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The Transcription Factor Fli-1 Modulates Marginal Zone and Follicular B Cell Development in Mice

Xian K. Zhang,2*, Omar Moussa,† Amanda LaRue,‡ Sarah Bradshaw,* Ivan Molano,* Demetri D. Spyropoulos,‡ Gary S. Gilkeson,*† and Dennis K. Watson*‡

Fli-1 belongs to the Ets transcription factor family and is expressed primarily in hematopoietic cells, including most cells active in immunity. To assess the role of Fli-1 in lymphocyte development in vivo, we generated mice that express a truncated Fli-1 protein, lacking the C-terminal transcriptional activation domain (Fli-1ΔCTA). Fli-1ΔCTA/Fli-1ΔCTA mice had significantly fewer splenic follicular B cells, and an increased number of transitional and marginal zone B cells, compared with wild-type controls. Bone marrow reconstitution studies demonstrated that this phenotype is the result of lymphocyte intrinsic effects. Expression of Igα and other genes implicated in B cell development, including Pax-5, E2A, and Egr-1, are reduced, while Id1 and Id2 are increased in Fli-1ΔCTA/Fli-1ΔCTA mice. Proliferation of B cells from Fli-1ΔCTA/Fli-1ΔCTA mice was diminished, although intracellular Ca2+ flux in B cells from Fli-1ΔCTA/Fli-1ΔCTA mice was similar to that of wild-type controls after anti-IgM stimulation. Immune responses and in vitro class switch recombination were also altered in Fli-1ΔCTA/Fli-1ΔCTA mice. Thus, Fli-1 modulates B cell development both centrally and peripherally, resulting in a significant impact on the in vivo immune response. The Journal of Immunology, 2008, 181: 1644–1654.

In mice, B cells are generated in the bone marrow after birth. Their stages of development are divided into pro-B, pre-B, and immature B cells by the sequential expression of cell surface markers and the ordered rearrangement of Ig H and L chain segments (1, 2). Progenitor B cells undergo rearrangement of the Ig H chain locus to become pro-B cells. Subsequently, expressed mu H chains associate with surrogate L chains and the signaling molecules Igα and Igβ, forming the pre-BCR complex (2). The pre-BCR complex plays a critical role in the clonal expansion of mu+ pro-B cells and differentiation to the pre-B cell stage (3, 4). Pre-B cells undergo Ig L chain rearrangement and the resultant L chains associate with the mu H chain to form membrane-bound IgM (3). After successful rearrangement of both H and L chain genes, the BCR, which includes Igα and Igβ, is formed and expressed on the surface of B cells. The BCR functions as an Ag-binding and signal transduction molecule during further B cell development (5).

Immature B cells emigrate from the bone marrow to the spleen, and there progress through transitional states. Transitional B cells enter splenic follicles, further differentiating to follicular (FO)3 B cells, which comprise the majority of the mature splenic B cell population and marginal zone (MZ) B cells (5, 6). The differentiation and maintenance of these mature B cells require signals derived from the BCR (7). Maintenance of transitional, FO, and MZ B cell subsets are controlled by their microenvironment and interactions with other cell types (5, 8). In mice, MZ B cells, together with metallophilic and marginal-sinus-associated macrophages, initiate a rapid first line of defense against blood-borne particulate Ags. MZ B cell responses were first thought to be primarily directed against T-independent Ags; however, recent reports indicate that MZ B cells play roles in both T-independent and -dependent immune responses. FO cells participate primarily in T-dependent Ab responses (8, 9). Progression through B cell development depends on the combined effects of multiple regulatory genes, including Pax-5, early B cell factor (EBF), the E2A factors E12 and E47, and the Ets transcription factor, Pu.1 (10). Members of the Ets gene family are found in genomes of diverse organisms, including Drosohplia, Xenopus, sea urchin, chicken, mouse, and human (11, 12). The transcription factor Fli-1 is a member of the Ets family and is expressed in hematopoietic cells and a variety of other tissues (13, 14). Like all members of the Ets gene family, Fli-1 has the conserved DNA-binding domain, the Ets domain. The Fli-1 gene encodes two protein products, the larger comprised of 452 aa (14, 15). The Ets (DNA-binding) domain of Fli-1 is located between amino acids 277 and 361. Deletion analysis has identified two transcriptional regulatory domains, designated ATA and CTA, for N-terminal transcriptional and C-terminal transcriptional activation domains, respectively (16). Ets proteins bind to DNA sequences that contain a consensus GGAA(A/T) core motif (Ets-binding site) and can function as either transcriptional activators or repressors (11, 12). Ets proteins regulate the expression of genes that are critical for the control of cellular proliferation, differentiation, and programmed cell death. Previous studies indicate that Fli-1 plays an important role in the regulation of megakaryocyte development (17–20).

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3 Abbreviations used in this paper: FO, follicular; MZ, marginal zone; CTA, C-terminal transcriptional activation domain; ChIP, chromatin immunoprecipitation; MZP, MZ precursor; KLH, keyhole limpet hemocyanin; BAFF, B cell-activating factor; TNP, 2,4,6-trinitrophenyl.

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The Journal of Immunology
Studies regarding the role of Fli-1 in immune function or dysfunction are limited. Several reports indicate that Fli-1 can bind specific Ets-binding sites and transcriptionally activate a number of genes including those encoding Egr-1, multiple megakaryocytic genes, bcl-2, mb-1, and hTERT (21–25). Fli-1 can also act as a repressor of Rb and collagen 1 expression (26–27). Targeted constitutive disruption of the Fli-1 gene resulted in hemorrhage into the neural tube and embryonic death due in part to thrombocytopenia (28–29). Two-fold overexpression of the Fli-1 protein in transgenic mice resulted in the development of a lupus-like disease, including progressive immune complex-mediated renal disease and ultimately premature death from renal failure. Hypergammaglobulinemia, splenomegaly, B cell peripheral lymphocytosis, and autoantibody production (antinuclear Ab and anti-dsDNA) were prominent in these transgenic mice (30). We previously reported that reduced expression of the Fli-1 protein in MRL/lpr mice, a murine model of lupus, significantly increased survival and decreased renal disease compared with wild-type littermates (31).

In 

Flow cytometry analysis and cell sorting
Single-cell suspensions were prepared from spleen, bone marrow, or thymus from mice at the age of 6–12 wk. The cells were stained with fluorochoirone- or biotin-conjugated Abs and analyzed on a FACScan/Calibur flow cytometer. Data were analyzed using CellQuest (BD Immunocytometry Systems) software. Transitional, FO, or MZ B cells were sorted by the MoFlo High-Performance Cell Sorter (DakoCyto) after staining with Abs and used for RNA preparation in real-time PCR. All Abs were purchased from BD Pharmingen.

Immunoblotting
Splenocytes, thymocytes, or purified B cells with negative selection kits (Miltenyi Biotec) from spleen cells were lysed by radioimmunoprecipitation assay buffer. Lysates were analyzed by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membrane was incubated with specific Fli-1 Ab and revealed by horseradish-conjugated goat-anti-rabbit Abs, following by the ECL detection system.

ELISA
ELISA were used to detect the Ig concentrations from mice as described previously (31).

Chromatin immunoprecipitation (ChIP) assays
Total bone marrow (4 × 10^8 cells) was isolated from wild-type and Fli-1ΔCTA/Fli-1ΔCTA mice. A ChIP assay was performed as described previously using anti-Fli-1 rabbit polyclonal Ab (21). The DNA was eluted from the DNA–protein–oligomer complexes by treatment with 5′-CCAGGAGCTCTGAGGAGGTCTCT-3′ and 5′-CTCTCACTGAGG CCACAACA-3′, specific for the proximal promoter region. Real-time PCR was conducted using a LightCycler (Roche) with the Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer’s instructions. PCR primers were used at a concentration of 250 nM. The cycling conditions for mb-1 were: preincubation at 95°C for 2 min, 95°C for 2 min, followed by 55 cycles of denaturation at 94°C for 10 s, annealing 57°C for 10 s, and extension at 72°C for 25 s, with a single data collection at the end of each extension. All ramping was done at 20°C per second. Relative expression analysis was conducted using the program LinRegPCR according to the suggested specifications.

Immunodetection and determination of Ig titers
Four 8-wk-old mice were immunized by i.p. injection of 50 μg of 2,4,6-trinitrophenyl (TNP) Ficoll or 50 μg of TNP-keyhole limpet hemocyanin (KLH; Biosearch Technologies) mixed with CFA. The mice immunized with TNP-KLH were given 50 μg of TNP-KLH mixed with IFA booster 1 wk after first immunization. Anti-TNP Abs were determined by ELISA with TNP-BSA as described previously (32).

Cell proliferation, culture, and stimulation
B cells were purified with B cell-negative selection kits from Invitrogen. The purity of B cells (over 90%) was confirmed by flow cytometry analysis. For CD23 induction, B cells were cultured in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, and 50 μM 2-ME at the concentration of 10^6 cells/ml. Cells were stimulated with 50 ng/ml IL-4 (R&D Systems) and 50 ng/ml IL-1β (R&D Systems) and expression of CD23 was measured after 24 h by flow cytometry after staining with anti-CD23-PE Abs. For in vitro class switch assays, B cells were cultured at 10^6 cells/ml with 50 μg of LPS (Sigma-Aldrich) and recombinant murine IL-4 at 50 ng/ml to induce switching IgG2a. The percentage of IgG1, IgG3, IgG2a, and IgG2b expression was measured by flow cytometric analysis after 4 days of stimulation and staining with anti-CD23-PE Abs. For in vivo class switch assays, B cells were cultured at 10^6 cells/ml with 50 μg of LPS (Sigma-Aldrich) and recombinant murine IL-4 at 50 ng/ml to induce switching to IgG1, with LPS alone to induce switching to IgG3 and IgG2b, or with LPS and IFN-γ at 10 ng/ml (eBioscience) to induce switching IgG2a. The percentage of IgG1, IgG3, IgG2a, and IgG2b expression was measured by flow cytometric analysis after 4 days of stimulation and staining with anti-anti-conjugated anti-mouse IgG1, IgG3, IgG2a, or IgG2b and PE-conjugated anti-B220.

In vivo proliferation assays
A total of 10^6 purified B cells were dispensed in a 96-well plate at 100 μl/well and cultured with RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, and 50 μM 2-ME. Cells were stimulated with 20 or 40 μg/ml anti-mouse IgM (goat F(ab’)2; Jackson ImmunoResearch Laboratories) for 72 h, and [3H]thymidine (1 μCi/well) was added to each well 24 h before harvest.

Ca2+ mobilization assay
B cells were purified as described above. B cells were incubated at 30°C for 30 min with Fluo-3 in RPMI 1640 with 10% FBS. Cells were washed before stimulation. Cells were warmed at 30°C for 5 min before stimulation and the basal Ca2+ concentration was measured for 30 s. Stimulation was performed with anti-mouse IgM F(ab’)2 (20 μg/ml; Jackson Immuno Research Laboratories) and the events were recorded for 220 s (33).

Immunofluorescent staining of spleen
Spleens were removed from the mice and frozen in liquid nitrogen. A section of spleen was cut at a thickness of 5 μm. After air-drying, tissue sections were fixed in 4% paraformaldehyde for 20 min. Immunohistochemical staining was done with FITC-conjugated rat anti-mouse metallophilic macrophage (MOMA-1; monoclonal; MCA947F; Serotec) and biotin-conjugated goat anti-mouse IgM (Southern Biotechnology Associates).
Table I. PCR primer sequences and GenBank accession numbers

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Ta, Annealing temperature.

after blocking with 10% rat serum for 1 h. Next, the sections were stained with Texas-Red streptavidin (Vector Laboratories). After being washed, sections were examined with an Olympus Fluoview IX70 confocal laser scanning microscope.

Bone marrow transplantation
Bone marrow cells were isolated from the femurs of wild-type or Fli-1ΔCTA/Fli-1ΔCTA B6 mice and were injected i.v. into sublethally irradiated recipient Ly5.1 mice (B6.SJL-PepRα−/−PepRβ/BoyJ) mice (600 Gy). The mice were sacrificed 12 wk later and splenic B cells were examined.

Real-time PCR
Total RNA was prepared from bone marrow, splenic cells, or purified B cells for real-time PCR analysis. A total of 2 μg of RNA was used to synthesize cDNA (SuperScript First-Strand Synthesis System; Invitrogen). Real-time PCR was performed in duplicate using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer’s instructions, with three independent RNA preparations. PCR was done using the LightCycler (Roche) and relative expression analysis was conducted using the program LinRegPCR according to the suggested specifications (34). The cycling conditions for all genes were: preincubation at 50°C for 2 min, 95°C for 2 min, followed by 30–50 cycles of denaturation at 94°C for 5 s, annealing at 1° below the lowest temperature for a given primer pair (Table I) for 5 s and extension for 45 s at 72°C, with a single data acquisition at the end of each extension. All ramping was done at 20°C/s.

Statistical analysis
Quantitative data are expressed as mean ± SD. Statistical analysis was performed using the two-tailed Student t test.

Results

Generation of Fli-1ΔCTA/Fli-1ΔCTA mice
Because complete disruption of the Fli-1 gene is embryonic lethal, we generated a new Fli-1 allele (Fli-1ΔCTA) that encodes a truncated Fli-1 protein (amino acids 1–384) lacking the CTA domain and used homozygous mutant mice to evaluate the role of Fli-1 in normal lymphocyte development. Removal of this domain of Fli-1 reduces overall in vitro transcriptional activation activity by 40–50% (16). We backcrossed Fli-1ΔCTA/Fli-1ΔCTA mice (B6 x 129 heterozygotes) with C57BL/6 (B6) mice for eight generations. Fli-1ΔCTA/Fli-1ΔCTA B6 mice were mated to generate two groups of mice, Fli-1ΔCTA/Fli-1ΔCTA and wild-type (Fli-1+/−/Fli-1−−) littermate controls for this study. A stable truncated Fli-1 protein was expressed in splenocytes, thymocytes, and isolated splenic B cells from Fli-1ΔCTA/Fli-1ΔCTA mice (Fig. 1A). Peripheral blood analysis demonstrated that Fli-1ΔCTA/Fli-1ΔCTA mice had a significant decrease in B220+ B cells (Table II). The difference in B cell number was dose-dependent, as Fli-1ΔCTA/Fli-1−− mice had fewer B cells than wild-type controls, but more than Fli-1ΔCTA/Fli-1ΔCTA mice (Table II).

The IgG1 and IgG3 concentrations in serum were decreased >50% in Fli-1ΔCTA/Fli-1ΔCTA mice compared with controls (for IgG1, wild type, 408.6 ± 75.0 μg/ml vs Fli-1ΔCTA/Fli-1ΔCTA mice, 188.3 ± 41.2 μg/ml, n = 14–15 in each group, p = 0.014; for IgG3, wild type, 97.2 ± 22.6 μg/ml vs Fli-1ΔCTA/Fli-1ΔCTA mice, 44.3 ± 12.2 μg/ml, n = 14–15 in each group, p = 0.045, Fig. 1B). Although the concentrations of serum IgM, IgG2a, and IgG2b in Fli-1ΔCTA/Fli-1ΔCTA mice were slightly lower than in wild-type mice, the difference was not statistically significant (Fig. 1B).

Fli-1 deficiency affects pre-B cell development in bone marrow
To further characterize the impact of Fli-1 CTA deficiency on B cell development, bone marrow cells were isolated from the femurs of wild-type and Fli-1ΔCTA/Fli-1ΔCTA mice and analyzed by flow cytometry. Pre-B cells and immature B cells (B220low CD43−) were significantly reduced in Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type mice as shown in Fig. 2 (wild type, 17.6 ± 2.0% vs Fli-1ΔCTA/Fli-1ΔCTA mice, 10.3 ± 1.1%, n = 5 in each group, p = 0.02). B220low CD43− cells were further subdivided into fractions D–F based on surface expression of IgM and IgD (35). Populations of fraction D (B220low IgM+ IgD−), fraction E (B220low IgM+ IgD−), and fraction F (B220low IgM+ IgD+) were all significantly lower in Fli-1ΔCTA/Fli-1ΔCTA mice than in wild-type controls (Fig. 2). There was no statistically significant difference in the number of pro-B cells (B220lowCD43+) from Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type mice (Fig. 2).

Fli-1 deficiency impacts FO and MZ B cell development
Next, we examined whether Fli-1 deficiency affected splenic B cell populations. The total number of spleen cells was significantly higher in Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type controls (wild type, 74.8 ± 10.4 × 106 cells, vs Fli-1ΔCTA/Fli-1ΔCTA, 105.7 ± 4.9 × 106, n = 8 in each group, p = 0.035, Fig. 3A) though spleen weights were similar (data not shown). The percentage of CD3+ T cells in spleens from Fli-1ΔCTA/Fli-1ΔCTA mice tended to be higher compared with wild-type control mice, but did not reach statistical significance (wild type, 28.1 ± 0.9% vs Fli-1ΔCTA/Fli-1ΔCTA, 32.3 ± 2.7%, n = 8, p = 0.18). The percentage of splenic B cells, however, was significantly decreased in
thymocytes, and B cells. Western blot of spleen and thymus tissue extracts prepared from wild-type, heterozygous Fli-1ACCTA/Fli-1ACCTA mice. A total of 30 μg of protein extract was resolved on a 12.5% acrylamide gel and probed with rabbit anti-Fli-1 polyclonal Ab. Blots were reprobed with anti-actin as a loading control. B, Serum Ig titers from Fli-1ACCTA/Fli-1ACCTA mice and wild-type controls. Sera were collected from Fli-1ACCTA/Fli-1ACCTA mice (n = 14) and littermate wild-type controls (n = 15) at the age of 8–12 wk. Ig concentrations were determined by ELISA.

**FIGURE 1.** Fli-1 expression in immune cells and impact on Ig levels. A, Immunoblot analysis of truncated Fli-1 expression in isolated total splenocytes, thymocytes, and B cells. Western blot of spleen and thymus tissue extracts prepared from wild-type, heterozygous Fli-1ACCTA/Fli-1ACCTA, and homozygous Fli-1ACCTA/Fli-1ACCTA mice. A total of 30 μg of protein extract was resolved on a 12.5% acrylamide gel and probed with rabbit anti-Fli-1 polyclonal Ab. Blots were reprobed with anti-actin as a loading control. B, Serum Ig titers from Fli-1ACCTA/Fli-1ACCTA mice and wild-type controls. Sera were collected from Fli-1ACCTA/Fli-1ACCTA mice (n = 14) and littermate wild-type controls (n = 15) at the age of 8–12 wk. Ig concentrations were determined by ELISA.

**FIGURE 2.** Pre-B and immature B cells are reduced in Fli-1ACCTA/Fli-1ACCTA mice. Fli-1ACCTA/Fli-1ACCTA and wild-type control mice were sacrificed at the age of 10 wk. Cells were isolated from bone marrow and analyzed for the expression of B220 and CD43 by flow cytometric analysis. Pre-B cells and pre-B/immature B cells are defined as B220+CD43− and B220+CD43+, respectively. Numbers reflect the percentage of the cells in each gated population. Data are representative of five mice per experiment from three independent experiments.

In contrast to the decreased percentage of FO B cells, MZ B cell percentage was significantly increased in Fli-1ACCTA/Fli-1ACCTA mice compared with wild-type controls (Fig. 3B, wild type, 5.5 ± 0.5% vs Fli-1ACCTA/Fli-1ACCTA, 8.3 ± 0.9%, n = 8; p = 0.0371). The significant increase in MZ B cells from Fli-1ACCTA/Fli-1ACCTA mice was further confirmed using additional markers for MZ B cells.

**Table II.** Flow cytometric analysis of peripheral blood cell from wild-type, Fli-1ACCTA/Fli-1ACCTA heterozygous, and Fli-1ACCTA/Fli-1ACCTA homozygous mice.

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<td>Fli-1ACCTA/Fli-1ACCTA</td>
<td>52.36 ± 4.82</td>
<td>41.40 ± 5.99a,c</td>
<td>32.58 ± 8.19b</td>
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The percentages of B220+ cells were assessed by flow cytometry. Values represent mean ± SD.

a Value of p < 0.0001 when compared to wild type.

b,c Value of p < 0.05 when compared to Fli-1ACCTA/Fli-1ACCTA homozygous mice.
cells, including CD1d and CD9 (Fig. 3D). The absolute number of MZ B cells in spleens from Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> mice was also significantly higher than in wild-type mice (Fig. 3A). Histological examination of the spleens showed an increase of IgM<sup>+</sup> B cells outside the MOMA-1<sup>+</sup> metallophilic macrophage region, which defines the boundary between the FO and MZ B cell zones (data not shown). These data, consistent with the flow cytometric data, demonstrate that Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> mice have an increased number of MZ B cells.

**Increased number of transitional B cells in Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> mice**

Upon arrival in the spleen, immature B cells emerging from the bone marrow are designated as transitional B cells and are divided into transitional stage 1 (T1) and transitional stage 2 (T2) B cells by Loder et al. (37). Recently, several studies demonstrated that these T2 cells are actually MZ precursors (MZP) cells (8, 38–39). To further delineate possible B cell developmental alterations in the spleens of Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> mice, the transitional B cell populations were examined. T1 B cells were characterized by B220<sup>+</sup>IgM<sup>hi</sup>CD21<sup>+</sup>CD23<sup>lo</sup> surface marker expression, and MZP B cells by the expression of B220<sup>+</sup>IgM<sup>hi</sup>CD21<sup>hi</sup>CD23<sup>lo</sup>. As shown in Fig. 4, significant increases in splenic percentage of T1 and MZP B cells were observed in Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> mice compared with wild-type mice (for T1 B cells, wild type, 4.7 ± 0.2%, vs Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup>, 11.2 ± 0.9%, n = 5, p = 0.0003; for MZP B cells, wild type, 6.8 ± 0.3%, vs Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup>, 8.2 ± 0.5%, n = 5, p = 0.0442, Fig. 4). The absolute number of T1 B cells in Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> mice is 10-fold higher than in wild-type controls (40.3 × 10<sup>5</sup> vs 4.0 × 10<sup>5</sup>, respectively, Fig. 3A). However, the absolute number of MZP B cells in the spleen is similar between Fli-1<sup>ΔCTA</sup>/Fli-1<sup>ΔCTA</sup> and wild-type control mice, despite the differences in percentage. These data suggest that efficient progression of T1 B cells to the FO B cell stage was...
decreased in Fli-1-deficient mice. The increase in MZ B cells may reflect preferential development of MZ B cells in Fli-1 deficiency due to a block in FO B cell development rather than a positive effect on MZ B cell development.

**B1 cell development is not affected in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice**

B1 cells are self-renewing, residing mainly in the peritoneal and pleural cavities (9). To examine whether Fli-1 deficiency also affected B1 cell development, we collected peritoneal lymphocytes from Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> and wild-type control mice. The cells were stained with anti-CD5 and -IgM. There was no significant difference in peritoneal B1 cell numbers between Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> and wild-type control mice (wild type, 14.7 ± 1.2%, vs Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> 14.2 ± 3.4%, n = 5, p = 0.8792).

**B cell-autonomous Fli-1 defect affects B cell development in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice**

The reduced number of FO B cells and increased number of transitional and MZ B cells could be the result of an intrinsic Fli-1 defect in the B cells themselves or due to influences of Fli-1 deficiency on the splenic microenvironment (non-B cells). To distinguish between these two possibilities, 1 × 10<sup>6</sup> bone marrow cells from wild-type or Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice with the Ly5.2 (CD45.2) genotype were transferred into sublethally irradiated B6 mice with the Ly5.1 (CD45.1) genotype. The mice were sacrificed 12 wk after transplantation and the spleen cells were analyzed by flow cytometry. As shown in Fig. 5A, over 94% of spleen cells from the recipients were CD45.2<sup>+</sup> indicating that the reconstituting B cells in the recipients were derived from donor bone marrow. B6 Ly5.1 recipients that received wild-type bone marrow had numbers of MZ B cells and FO B cells similar to those of wild-type mice (Fig. 5B). In contrast, the B6 Ly5.1 mice that received Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> bone marrow cells had an increased number of MZ B cells with a decreased number of FO B cells, similar to unmanipulated Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice (for FO B cell, wild-type bone marrow recipients, 77.0 ± 0.5% vs Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> bone marrow recipients, 49.5 ± 1.6%, n = 5; p < 0.0001, for MZ B cells, wild-type bone marrow recipients, 4.5 ± 0.5% vs Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> bone marrow recipients, 14.0 ± 1.1%, n = 5, p < 0.0001). These data indicated that the reduction of FO B cells and increase of transitional B cells and MZ B cells results from lymphocyte/bone marrow-intrinsic Fli-1 deficiency and not stromal cell effects.

**Altered B cell gene expression in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice**

Because previous reports suggested Fli-1 may be involved in regulation of Igα (mb-1) expression (23), we first examined the expression of Igα on B cells from Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice. As shown in Fig. 6A, the surface expression of Igα on B cells from Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice was decreased compared with wild-type mice. Real-time RT-PCR analysis of mb-1 mRNA demonstrated significantly reduced expression in bone marrow from Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice compared with that from wild-type mice. (Fig. 6B).

Thus, both at the protein and transcript level, Igα expression was decreased in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice.

A limited set of transcription factors (e.g., ikaros, PU.1, E2A, EBF, Pax-5) regulate expression of B-lineage-specific genes. B cell development is dependent upon proper expression of multiple transcription factors, including E2A (E12 and E47), Id1, and Id2 (10, 40–41). Real-time RT-PCR was used to examine the expression of multiple transcripts in mRNA prepared from total bone marrow and purified B cells (Fig. 6B). Id proteins are negative regulators of transcription factors E2A and Pax-5 (40, 41). Although Pu.1 and EBF were not significantly altered in the Fli-1-deficient mice, the expression of E2A and Pax-5 mRNA was significantly reduced and Id1 and Id2 mRNA was significantly increased in RNA prepared from bone marrow of Fli-1-deficient vs wild-type mice. (Fig. 6B). E2A transcript levels were reduced in RNA prepared from splenic B cells of the Fli-1-deficient mice, although the values did not reach statistical significance. Id2 transcripts were statistically significantly increased in total RNA prepared from bone marrow and purified splenic B cells (Fig. 6B) from Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> compared with wild-type mice. Egr-1 is required for B cell maturation and is regulated by Fli-1 (25, 42). Quantitative real-time RT-PCR assessment of Egr-1 mRNA levels in RNA prepared from bone marrow and purified B cells demonstrated a consistent reduction in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice compared with wild-type mice. Transitional, FO, and MZ B cells were isolated with a FACSVantage cell sorter for analysis of gene expression. Reduced expression of mb1 and Pax-5 in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice compared with wild-type controls was observed in these isolated populations (Fig. 6C). Transitional B cells demonstrated higher expression of Fli-1 than MZ and FO B cells (Fig. 6C).

Programmed cell death is a critical process in B lymphocyte development. We found that Bcl-2 and Bcl-2 expression was decreased in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice compared with wild-type controls (Fig. 6B). We did not, however, detect differences in in vivo or in vitro apoptosis between wild-type and Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice (data not shown).

As noted above (Fig. 3C), cell surface CD23 protein expression was reduced in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice. Consistent with this observation, CD23 mRNA was also reduced in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> bone marrow and isolated B cells. The CD23 mRNA reduction was demonstrated in the spleen was over 2-fold (Fig. 6B). Furthermore, CD19 transcripts were reduced in Fli-1<sup>CTA</sup>/Fli-1<sup>CTA</sup> mice.
FIGURE 6. Altered B cell gene expression in Fli-1ΔCTA/Fli-1ΔCTA mice. A, Decreased Igα expression in B cells from Fli-1ΔCTA/Fli-1ΔCTA mice. Spleen cells were stained with Abs against B220 and Igα and analyzed by flow cytometry. Igα expression in Fli-1ΔCTA/Fli-1ΔCTA mice was reduced compared with wild-type mice. Data are representative of five mice per experiment from a total of four independent experiments. B, Altered gene expression in bone marrow and splenic B cells from Fli-1ΔCTA/Fli-1ΔCTA mice. Total RNA was prepared from bone marrow or purified B cells for real-time PCR analysis. Total RNA was converted to cDNA with the SuperScript First-Strand Synthesis System (Invitrogen). Real-time PCR was done in triplicate with the appropriate primers. *, Statistically significant differences in expression. C, Altered gene expression in transitional B cells (TB), FO B cells (FO), and MZ B cells (MZ) from Fli-1ΔCTA/Fli-1ΔCTA mice. Total RNA was prepared from sorted transitional, FO, or MZ B cells for real-time PCR analysis. D, ChIP analysis of Fli-1 binding to the mb-1 promoter. Total bone marrow (4 × 10⁶ cells) was isolated from wild-type and Fli-1ΔCTA/Fli-1ΔCTA mice. The −160 to +34 region of the mouse mb-1 promoter was amplified by real-time PCR using mb-1 primers specific for the proximal promoter region as outlined in Materials and Methods. Relative expression analysis was conducted using the program LinRegPCR according to the suggested specifications. As a positive control, 5% of the input chromatin was used as a template. Normal rabbit IgG was used as a negative control.

BCR signaling and proliferation of B cell in Fli-1ΔCTA/Fli-1ΔCTA mice

Because Igα expression was decreased in Fli-1ΔCTA/Fli-1ΔCTA mice, we isolated B cells and measured proliferation after cross-linking the BCR with anti-IgM. [3H]Thymidine uptake by B cells from Fli-1ΔCTA/Fli-1ΔCTA mice following anti-IgM cross-linking was significantly less than that from wild-type controls (Fig. 7A) indicating that B cells from Fli-1ΔCTA/Fli-1ΔCTA mice have decreased proliferative capacity compared with wild-type B cells. To determine whether the decreased proliferation was due to early components of BCR signaling, we assessed intracellular Ca²⁺ levels following anti-IgM cross-linking. Despite the clear impact of Fli-1ΔCTA/Fli-1ΔCTA on B cell proliferation, intracellular Ca²⁺ increase after anti-IgM stimulation of B cells from Fli-1ΔCTA/Fli-1ΔCTA mice was similar to that of wild-type controls (Fig. 7B). Thus, the decreased proliferative capacity following BCR cross-linking of Fli-1ΔCTA/Fli-1ΔCTA B cells was not due to early defects in BCR signaling, despite the decrease in Igα expression.

Immune responses of Fli-1ΔCTA/Fli-1ΔCTA mice

FO and MZ B cells have different functions in immune responses (6, 8). A decrease in FO B cells and an increase of MZ B cells in Fli-1ΔCTA/Fli-1ΔCTA mice may affect in vivo immune responses. Thus, to determine whether the lack of Fli-1 CTA had a significant in vivo effect, we examined the humoral immune responses to T
Reduced in vitro IgG1 class switch of B cells from Fli-1AC/Ta/Fli-1AC/Ta mice

Because the level of IgG1 Abs was lower in Fli-1AC/Ta/Fli-1AC/Ta mice after immunization compared with wild-type controls, next we investigated whether the IgG1 class switch of B cells from Fli-1AC/Ta/Fli-1AC/Ta mice was alternated. B cells were purified from spleen and stimulated with 50 μg of LPS and recombinant murine IL-4 at 50 ng/ml to induce switching to IgG1, with LPS alone to induce switching to IgG3 and IgG2b, or with LPS and IFN-γ at 10 ng/ml to induce switching IgG2a. The percentage of IgG1-expressing B cells was significantly lower in Fli-1AC/Ta/Fli-1AC/Ta mice compared with wild-type mice (Fig. 9, wild type, 26.9 ± 1.6%, vs Fli-1AC/Ta/Fli-1AC/Ta, 20.9 ± 1.0%, n = 5, p = 0.029). There was no significant difference in IgG3 expression between B cells from Fli-1AC/Ta/Fli-1AC/Ta mice and that from wild-type controls (Fig. 9, wild type, 11.4 ± 1.2%, vs Fli-1AC/Ta/Fli-1AC/Ta, 10.2 ± 0.4%, n = 5, p = 0.1806). Percentages of IgG2a- and IgG2b-expressing B cells were also not significantly different between B cells from wild-type mice and Fli-1AC/Ta/Fli-1AC/Ta mice after stimulation (data not shown).

FIGURE 7. Impact of Fli-1 on responses to BCR cross-linking. A, Impaired proliferation of FO B cells from Fli-1AC/Ta/Fli-1AC/Ta mice. FO B cells were purified from Fli-1AC/Ta/Fli-1AC/Ta mice and wild-type controls and stimulated by cross-linking with anti-IgM. Proliferation responses were determined by [3H]thymidine incorporation. Data are representative of two independent experiments. B, Ca2+ flux is not affected in Fli-1AC/Ta/Fli-1AC/Ta mice. FO B cells were preloaded with Fluo-3 and stimulated with anti-IgM F(ab')2. Data shown are the change in relative intracellular Ca2+ concentrations. The arrow shows the point of stimulation. Data are representative of four independent experiments.

FIGURE 8. Altered humoral immune responses in the Fli-1AC/Ta/Fli-1AC/Ta mice. Mice were challenged with T cell-independent Ag TNP-Ficoll or T cell-dependent Ag TNP-KLH at the age of 10 wk. The concentration of TNP-specific Abs on days 7, 14, and 21 after immunization were examined by ELISA and are shown for four littermates from each phenotype. Data are representative of two independent experiments. *, Statistically significant changes.

FIGURE 9. Decreased in vitro IgG1 class switch recombination efficiency in B cells from Fli-1AC/Ta/Fli-1AC/Ta mice. The purified B cells were cultured with 50 μg of LPS (Sigma-Aldrich) and recombinant murine IL-4 at 50 ng/ml to induce switching to IgG1, with LPS alone to induce switching to IgG3 and IgG2b, or with LPS and IFN-γ at 10 ng/ml to induce switching IgG2a. The percentage of IgG1-expressing B cells was significantly lower in Fli-1AC/Ta/Fli-1AC/Ta mice compared with wild-type mice (Fig. 9, wild type, 26.9 ± 1.6%, vs Fli-1AC/Ta/Fli-1AC/Ta, 20.9 ± 1.0%, n = 5, p = 0.029). There was no significant difference in IgG3 expression between B cells from Fli-1AC/Ta/Fli-1AC/Ta mice and that from wild-type controls (Fig. 9, wild type, 11.4 ± 1.2%, vs Fli-1AC/Ta/Fli-1AC/Ta, 10.2 ± 0.4%, n = 5, p = 0.1806). Percentages of IgG2a- and IgG2b-expressing B cells were also not significantly different between B cells from wild-type mice and Fli-1AC/Ta/Fli-1AC/Ta mice after stimulation (data not shown).

T cell development was not affected in thymus in Fli-1AC/Ta/Fli-1AC/Ta mice

Finally, we investigated whether T cell development was affected in Fli-1AC/Ta/Fli-1AC/Ta mice. Thymocyte populations were isolated from Fli-1AC/Ta/Fli-1AC/Ta and wild-type mice and...
analyzed by flow cytometry. There were no significant differences in mature CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes between Fli-1ΔCTA/Fli-1ΔCTA and wild-type mice (data not shown). Furthermore, the numbers of CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes in Fli-1ΔCTA/Fli-1ΔCTA mice were similar to those found in wild-type mice (data not shown).

Discussion

Prior experiments in Fli-1-transgenic and heterozygous knockout mice indicated that Fli-1 has significant effects on immune function, specifically B cell function (30, 31). Because complete deficiency of Fli-1 is embryonic lethal, to determine the impact of Fli-1 deficiency on B cell development, we developed a mouse that expressed a truncated Fli-1 protein and demonstrated conclusively that Fli-1 plays a critical role in B cell development. Specifically, we found a significant decrease in splenic FO B cells and an increase in transitional and MZ B cells in Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type controls. Although bone marrow pro-B cell number was not affected, the reduction of bone marrow pre-B cells observed in Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type mice supports the proposed model that wild-type Fli-1 function is required for maximum efficiency of early B cell differentiation.

The pre-B cell population was significantly lower in Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type mice, suggesting that Fli-1 plays a critical role in the differentiation of pro- to pre-B cells. Formation of the pre-BCR is required for the clonal expansion of pro-B cells and differentiation to the pre-B cell stage (3, 4). Disruption of pre-BCR signaling resulted in a significant reduction of pre-B cells in mice (43). Complete knockout of the Pax-5 gene resulted in arrest at the early pro-B cell stage and lack of pro- to pre-B cell differentiation (44, 45). Elevated Id1 contributed to a reduced pre-B cell population (46, 47). Disruption of Egr-1 and E2A genes also significantly reduced pre-B cell populations (42, 48, 49). We found that expression of mb-1, Pax-5, E2A, and Egr-1 was significantly reduced and expression of Id1 was increased in bone marrow-derived cells from Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type controls. The decrease in pre-B cells in Fli-1ΔCTA/Fli-1ΔCTA mice is likely due to the combination of decreased expression of Igα (pre-BCR), Erg-1, Pax-5, and E2A or increased expression of Id1 (Fig. 10). The decreased proliferative response of Fli-1-deficient B cells to BCR cross-linking indicates that a Fli-1-regulated factor is required for full BCR responsiveness. We believed that the decreased expression of Igα would result in decreased intracellular Ca²⁺ flux following BCR cross-linking. The similar response of Fli-1-deficient B cells and wild-type B cells to calcium indicates that the impact of Fli-1 expression is not at the earliest phases of BCR signaling, but further down the signal transduction pathway. Thus, it is unlikely that the decreased expression of Igα alone is sufficient to explain the B cell phenotype observed in the Fli-1-deficient mice.

The significant increase of T1 B cells in Fli-1ΔCTA/Fli-1ΔCTA mice indicated that Fli-1 is critical in the progression of B cells through the transitional stages to mature B cell subsets. A truncated cytoplasmic tail of Igα or Igβ resulted in failure of T1 B cells to progress to mature B cells. These results suggested that the BCR is also important in the transition of T1 to mature B cells (37, 50). In addition to being an early response gene following AgR engagement on mature B cells, a role for Egr-1 in promotion of transitional B cells to mature B cell subsets was demonstrated (42). We have found that expression of Egr-1 is reduced in splenic B cells in Fli-1ΔCTA/Fli-1ΔCTA mice. The decreased expressions of Igα and Egr-1 in Fli-1-deficient mice likely contribute to the decreased transition of T1 B cells to mature B cells (Fig. 10).

In this study, we found a significant decrease in FO B cells and increased MZ B cells in Fli-1ΔCTA/Fli-1ΔCTA mice. The BCR-signaling pathway is a major factor in determining the fate of mature B cells (5, 8, 51). Disruption of BCR-signaling components blocks transition of T2 B cells to FO B cells (43, 52, 53). Mice in which BCR signaling was impaired because of the presence of a knocked-in L chain mutation had increased numbers of MZ B cells (51). A model was proposed to explain the defects observed in MZ and FO B cell development in these gene-disrupted animals (5, 8). In this model, strong BCR signaling leads immature B cells to differentiate to FO B cells, while weak BCR signaling leads immature B cells to differentiate to MZ B cells. Igα is one of the signaling components of the BCR and plays a very important role in the BCR-signaling pathway as noted above. Disruption of the Igα cytoplasmic tail blocked generation of mature B cells (53). Mice in which the tyrosine residues in the Igα ITAM were mutated to phenylalanine exhibited enhanced Ca²⁺ signaling downstream of the BCR and resultant decreased number of MZ B cells (52). Fli-1 has been reported to coregulate Igα protein and mRNA in B cells from Fli-1ΔCTA/Fli-1ΔCTA mice compared with wild-type controls. This reduced expression may contribute to “weak” BCR signaling and...
concomitant decreased number of FO B cells and increased number of MZ B cells observed in these mice. We also found that expression of Id genes were higher in B cells from Fli-1AC/CTA/Fli-1AC/CTA mice. Relative to BCR signaling, Id1 expression is negatively correlated with mb-1 expression (54). Thus, elevated Id1 may contribute to the reduced FO B cell population. Notch-RBP-J signaling was reported to be involved in the fate determination of MZ B cells (55, 56). Because we did not find significant differences in the expression of Notch 1 or its negative regulators, NUMB, Nrarp, MINT, and sel-10 (data not shown), it is unlikely that Fli-1 affects MZ B cell development via aberrant Notch signaling. B cell-activating factor (BAFF) plays an important role in B cell development. BAFF-deficient mice lost all FO and MZ B cells, but B1 cell development was not affected (57, 58). We found that there was no difference in the BAFF concentrations in the sera from Fli-1AC/CTA/Fli-1AC/CTA mice compared with that from wild-type mice (data not shown). However, in this study, we did not investigate whether the expression of BAFF receptors in Fli-1AC/CTA/Fli-1AC/CTA mice was affected, nor did we investigate signaling through the BAFF receptor. Such an alternation of BAFF receptor signaling could contribute to the lower numbers of FO B cells in Fli-1AC/CTA/Fli-1AC/CTA mice.

Fli-1 is also reported to regulate the expression of Bcl-2 (22, 59). Bcl-2 is involved in the development of FO and MZ B cells (60). Bcl-2-transgenic mice have no MZ B cells, whereas Bcl-2-knockout mice have an increased number of MZ B cells (60). We found that Bcl-2 and Bcl-xL expression was decreased in Fli-1AC/CTA/Fli-1AC/CTA mice compared with wild-type controls. Thus, decreased expression of these antiapoptotic genes may partially contribute to the increase of MZ B cells, although we could not demonstrate a significant impact of Fli-1 genotype on measures of apoptosis in the spleen (data not shown).

We and others have demonstrated that Fli-1 is highly expressed in T cells, and expression of Fli-1 is decreased after T cell activation (61, 62). Although the T cell population was higher in the spleen, we did not find significant differences in the stages of T cell development in the thymus in Fli-1AC/CTA/Fli-1AC/CTA mice. Because only the CTA domain was removed in Fli-1AC/CTA/Fli-1AC/CTA mice, it remains possible that, while Fli-1 is involved in the development of T cells, the CTA domain is not critical for these functions. In addition, these results suggest that this allele does not seem to regulate T cell lymphocyte lineage choice. These results also suggest that the B cell lineage differences are not secondary to T cell effects, but are rather intrinsic to B cells.

Several studies have demonstrated that stromal cells play an important role in lymphocyte development, and that gene-deficient stromal cells affect normal lymphocyte development (63, 64). Our bone marrow transplantation study clearly demonstrated that the observed reduction of FO B cells and increase of MZ and transitional B cells resulted from bone marrow cell-intrinsic Fli-1 deficiency, not stromal cell Fli-1 deficiency.

Fli-1 deficiency impacts in vivo immune response phenotypes. Serum IgG1 concentrations were significantly lower in Fli-1AC/CTA/Fli-1AC/CTA mice compared with wild-type controls, probably due to the decreased number of FO B cells and their decreased abilities to switch IgG1 in these mice. It is also notable that the serum IgG3 concentrations were also significantly lower in Fli-1AC/CTA/Fli-1AC/CTA mice, even though the total number of MZ B cells was higher in these mice. MZ B cells participate in early T cell-independent immune responses and interact with blood-borne Ags by production of IgM and IgG3. MZ B cells also play a role in T cell-dependent immune responses, but mature FO B cells are the principal effectors of these responses via IgG1 production (6, 8). We found that Fli-1AC/CTA/Fli-1AC/CTA mice had significantly less IgG1, but increased IgM Ab production, following challenge with T cell-dependent and -independent Ags, compared with wild-type control mice. These data are consistent with Fli-1AC/CTA/Fli-1AC/CTA resulting in increased MZ B cells and decreased FO B cells and indicate that Fli-1 induced changes in splenic B cells are of in vivo physiologic relevance.

In summary, the lack of a CTA domain of Fli-1 resulted in a novel profound impact on B cell development. The transitions from pro- to pre-B cell, pre-B to immature B cells, and T1 B cells to FO or MZ B cells appear primarily affected. A number of genes known to affect B cell development showed altered expression in Fli-1AC/CTA/Fli-1AC/CTA mice (Fig. 10). The impact of Fli-1 on B cell development is, thus, likely multifactorial, implicating BCR signaling and expression of transcription factors important for B cell lineage development/differentiation. Further studies, such as genetic reconstitution and generation of B cell-specific Fli-1-null mice, are needed to dissect the distinct mechanisms underlying the B cell effects noted. The in vivo importance of these findings in normal immunity is evidenced by the altered response to immunization of mice deficient in Fli-1 CTA expression. Furthermore, our previous report of a profound effect of Fli-1 heterozygosity on lupus development in MRL/lpr mice underscores the in vivo implications of these studies.

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Disclosures

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