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The Fas-Associated Death Domain Protein Is Required in Apoptosis and TLR-Induced Proliferative Responses in B Cells

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The Fas-associated death domain protein (FADD)/Mort1 is a signaling adaptor protein which mediates the activation of caspase 8 during death receptor-induced apoptosis. Disruption of FADD in germ cells results in death receptor-independent embryonic lethality in mice. Previous studies indicated that in addition to its function in apoptosis, FADD is also required in peripheral T cell homeostasis and TCR-induced proliferative responses. In this report, we generated B cell-specific FADD-deficient mice and showed that deletion of FADD at the pro-B cell stage had minor effects on B cell development in the bone marrow, and resulted in increased splenic and lymph node B cell numbers and decreased peritoneal B1 cell numbers. As in T cells, a FADD deficiency inhibited Fas-induced apoptosis in B cells. However, B cell-proliferative responses induced by stimulation of the BCR and CD40 using anti-IgM or anti-CD40 Abs were unaffected by the absence of FADD. Further analyses revealed that FADD-deficient B cells were defective in proliferative responses induced by treatments with dsRNA and LPS which stimulate TLR3 and TLR4, respectively. Therefore, in addition to its apoptotic function, FADD also plays a role in TLR3- and TLR4-induced proliferative responses in B cells. The Journal of Immunology, 2006, 176: 6852–6861.
from transitional B cells (38), and reside primarily around the periphery of the splenic lymphoid nodules. The development of these conventional (or B2) B cells is initiated in the bone marrow and continues in peripheral lymphoid organs such as the spleen (39). Finally, B1 B cells are believed to be fetal liver-derived, long-lived and present mainly in the peritoneal and pleural cavities (40, 41).

The BCR induces intracellular signaling processes analogous to those induced by the TCR. Given the proliferation defect present in FADD-deficient T cells, it was of interest to determine whether FADD is required for proliferative responses induced by BCR signaling. B cells can also be induced to proliferate by stimulation of CD40 and by certain macromolecules present in microbial pathogens which can trigger intracellular signaling through TLRs. These evolutionarily conserved “pattern recognition” receptors play a critical role in innate immune responses (42, 43). TLRs contain an intracellular TLR/IL-1R (TIR) domain which interacts with the TIR domain of the adaptor protein MyD88. The DD of MyD88 binds that of IRAK serine/threonine kinases (44, 45). Although MyD88 is believed to be used by all TLRs during signaling, other adaptor proteins, such as TRIF (TIR domain-containing adaptor protein-inducing IFN-β), mediate alternative pathways, particularly those induced by TLR3 and TLR4 (46).

In this study, we generated B cell-specific FADD-deficient mice using the Cre-loxP system to determine the temporal requirement of FADD in B cell lymphopoiesis and the FADD function in apoptosis and proliferation responses in B cells. Analysis of these mice revealed that FADD is required for Fas-induced apoptosis in B cells to maintain homeostasis in the spleen and lymph nodes. Furthermore, FADD plays a role in B cell proliferative responses induced by TLR3 and TLR4.

Materials and Methods

Generation of B cell-specific FADD−/− mice

FADD+/− and FADD:GFPflox mice were reported previously (22, 26) and crossed to obtain FADD+/− FADD:GFPflox mice, which were then backcrossed to FADD−/− mice to produce viable FADD+/− FADD:GFPflox mice. To generate B cell-specific FADD-deficient mice, CD19-Cre transgenic mice, provided by Dr. K. Rajewsky (Harvard Medical School, Boston, MA), were crossed with FADD−/− mice. The resulting FADD−/− CD19-Cre mice were backcrossed to B6 mice. Co-segregation of the FADD knockout allele and CD19-Cre allele in the offspring indicates that crossovers on the chromosome 7 resulted in linkage of these two gene alleles on the same chromatin in the parental mice. These were then crossed with FADD+/− FADD:GFPflox mice to generate B cell-specific FADD-deficient FADD−/− FADD:GFPflox CD19-Cre mice. Messenger RNAs with and without FADD alleles were determined by Southern blot analyses using mouse tail DNA and a 0.4-kb probe to detect the endogenous and knockout FADD alleles and FADD:GFP as different sizes of EcoRI fragments as described elsewhere (26). The CD19-Cre gene was detected by PCR using oligo primer DNA sequences (GTCTGAAAGCTTCCGACCGG and CTG CGTGCAATCCATCTGTGTT) and a protocol (1 min at 94°C, 1 min at 63°C, and 2.5 min at 72°C for 35 cycles) provided by Dr. R. Richert (Bournham Institute, San Diego, CA). All animal studies were approved by the Institutional Review Board at Thomas Jefferson University.

Flow cytometry

Single-cell suspensions were prepared from the bone marrow, spleen, lymph nodes, or peripheral blood. RBC were depleted by hypotonic lysis. To determine GFP expression, cells were subjected to flow cytometric analysis using a Coulter Epics XL analyzer (Beckman Coulter). For cell surface protein staining, appropriate fluorochrome-conjugated Abs were added to single-cell suspensions in PBS with 1% FBS and 0.1% sodium azide, followed by incubation on ice for 20–30 min and two washes with PBS. Peritoneal cavity cells were harvested from individual mice by flushing two times with 5 ml each of RPMI 1640 medium (Mediatech). After counting, cells were resuspended in staining buffer (3% BSA, 0.5 mM EDTA, 0.05% sodium azide) and stained with appropriate Abs. The following Abs were used: Tri-Color-conjugated anti-CD4, CD8, B220, and biotinylated anti-CD23 Abs (Caltag Laboratories); PE-conjugated anti-AA4.1, and TLR-4 Abs (eBioscience); PE-conjugated anti-CD3, CD21, CD25, e-Kit, and biotinylated anti-CD19 (clone 1D3), CD5 (clone 53-7.3), CD11b (Mac-1, clone M1/70) Abs, streptavidin-CyChrome (BD Biosciences); PE-conjugated anti-mouse IgM Abs (clone II/41; Jackson ImmunoResearch Laboratories); biotinylated anti-IgD (clone 11-26; Southern Biotechnology Associates). Cells were analyzed using a Coulter Epics XL cytometer (Beckman Coulter), and data analyzed with WinMDI (C. J. Trotter, The Scripps Institute, La Jolla, CA) and FlowJo software (Tree Star). To purify B cells by sorting, single-cell suspensions were prepared as described above and stained with Tri-Color-anti-B220 or PE-anti-CD19 Abs. CD19+ GFP− or B220+ GFP− cells were isolated using a MoFlo high-speed cell sorter (DakoCytomation).

Immunohistology

Spleen and lymph nodes cryostat sections (5–6 μm) were prepared and immunohistology was performed as previously described (47). Abs were detected using the Vector Blue Alkaline-phosphatase Substrate kit III and the Vector NovoRed kit for peroxidase (Vector Laboratories). HRP-anti-CD4 (clone GK1.5) Abs were made in-house. The stained sections were analyzed using a light microscope (Leitz Diaplan, Axioplan Universal Microscope; Carl Zeiss MicroImaging), and digital images were captured using an Eastman Kodak camera.

Cell culture

B cells were cultured at 37°C in a 5% CO2 incubator in RPMI 1640 (Mediatech) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM glutamine, 50 μM 2-ME (Sigma-Aldrich), and 10% FBS (HyClone Laboratories).

Fas-induced apoptosis assay

Sorted mutant and control B cells were seeded to 96-well plates (105/well) in 100 μl of RPMI 1640 medium. Soluble Fasl (sFasl; Alexis Biochemical) was added at various concentrations in triplicates. Anti-FLAG Abs M2 (Sigma-Aldrich) were then added (1 μg/ml) to the culture. After a 16-h incubation, cells were transferred to flow tubes and subject to analysis using a flow cytometer after addition of propidium iodide (PI) (1 μg/ml).

Serum Igs

Concentrations of serum Igs were determined by ELISA. Ninety-six-well flat-bottom plates (Thermo Labsystems) were coated with 10 μg/ml goat anti-mouse capture Ab (Southern Biotechnology Associates) in PBS overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS and blocked with 1% BSA in PBS for at least 1 h at room temperature. After washing, serum dilutions of 1/1,000, 1/3,000, 1/900, and 1/270,000 were made and added to appropriate wells for 1 h at room temperature. After washing, HRP-conjugated goat anti-mouse Igs were added for 1 h at room temperature, then thoroughly washed. Substrate solution was prepared by mixing 10 ml of citrate substrate buffer (525 mg of citric acid in 50 ml of H2O) with 0.2 ml of 2.2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) stock solution and 100 μl of 3% H2O2. Substrate solution was added to the wells and absorbance was measured at 405 nm at 10 and 20 min. Ig concentrations were determined by using a standard curve obtained from every ELISA plate.

B cell proliferation and activation marker assays

For [3H]thymidine incorporation assays, sorted B cells were plated in triplicates in 96-well round-bottom plates (Thermo Labsystems) coated with 10 μg/ml goat anti-mouse capture Ab (Southern Biotechnology Associates) in PBS overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS and blocked with 1% BSA in PBS for at least 1 h at room temperature. After washing, serum dilutions of 1/1,000, 1/3,000, 1/900, and 1/270,000 were added for 1 h at room temperature. After washing, HRP-conjugated goat anti-mouse Igs were added for 1 h at room temperature, then thoroughly washed. Substrate solution was prepared by mixing 10 ml of citrate substrate buffer (525 mg of citric acid in 50 ml of H2O) with 0.2 ml of 2.2’-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) stock solution and 100 μl of 3% H2O2. Substrate solution was added to the wells and absorbance was measured at 405 nm at 10 and 20 min. Ig concentrations were determined by using a standard curve obtained from every ELISA plate.

To analyze cell division by CFSE labeling, B220+ GFP− B cells were sorted from the spleen and lymph nodes, labeled with 5 μM CFSE (Molecular Probes) in PBS plus 5% FBS for 5 min at 37°C in dark, and washed three times with 5 ml of RPMI 1640–10% FBS. These labeled cells were then stimulated with LPS (10 μg/ml) or anti-IgM Abs (10 μg/ml) for 72, 96, and 120 h, and analyzed using a flow cytometer. 7-Aminoactinomycin D (7-AAD; BD Pharmingen) was added (0.83 μg/ml) to CFSE-labeled B cells at 72 h after stimulation with LPS, to detect cell death by two-color flow cytometry. To detect CD54, CD86, and MHC class II up-regulation, sorted mutant and control B cells were cultured with LPS (10 μg/ml) in 96-well round-bottom plates as indicated above. After 16 h, cells were washed once with 1 ml of staining buffer, and then stained on ice for 30 min with CD86-PE.
FADD FUNCTION IN B CELLS AND TLR SIGNALING

B cell survival/death assays

B cells were isolated from the spleen and lymph nodes by high-speed sorting, resuspended in complete medium with or without LPS, and seeded (10⁶ cell/well) into 96-well round-bottom plates. At indicated times, live cells were detected as those excluding PI (1 μg/ml) in flow cytometry assays.

Western blot analysis

To detect NF-κB, ERK, JNK, and Akt activation, sorted B cells (2–5 × 10⁶) were stimulated at 37°C in 0.5 ml of RPMI 1640 medium-10% FBS and LPS (10 μg/ml) for the times indicated. Cells were washed once with ice-cold PBS and lysed for 3 min in an ice-cold buffer (1% Triton X-100, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 20 mM EDTA, 1 mM Na3VO4, 1 mM NaF, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 0.7 μg/ml pepstatin, and complete protease inhibitor mixture). Total proteins (10–25 μg) were denatured by boiling for 3 min in SDS-containing sample buffer, separated by 10% SDS/PAGE, and blotted onto nitrocellulose membranes. Blots were incubated with 5% BSA-TBST (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1% Tween 20) for 1 h at room temperature, and then overnight at 4°C with Abs specific for IκB, ERK1/2, or phosphorylated forms of ERK1/2, Akt (clone 193H12), and JNK (Cell Signaling Technology) at 1/1000 dilution per manufacturer’s recommendation. After three washes, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (PerkinElmer) was used for signal detection with x-ray films (Kodak). Anti-TLR3 Abs were obtained from Santa Cruz Biotechnology.

Results

Generation of B cell-specific FADD-deficient mice

Inactivation of the mouse FADD gene upon deletion of the pro-motormer and the first coding exon (middle, Fig. 1A) results in homozygous FADD knockout (FADD<sup>−/−</sup>) mice that die during midgestation (22). We recently showed that a FADD knockoout allele (FADD:GFP<sup>−/−</sup>) results in loss of the coding region (middle, Fig. 1A) and are representative of 14 independent experiments.

FADD:GFP<sup>−/−</sup> mice were used as controls. The absence of GFP<sup>−/−</sup> B cells (right) from FADD:GFP<sup>−/−</sup>CD19-Cre mice were analyzed by Western blotting using anti-FADD Abs. FADD<sup>+/−</sup>, FADD<sup>+/+</sup>:FADD:GFP<sup>+</sup>, FADD<sup>−/+</sup>:FADD:GFP<sup>+</sup>, FADD<sup>−/−</sup>:FADD:GFP<sup>+</sup>, FADD<sup>−/−</sup>:FADD:GFP<sup>+</sup> CD19-Cre mice were used as controls. The absence of FADD:GFP<sup>−/−</sup> cells were confirmed in three independent experiments.

FIGURE 1. A, Diagrammatic scheme in generating B cell-specific FADD-deficient mice. The CD19 locus is closely linked to FADD on mouse chromosome 7 with a ~10 centimorgan distance (top). The CD19-Cre allele was crossed to the FADD knockout allele (22) (middle) by mouse mating to delete FADD:GFP<sup>loxP</sup> specifically in B cells in FADD<sup>−/−</sup> mice. The promoter of CD19 (arrows), the neomycin-resistant gene (neo), exons of FADD (boxes) and GFP are indicated. Drawings are not to scale. B, Flow cytometric analysis of FADD:GFP deletion in B cells. Single-cell suspensions were prepared from the bone marrow, spleen, lymph nodes, and peripheral blood, and stained for CD19. The indicated percentages of GFP<sup>−</sup> cells in the CD19<sup>+</sup> population of FADD<sup>−/−</sup>:FADD:GFP<sup>loxP</sup> CD19-Cre mice were determined by flow cytometry. Cells from FADD<sup>+/−</sup>CD19-Cre and FADD<sup>−/−</sup>:FADD:GFP<sup>loxP</sup> mice were used as GFP<sup>−</sup> and GFP<sup>+</sup> controls, respectively. Histograms are from one experiment using one mouse of each genotype, and are representative of 14 independent experiments. C, Total thymocytes and splenocytes (left) or sorted GFP<sup>−/−</sup> B cells (right) from FADD<sup>−/−</sup>:FADD:GFP<sup>loxP</sup>CD19-Cre mice were analyzed by Western blotting using anti-FADD Abs. FADD<sup>+/−</sup>, FADD<sup>+/+</sup>:FADD:GFP<sup>+</sup>, FADD<sup>−/+</sup>:FADD:GFP<sup>loxP</sup>, FADD<sup>−/−</sup>:FADD:GFP<sup>+</sup>, FADD<sup>−/−</sup>:FADD:GFP<sup>loxP</sup> CD19-Cre mice were used as controls. The absence of FADD:GFP<sup>−/−</sup> cells were confirmed in three independent experiments.
(76%), and blood (82%; Fig. 1B). The gradually decreasing GFP+ and GFPlow B cell populations indicate continuing FADD:GFP gene deletion and FADD:GFP protein turnover during B cell development and maturation. Western blot analyses of total cells from the spleen revealed reduced levels of the FADD:GFP fusion protein in FADD−/−FADD:GFPfloxF19-Cre mice, in comparison to control mice lacking CD19-Cre (lanes 3 and 4 on the left, Fig. 1C). As expected, the FADD:GFP protein was not reduced in thymocytes of FADD−/−FADD:GFPfloxF19-Cre mice (lane 8 on the left, Fig. 1C). Western blot analyses of GFP+ B cells purified by high-speed sorting indicated undetectable levels of FADD:GFP (lane 3 on the right, Fig. 1C), in comparison to control GFP+ and GFP+ B cells isolated from FADD−/−CD19-Cre and FADD−/−FADD:GFPfloxF mice, respectively. Therefore, FADD−/−FADD:GFPfloxF19-Cre mice contain FADD−/− B cells and are referred to as B cell-specific FADD-deficient (or FADD−/−) mice hereafter. FADD−/−FADD:GFPfloxF19-Cre (FADD−/−) mice containing one allele of the endogenous FADD and CD19 genes were used as controls in the described analyses to follow.

**B cell development in the bone marrow and periphery in B cell-specific FADD−/− mice**

Cre gene expression is presumably initiated as early as in the pro-B cell stage, because it is integrated into a site immediately downstream of the CD19 gene promoter (48). To determine the deletion of FADD:GFP in various B cell subsets, total bone marrow cells were isolated from B cell-specific FADD−/− mice, stained for the B lineage-specific marker CD19 and stage-specific markers (c-Kit, CD25, IgM, and IgD), and analyzed by flow cytometry. Few GFP− cells (5.4%) were detected in the pro-B population (CD19/c-Kit+) (Fig. 2A). The percentages of GFP− cells were increasingly higher in the CD19CD25− pre-B (56.1%), IgM+IgD− immature (59%), and IgM+IgD− mature (78.5%) populations, accompanied by a gradual decrease of GFP+ and GFPlow cell numbers in each (Fig. 2A). To analyze the effect of FADD deficiency on B cell development, the bone marrow B cell profile was determined by flow cytometry. When analyzed using stage-specific markers, B cell-specific FADD−/− mice were found to contain pro-B (CD19+c-Kit+), pre-B (CD19CD25+), immature (IgM+IgD−), and transitional (CD19AA4.1+) B cells at levels similar to those in the bone marrow in FADD−/− mice (Fig. 2B). There appeared to be lower percentages of recirculating IgD+IgM+ mature B cells in the bone marrow of B cell-specific FADD−/− mice than that in FADD−/− control mice (Fig. 2B). Therefore, B cell development in the bone marrow was not significantly affected in B cell-specific FADD−/− mice.

As discussed above (Fig. 1B), B cell-specific FADD−/− mice contained a large proportion (70–80%) of FADD−/− (GFP+) B cells in the peripheral lymphoid organs. The spleen and lymph nodes of B cell-specific FADD−/− mice were generally larger in size (data not shown), and the total cellularity in these organs was significantly higher in mutant mice when compared to control FADD−/− mice (left, Fig. 3A). When analyzed by flow cytometry after staining for CD19 and the T cell marker CD3, B cell-specific FADD−/− mice were found to contain slightly higher percentages of splenic and lymph node B cells than that in FADD−/− mice (Fig. 3B). When total B cell numbers were compared, there was a significant increase of B cells in the spleen and lymph nodes of FADD−/− mice than in these peripheral lymphoid organs of FADD−/− mice (right, Fig. 3A). Peripheral T cell numbers in B cell-specific FADD−/− mice appeared to be higher in comparison to FADD−/− mice (data not shown). However, this phenotype does not appear to be statistically significant. Because of these phenotypes, B220 and CD4 immunohistochemistry was performed on sections of the spleen and lymph nodes isolated from mutant and control mice to examine the microarchitecture of these peripheral lymphoid organs. The B cell follicles in mutant mice were generally larger, particularly in lymph nodes, than in control mice (blue B220, Fig. 3C), which is consistent with the data showing the increased number of B cells in these mutant peripheral lymphoid organs (Fig. 3, A and B). However, the T cell compartment appears to be unaffected by a lack of FADD in B cells (brown-CD4, Fig. 3C).

**To analyze B cell development in the periphery, splenocytes** were isolated and analyzed by flow cytometry. The T1 (IgDlowIgMhigh) and T2 (IgDhighIgMhigh) transitional B cells were present in B cell-specific FADD−/− mice in percentages similar to that in FADD−/− mice (see Fig. 4A). The percentages of marginal zone and follicular B cells in the mutant spleen were also similar to that in control mice, as determined by staining for IgD and IgM, or CD21 and CD23 (see Fig. 4, A and B). These results indicate that the FADD deficiency did not affect B cell development in the periphery. Because the percentages of transitional, marginal zone, and follicular B cells in the periphery of B cell-specific FADD−/− mice are similar to those of FADD−/− mice (see Fig. 4, A and B), the higher total B cell numbers as seen in the spleen in FADD−/− mice (Fig. 3A) is likely due to increases in every B cell subpopulation, and not because of selective expansion of a particular B cell population. To analyze the effect of FADD deficiency on the B1 cell population, peritoneal cells were isolated from mutant and control mice and analyzed by flow cytometry. As shown in Fig. 4C, the percentage of IgM+M1c+ B1 cells in B cell-specific FADD−/− mice (9.55 ± 3.84%; n = 10) was ~60% of that in control FADD−/− mice (16.46 ± 3.61%; n = 10).
Apoptosis and serum Ig analysis

FADD is critical for Fas-induced apoptosis in fibroblasts and T cells (16, 26). To determine Fas-induced cell death responses, B cells were sorted from the spleen and lymph nodes and cultured in the presence of FLAG-tagged sFasL, which was then cross-linked by using anti-FLAG Abs. Both FADD−/− B cells and those lacking the endogenous FADD but expressing FADD:GFP isolated from FADD−/− FADD:GFPfloxCD19-Cre mice (FADD−/−) were compared with that of control FADD+/− FADD:GFPfloxCD19-Cre mice (FADD+/−). Error bars indicate SD from analysis of seven mice of each indicated genotype. B. Representative dot plots from flow cytometric analyses of CD3+ T cells and CD19+ B cells in 15 mice of each genotype indicated. C. Immunohistological analysis of cryosections of the spleen and lymph nodes. B cells were stained with anti-B220 Abs (blue) and T cells were stained with anti-CD4 Abs (brown). Data shown are representative of analysis of five mice of each indicated genotype.

FIGURE 3. Analysis of the peripheral lymphoid system in B cell-specific FADD−/− mice. A, Total (left) and CD19+ B (right) cell numbers in the spleen and lymph nodes from FADD−/− FADD:GFPfloxCD19-Cre mice (FADD−/−) were compared with that of control FADD+/− FADD:GFPfloxCD19-Cre mice (FADD+/−). Error bars indicate SD from analysis of seven mice of each indicated genotype. B, Representative dot plots from flow cytometric analyses of CD3+ T cells and CD19+ B cells in 15 mice of each genotype indicated. C, Immunohistological analysis of cryosections of the spleen and lymph nodes. B cells were stained with anti-B220 Abs (blue) and T cells were stained with anti-CD4 Abs (brown). Data shown are representative of analysis of five mice of each indicated genotype.

Apopotosis and serum Ig analysis

FADD is critical for Fas-induced apoptosis in fibroblasts and T cells (16, 26). To determine Fas-induced cell death responses, B cells were sorted from the spleen and lymph nodes and cultured in the presence of FLAG-tagged sFasL, which was then cross-linked by using anti-FLAG Abs. Both FADD−/− B cells and those lacking the endogenous FADD but expressing FADD:GFP isolated from FADD−/− FADD:GFPfloxCD19-Cre mice were killed in a dose-dependent manner by this treatment (Fig. 5A), indicating that FADD:GFP functions similarly to the endogenous FADD. In contrast, FADD−/− B cells were resistant to cell death induced by sFasL at various concentrations (Fig. 5A). Defective Fas signaling may lead to autoimmune diseases such as arthritis, higher levels of serum IgS, and increased mortality in aged mice. Although B cell-specific FADD−/− mice contained increased numbers of B cells defective in Fas-induced apoptosis, they showed no obvious joint swelling, a symptom of arthritis, and had survival rates similar to that of control littermates even when aged. We also collected sera of mice aged 2–10 mo and assayed for Ig levels. The data in Fig. 5B indicate that B cell-specific FADD−/− mice aged from 2 to 10 mo contained lower average serum Ig levels in comparison with those in FADD+/− control mice. Therefore, FADD is essential for B cell apoptosis induced by Fas-induced signaling, yet a lack of FADD in B cells is not sufficient for induction of autoimmune diseases in mice.

Proliferation responses in FADD−/− B cells

T cell-specific deficiency of FADD resulted in not only defective Fas-induced apoptosis, but also abnormal TCR-induced proliferation responses (26). We therefore examined FADD−/− B cell proliferation in response to various stimuli. Signaling through the BCR can be induced by treatment with F(ab′)2 anti-IgM Abs. FADD−/− and FADD−/− B cells were isolated by sorting for the GFP+ population from the spleen and lymph nodes, and treated with increasing concentrations of anti-IgM Abs. As shown in Fig.
To further analyze the proliferation defects in FADD\(^{-/-}\) B cells, cell division kinetics were determined using CFSE to label intracellular molecules, as CFSE fluorescence intensity halves upon each cell division. FADD\(^{-/-}\) and FADD\(^{+/+}\) B cells were sorted from the spleen and lymph nodes, labeled with CFSE, and stimulated with LPS or anti-IgM Abs to induce proliferation. As shown in Fig. 7A (top), cell division potentials in FADD\(^{-/-}\) and FADD\(^{+/+}\) B cells were equivalent in response to BCR stimulation with anti-IgM Abs, a result in agreement with \(^{[3}H\)thymidine-labeling assays (Fig. 6A). However, FADD\(^{-/-}\) B cells had a profound defect in cell division in comparison with control FADD\(^{+/+}\) B cells in cultures stimulated with LPS for 72, 96, and 120 h (bottom, Fig. 7A). To analyze any intrinsic defects in survival due to FADD deletion, B cells were cultured in complete medium without any stimulation and cell survival was determined by PI exclusion and flow cytometry. No consistent difference between FADD\(^{-/-}\) and FADD\(^{+/+}\) B cells in survival capability was detected over a period of 2 days in culture in several independent experiments (Fig. 7B). We further analyzed B cell death during stimulation with LPS by PI uptake and flow cytometry. Similar cell death rates were detected in both mutant and control B cells during an initial 12-h stimulation (Fig. 7B). In the FADD\(^{-/-}\) B cell culture stimulated with LPS, cell death peaked at 24 h and gradually decreased thereafter, whereas higher percentages of cell death were detected in FADD\(^{-/-}\) B cells at these time points during the 2-day stimulation (Fig. 7B). In a different assay, we performed two-color flow cytometric analyses by staining the CFSE-labeled B cell with 7-AAD. Cell death in the B cell cultures were indicated by 7-AAD-positive populations. As shown in Fig. 7C, there was more cell death in FADD\(^{-/-}\) than in FADD\(^{+/+}\) B cell cultures stimulated with LPS. These results indicate that FADD\(^{-/-}\) B cells do not appear to have intrinsic defects in ex vivo survival, but are not capable of complete division induced by LPS stimulation, thus resulting in increased cell death.

**Analysis of activation markers and signaling processes induced by LPS stimulation**

In normal B cells, LPS induces expression of CD54 (ICAM-1), the costimulatory receptor ligand B7.2 (CD86), and MHC class II molecules. The expression of these activation markers was analyzed by flow cytometry. Freshly isolated FADD\(^{-/-}\) B cells from the spleen and lymph nodes express basal levels of CD54 and CD86 on their surface, similar to that on FADD\(^{+/+}\) B cells (Fig. 8A). Within 16 h after stimulation by LPS, these two proteins were up-regulated equivalently in both mutant and control B cells (Fig. 8A). Resting B cells acting as professional APCs expressed high levels of MHC class II, which was further up-regulated during stimulation by LPS for 16 h in both FADD\(^{-/-}\) and FADD\(^{+/+}\) B cells (Fig. 8A). Therefore, a FADD deficiency did not appear to affect expression of activation markers induced by LPS stimulation.

Stimulation of TLRs induces activation of NF-κB and MAPKs such as ERKs and JNK (42). Upon signaling, the inhibitory subunit of NF-κB, IκB, is targeted for ubiquitination and degradation by the proteosome, thus releasing NF-κB for translocation to the nucleus (49). To analyze NF-κB activation, Western blotting was used to detect IκB. As demonstrated in Fig. 8B, the IκB protein was present in unstimulated B cells (0 min) and was reduced at later time points (15, 30, 45 min), indicating degradation of IκB was initiated in both FADD\(^{-/-}\) and FADD\(^{+/+}\) B cells upon stimulation with LPS. Phosphorylation of IκB, as determined by Western blot analyses using phospho-IκB-specific Abs, was not affected in FADD\(^{-/-}\) B cells stimulated with LPS (data not shown). To analyze activation of MAPKs, Western blotting was used to detect
phosphorylation of ERK1/2. In FADD+/− B cells, activation of ERK1/2 was evident, as indicated by the presence of the phosphorylated ERK1/2 within 15 min following LPS stimulation (Fig. 8B). Similar ERK1/2 activation kinetics were observed in FADD−/− B cells. Activation of JNK was also analyzed by Western blotting using phosphospecific Abs, and no apparent difference between FADD+/− and FADD−/− B cells stimulated by LPS was detected (Fig. 8B). PI3K can be activated by LPS, leading to the activation of the oncogenic serine/threonine kinase Akt, also known as protein kinase B (50, 51). As shown in Fig. 8B, Akt activation was readily achieved by LPS stimulation in both FADD+/− and FADD−/− B cells, as indicated by phosphorylation of Akt.

Discussion
The FADD protein was initially identified as an adaptor molecule required for cell death signaling induced by DD-containing receptors such as Fas, TNFR1, and TRAILR. These death receptors play an important role in regulating the function of the immune system and possibly in tumor surveillance, but are dispensable during embryonic development and hemopoiesis. FADD has additional functions because its absence in germ cells results in early embryonic lethality. In this study, we analyzed the function of FADD in the B cell lineage using a conditional FADD mutant mouse model, and demonstrated that the deletion of FADD specifically in B cells at the pre-B stage had no major effect on B cell development (Figs. 2 and 3). A FADD deficiency inhibited Fas-induced apoptosis (Fig. 5), resulting in an increased number of splenic and lymph node B cells (Fig. 3). Interestingly, peritoneal B1 cells were reduced in these B cell-specific FADD-deficient mice (Fig. 4). Unlike FADD−/− T cells which are defective in Ag receptor-induced proliferation responses (26), FADD−/− B cells appear to proliferate normally in response to stimulation of the BCR (Fig. 6). In addition, proliferation of B cells induced by CD40 or TLR9 stimulation was not affected in the absence of FADD. Surprisingly, however, FADD−/− B cells have a profound defect in proliferation induced by stimulation of TLR3 and TLR4. These results have thus revealed a novel function of FADD in the innate immune responses mediated by TLRs.
During the early stages of B lymphocyte development in the bone marrow, lymphoid progenitor cells give rise to fully committed B lymphocytes after a process of sequential recombination and assembly of Ig gene segments. In a previous study using viable mutant chimeras generated using FADD and Rag-1−/− blastocysts, FADD was absent through embryonic and lymphoid development (22). These chimeras contained few T cells and undetectable levels of B cells in the periphery. In this study, an absence of FADD at the pre-B and subsequent stages still allowed the generation of immature and mature B cells in the bone marrow and periphery at levels similar to FADD-expressing control mice (Figs. 2–4). Therefore, it is likely that FADD plays a more important role in earlier progenitor cells before development reaches the pre-B and later stages. B1 cells represent a unique population of B lymphocytes and can be distinguished from other B cells by their surface phenotype (39, 40). They constitute a substantial fraction of B1 cells in the peritoneal cavity and express Mac-1 (CD11b/CD18 or complement receptor type 3). The B1 lineage is presumably derived from precursors in the fetal liver and would persist for the life of the animal by self-renewal in adults. Mutations in several genes in mice such as CD19, B cell linker protein, Bruton’s tyrosine kinase, Vav, phospholipase Cγ2, the PI3K p85 subunit, and protein kinase C result in reduced B-1 cells (40). In this study, B cell-specific FADD−/− mice also contained reduced numbers of B1 cells, indicating that FADD may play a role in the development of B1 B cells.

It is interesting that similar to FADD, caspase 8 and the caspase 8-like regulatory protein c-FLIP are also essential for embryonic development (24, 25). T cell-specific caspase 8−/− mice have no major defect in thymocyte development, but contain reduced numbers of peripheral T cells (30). B cell-specific caspase 8−/− mice have no major defect in bone marrow B cell development, but contain increased number of peripheral conventional B cells and reduced B1 B cells (52). These phenotypes are strikingly similar to those of lymphocyte-specific FADD−/− mice as described in previous studies and this study (26). In the analysis of c-FLIP function, deletion of the c-FLIP gene in ES cells or lymphocytes resulted in phenotypes similar to those of FADD gene deletion in ES
cells or lymphocytes. Whereas c-FLIP−/−→Rag-1−/− chimeras generated from c-FLIP−/−→ES cells have severe defects in T and B cell development (33), T cell-specific deletion of c-FLIP has no obvious effect on thymocyte development, but inhibits peripheral T cell production (32). Additional Cre systems could be used in future studies to help determine the temporal requirement of FADD, caspase 8, and c-FLIP during development of lymphoid progenitors.

The phenotype of increased B cells in the spleen and lymph nodes of B cell-specific FADD−/− mice is distinct from that of T cell-specific FADD−/− mice, which contain a reduced peripheral T cell pool (26). This phenotypic difference may be due to the differential roles of FADD in these two distinct cell lineages. Whereas FADD is essential for Ag receptor signaling in T cells, it is dispensable in BCR signaling (Fig. 6). Defective TCR signaling resulted in a reduced capacity for homeostatic expansion, a possible cause for T lymphopenia in T cell-specific FADD−/− mice (26). The increased peripheral B cell numbers in B cell-specific FADD−/− mice may be due in part to defective Fas-induced apoptosis (Fig. 5), which also regulates B cell homeostasis. Unexpectedly, a FADD deficiency in B cells did not appear to result in an elevated serum Ig concentration or ALPS, even in aged mice (Fig. 5), unlike that caused by the Fas deficiency in mice. This phenotype may not be surprising, given the results from a recent study showing that a B cell-specific Fas deficiency did not result in autoimmune diseases in mice (53). However, an lpr-like disease becomes obvious when Fas is deleted simultaneously in both lymphoid and nonlymphoid cells. Therefore, a lack of FADD in B cells along with other cell types may result in autoimmune pathology. It would be of interest to determine whether this is the case when additional Cre systems are used to delete FADD in multiple cell types in adult mice.

The “pattern recognition” receptors, TLRs, play important roles in innate immune responses against various microbial pathogens. MyD88 via TIR-TIR interaction, leading to the activation of IL-1R-associated kinases. Later studies revealed a MyD88-independent pathway mediated by another TIR-containing adaptor, TRIF, particularly in TLR3 and TLR4 signaling (46). In this study, we showed that FADD deficiency abrogated proliferation of B cells stimulated by either dsRNA or LPS, but not by CpG-containing DNA, thus introducing FADD as a new component of TLR3- and TLR4-induced signaling pathways. Interestingly, the recently reported B cell-specific caspase 8-deficient mice also have a defect in B cell proliferative responses induced by TLR3 and TLR4 (52). TLRs are capable of inducing signal transduction pathways involving NF-κB and MAPKs such as ERK and JNK. Inactivation of MyD88 still allows for the activation of these pathways in response to LPS, albeit with delayed kinetics (55). Disruption of TRIF has no obvious effect on the activation of MAPKs and NF-κB induced by LPS, but inhibits up-regulation of CD54, CD86, and MHC class II molecules (46, 56). However, deletion of both MyD88 and TRIF completely inhibited LPS-induced activation of NF-κB and MAPKs (46). Previous reports suggested that FADD was able to activate the NF-κB and MAPK pathways (57, 58). Another early signal required for survival during B cell proliferation is the activation of PI3K (50, 51). Stimulation of TLR4 by LPS can activate PI3K in B cells, leading to the activation of the serine/threonine kinase Akt. A recent study has shown that the receptor interacting kinase protein RIP plays a role in TLR4 signal-induced activation of Akt in B cells (59). In this study, LPS- and dsRNA-induced proliferative responses were defective in FADD−/− B cells in [3H]thymidine incorporation and cell division kinetics assays (Figs. 6 and 7). However, LPS-induced activation of NF-κB and MAPKs appeared to be unaffected in FADD−/− B cells (Fig. 8B). Furthermore, Akt activation in FADD−/− B cells appeared to be normal following LPS stimulation (Fig. 8B). LPS-induced up-regulation of MHC class II proteins as well as the activation markers CD54 and CD86, which are dependent on the TRIF pathway (46, 56), were not affected by a lack of FADD in B cells (Fig. 8B). In the recently reported B cell-specific caspase 8-deficient mice, B cells did not appear to have defects in activation marker up-regulation or the activation of NF-κB and MAPKs (52). Therefore, it is likely that FADD and caspase 8 mediate additional pathways induced by TLR3 or TLR4 signaling.

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Disclosures

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