

The Requirements for Fas-Associated Death Domain Signaling in Mature T Cell Activation and Survival¹

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Fas-associated death domain (FADD) is a death domain containing cytoplasmic adapter molecule required for the induction of apoptosis by death receptors. Paradoxically, FADD also plays a crucial role in the development and proliferation of T cells. Using T cells from mice expressing a dominant negative form of FADD (FADD^{ddd}), activation with anti-TCR Ab and costimulation or exogenous cytokines is profoundly diminished. This is also seen in wild-type primary T cells transduced with the same transgene, demonstrating that FADD signaling is required in normally differentiated T cells. The defective proliferation does not appear to be related to the early events associated with TCR stimulation. Rather, with a block in FADD signaling, stimulated T cells exhibit a high rate of cell death corresponding to the initiation of cell division. Although CD4 T cells exhibit a moderate deficiency, this effect is most profound in CD8 T cells. In vivo, the extent of this defective accumulation is most apparent; lymphocytic choriomeningitis virus-infected FADD^{ddd}-expressing mice completely fail to mount an Ag-specific response. These results show that, in a highly regulated fashion, FADD, and most likely caspases, can transduce either a signal for survival or one that leads directly to apoptosis and that the balance between these opposing outcomes is crucial to adaptive immunity. *The Journal of Immunology*, 2003, 171: 247–256.

A subset of the TNFR family is collectively known as “death receptors” (DR)³ and each of these receptors propagates signals in a distinct manner conditionally promoting apoptosis (1, 2) or cell survival (3, 4). In mice and human beings with deficiencies in the DR Fas/Apo-1/CD95, severe lymphadenopathies and autoimmune pathologies develop, presumably as a consequence of a diminished capacity for activated cell death (5, 6). Fas-associated death domain (FADD) is an adapter protein that is required for apoptosis induced by all known DRs (including Fas, TNF-R1, DR3, TRAIL receptors, and DR6) (7–9). This adapter contains a carboxyl-terminal death domain that allows binding to other death domain-containing proteins and an amino-terminal death effector domain that participates in homotypic binding to propagate signaling through caspases. Residing in the cytoplasm, FADD is recruited to DRs following binding by extracellular DR ligands, either by direct binding of FADD to the death domain of the DR or via indirect binding via a second adapter known as TNFR-associated death domain protein (10, 11).

Although a number of other proteins are recruited into complexes that form around the DR cytoplasmic tail, FADD is thought

to exclusively activate apoptosis by directly binding to and promoting the autoproteolysis of caspases-8 and -10 (12). This hypothesis is borne out in experiments with FADD-deficient fibroblasts which are apoptotically insensitive to Fas ligand, TNF, and TRAIL (7, 13). Similarly, T cells with a germline deficiency in FADD or those carrying a dominant-interfering form (FADD^{ddd}) are known to be resistant to Fas-induced apoptosis (9, 14–16). Therefore, a prediction was that mice with a deficiency in FADD would lack all forms of DR-mediated apoptosis and manifest severe lymphadenopathy. Paradoxically, such mice fail to develop lymphoproliferative diseases and further studies indicated an unexpected defect in T cell activation. FADD mutant T cells fail to proliferate in response to a variety of mitogens, suggesting that FADD participates in a nonapoptotic pathway necessary for clonal expansion. Furthermore, thymopoiesis in FADD mutant mice is partially defective (14, 17, 18). In these mice, thymocytes transit through the β selection stage with a reduced level of proliferation. The receptor-mediated positive and negative selection events appear to be unaffected. These results are further reinforced by a recent report showing that human beings with a deficiency in caspase-8 are highly immunodeficient (19).

One proposal is that FADD directly regulates the cell cycle in T cells and thymocytes (20). A related possibility is that rather than directly regulating the cell cycle, FADD constitutes part of a signaling cascade that promotes survival at one or more cell cycle checkpoints in activated T cells. In this report, we show that when FADD signaling is blocked with FADD^{ddd}, T cells initiate cell division but undergo cell death at a high rate. There was found to be little accumulation of dividing T cells in cultures stimulated with either anti-TCR Abs or specific Ags. In CD8⁺ cells derived from MHC class I-specific TCR-transgenic mice, Ag-driven T cell expansion and survival were severely diminished; however, in MHC class II-specific TCR-transgenic mice, FADD^{ddd} expression failed to block such proliferation. These discrepancies were not the result of differential activation-induced down-regulation of FADD^{ddd} in these subsets or the manifestation of aberrant T cell development. Enforced retroviral expression of FADD^{ddd} in mature T

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³ Abbreviations used in this paper: DR, death receptor; FADD, Fas-associated death domain; GFP, green fluorescent protein; OVAp, OVA peptide; LCMV, lymphocytic choriomeningitis virus; 7AAD, 7-aminoactinomycin D; PCC pigeon cytochrome c; MSCV, murine stem cell virus; IRES, internal ribosome entry site.

cells produced similar results. These findings support the hypothesis that FADD regulates survival as part of the progression through the cell cycle. Moreover, these studies may highlight differences in the signaling cascades necessary for Ag-driven proliferation and accumulation of CD4⁺ and CD8⁺ cells.

Materials and Methods

Mice

All mice were bred and maintained in either the animal care facilities at the University of California, San Diego or University of California, Irvine. All mice were also maintained on the C57BL/6 (B6) background, unless otherwise indicated. *FADD* transgenic mice have been described previously (14). *OT-I* mice (21) and *OT-II* mice (22) were obtained from C. Surh (The Scripps Institute, San Diego, CA). *AND* mice (23) have been described previously. *P14* mice (24) and B6.PL mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell culture

All cell culture was performed in RPMI 1640 (Irvine Scientific, Irvine, CA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM glutamine, nonessential amino acids (Life Technologies, Gaithersburg, MD), 0.11 mg/ml sodium pyruvate (Life Technologies) 5.5×10^{-5} M 2-ME, and 10% FBS (HyClone Laboratories, Logan, UT). Cells were cultured at 37°C in a 5% CO₂ buffered incubator. Recombinant human IL-2 was a kind gift from Cetus (Emeryville, CA) and used at 25 U/ml. Recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ) was used at 100 ng/ml. Anti-IL-2 (eBiosciences, San Diego, CA) was used at 5 µg/ml to block autocrine signaling where indicated.

In vitro proliferation

Spleen or lymph node cells were cultured in either medium alone or with increasing concentrations of OVA peptide (OVAp; Bachem, Switzerland) for 2 days (*OT-I* transgenics) or pigeon cytochrome *c* (PCC) peptide (Genemed Synthesis, South San Francisco, CA) for 3 days (*AND* transgenics). Cells were pulsed with 1 µCi [³H]thymidine (PerkinElmer Life Sciences, Boston, MA) for the last 8 h of culture and cpm were determined for each culture condition in triplicate. Alternatively, cells were first labeled with 5 µM CFSE (Molecular Probes, Eugene, OR) for 7 min at room temperature, washed twice with 10 ml of PBS, and cultured for 2 or 3 days. Proliferation was then assessed by flow cytometry.

Flow cytometry

Spleen or lymph node cells were incubated with the appropriate primary Abs for 15–30 min in 1× PBS with 1% FBS and 0.01% sodium azide. FITC-, PE-, PerCP-, allophycocyanin-, or biotin-conjugated CD3, CD4, CD8, Vα2, Vα11, Vβ5, Vβ3, Vβ8, CD25, CD69, CD44, and CD62L were obtained from BD PharMingen (San Diego, CA). Abs conjugated to the same molecules specific for CD4, Thy1.1, Thy1.2, IFN-γ, and IgG1 were purchased from eBioscience. PE-conjugated recombinant annexinV was purchased from Caltag Laboratories (Burlingame, CA). Streptavidin-allophycocyanin or streptavidin-PerCP were used to detect biotinylated Abs. 7-actinomycin D (7AAD) was purchased from Calbiochem (San Diego, CA) and used on nonpermeabilized cells. After washing once with PBS, cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA) and FlowJo software (Treestar, San Carlos, CA).

Western blotting

To assay for expression of the transgenic *FADD* in CD4 and CD8 subsets, splenocytes were harvested and activated as indicated. Following activation, CD4 and CD8 T cells were isolated using anti-CD4- and anti-CD8-coated Dynabeads (DynaL Biotech, Lake Success, NY), followed by lysis and Western blotting onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). The blots were then probed with anti-FADD polyclonal (BD Transduction Laboratories, Lexington, KY), followed by incubation with anti-rabbit-HRP (Vector Laboratories, Burlingame, CA) and ECL visualization (Amersham, Piscataway, NJ). For STAT5 activation Western blots, T cells were obtained from *OT-I*/*FADD* mice using a T cell isolation kit (Miltenyi Biotec, Auburn, CA). Isolated cells were incubated with anti-CD3 as indicated. In some cases, anti-IL-2 was added during the initial activation to eliminate autocrine stimulation of the IL-2R signaling pathway. Fifteen minutes before lysis, cells were washed once with PBS and 25 U/ml human rIL-2 was added. The lysates were transferred to polyvinylidene difluoride membranes as above, probed with anti-

phosphotyrosine STAT5 Ab (Cell Signaling Technology, Beverly, MA), and visualized as above. To assay total STAT5 expression, the blots were stripped and reprobed with anti-STAT5a polyclonal Abs.

Cell death assays

For all cell death assays, cells were cultured either with medium alone or 1×10^{-9} M OVAp for 24 h at 37°C. Dexamethasone (Sigma-Aldrich, St. Louis, MO) was added to some of the cultures for the last 12 h as a positive control. Annexin V staining was performed in HBSS containing calcium and magnesium for 10 min at 37°C. TUNEL staining (25) was performed using a kit from Calbiochem (San Diego, CA) per included instructions with minor modifications. Cells were first stained with anti-Vα2 PE and anti-CD8 allophycocyanin. Cells were then washed once, fixed for 15 min with 1% paraformaldehyde, and permeabilized with 0.5% Tween 20 for 15 min. The rest of the procedure was performed per provided instructions and analyzed by flow cytometry.

Lymphocytic choriomeningitis virus (LCMV) infection and analysis

Six- to 12-wk-old C57BL/6 and *FADD* mice were infected with 2.5×10^5 PFU of LCMV Armstrong in 0.5 ml of PBS i.p. At the indicated time points, spleens were harvested from infected mice as well as uninfected controls. Splenocytes were either analyzed for their CD4/CD8 profiles or stimulated with peptides derived from gp33 or gp61 (Genemed Synthesis) in the context of MHC class I or class II, respectively. Intracellular IFN-γ staining was performed as described previously (26). Briefly, 1×10^6 spleen cells were cultured in 96-well round-bottom plates (Costar, Cambridge, MA) with 10 U/well recombinant human IL-2 and 1 µl/ml brefeldin A (GolgiStop; BD PharMingen) either with or without dominant peptide epitopes. Intracellular staining was performed using a Cytofix/Cytoperm kit according to manufacturer's instructions (BD PharMingen).

Retroviral constructs and transduction

FADD (aa 80–208 of human FADD) was subcloned into MiG murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP and MiT (MSCV = iThy1-1). These constructs were cotransfected with the ΨEco helper virus construct into 293T cells. Following transfection, supernatants were isolated at various times and subsequently titered by infection of murine NIH3T3 cells. To transduce T cells, spleens and lymph nodes were harvested from 6- to 8-wk-old mice, and single-cell suspensions were incubated with anti-CD3 and IL-2 to initiate cell cycle. After 24 h, retroviral supernatants were added, along with polybrene, and the cells were spun for 1 h to enhance infection. At various times following transduction, cells were isolated and stained for FACS. For MiG-transduced cells, cells were stained with anti-CD8 PerCP (BD Biosciences) and anti-CD4 allophycocyanin (eBioscience). For MiT, cells were stained similarly, but also with anti-Thy1.1-PE (eBioscience). In each case, mock-transduced cells served as negative controls for the transduction marker (e.g., green fluorescent protein (GFP) or Thy1.1).

Results

Differential requirements for FADD in CD4⁺ and CD8⁺ T cells

Extensive studies of the lymphocyte cellularity of peripheral lymphoid organs of *FADD* mice revealed a more profound reduction in the number of CD8⁺ cells than CD4⁺ cells when compared with wild-type mice. This is seen in differences in the ratios of CD4:CD8 cells; in wild-type mice this ratio was typically 2:1 in the spleen and lymph nodes, whereas this ratio was roughly 4:1 in *FADD* mice (data not shown). This observation suggested that CD4 and CD8 cells may have intrinsic differences in their requirements for FADD. To test this, we analyzed T cell activation using anti-CD3 under conditions that require exogenous costimulation or cytokines. T cells were first separated into CD4⁺ and CD8⁺ populations, labeled with CFSE, and activated for 3 days under the indicated conditions. Analysis by flow cytometry was conducted by collecting a set proportion of each group of cultures. In this way, the magnitude of the peaks revealed the recovery of cells and the histograms revealed the distribution of cell divisions under different culture conditions (Fig. 1A). The data show that CD4 T cells from *FADD* mice accumulate at a rate only slightly reduced from that of wild-type T cells, whereas CD8 T cells from

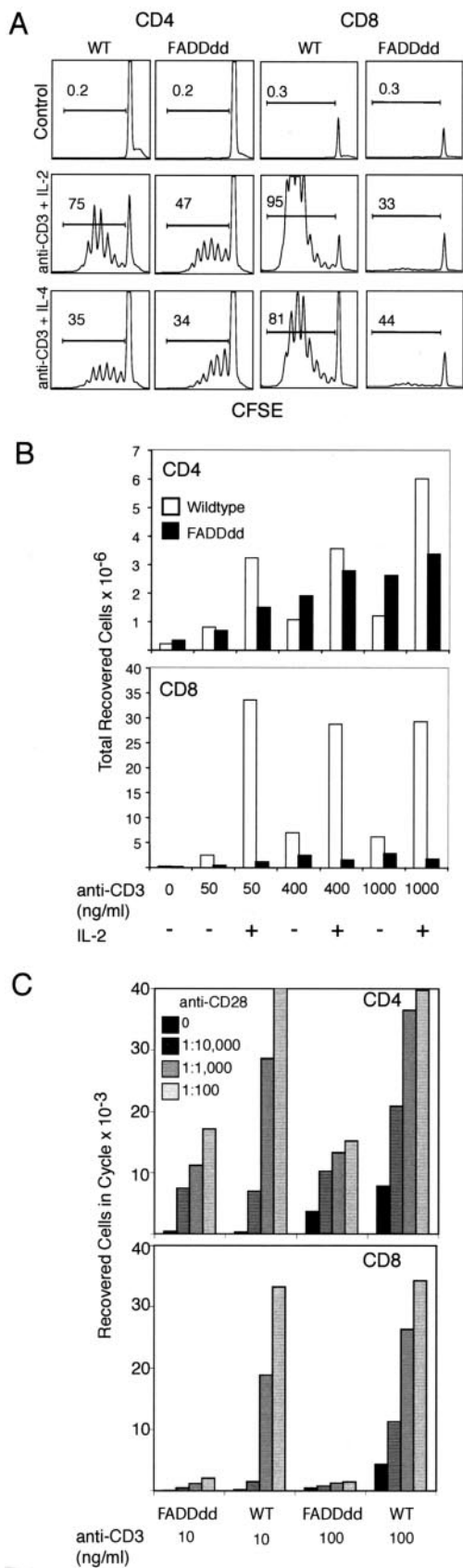


FIGURE 1. Differential requirements for FADD function in CD4⁺ and CD8⁺ T cells. *A*, CFSE analysis of CD4- and CD8-gated T cells treated with plate-bound anti-CD3 (145-2C11) at 50 ng/well along with IL-2 (25 U/ml) or IL-4 (100 ng/ml). Cells were analyzed after 3 days of culture under these conditions. *B*, Differential accumulation of CD4⁺ and CD8⁺ T cells following mitogenic stimulation. Splenocytes from *FADDdd* and

FADDdd mice accumulate poorly. Some cells apparently divided, since there is a significant percentage of cells with reduced fluorescence; however, unlike T cells from wild-type mice, the population did not expand. Addition of exogenous IL-4 in place of IL-2 revealed similar findings, indicating that the faulty proliferation observed was not selective for IL-2. This was further examined by titrating the dose of anti-CD3 in culture with or without the addition of IL-2 (Fig. 1*B*). At all concentrations of anti-CD3, the addition of exogenous IL-2 promoted accumulation of CD8 wild-type cells, whereas the accumulation of CD8 *FADDdd* T cells was dramatically inhibited. Once again, the accumulation of CD4 *FADDdd* T cells was only modestly affected. We note that the accumulation of CD4 T cells under these conditions was dependent on the amount of anti-CD3 cross-linking, but less dependent on the addition of exogenous IL-2 when compared with CD8 T cells from the same culture.

To determine whether the defect in proliferation also applied to T cells stimulated through the CD28 coreceptor, T cells were activated with plate-bound anti-CD3 supplemented with varying concentrations of anti-CD28 Ab (Fig. 1*C*). The total number of cells that had undergone at least one division was plotted vs the amount of anti-CD28 added. As shown, the number of cells in cycle was directly proportional to the amount of costimulation provided by anti-CD28. For the CD4 subset, the *FADDdd* T cells exhibited a diminished accumulation, although the response was substantial. In contrast, *FADDdd* CD8 T cells were almost completely unable to accumulate in culture regardless of the amount of stimulation through the CD28 coreceptor. These data show that the survival signals provided by the CD28 coreceptor are not sufficient to overcome the deficit associated with the expression of FADDdd. Whether proliferation was promoted by exogenous cytokines or potent costimulation, CD8⁺ *FADDdd* T cells exhibited an extreme deficiency in their ability to accumulate in culture following cell division.

Enhanced death of proliferating FADDdd T cells

Previous experiments conducted with T cells expressing a *FADDdd* transgene or deficient in *FADD* showed that early activation was equivalent to wild-type cells (9, 15, 27). The levels of IL-2 production, CD69 up-regulation, or CD25 up-regulation at 18 h of culture were all unchanged in T cells from *FADDdd* mice, regardless of the manner in which they were stimulated (data not shown). These results suggest that the early events in T cell activation occur normally, but as cells progress through the cell cycle, there is a deficit in survival. This was illustrated by an analysis of cell death vs cell proliferation (Fig. 2). T cells were labeled with CFSE and cultured with anti-CD3 with or without cytokines, and then labeled for CD4, CD8, and annexin V. Annexin V binds to phosphatidyl serine, either because the cells are apoptotic and have lost plasma membrane asymmetry or because they are necrotic and thus permeable to the annexin V protein. The caveat to these experiments is that dead cells can be recorded in culture for only a

wild-type mice were treated with plate-bound anti-CD3 at the indicated concentrations, without and with exogenous IL-2 (20 U/ml), and then cultured for 5 days. Cultures were then collected, stained with anti-CD4 and anti-CD8, and analyzed by FACS. Numbers of live cells are shown for live-gated cells obtained after a 20-s collection. *C*, T cells were isolated from wild-type and *FADDdd* mice and were labeled with CFSE. The cells were cultured in the presence of increasing doses of anti-CD28 Ab in the presence of 10 or 100 ng anti-CD3 Ab. Shown are results for T cells electronically gated for CD4 and CD8 expression. To calculate the numbers of recovered cells in cycle, the total number of cells was multiplied by the proportion of cells having undergone at least one division, based on CFSE dilution.

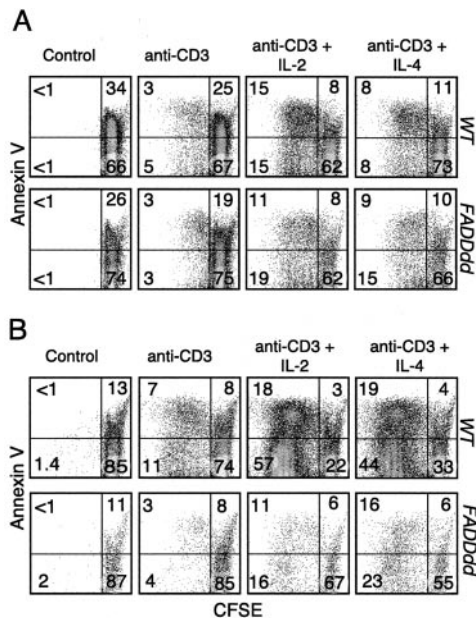


FIGURE 2. Increased cell death in *FADDdd*-transgenic CD8 but not CD4 populations. Lymph node cells were labeled with CFSE and cultured for 5 days under the indicated conditions. After 5 days, cells were stained for Annexin V, CD4, and CD8 and analyzed by FACS. Shown are plots for CFSE vs Annexin V of CD4 (A) and CD8 (B) populations gated separately. Upper and lower left quadrants represent proportion of cultures that have divided and are dead or alive, respectively.

limited span of time, after which they disintegrate or they are cleared by phagocytic cells. The analysis does not so much reveal the accumulation of dead cells in culture but rather a snapshot of dying cells at any one time. As shown in Fig. 2, there is an accumulation of dividing CD4 cell cultures containing either wild-type or *FADDdd* T cells. In fact, we note that among the cells that have divided at least once, the ratio of live cells to dying cells is enhanced for *FADDdd* CD4 T cells, presumably because FADDdd is blocking DR-mediated activation-induced cell death. An examination of the CD8 T cells from these cultures reveals the opposite result. Again, there is little accumulation of dividing cells, but, in addition, the proportion of dying to live cells is increased in cultures containing *FADDdd* T cells. The reduced numbers of cells plotted in this dot plot attests to the minimal recovery of CD8 cells in this assay. These results support the notion that the presence of FADDdd blocks the survival of dividing CD8 T cells.

One explanation for the differential requirement for FADD in CD4 vs CD8 T cells would be that expression of the *FADDdd* transgene is preferentially lost in CD4 T cells following activation, thus removing this FADD signaling inhibition (27). To address this, we treated T cells from *FADDdd* and wild-type mice with low and high levels of plate-bound anti-CD3 and IL-2 and then collected the cultures after different lengths of time in culture. CD4⁺ and CD8⁺ T cells were isolated using Ab-coated magnetic beads, then lysed and analyzed by Western blotting with an anti-FADD Ab (Fig. 3). When compared with the level of endogenous FADD, the level of the transgenic FADDdd did not change appreciably in CD4⁺ T cells following activation in the first 24 h. There does appear to be somewhat less FADDdd at 72 h following high-level CD3 cross-linking, but this loss was more apparent in CD8 T cells which have a more striking proliferative defect. Transgenic FADDdd in these experiments appeared to run as a doublet on western blots, and this is probably due to phosphorylation (28–30). Taken together, these data demonstrate that distinct effects of the *FADDdd* transgene on CD4⁺

and CD8⁺ T cells cannot be ascribed to a preferential loss of transgene expression in activated CD4 T cells.

Normal development but defective survival of Ag-specific CD8 T cells

Although results using anti-CD3 and IL-2 revealed clear differences between *FADDdd*-transgenic CD4⁺ and CD8⁺ T cells, we wished to verify this in an Ag-mediated activation assay. To this end, we crossed *FADDdd*-transgenic mice to *OT-I* TCR-transgenic mice (21) and compared the development and proliferation of the resulting double-transgenic T cells. *OT-I* mice express a transgenic TCR that recognizes OVA_p bound to H-2D^b. Similar to *FADDdd* single-transgenic mice, analyses of the CD4 and CD8 profiles showed that *FADDdd* did not alter the steady-state populations of thymic CD8 *OT-I* T cells (Fig. 4, A and B). In contrast, in peripheral lymphoid organs of *OT-I*; *FADDdd* mice, the CD8 T cell population was reduced by ~50%. To investigate the proliferative capacity of these T cells, splenocytes were CFSE labeled, treated with OVA_p, and cultured for 2 days. Following culture, gated CD8⁺ T cells were analyzed for CFSE dilution. The profiles show that T cells from *OT-I* and *OT-I*; *FADDdd* cells underwent extensive cell division; however, as we observed for anti-CD3, Ag-stimulated *OT-I*; *FADDdd* T cells accumulated at a dramatically reduced rate compared with *OT-I* T cells (Fig. 4C). This finding was reinforced by investigation of Ag dose-response curves assayed by the incorporation of [³H]TdR (Fig. 4D). The dose-response curves were similar, showing that transgenic FADDdd does not affect Ag responsiveness, but the plateau achieved by T cells expressing FADDdd was considerably reduced. *OT-I*; *FADDdd* T cells responded to OVA_p since CD69 was strongly expressed after 18 h of stimulation (Fig. 4E). Consistent with our findings in non-TCR-transgenic CD8⁺ T cells, activation led to a loss of viability, as determined by Annexin V staining (Fig. 4E). Similar results have been obtained with *FADDdd*-transgenic mice bred to express the P14 gp33-specific TCR (31) and the H-Y-specific TCR (32) (data not shown). These results demonstrate that, regardless of stimulus, the survival of activated CD8 T cells is substantially reduced by inhibition of FADD signaling.

The death induced in response to Ag was characterized in more detail. After only 24 h of culture, the number of T cells recovered was markedly diminished in the presence of FADDdd (Fig. 5A). As shown, even without Ag stimulation, there are fewer CD8 T cells and this reflects the reduced starting population. In response to Ag and before cell division, the number of *FADDdd* T cells was markedly reduced, whereas wild-type cells actually exhibit enhanced survival over the no-Ag control. As shown in Fig. 5B, this

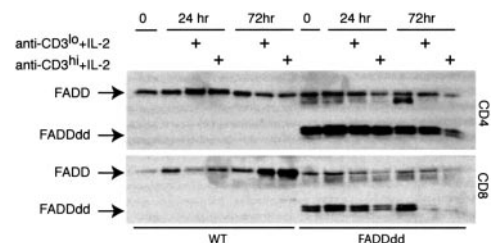


FIGURE 3. *FADDdd* transgene expression in activated *FADDdd* T cell subsets. Pooled lymph node and splenic T cells from wild-type and *FADDdd* mice were activated for 24 and 72 h with anti-CD3 and IL-2 or left untreated and then purified using anti-CD4 or anti-CD8 magnetic beads. Lysates were resolved on SDS-PAGE and analyzed by Western blotting with anti-FADD Ab. Shown for each Western blot are the full-length endogenous FADD band and the transgenic FADD dominant-negative doublet (FADDdd). CD3^{low}, 50 ng/well anti-CD3; CD3^{high}, 1000 ng/well anti-CD3.

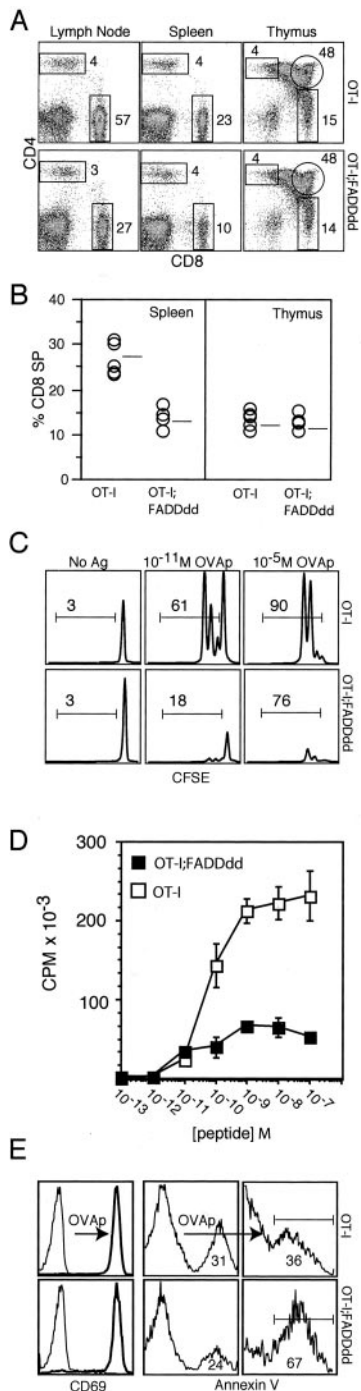


FIGURE 4. Normal thymopoiesis but defective proliferation and maintenance of peripheral *OT-I;FADDdd* CD8⁺ T cells. *A*, CD4 vs CD8 dot plots of *OT-I* single and *OT-I;FADDdd* double-transgenic T cells. *B*, The percentage of single- or double-transgenic thymocytes and splenocytes staining positive for CD8 is shown below. *C*, CFSE analyses of *OT-I* and *OT-I;FADDdd* T cells treated with low (10⁻¹¹ M)- and high (10⁻⁵ M)-dose OVAp after 2 days. Plots are scaled for an absolute number of cells to indicate the extent of the loss of live cells after activation of *OT-I;FADDdd* T cells. *D*, Response curve of *OT-I;FADDdd* T cells to varying doses of OVAp. *E*, Expression of CD69 and annexin V following activation of *OT-I* and *OT-I;FADDdd* T cells after 18 h.

loss of *FADDdd* T cells is reflected in a substantially increased proportion of annexin V-positive T cells, at least 3-fold over the wild-type T cell population. The binding of annexin V to cells can either reflect a loss of membrane asymmetry associated with ap-

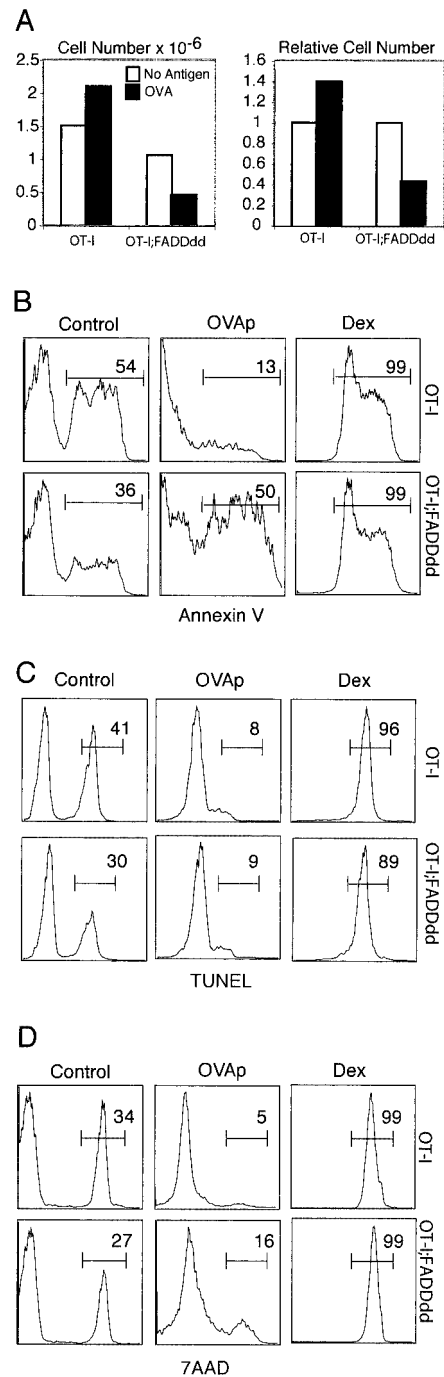


FIGURE 5. Induction of cell death without DNA fragmentation in activated *OT-I;FADDdd* T cells. Splenic T cells from *OT-I;FADDdd* mice were activated for 18 h with OVAp or dexamethasone. *A*, The total and relative numbers of recovered CD8 T cells with and without Ag stimulation were plotted. *B*, Live CD8 T cells were stained with annexin V. *C*, Fixed and permeabilized CD8 T cell cells were stained for DNA fragmentation using TUNEL. *D*, Live CD8 T cells were stained with 7AAD, a DNA intercalating agent that only stains cells with a loss of membrane integrity.

optosis or it can reflect the loss of membrane integrity that allows annexin V to bind intracellular phosphatidyl serine. To determine whether *FADDdd* T cells retain membrane integrity at this time after Ag stimulation, we stained T cells with 7AAD, a DNA intercalating dye that is excluded from viable cells. As shown in Fig. 5D, the number of 7AAD-staining cells did not account for the

50% annexin V staining cells, indicating that the annexin V staining cannot be accounted for by a loss of membrane integrity. Finally, we analyzed the cell population for DNA fragmentation, a hallmark of apoptotic cell death (33). Oligonucleosomal DNA fragmentation occurs via caspase-dependent cleavage of ICAD/DFF45, a cytoplasmic inhibitor of the nuclease CAD/DFF40 (34–38). To test for the activation of this pathway, we stained activated *OT-I;FADDdd* T cells using TUNEL, a sensitive method to detect free 3' DNA ends (25). Surprisingly, treatment of both *OT-I* and *OT-I;FADDdd* T cells with OVAp produced low levels of TUNEL-positive cells, although the overt induction of apoptosis using dexamethasone converted the entire population into TUNEL-positive cells (Fig. 5C). The treatment with antigenic peptide actually reduced the levels of annexin V- and TUNEL-positive cells, consistent with the notion that signals derived via the TCR can rescue T cells from death by “neglect” (39). Paradoxically, these data appear to indicate that, at least after 24 h, cells are dying of apoptosis without inducing DNA fragmentation. In any case, the defect conferred by inhibiting FADD activity is to cause Ag-activated T cells to die at a high rate such that there is very little proliferation.

Given that CD8⁺ T cells are exquisitely dependent upon cytokine-induced signals for proliferation and survival (Fig. 1 and Ref. 40), we considered that *FADDdd* CD8⁺ T cells might possess a gross deficit in cytokine-dependent signal transduction. During in vitro stimulation of T cells via Ag or Ag mimics, the primary cytokine that drives T cell proliferation is IL-2. One signal delivered through the IL-2R is propagated by Janus kinase 3 activation, resulting in STAT5 phosphorylation and nuclear localization. This event has been shown to be crucial for T cell proliferation and survival (41–43). To determine whether this signaling is overtly defective in cells expressing *FADDdd*, purified CD8 cells from *OT-I*-transgenic mice were activated with anti-CD3 Abs, and STAT5 tyrosine phosphorylation was induced by the addition of IL-2 for 15 min. The level of total STAT5 was highly induced by stimulation through CD3 in the absence of IL-2 signaling, whereas there was a large induction of phospho-STAT5 with IL-2 stimulation (Fig. 6). This stimulation was unchanged by the presence of the *FADDdd* transgene. Although other signals, such as phosphoinositide 3-kinase and p56^{lck}, are engendered by the IL-2R (44), this result indicates that T cells from *FADDdd* mice express a functional IL-2R and transmit at least one important signal. Whether there are specific differences in other signaling pathways is currently under investigation.

Normal proliferation and survival of *AND;FADDdd* T cells

The results from the experiments presented above indicate that CD8 T cells die at a high rate upon Ag induction, whereas CD4 T cells are somewhat less affected. We therefore predicted that an

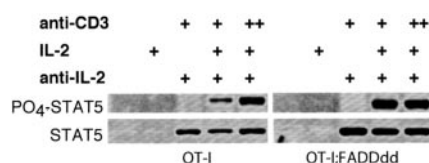


FIGURE 6. Normal STAT5 phosphorylation of *OT-I;FADDdd* T cells following treatment with anti-CD3 and IL-2. Purified T cells were treated with 25 ng (+) or 100 ng (++) plate-bound anti-CD3 for 24 h in the presence of anti-IL-2. Then the cultures were washed and IL-2 was added to indicated cultures for an additional 15 min before lysis. The Western blots were probed with an Ab specific for tyrosine-phosphorylated STAT5. To control for STAT5 expression, the Western blots were reprobbed with an Ab that recognized total STAT5a.

Ag-induced CD4 response would be relatively normal. To address this question, we crossed *FADDdd* mice to produce cytochrome *c*-specific *AND;FADDdd* mice. *AND* mice express a TCR specific for PCC peptide (88–104) bound to MHC class II H-2E^k molecules (23). Examination of T cell subsets based on CD4 and CD8 expression in the thymus, spleen, and lymph nodes revealed that in contrast to CD8 T cells from MHC class I-specific TCR-transgenic mice, *FADDdd* expression manifested no effect on steady-state levels of these subsets (Fig. 7, *a* and *b*). Western blotting showed that the *FADDdd* transgene was nonetheless expressed at high levels in the CD4 T cells from *AND* mice (data not shown). Stimulation of *AND* or *AND;FADDdd* splenocytes revealed identical levels of activation whether we assayed for the dilution of CFSE in culture or the incorporation of [³H]TdR (Fig. 7, *C* and *D*). Likewise, *FADDdd* expression did not reduce the early activation nor the viability of CD4⁺ T cells responding to PCC peptide (Fig. 7E). Identical results were obtained with T cells from *OT-II;FADDdd* mice (45) stimulated with OVA_{323–339} (data not shown). We conclude that there is no net effect of *FADDdd* expression in the Ag response of CD4 T cells.

Retroviral transduction of *FADDdd* into wild-type T cells

In addition to defective T cell proliferation, mice carrying a *FADDdd* transgene or those carrying a germline ablation of the *FADD* gene are known to have aberrant thymopoiesis. Previous studies have revealed these mice exhibit a defect in the expansion associated with the β selection step and reduced thymus cellularity (9, 14, 15). Furthermore, paradoxically, the *FADDdd* transgene rescues the development of double-positive thymocytes in RAG2-deficient mice (17). Thus, it is plausible that the observed defects in proliferation and the differential requirements for FADD function in CD4⁺ and CD8⁺ T cells are consequences of this aberrant development. To specifically address this point, we ectopically expressed *FADDdd* in mature T cells from wild-type mice. The *FADDdd* transgene was subcloned into MiG, a helper-dependent murine stem cell virus construct containing an MSCV long terminal repeat driving expression of the gene of interest followed by an IRES and GFP (46). T cells were activated with anti-CD3 and IL-2 for 24 h and then transduced with MiG-*FADDdd* or MiG. At different times following transduction, the IL-2-stimulated cultures were harvested, stained for CD4 and CD8 expression, and analyzed by flow cytometry. The transduced cells were identified by the expression of GFP. With time, the viability of CD8 T cells was dramatically reduced, and this is exemplified by an analysis of the viable CD4 and CD8 T cells at 5 days (Fig. 8A). A plot of GFP⁺ CD4 and CD8 T cell recovery over 5 days reveals that CD4 T cell recovery was somewhat reduced by the expression of *FADDdd*, whereas the proportion of GFP-expressing CD8⁺ cells was markedly reduced after initial infection (Fig. 8B). The increased proportion of GFP⁺, MiG-transduced cells observed in these assays during the course of the culture likely reflects the fact that MSCV preferentially infects dividing cells; nondividing cells in this assay likely die off over time and contribute a decreasing proportion of cells in culture (47). Although the kinetics and initial infectivity varied in three independent experiments, we consistently observed a dramatic reduction over the period of the experiment in the proportion of CD8⁺GFP⁺ cells expressing *FADDdd*.

To demonstrate the generality of this effect and to rule out vector-specific artifacts, we generated a second retroviral construct by subcloning *FADDdd* into MiT. MiT is a retroviral construct based on MiG, but with the IRES-GFP replaced with an IRES-Thy1.1 sequence (47). Transduced cells can be identified based on the expression of Thy1.1 rather than GFP. Since T cells derived from C57BL/6 mice express Thy1.2, staining with labeled anti-Thy1.1 provides a

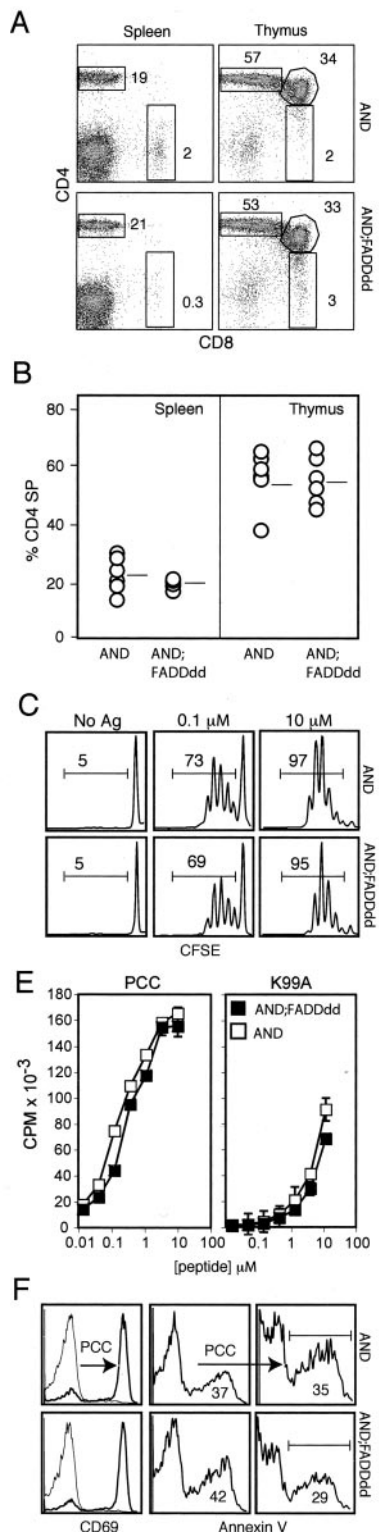


FIGURE 7. Normal thymopoiesis, proliferation, and maintenance of peripheral *AND;FADDdd* CD4⁺ T cells. *A*, CD4 vs CD8 dot plots of *AND* single- and *AND;FADDdd*-transgenic T cells. *B*, The percentage of single- or double-transgenic thymocytes and splenocytes staining positive for CD4 is shown below. *C*, Normal proliferation of *AND;FADDdd* CD4⁺ T cells as measured by CFSE dilution. Following isolation and labeling with CFSE, cultures were treated with low (0.1 mM) and high (10 mM) doses of PCC peptide. Data are shown scaled equivalently. *D*, Proliferation of *AND* and *AND;FADDdd* T cells as measured by incorporation of [³H]thymidine after a 3-day stimulation with the indicated concentrations of PCC peptide or the altered PCC peptide ligand K99A. *E*, Expression of CD69 and annexin V following antigenic stimulation of *AND;FADDdd* T cells.

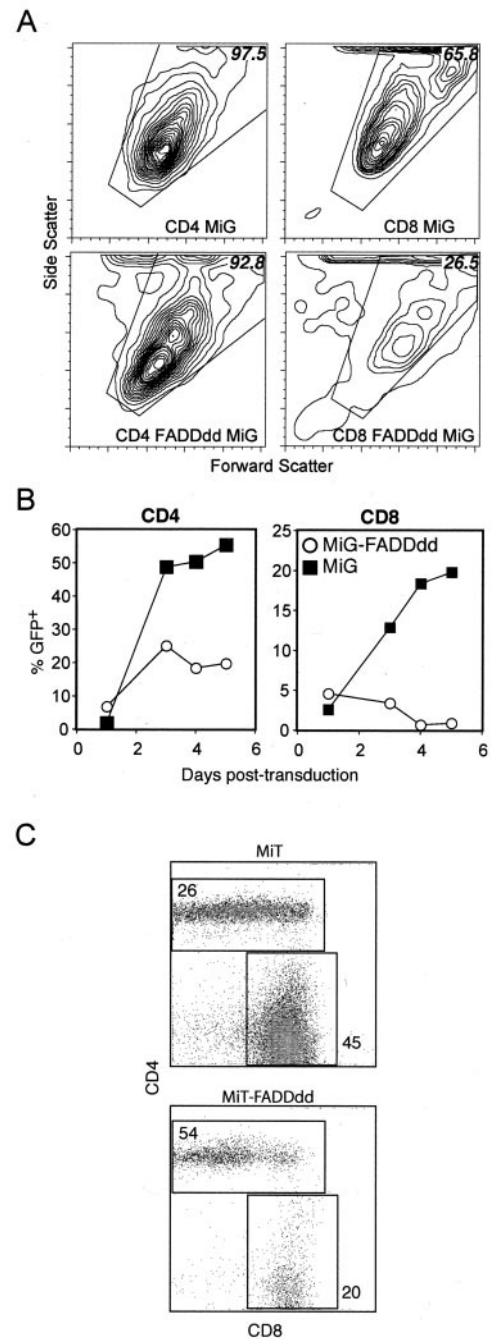


FIGURE 8. Decreased survival and accumulation of wild-type CD8⁺ T cells transduced with FADDdd-expressing retroviruses. *A*, Activated wild-type T cells were transduced with MiG-FADDdd or MiG and GFP expression was analyzed on the indicated days following spin transduction. Before FACS analysis, cultures were stained with anti-CD4 and anti-CD8 Abs and the results represent cells electronically gated for these markers. *B*, Reduced survival of MiG-FADDdd-transduced CD8⁺ T cells following infection. Shown are GFP⁺CD4⁺ or GFP⁺CD8⁺ cells transduced with MiG-FADDdd or MiG empty vector. Percentages represent the proportion of GFP⁺ cells within a live gate. *C*, Cells were transduced with a Thy1.1-expressing retrovirus (MiT) or a clone containing both Thy1.1 and *FADDdd* (MiT-FADDdd) and an IRES. Five days after transduction, the cells were stained for Thy1.1, CD4, and CD8. Shown are the percentages of Thy1.1⁺ cells staining for CD4 or CD8.

means to identify infected cells in the culture. Infection time course experiments revealed similar results with MiT-FADDdd as had been seen previously with the MiG-FADDdd retrovirus (data not shown).

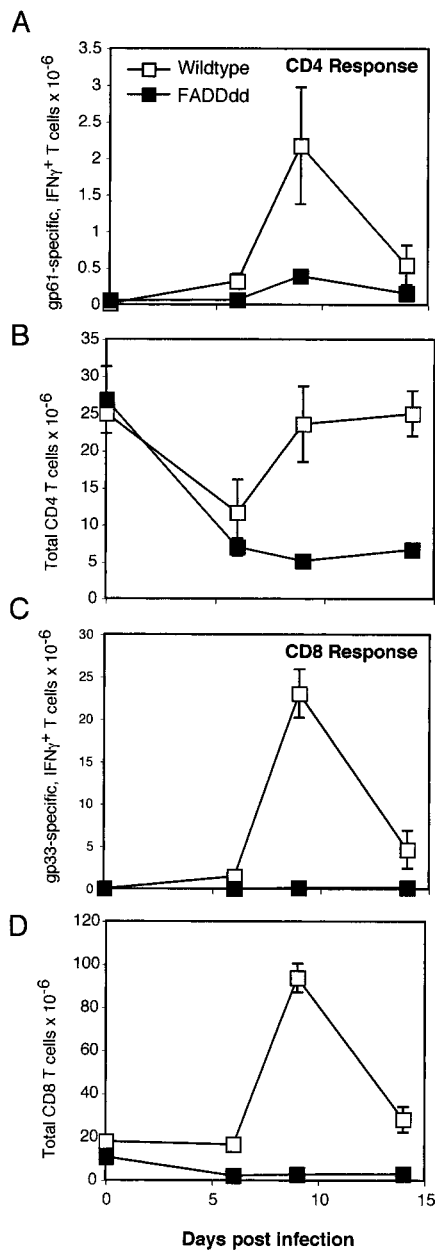


FIGURE 9. Defective CD4 and CD8 T cell responses to LCMV challenge in *FADDdd* mice. Following i.p. infection of wild-type and *FADDdd* mice, total cells were recovered from the spleen on indicated days and analyzed by flow cytometry for CD8 and CD4 expression. The total numbers of CD4⁺ (B) and CD8⁺ (D) cells are shown at days 0, 6, 9, and 14 after infection. LCMV-specific T cells were determined by intracellular IFN- γ staining. Splenocytes were stimulated with either gp61 and stained with anti-CD4 and anti-CD44 (A) or gp33 and stained with anti-CD8 and anti-CD44 (C). Graphs represent total number of CD4 or CD8 T cells secreting IFN- γ to gp61 or gp33 peptides, respectively.

We also analyzed the CD4:CD8 ratio of Thy1.1⁺ cells 5 days after activation and retroviral transduction (Fig. 8C). Although splenic naive T cells typically display a 2:1 CD4:CD8 ratio, we observed that activation with anti-CD3 and IL-2 led to preferential expansion of the CD8⁺ subset. However, cultures transduced with MiT-FADDdd contained a greatly reduced proportion of CD8⁺ cells. Taken together, these results demonstrate that FADD function is required to maintain the proliferation of normally differentiated mature T cells. Furthermore, these results are consistent with results obtained in *FADDdd*-transgenic T cells and we conclude that FADDdd blocks survival

associated with progression through the cell cycle; unexpectedly, this requirement is more strictly enforced in CD8⁺ T cells responding to antigenic stimulation.

FADD signaling is required for in vivo LCMV clearance

Finally, to verify that the effects of FADDdd observed in vitro can extend to a pathogen-induced immune response, wild-type and *FADDdd* mice were infected with LCMV Armstrong (48). Mice were examined at various times after infection for total CD4 and CD8 T cells in the spleen and also for the number of IFN- γ ⁺ T cells after stimulation in vitro with the dominant T cell epitope from the viral gp61 (CD4 T cells) and gp33 (CD8 T cells) peptides. The peak of the response to LCMV has been shown to occur about 8 days following infection (26), and the results depicted in Fig. 9 are consistent with the published reports. The total number of splenic CD4 T cells decreased and then recovered by day 9, and this corresponded with a rise in gp61-responsive CD4 T cells. In *FADDdd* mice, in contrast, the CD4 T cells did not recover during the course of the experiment and the number of gp61-specific T cells was greatly diminished. A similar kinetics was observed for the CD8 T cell response corresponding with a 5-fold increase in total CD8 T cells and a dramatic increase in the number of gp33-specific CD8 T cells 9 days after infection. In harmony with all other aspects of the CD8 T cell response in *FADDdd* mice, there was no increase in gp33-specific CD8 T cells nor total T cells in *FADDdd* mice. Consistent with the idea that T cells expressing FADDdd can respond but not accumulate, the percentage of both CD4 and CD8 T cells that converted to a CD44^{high}CD62L^{low} phenotype was equivalent or even slightly higher for splenocytes from *FADDdd* mice compared with wild-type mice (data not shown). Thus, the analysis of an actual immune response to an infectious agent reveals a profound deficiency in T cells expressing a *FADDdd* transgene to accumulate after Ag-mediated activation.

Discussion

The analysis of mice with defective signaling through the FADD adaptor has revealed an unexpected duality in its function. Although it is certainly essential for mediating the activation of proapoptotic caspases downstream of DRs, it is apparently also involved in regulating T cell responses. The essential problem is to characterize the mode of control and the mechanisms responsible for FADD-mediated regulation. From such information the logic underlying this form of T cell regulation may be revealed.

In this study, we have used a dominant interfering form of FADD in two ways to show that FADD acts by affecting the survival of cycling T cells. In the presence of FADDdd, T cell activation is accompanied by little or no proliferation. The proximal signals involved in T cell activation appear to be unaffected and T cells appear to respond to cytokines and enter the cell cycle normally. In fact, by visualizing the distribution of cell divisions using CFSE, we find that T cells from *FADDdd* mice are capable of multiple cell divisions. The difference is that the percentage of cells that die at each round of division is increased and the cumulative result is a lack of proliferation (Figs. 2, 4, and 5). The survival defect was not overcome by stimulation through the CD28 coreceptor or the addition of large concentrations of IL-2 or IL-4 (Fig. 1).

Additional insight into the nature of the requirement for FADD signaling is provided by the aforementioned retrovirus experiments. Since FADD is known to impart cues for the development of thymocytes at the TCR β -chain selection stage (14, 15, 17, 18), we investigated whether the presence of a fully rearranged TCR at this stage might rescue developing thymocytes such that the resulting T cells, expressing FADDdd, would complete maturation

and exhibit a normal response. Clearly this did not occur and, furthermore, the acute expression of FADD^{dd} by retroviral transduction revealed the same type of survival defect. We conclude that despite an important role for FADD in early thymocyte differentiation before positive selection, defective proliferation and survival of FADD^{dd} T cells is not simply due to aberrant thymocyte development. Signaling that can be blocked by FADD^{dd} is clearly important for the expansion of mature T cells following antigenic stimulation.

Yet another unexpected aspect of FADD activity is our observation that *FADD^{dd}* expression appears to almost completely abolish CD8 T cell proliferation in every assay, whereas its effect on CD4 T cell proliferation was variable depending on the type of analysis. First, we found a marked increase in the steady-state CD4:CD8 ratio in *FADD^{dd}* mice when compared with wild-type counterparts, and this was largely a function of CD8 T cell loss. Second, we found that *FADD^{dd}* had no apparent effect on the in vitro Ag-mediated proliferation of CD4 T cells from two different TCR-transgenic mice (*AND* and *OT-II*), whereas it completely abolished proliferation of CD8 T cells from three other TCR-transgenic mice (*OT-I*, *H-Y*, and *P14*) (data not shown) (21, 32); Newton et al. (27) have reported an analogous defect in the in vivo response of *OT-I*; *FADD^{dd}* T cells injected into RIP-mOVA mice (27). Third, retrovirus-mediated transduction experiments showed that both CD4 and CD8 T cells were affected by expression of FADD^{dd}, although the magnitude of the effect on CD8 T cells was much greater. Fourth, in other in vitro culture assays, the activation of T cells via anti-CD3 and either anti-CD28 or cytokines revealed only a moderate, albeit detectable CD4 defect. In contrast, the response to LCMV showed that in an immune response to an infectious agent, the accumulation of Ag-responsive CD4 T cells was strongly attenuated; however, even in this assay, the effect on CD8 T cells was more pronounced.

Although we do not understand this dichotomy, one possibility is that CD4 T cells undergo Fas-mediated cell death in response to Ag and cytokine stimulation, and this would be blocked by transgenic FADD^{dd}. Thus, there may be opposing effects mediated by FADD^{dd} that can produce variable results depending on the method of stimulation. Ag-mediated activation in culture may include a large component of Fas-mediated cell death, whereas LCMV-mediated T cell expansion in vivo may not (49, 50). We find no evidence for the trivial explanation that FADD^{dd} expression is lessened in CD4 T cells as compared with CD8 T cells and this possibility is further diminished by the results of the retroviral experiments where the presence of the FADD^{dd} transgene is revealed by GFP or Thy1.1 expression. The possibility exists that the competitive blocking of FADD is incomplete, but this only underscores the relative difference in the requirement for FADD in these two subsets. On the other hand, FADD^{dd} could bind to and block death domain molecules in addition to FADD, but in that case such molecules must be specifically required for CD8 T cells to survive a cell cycle check point. Whether a similar dichotomy would be found for T cells that are truly FADD-deficient is presently unknown. Working with similar FADD^{dd}-expressing mice, Newton et al. did not observe significant differences between CD4 and CD8 T cells responding to mitogenic stimulation (15, 27). We are uncertain about this discrepancy, but one possibility is that their transgene was expressed at a different level and affected Fas-mediated cell death to a lesser extent. Thus, although the distinctions observed with TCR-transgenic mice may serve to focus our attention on signaling pathways that are specifically activated in CD4 and CD8 cells, our overarching conclusions are in accord with the notion that FADD signaling plays a fundamental role in T cell proliferation and adaptive immunity in general.

These results are consistent, in part, with a previous report showing a defect in cell cycle progression in FADD-deficient T cells. Zhang and coworkers (20) examined FADD-deficient T cells produced by the complementation of RAG1-deficient embryos with FADD-deficient embryonic stem cells. These T cells were found to be spontaneously cycling, perhaps due to the lymphopenic state of these mice, and their profile of cell cycle control molecules was abnormal. Although we agree that FADD is involved in the regulation of the cell cycle in T cells, our data show that *FADD^{dd}*-expressing T cells can be activated and progress through the cell cycle albeit with a diminished probability of survival.

A likely candidate for the proximal mediator of FADD is caspase-8 and perhaps downstream caspases (51, 52). Similar defects in T cell activation have been observed when the caspase inhibitors Z-Val-Ala-Asp(*O*-methyl)-fluoromethyl ketone and Ile-Glu-Thr-Asp-FMK are added to cultures (Refs. 51 and 52 and data not shown), and recent evidence has demonstrated a requirement for caspase-8 in the function of human T cells (19). Furthermore, addition of Z-Val-Ala-Asp(*O*-methyl)-fluoromethyl ketone to in vitro activation cultures does not rescue *FADD^{dd}* CD8 cells (data not shown). Given these results and the lack of an increase in TUNEL staining following activation (Fig. 6), we propose that *FADD^{dd}* T cells may die by a mechanism that is not prototypically apoptosis, but neither is it necrosis (53, 54). An attractive hypothesis is that FADD, via caspase-8, may transduce broad range signals that are required for T cell survival or metabolism. Notwithstanding our bias that the death is caspase independent, such prosurvival signals might be mediated by NF- κ B and extend to the inhibition of proapoptotic factors, such as p73 (55), or the enhancement of prosurvival factors such as Bcl-2 family members and inhibitors of apoptosis proteins.

What is the logic underlying the dual role of FADD in caspase-mediated cell death and cell cycle survival? One possibility is that this type of mechanism inextricably links the expansion and contraction of T cells during an immune response. In order for T cells to achieve expansion, the FADD death pathway has to be "locked and loaded." If the FADD pathway is not activated, T cells die during the progression of the cell cycle. If it is activated, they eventually die via caspase-8 activation and/or enhanced expression of proapoptotic Bcl-2 family members such as Bim (56). Either way, T cells are prevented from proliferating uncontrollably. A solution to the problem of FADD-mediated T cell survival will illuminate a novel mechanism of T cell regulation.

Addendum. During the review of this article, Selemena et al (57) published a study of mice in which caspase-8 was deleted in T cells. The results closely match those described here even to the extent that there is an increase in the CD4:CD8 ratio in peripheral lymphoid organs. We conclude that T cell survival under conditions of proliferation requires the FADD-mediated activation of caspase-8.

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