not affect thymocyte development (46). Because of the potential involvement of FADD during early hematopoiesis and/or thymic development, it becomes uncertain whether the defect detected in FADD−/−→Rag-1−/− chimeras is indicative of a role for FADD in peripheral T cell proliferation or a secondary effect resulting from abnormal development. Likewise, the FADD−/−→Rag-1−/− chimeras, which are lymphopenic, may not be a plausible system for determining the function of FADD in T cell apoptotic responses. The tFADD mice reportedly contain mostly tFADD-expressing T cells and undetectable FADD-deficient T cells because there was no obvious reduction of the FADD protein after the expression of Lck-Cre (45). The implication of this phenotype is unclear, and further analysis is needed to determine whether FADD deficiency in the thymus completely blocks development and/or results in immediate T cell death. To address these issues, it is necessary to establish viable mutant mice, which contains detectable FADD-deficient T cells by inducing stage-specific deletion of FADD during development, and to analyze the effect of a FADD deficiency induced in normally differentiated T cells.

In this study, we generated novel mutant mice in which efficient deletion of a GFP-tagged FADD gene was induced specifically in DN or DP thymocytes by using the Lck-Cre or CD4Cre transgenes. In addition, deletion of the FADD-GFP fusion gene in CD4+ or CD8+ single-positive (SP) T cells was induced using retrovirus-mediated delivery of the Cre recombinase. High percentages of FADD-deficient T cells were readily detected as the GFP-negative (GFP−) population in the thymus and periphery by flow cytometric analysis. The data obtained from analysis of these T cell-specific FADD-deficient mice demonstrate that FADD deficiency has no obvious effect in thymocyte development, but blocks cell death responses, and impairs peripheral homeostasis as well as TCR-induced proliferation responses in mature T cells.

Materials and Methods

Generation of T cell-specific FADD-GFP-deficient mice

The 12-kb EcoRV genomic DNA fragment containing two coding exons of the mouse FADD gene was isolated from a cosmid clone described elsewhere (20). The GFP gene coding region was amplified by PCR from the pEGFP-C1 plasmid (BD Clontech) and fused to the 3′ end of the FADD coding region in the 12-kb EcoRV fragment. Two LoxP sites were inserted to flank the FADD-GFP coding region. The resulting FADD-GFP fusion construct was injected into mouse embryos to generate transgenic mice at the Kimmel Cancer Center Facilities of Thomas Jefferson University. Mouse ear tissue lysates were prepared and used in genotyping for FADD-GFP-positive (GFP+) founders by PCR using GFP-specific primers. Tail genomic DNA was digested with EcoRI for genotyping by Southern blot analysis. A 0.4-kb DNA fragment was used as a probe to hybridize to the second exon of FADD-GFP for genotyping. FADD:GFP mice were crossed 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 Cd4Cre+ transgenes were obtained from Taconic, and were crossed with FADD−/− mice. The resulting FADD−/−/Lck-Cre+ or FADD−/−/Cd4Cre+ mice were crossed with FADD−/−/FADD-GFP+→Lck-Cre+ or FADD−/−/FADD-GFP+→Cd4Cre+ 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 Cd4Cre transgenes were detected by PCR-based genotyping using mouse ear tissue lysates and Cre-specific primers (5′-CCAGCTAAACATGCTTCATCGTC-3′ and 5′-CCTGATCCTGGCAATTTCGG-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 fluorochrome-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), proteinase inhibitor mixture (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 on 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 (100 μg/ml). All of the growth factors 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

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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 in triplicate to wells (10^6 of thymocytes/well) in a 96-well plate. Sixteen hours after treatment, cell death was determined by propidium iodide (PI) uptake.

FADD-GFP

(Food) GFP was viable and develop normally only when

with Con A (2.5 μg/ml/H9262) stimulated for 2 days with coated anti-CD3 plus soluble anti-CD28 Abs or

FADD-GFP

(Food) WT

FIGURE 1.

The Cre gene was amplified 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 (Imgenex) 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 (1/5000 dilution) and anti-CD28 (1/1000 dilution) Abs.

Adoptive 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-GFP Lck-Cre and 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 × 10^7/ml in PBS + FBS (5%), and mixed with equal volumes of CFSE (20 μM; Molecular Probes) in PBS + FBS. After incubation at 25°C for 5 min, the labeled cells were washed three times with PBS + FBS, once with PBS, and were injected i.v. (4 × 10^7 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 antibodies (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 various concentrations in triplicate. 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 to a 6-well plate (4 ml/well) and stimulated with soluble anti-CD3 (1/5000 dilution) and anti-CD28 (1/1000 dilution) Abs.

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 FADD 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.

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−/− 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, spleenocytes, and lymph node cells from mice of various genotypes by Western blot analyses. FADD-GFP differs in size from the endogenous FADD, which is absent in FADD−/−:FADD-GFP mice.
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+ histogram 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 littermates 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.** 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−/− FADD:GFP+ Lck-Cre+ and FADD−/− FADD:GFP− Lck-Cre+ 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 (data not shown). When total peripheral lymphocytes were analyzed by Western blots, reduction of the FADD:GFP protein in FADD−/− FADD:GFP+ Lck-Cre+ or FADD−/− FADD:GFP− Lck-Cre+ mice was not as obvious as that detected in thymocytes (Fig. 3A), probably due to the presence of higher percentages of GFP+ cells in the spleen (84%) and lymph nodes (56%) (Fig. 2). These peripheral GFP+ populations are mostly B220-expressing B cells (data not shown). GFP− 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− T cells contain undetectable levels of the FADD:GFP protein in comparison with control GFP+ T cells isolated from FADD−/− FADD:GFP+ mice. After activation and growth in culture for several days, the FADD:GFP protein remains undetectable in mature GFP− T cells (Fig. 3B). Thus, the GFP+ population in FADD−/− FADD:GFP+ Lck-Cre+ mice represents FADD-deficient (FADD−/−) T cells.

We also introduced into FADD−/− FADD:GFP+ mice the CD4-Cre transgene, which is expressed at the DP stage in the thymus (47). Unlike FADD−/− FADD:GFP+ Lck-Cre+ mice, which contain a discrete GFP+ population in the thymus, FADD−/− FADD:GFP+ CD4-Cre+ 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− cells were detected as a distinct population in the spleen (43%) and lymph nodes (23%) in FADD−/− FADD:GFP+ CD4-Cre+ mice (Fig. 2). The FADD:GFP protein was undetectable in sorted peripheral GFP− 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−/− FADD:GFP+ Lck-Cre+ mice and found to contain 80–97% of GFP+ 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−/− FADD:GFP+ 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+ T cells were isolated from the spleen and lymph nodes in FADD−/− FADD:GFP+ mice, and these cells were killed in a dose-dependent manner by sFasL (FADD:GFP+; Fig. 4B). In contrast, GFP− (FADD−/−) T cells isolated from the spleen and lymph nodes in FADD−/− FADD:GFP+ Lck-Cre+ 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. To determine cell death responses 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− and GFP+ T cells were isolated by FACS from the spleen and lymph nodes in FADD−/− FADD:GFP+ 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 response in control FADD:GFP-expressing cells (FADD:GFP+), but not in activated GFP− FADD-deficient T cells (FADD−/−; Fig. 4C). TNF does not have much effect on resting T cells but can induce detectable 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+) and <5% cell death in activated GFP− T cells (FADD−/− FADD:GFP−).
death in mutant-activated T cells (FADD<sup>−/−</sup>; 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<sup>+</sup> 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-mo-old FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> 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<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> or CD4-Cre<sup>+</sup>) (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<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> mice in a pattern similar to that detected in littermate control FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> mice (Fig. 5). Normal thymocyte subsets were also detected in FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> CD4-Cre<sup>+</sup> mice (Fig. 5). When peripheral T cells were analyzed by flow cytometry, a reduction in the number of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells was detected in the spleen and lymph nodes from FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> mice containing either Lck-Cre or CD4-Cre (Fig. 5). Among the FADD<sup>−/−</sup> FADD:GFP<sup>+</sup> Lck-Cre<sup>+</sup> mice analyzed, there is an average 46% reduction in the number of peripheral T cells.
FIGURE 5. Flow cytometric analysis of T cell subpopulations. Cells isolated from the thymus, spleen, and lymph nodes were stained for the CD4 and CD8 coreceptors. T cell-specific gene deletion in FADD−/− FADD:GFP+ mice induced by either Lck-Cre or CD4-Cre did not alter thymocyte populations (top), but resulted in reduced T cell populations in the spleen and lymph nodes (middle and bottom). Littermate FADD+/− FADD:GFP+ mice containing Lck-Cre or CD4-Cre were used as controls.

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

| Genotype | CD4 (×10^6) | CD8 (×10^6) |
|----------|-------------|-------------|
| Thymus   | 4.6 ± 1.1   | 0.9 ± 0.4   |
| SPLN†    | 14.4 ± 3.2  | 6.1 ± 0.7   |
| LN‡      | 7.9 ± 1.9   | 3.1 ± 0.9   |

† 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

| Genotype | CD4 (×10^6) | CD8 (×10^6) |
|----------|-------------|-------------|
| Thymus   | 14.1 ± 2.1  | 3.8 ± 1.8   |
| SPLN†    | 15.5 ± 2.4  | 6.9 ± 1.5   |
| LN‡      | 15.3 ± 3.8  | 9.1 ± 2.3   |

† SPLN, Spleen; LN, lymph node.
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).

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 protein by Western blot analysis (data not shown). GFP+ T cells were isolated from control MSCV-infected cultures. These purified proliferating cells were cultured in medium containing IL-2, and growth curves were generated by determining the cell number in each culture each day. As indicated in Fig. 7C, the number in the culture of activated FADD-deficient T cells decreased gradually 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 isolated 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 tFADD mouse model was generated in an attempt to disrupt FADD specifically in the T cell lineage (45). In these tFADD 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 tFADD mice (45). It is possible that T cell-specific deletion of tFADD was ineffective, because a reduction of FADD protein expression in T cells was not detectable in tFADD 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 naïve 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 tFADD mice, which appears to contain undetectable peripheral FADD-deficient T cells (45). Using FADD:

![Figure 7](image-url)
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−/− FADD:GFP−/− littermates. CFSE-labeled T cells were activated by stimulation with anti-CD3 and anti-CD28 Abs, and analyzed by flow cytometry at 72, 96, and 120 h poststimulation. The percentages of cells that underwent various divisions are indicated in brackets on the graph.

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 T cell-specific FADD-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
DRs, which regulates embryogenesis and peripheral T cell function.

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Disclosures

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