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Regulation of Follicular B Cell Differentiation by the Related E26 Transformation-Specific Transcription Factors PU.1, Spi-B, and Spi-C

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Splenic B-2 cells can be divided into two major subsets: follicular (FO) and marginal zone (MZ) B cells. FO and MZ B cells are generated from immature transitional B cells. Few transcription factors have been identified that regulate FO B cell differentiation. The highly related proteins PU.1, Spi-B, and Spi-C are transcription factors of the E26-transformation-specific family and are important for B cell differentiation and function. To determine whether these proteins play a role in the differentiation of FO B cells, we performed a detailed analysis of splenic B cells in mice with inactivating mutations in the genes encoding PU.1 (*Sfpi1*) or Spi-B (*Spib*). *Sfpi1*$^{+/-}$ *Spib*$^{-/-}$ (*PUB*) mice had a 9-fold reduction in the frequency of CD23^{+} FO B cells compared with that of wild-type mice. In contrast, *PUB* mice had a 2-fold increase in the frequency of MZ B cells that was confirmed by immunofluorescence staining. Expression of Spi-C in Eμ-Spi-C transgenic *PUB* mice partially rescued frequencies of CD23^{+} B cells. Gene expression analysis, in vitro reporter assays, and chromatin immunoprecipitation experiments showed that transcription of the *Fcer2a* gene encoding CD23 is activated by PU.1, Spi-B, and Spi-C. These results demonstrate that FO B cell differentiation is regulated by the E26-transformation-specific transcription factors PU.1, Spi-B, and Spi-C. *The Journal of Immunology*, 2010, 185: 7374–7384.

Human circulating and splenic B-2 cells can be divided into two subsets known as "naive" and "memory." Memory B cells compose up to 40% of circulating B cells, which includes a large frequency of B cells that express CD27, IgM, IgD, and have mutated *Ig* genes. Memory B cells can respond to Ag stimulation without T cell help and are thought to be in charge of T-independent Ab responses to bacterial polysaccharide Ags (1). In the human spleen, memory B cells are located in the marginal zones (MZs) of lymphoid follicles and are therefore likely equivalent to MZ B cells in the mouse, whereas naive B cells are equivalent to follicular (FO) B cells in the mouse (2, 3). Studies of patients with primary B cell immunodeficiency suggest distinct developmental requirements for naive and MZ/memory B cells (1, 2). Taken together, these studies suggest that naive and memory B cells are separate lineages with different genetic determinants that control differentiation. Therefore, identifying factors that control FO versus MZ B cell differentiation will have implications for human health.

The mouse model has been used to study factors involved in controlling FO versus MZ B cell differentiation (4). These studies revealed that splenic transitional B cells are the precursors for both FO and MZ B cells (5, 6). Transitional B cells that enter the spleen from the blood express B220 and CD93 (AA4.1), and can be subdivided into transitional-1 (T1)–transitional-3 (T3) stages by differential expression of the cell surface markers CD23 (low-affinity IgE receptor) and surface IgM (5–7). Transitional-2 (T2) B cells are precursors for both FO and MZ B cells (8). T1 and T2 cells are highly sensitive to tolerance induction as a consequence of high-affinity interaction of a self-reactive BCR with self-Ag (5, 9). T3 cells are the product of T2 cells that have been "anergized" as a consequence of interaction with self-Ag (9). Signaling through the BCR is important for determining the developmental fate of transitional B cells. In general, strong BCR signaling drives differentiation of transitional B cells toward the FO B cell fate, whereas weak BCR signaling favors differentiation of transitional B cells into MZ B cells (4). Mutations in genes encoding proteins important for BCR signaling, such as Btk or PLCγ2, cause reductions in the frequency of FO B cells in the spleen and increases in the frequency of MZ B cells (10, 11). However, few transcriptional regulators of the FO versus MZ cell fate decision have been identified. MZ B cell development is impaired in the absence of Notch2 signaling (12, 13) as well as by mutation of the E26 transformation-specific (Ets) transcription factor Ets-1 (14). FO B cell development is moderately impaired in the absence of the Ets transcription factor Fli-1 (15). However, more work needs to be done to determine what transcription factors are critical for FO versus MZ B cell differentiation.

PU.1, Spi-B, and Spi-C are highly related members of the Ets family of transcription factors. PU.1 is encoded by the *Spi-1* gene in humans and by the *Sfpi1* gene in mice. PU.1 is required to

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Abbreviations used in this paper: ChIP, chromatin immunoprecipitation; Ets, E26 transformation-specific; FO, follicular; Mef2c, myocyte-enhancer factor 2c; MZ, marginal zone; *PUB*, *Sfpi1*$^{+/-}$ *Spib*$^{-/-}$; qPCR, quantitative PCR; RT-qPCR, reverse transcription-quantitative PCR; T1, transitional-1; T2, transitional-2; T3, transitional-3; TSS, transcription start site; WT, wild-type.

generate lymphoid progenitors (16). Despite this early requirement, deletion of *Sfpi1* after commitment to the B cell lineage permits the generation of mature B cells (17). Spi-B–deficient B cells have abnormal signaling through the BCR, proliferate poorly, fail to generate specific Abs, and do not form germinal centers (18, 19). Reduction of PU.1 levels by 50% makes the phenotype of Spi-B KO mice much more severe (*Sfpi1*$^{+/-}$ *Spib*$^{-/-}$ [*PUB*] mice) (20). Peripheral B cell differentiation has not been examined in detail in *PUB* mice, raising the issue of whether the immunodeficiency of these mice might be explained in part by selective loss of a particular B cell subset. Spi-C is expressed in mature B cells and in a subset of splenic macrophages and is highly expressed in splenic transitional B cells (21–24). Mutation of the gene encoding Spi-C (*Spic*), or transgenic overexpression of Spi-C, causes various B cell differentiation abnormalities (24, 25).

The objective of this study was to determine whether the Ets transcription factors PU.1 and Spi-B play roles in FO versus MZ B cell development. We found that FO B cell differentiation was dramatically impaired in *PUB* mice. In contrast, *PUB* mice had an increase in the frequency of MZ B cells. Transgenic expression of Spi-C in *PUB* mice partially restored frequencies of CD23$^+$ B cells. Analysis of gene expression, reporter assays, and chromatin immunoprecipitation (ChIP) experiments showed that PU.1, Spi-B, and Spi-C activate CD23 transcription. In conclusion, these results suggest that PU.1, Spi-B, and Spi-C are important transcriptional regulators of FO B cell differentiation.

Materials and Methods

Generation and breeding of mice

Mice were housed in the University of Western Ontario Health Sciences animal facility (London, Ontario, Canada). Animal husbandry and breeding was done in compliance with animal protocols approved by the University of Western Ontario University Council on Animal Care. C57BL/6 mice were purchased as required from Charles River Laboratories (Pointe-Claire, Quebec, Canada). *Sfpi1*$^{+/-}$, *Spib*$^{-/-}$, *PUB*, and Eμ–Spi-C transgenic mice were previously generated and characterized, and genotyping was performed by PCR as described (18, 20, 24, 26). To generate Eμ–Spi-C$^+$ *PUB* mice, Eμ–Spi-C transgenic stud males were bred with *PUB* females to generate Eμ–Spi-C$^+$ *Sfpi1*$^{+/-}$ *Spib*$^{+/-}$ mice, which were back-crossed to *PUB* mice to generate Eμ–Spi-C$^+$ *PUB* mice. Mice were generated for the experiments described in this paper by continuous pair mating of Eμ–Spi-C$^+$ *PUB* males with *PUB* females. All experiments in this report were performed using female mice aged 6–12 wk.

Cell culture

The WEHI 279 B cell lymphoma cell line (27) was obtained from the American Type Culture Collection (Cedarlane Laboratories, Burlington, Ontario, Canada). WEHI-279 cells were maintained by continuous passage in DMEM (4.5 g/l glucose; Lonza, Shawinigan, Quebec, Canada) supplemented with 10% FBS (BioWest, Miami, FL), penicillin/streptomycin/L-glutamine stock (Mediatech, Manassas, VA), 5×10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO), and 5 mM HEPES buffer (Sigma). Cells were maintained at 37˚C with 5% CO_2 atmosphere.

Cloning and plasmids

To generate MIG–3XFLAG–Spi-C, PCR was used to amplify the 3XFLAG-coding sequence (28) from the plasmid vector pBICEP–CMV-2 (Sigma-Aldrich, St. Louis, MO) to introduce a 5′ XhoI site and retain an in-frame 3′ NotI site. This DNA fragment was cloned, sequenced, and ligated into the retroviral vector MIG–HA–Spi-C using XhoI and NotI sites to replace the hemagglutinin epitope tag sequence of this vector (29). The *Fcer2a* promoter was amplified from C57BL/6 genomic DNA by PCR using LA-TAQ (TaKaRa; Thermo-Fisher Scientific, Ottawa, Ontario, Canada) and cloned into the vector pSC-A using a PCR cloning kit (Agilent Technologies, La Jolla, CA). The cloned *Fcer2a* promoter fragment was PCR amplified a second time using the same forward primer combined with a reverse primer containing a HindIII site and cloned into pSC-A. The *Fcer2a* promoter was cut from pSC-A using HindIII digestion and ligated into the HindIII site of the luciferase reporter vector pGL3-basic (Promega, Madison, WI). A predicted Ets binding site was mutated using the QuickChange II XL site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies). All constructs were verified by DNA sequencing. Transfection-quality plasmid DNA was prepared by expression in DH5α bacteria and purification with a plasmid maxi kit (Qiagen, Alameda, CA). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). Primer sequences are listed in Supplemental Table I.

Bioinformatic analysis

A transcription start site (TSS) was previously described for the human *Fcer2a* promoter (30). DNA sequences for the human and murine *Fcer2a* promoter regions were obtained from the Ensembl database and compared using MacVector 9.0 (Accelrys, San Diego, CA). A highly conserved region proximal to the *Fcer2a* TSS was identified and analyzed for predicted transcription factor binding sites using MatInspector (Genomatix, Ann Arbor, MI). Predicted binding sites with a score >0.9 were selected for further analysis.

Retroviral infection

MIG–3XFLAG–Spi-C retrovirus was generated by transient transfection of Plat-E packaging cells as previously described (31). WEHI-279 cells in early log-phase growth were infected by "spinoculation" in the presence of 20 μg/ml polybrene (Sigma-Aldrich) as described (31). Infected cells were expanded in culture for 1 wk, analyzed for GFP expression, and GFP$^+$ cells sorted as described above.

Immunoblotting and flow cytometry

Whole-cell lysates from uninfected or MIG–3XFLAG–Spi-C–infected WEHI-279 cells were probed with HRP-conjugated anti-FLAG M2 Ab (Sigma-Aldrich) and visualized with SuperSignal West Pico reagent (Thermo-Fisher Scientific). For flow cytometry, mouse splenocytes were washed after hypotonic lysis with ammonium chloride solution and stained with allophycocyanin-conjugated anti-B220 (RA3-6B2), PE-conjugated anti-CD19 (1D3), PE-Cy5–conjugated anti-IgM (II/41), PE-conjugated anti-CD93 (AA4.1), FITC-conjugated anti-IgD (11–26), FITC-conjugated anti-CD23 (B3B4), FITC-conjugated anti-CD21 (eBio8D9), FITC-conjugated anti-CD1d (1B1), or biotin-conjugated anti–IL-21R (eBio4A9). All Abs were purchased from eBioscience (San Diego, CA). Analysis of stained cells was done using a BD FACSCalibur (San Jose, CA) system, and sorting was performed using a FACSAria II system, both located in the London Regional Flow Cytometry core facility (London, Ontario, Canada). The purity of sorted cells was confirmed to be ≥95% using the FACSAria II system.

Immunofluorescence staining

Spleens were embedded in Tissue-Tek OCT compound (Sakura-Finetec, Torrance, CA) and frozen on dry ice. Spleen sections 5 μm thick were fixed in cold methanol and blocked with 5% BSA. After blocking, the sections were incubated with primary Abs anti-mouse CD22-FITC (Cy34; BD Biosciences, San Diego, CA) and anti-Moma-1 (Serotec, Raleigh, NC). After washing, sections were incubated with fluorescent (Alexa Fluor 568) secondary Abs (anti-rat IgG). Sections were mounted in Vestashield mounting medium (Vector Labs, Burlingame, CA) and photographed with a Nikon (Melville, NY) Eclipse 80i microscope attached to an X-cite 120 fluorescence illumination unit. Fifteen follicles were examined per mouse for each indicated genotype. Diameters of follicles and MZ were measured using SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI).

Transient transfection analysis

WEHI-279 cells in early log phase growth were washed three times in serum-free DMEM with 4.5g/l glucose (Lonza). Triplicate sets of cells were incubated for 10 min at room temperature with 10 μg of each luciferase reporter plasmid and 0.5 μg of pRL-TK (Promega). Cell-DNA mixtures were electroporated at 220 V and 950 μF using 4-mm gap cuvettes and a GenePulser II instrument with Capacitance Extender (Bio-Rad, Mississauga, Ontario, Canada). After an additional 10 min of incubation at room temperature, transfected cells were transferred to complete media and incubated at 37˚C for 24 h. Luciferase assays were performed on cell lysates using the Dual-Luciferase Reporter Assay System (Promega). Light production was measured using a Lumat LB 9507 tube luminometer (Berthold Technologies, Oak Ridge, TN).

Reverse transcription-quantitativePCR

Total RNA was isolated from sorted FO B cells or WEHI-279 B cells using RNA-Bee reagent (Tel-Test, Friendswood, TX). cDNA was synthesized from total RNA using an iScript cDNA Synthesis Kit (Bio-Rad). Quan-

titative PCR (qPCR) was performed using the iQ SYBR Green Supermix kit (Bio-Rad) and a Rotor-Gene 6000 instrument (Corbett Life Sciences, Valencia, CA). Relative mRNA transcript levels were normalized to GAPDH as a reference gene and compared between samples using the comparative threshold cycle method (32). Results are presented as the mean and SD of triplicate experiments. Primer sequences are listed in Supplemental Table I.

ChIP experiments

MIG–3XFLAG–Spi-C–infected WEHI-279 cells were cross-linked with 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated with 125 mM glycine, then cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS) containing ProteoBlock protease inhibitor mixture (Fermentas, Burlington, Ontario, Canada). Chromatin solutions were sonicated to yield DNA fragments in the range 300–700 bp using a Bioruptor UCD-200TM-EX waterbath sonicator (Diagenode, Sparta, NJ). Sonicated chromatin was incubated with monoclonal mouse M2 anti-FLAG Ab (Sigma-Aldrich) conjugated to protein G DynaBeads (Invitrogen, Burlington, Ontario, Canada) overnight at 4°C. As a control, sonicated chromatin was incubated with normal mouse IgG conjugated to protein G DynaBeads. Magnetic bead-bound complexes were enriched using a Dynal magnetic particle concentrator (Invitrogen). Bound beads were washed once with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, 150 mM NaCl), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-Cl), and twice with Tris-EDTA buffer at pH 8. Immunocomplexes were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Cross-links were reversed in a final volume of 300 μl, containing 200 mM NaCl, overnight at 65°C. DNA was purified using a Wizard SV Gel and PCR purification Kit (Promega). Enrichment was measured using qPCR of DNA immunoprecipitated with anti-FLAG or mouse IgG, using primers indicated in Supplemental Table I. Fold enrichment was calculated using the comparative threshold cycle method (32).

Statistical analysis

All averages shown were means of *n* mice as indicated in the legends of the figures of this paper. All error bars are SD of the mean. Statistical significance was measured using Student *t* test. For all figures, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

PUB *mice have reduced frequencies of splenic CD23$^+$ FO B cells*

PUB mice were previously shown to have moderately reduced frequencies of splenic B cells with severely impaired signaling in response to anti-IgM or anti-CD40 stimulation but relatively normal responses to LPS (20). We hypothesized that this defect might be accounted for by a reduction of FO B cells in *PUB* mice. To test this hypothesis, we performed detailed analyses of B cell subsets in *PUB* mice. Analysis of *PUB* mice confirmed a moderate but not statistically significant reduction in the overall frequency of B220$^+$ CD19$^+$ B cells as well as a corresponding increase in the frequency of splenic T cells and myeloid cells (data not shown). Mouse FO B cells express the low-affinity IgE Fc receptor FcεRII (CD23), whereas MZ B cells do not express CD23 (5). B220$^+$ CD93$^-$ IgM$^+$ CD23$^+$ cells represent FO cells, whereas B220$^+$ CD93$^-$ IgM^{hi} CD23$^-$ cells represent MZ cells (5). FO B cells were the majority of B220$^+$ CD93$^-$ B cells in the spleen of adult female C57BL/6 mice (33) (Fig. 1*A*, *left panel*). However, in *PUB* mice, splenic FO B cells were reduced to an average of ~7% of mature B cells compared with ~62% in wild-type (WT) mice (Fig. 1*A*, 1*B*). The frequency of B220$^+$ CD93$^-$ IgM$^+$ CD23$^-$ cells was increased ~4-fold, from 5.1% in WT mice to 18.3% in *PUB* mice ($n = 5$, $p = 0.01$). However, this change was not sufficient to account for the ~9-fold reduction in CD23$^+$ FO B cell frequencies in *PUB* mice. CD23$^+$ FO B cell frequencies were also significantly reduced in *Sfpi1*$^{+/-}$ mice and in *Spib*$^{-/-}$ mice (Fig. 1*B*). The reduction in CD23$^+$ FO B cell frequencies in *PUB* mice was accompanied by an increase in the frequency of B220$^+$ CD93$^+$ IgM^{hi} CD23$^-$ cells, suggesting an increase in MZ B cell frequencies (Fig. 1*A*, 1*C*). The mean CD23 staining intensity (Fig. 1*D*, 1*E*) and frequency of B220$^+$ CD23$^+$ cells (Fig. 1*D*, 1*F*) was dramatically reduced in *PUB* mice compared with that in WT mice. In summary, these data show that the frequency of CD23$^+$ FO B cells is dramatically reduced as a consequence of loss of PU.1 and/or Spi-B. These results suggest that PU.1 and Spi-B are important for the development of CD23$^+$ FO B cells.

To confirm that FO B cells were reduced in frequency in the spleens of *PUB* mice, splenic B cell subsets were examined by several alternative methods. FO B cells express high levels of cell surface IgD and low levels of cell surface IgM (IgD^{hi} IgM^{lo}) (34). As shown in Fig. 2*A* and 2*B*, IgD^{hi} IgM^{lo} FO B cells were significantly reduced in frequency in the spleens of *PUB* mice compared with that in WT mice. In addition, IgD^{hi} IgM^{lo} B cells from *PUB* spleen expressed very low levels of CD23 compared with that in WT IgD^{hi} IgM^{lo} B cells (data not shown). IgD^{lo} IgM^{hi} B cells, which represent MZ B cells, were significantly increased in frequency in *PUB* mice compared with that in WT mice (Fig. 2*A*, *right panel*, Fig. 2*C*). Next, we determined the frequency of cells expressing high levels of the receptor for IL-21 (IL-21R). Most B cells express IL-21R, but FO B cells express high levels whereas MZ B cells express low levels (35). The spleens of PUB mice contained reduced frequencies of IL-21R^{hi} B cells compared with that in WT mice (60% compared with 92%; Fig. 2*D*). Taken together, these results confirm that the frequencies of FO B cells were reduced in the spleens of *PUB* mice compared with that in WT mice.

Increased frequency of MZ B cells in spleens of PUB *mice*

In contrast with reduced frequencies of FO B cells in *PUB* mice, the relative frequency of IgM^{hi} CD23$^-$ B cells was significantly increased in *PUB* mice compared with that in WT mice (Fig. 1*A*, 1*C*). Furthermore, the frequencies of IgD^{lo} IgM^{hi} B cells and IL-21R^{lo} B cells were increased in *PUB* mice compared with those in WT mice (Fig. 2*C*, 2*D*). These results suggest that MZ B cells are increased in frequency in *PUB* mice compared with that in WT mice. To determine if this is true, the frequency of MZ B cells was determined using several alternative combinations of cell surface markers. First, we measured the frequency of B220$^+$ CD93$^-$ IgM^{hi} CD21^{hi} cells, previously shown to represent MZ B cells (36). The frequency of B220$^+$ CD93$^-$ IgM^{hi} CD21^{hi} MZ B cells in the spleens of adult *PUB* mice was approximately double that of WT mice (Fig. 3*A*, 3*B*). MZ B cells express high levels of CD1d on their cell surfaces (37). The frequency of B220$^+$ IgM^{hi} CD1d^{hi} MZ B cells in *PUB* spleen was double that of WT spleen (data not shown). In addition, we found that the same IgM^{hi} cell population expressed high levels of both CD1d and CD21 (data not shown). Notably, the frequency of MZ cells was not significantly increased in either *Sfpi1*$^{+/-}$ or *Spib*$^{-/-}$ mice (Fig. 3*B*). Taken together, these data suggest that *PUB* mice have significantly increased frequencies of MZ B cells.

To determine if the size and composition of splenic follicles were altered in *PUB* mice, we performed immunofluorescence staining of frozen spleen sections. Anti-CD22 Abs were used to identify B cells, and anti–MOMA-1 Abs were used to identify metallophilic macrophages marking the border between the follicle and the MZ (14). Compared with WT mice, MZ diameters were significantly increased in the follicles of *PUB* mice (Fig. 3*C*). B cell follicle diameters were decreased in *PUB* compared with that in WT mice (Fig. 3*C*). Ratios of MZ to follicle diameters were significantly increased in *PUB* mice compared with those in WT mice (Fig. 3*D*). *Sfpi1*$^{+/-}$ or *Spib*$^{-/-}$ mice had intermediate phenotypes that were not significantly different from those of WT

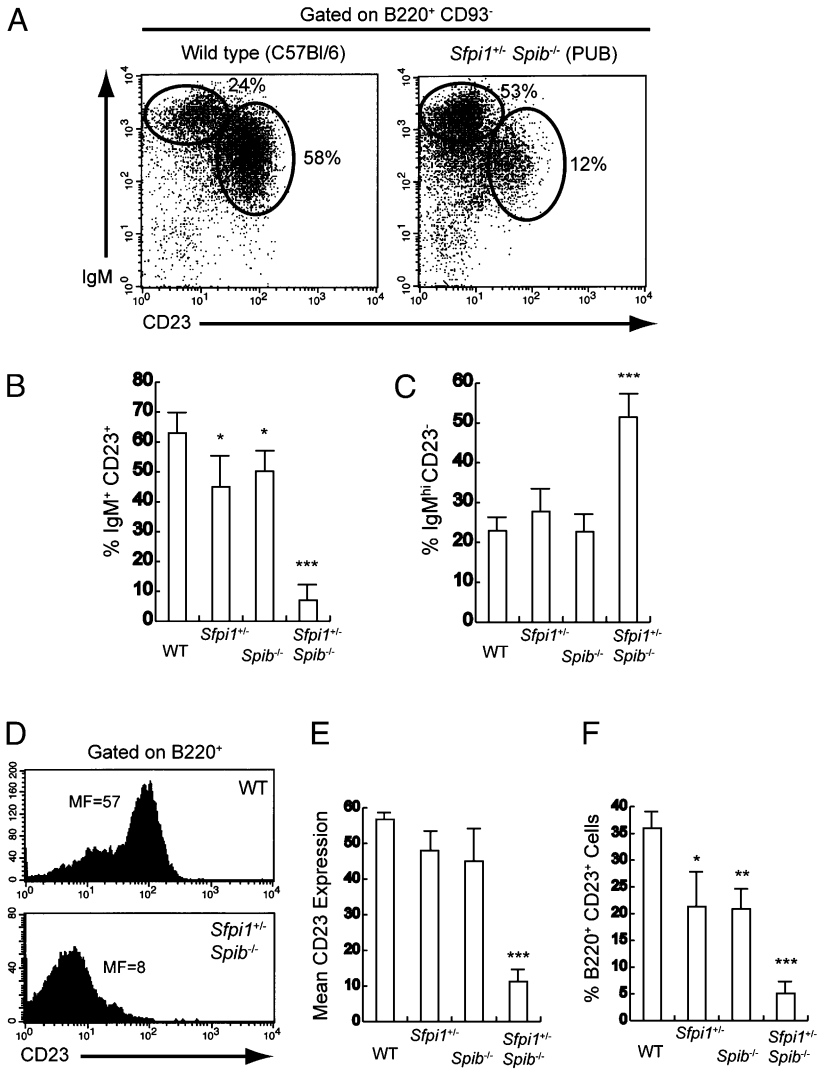

FIGURE 1. The frequency of CD23$^+$ FO B cells is reduced in spleens of *PUB* mice. *A*, Frequency of CD23$^+$ FO B cells in WT and *PUB* mice. Splenocytes from adult WT (*left panel*) and *PUB* (*right panel*) mice were stained with anti-B220, anti-CD93, anti-IgM, and anti-CD23 Abs and analyzed by flow cytometry. The indicated results are gated on B220$^+$ CD93$^-$ lymphocytes. The *upper left oval* in each panel represents B220$^+$ CD93$^-$ IgM^{hi} CD23$^-$ MZ B cells, and the *lower right oval* in each panel represents B220$^+$ CD93$^-$ IgM$^+$ CD23$^+$ FO B cells. *B*, Quantitation of data shown in the *lower right oval* (CD23$^+$ FO B cells) of panels in *A* showing mean and SD for five mice per group. In addition to WT and *PUB* splenocytes, *Sfpi1*$^{+/-}$ and *Spib*$^{-/-}$ splenocytes were also analyzed (*center bars*). *C*, Quantitation of data shown in the *upper left oval* (MZ B cells) of panels in *A* showing mean and SD for five mice per group. In addition to WT and *PUB* splenocytes, *Sfpi1*$^{+/-}$ and *Spib*$^{-/-}$ splenocytes were also analyzed (*center bars*). *D*, Reduction in CD23 expression on B220$^+$ B cells from WT and *PUB* spleen. *E*, Quantitation of data shown in *D* for five mice per group. In addition to WT and *PUB* splenocytes, *Sfpi1*$^{+/-}$ and *Spib*$^{-/-}$ splenocytes were also analyzed (*center bars*). *F*, Quantitation of the total frequency of B220$^+$ CD23$^+$ cells in the spleens of WT, *Sfpi1*$^{+/-}$, *Spib*$^{-/-}$, and *PUB* mice for five mice per group. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

mice (Fig. 3*C*, 3*D*). Taken together, these results show that the diameter of the MZ as well as the frequency of MZ B cells were increased by reduction of PU.1 and Spi-B in *PUB* mice.

An Spi-C transgene rescues frequencies of CD23$^+$ B cells in spleens of PUB *mice*

To determine if the *PUB* phenotype could be complemented by ectopic expression of a closely related Ets transcription factor, we crossed *PUB* mice to Eμ–Spi-C transgenic mice (24). Spi-C is highly related to PU.1 and Spi-B and interacts with similar cognate DNA sequences (29, 38). Eμ–Spi-C mice express ~2-fold increased levels of Spi-C protein in spleen cells compared with that in WT mice (24). Eμ–Spi-C$^+$ *PUB* mice were viable and fertile, although smaller than *PUB* littermates as previously described for Eμ–Spi-C mice (24). Notably, MZ diameters and MZ/follicle diameter ratios were smaller in Eμ–Spi-C$^+$ *PUB* mice than in PUB mice (Fig. 3*C*, 3*D*). The spleens of Eμ–Spi-C$^+$ *PUB* mice were ~50% of the size of *PUB* littermates (Fig. 4*A*) and contained lower frequencies and absolute numbers of B cells, as was previously found for Eμ–Spi-C mice (data not shown) (24). CD23 expression on B cells (Fig. 4*B*) and frequencies of B220$^+$ CD23$^+$ cells (Fig. 4*C*) were substantially increased in Eμ–Spi-C$^+$ *PUB* mice compared with that in *PUB* mice. The frequency of splenic B220$^+$ CD93$^-$ IgM$^+$ CD23$^+$ cells in *PUB* mice was partially rescued by expression of the Spi-C transgene (Fig. 4*D*, 4*E*). The frequency of splenic MZ B cells (either B220$^+$ CD93$^-$ IgM^{hi} CD23$^-$ or B220$^+$ CD93$^-$ IgM^{hi} CD21^{hi}) was significantly reduced by expression of the Spi-C transgene (Fig. 4*D* and data not shown). Expression of the Spi-C transgene did not increase the frequency of IgD^{hi} IgM^{lo} B cells in *PUB* mice; however, CD23 expression promoted by Spi-C was located within the IgD^{hi} IgM^{lo} and IgD^{hi} IgM^{hi} subsets and not the IgD^{lo} IgM^{hi} MZ B cell subset (data not shown). Taken together, these data suggest that Spi-C partially complements the phenotype of *PUB* mice by promoting CD23 expression and reducing MZ B cell frequencies.

PUB *mice have a reduced frequency of CD23$^+$ T2 cells that is partially rescued by Spi-C*

Transitional B cells entering the spleen from the blood express CD93 (AA4.1), a marker of immature hematopoietic cells (5). T1 and T2 B cells can be distinguished by their expression of CD23 (5, 6). We determined the frequencies of transitional B cells in the spleens of WT, *Sfpi1*$^{+/-}$, *PUB*, and Eμ–Spi-C$^+$ *PUB* mice. The frequency of B220$^+$ CD93$^+$ transitional cells was not significantly different between WT, *Sfpi1*$^{+/-}$, and *PUB* mice, although frequencies of transitional cells were reduced by ~50% in Eμ–Spi-C$^+$ *PUB* mice, as previously reported (data not shown) (24). In WT mice, IgM$^+$ CD23$^-$ T1 cells represented (on average) 19% of B220$^+$ CD93$^+$ splenocytes, and IgM$^+$ CD23$^+$ T2 cells represented 43% and IgM^{lo} CD23$^+$ T3 cells represented 11% (Fig. 5). In *PUB* mice, the frequency of CD23$^+$ T2 and T3 cells was strikingly reduced, such that T1 cells represented on average 62%, T2 cells

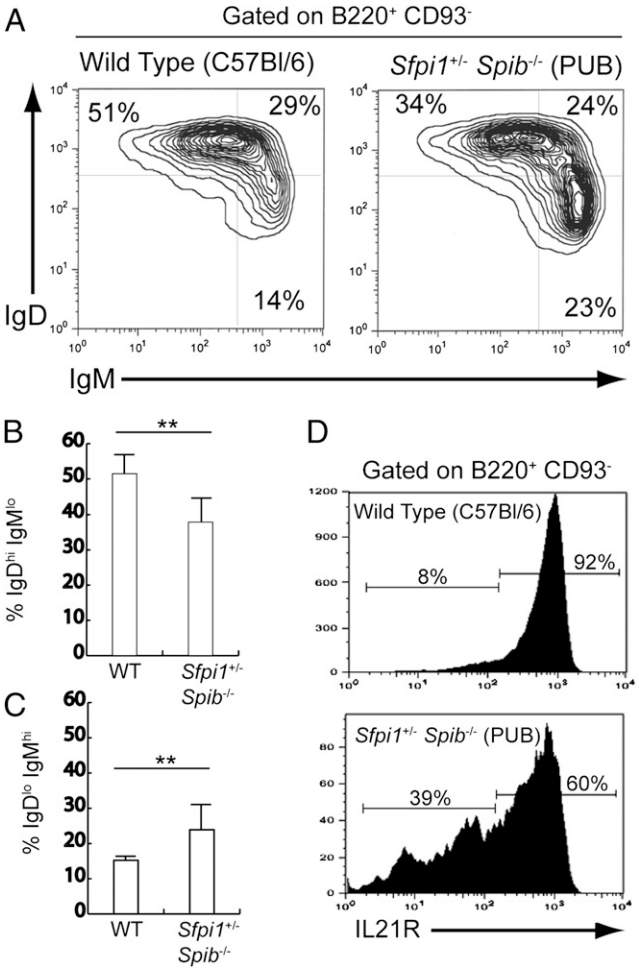

FIGURE 2. Reduction in the frequency of IgD^{hi} IgM^{lo} FO B cells in the spleens of *PUB* mice. *A*, The frequency of IgD^{hi} IgM^{lo} "follicular-1" B cells is reduced in *PUB* mice. Splenocytes from adult WT (C57BL/6, *left panel*) and *PUB* (*right panel*) mice were stained with anti-B220, anti-CD93, anti-IgD, and anti-IgM Abs and analyzed by flow cytometry. The results shown are gated on $B220^+$ $CD93^-$ lymphocytes. *B*, Quantitation of the frequency of IgD^{hi} IgM^{lo} cells (panels in *A*, *upper left quadrants*) for seven mice per group. *C*, Quantitation of the frequency of IgD^{lo} IgM^{hi} B cells (panels in *A*, *lower right quadrants*) for seven mice per group. *D*, IL-$21R^{hi}$ B cells are reduced in frequency in *PUB* mice. Splenocytes from adult WT (C57BL/6, *upper panel*) and *PUB* (*lower panel*) mice were stained with anti-B220, anti-CD93, and anti–IL-21R Abs and analyzed by flow cytometry. The results shown are gated on $B220^+$ $CD93^-$ B lymphocytes. The frequencies of IL-$21R^{hi}$ and IL21-R^{lo} cells are indicated on the histograms. $^{**}p < 0.01$.

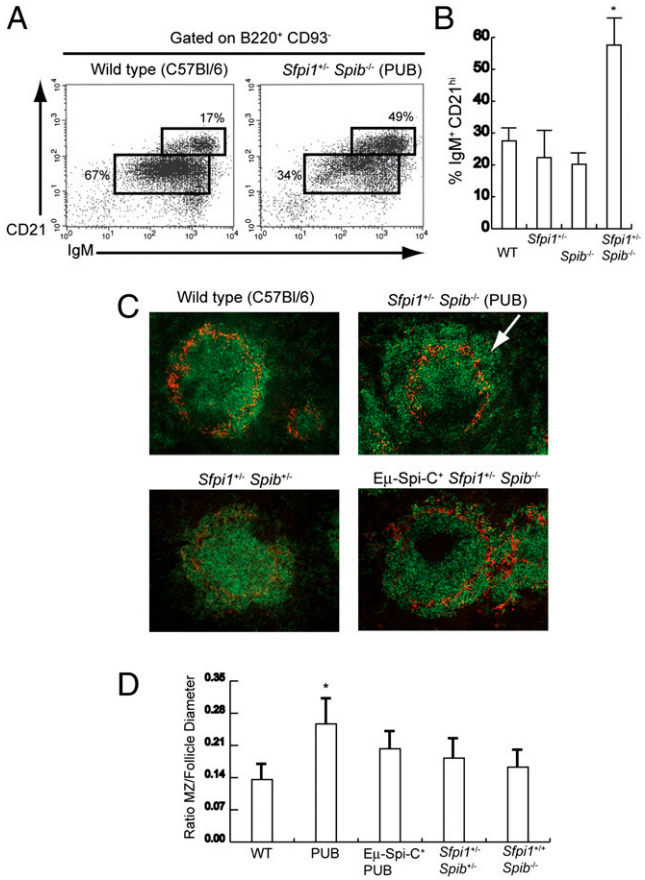

FIGURE 3. The frequency of MZ B cells is increased in the spleens of *PUB* mice. *A*, Increase in frequency of IgM^{hi} $CD21^+$ MZ B cells in *PUB* mice. Splenocytes from adult WT (C57BL/6) (*left panel*) and *PUB* (*right panel*) mice were stained with anti-B220, anti-CD93, anti-CD21, and anti-IgM Abs and analyzed by flow cytometry. The *lower left box* in each panel indicates IgM^+ $CD21^{low}$ FO B cells, and the *upper right box* indicates IgM^{hi} $CD21^+$ MZ B cells. The results shown are gated on $B220^+$ $CD93^-$ lymphocytes. *B*, The frequency of IgM^{hi} $CD21^+$ MZ B cells is increased in the spleens of *PUB* mice. Shown is the quantitation of the data shown in *A* indicating mean and SD for five mice per group. In addition to WT and *PUB* splenocytes, $Sfpi1^{+/-}$ and $Spib^{-/-}$ splenocytes were also analyzed (*center bars*). *C*, MZ diameters are increased in *PUB* mice. Immunofluorescence staining was performed on frozen sections of mouse spleen of the indicated genotypes using anti-CD22 Ab (green) to visualize B cells and anti–MOMA-1 (red) to visualize metallophilic macrophages (original magnification ×20). The arrow in the *upper right panel* indicates an enlarged MZ in a section prepared from *PUB* spleen. *D*, Increased ratio of follicle to MZ diameters in *PUB* mice. Measurements were taken of follicle or MZ diameter in 15 follicles in each of five mice of the indicated genotype. The *y*-axis represents the ratio of follicle to MZ diameter for each sample. $^*p < 0.05$.

13%, and T3 cells 0.1% (Fig. 5). Notably, even though overall transitional cell numbers were reduced, in Eμ–Spi-C^+ *PUB* mice the frequency of $CD23^+$ cells was substantially increased, such that the frequency of T1 cells was on average 44%, T2 cells was 24%, and T3 cells was 6% (Fig. 5). Finally, the phenotype of $Spi1^{+/-}$ or $Spib^{-/-}$ transitional B cells was intermediate between WT and *PUB* mice (data not shown). We conclude that reduced PU.1 and Spi-B levels have a dose-dependent effect on the frequency of splenic $CD23^+$ T2 and T3 B cells and that ectopic expression of Spi-C partially "rescues" CD23 expression on *PUB* transitional cells.

CD23 expression is activated by PU.1, Spi-B, and Spi-C

To identify potential target genes of related Ets transcription factors in FO B cells, we used cell sorting to enrich $B220^+$ $CD93^-$ IgD^{hi} IgM^{lo} cells (representing FO-I B cells) or $B220^+$ $CD93^-$ IgD^{hi} IgM^{hi} cells (representing FO-II/MZ-P B cells) (34) from spleens of WT, *PUB*, and Eμ–Spi-C^+ *PUB* mice. RNA and cDNA were prepared from sorted cells and used as the template for reverse transcription-quantitative PCR (RT-qPCR) to determine the relative steady-state levels of mRNA transcripts. Transcript levels were normalized to *Gapdh* transcript levels. As shown in Fig. 6*A*, *Cd19* transcripts (encoding the CD19 cell surface marker) were expressed in both FO-I and FO-II/MZ-P B cell subsets. Myocyte-enhancer factor 2c (Mef2c) is a MADS-box transcription factor that is important for B cell differentiation and function (39, 40). We recently showed that *Mef2c* is directly activated by PU.1 in B cells (39). As would be predicted by this observation, *Mef2c*

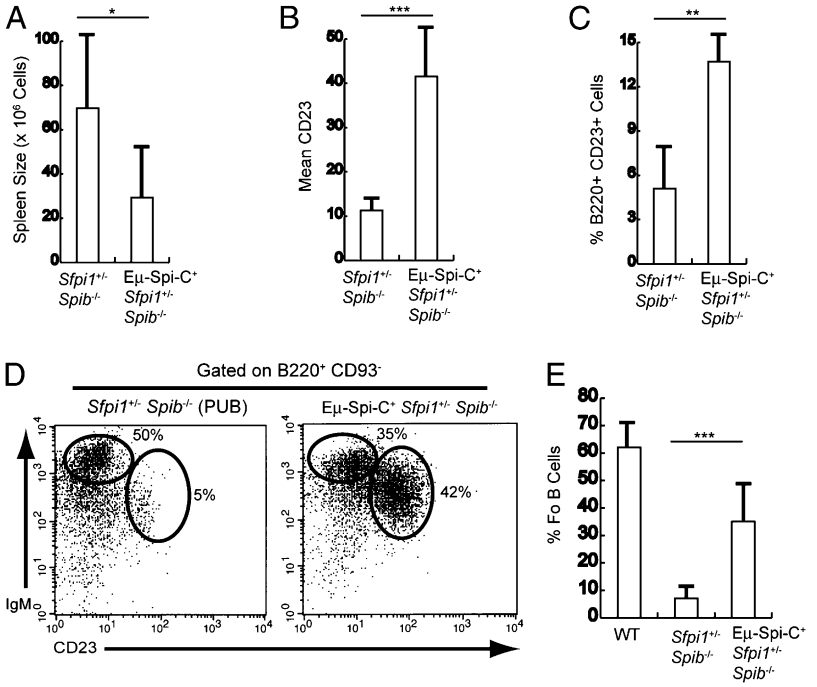

FIGURE 4. An Spi-C transgene "rescues" CD23$^+$ B cells in *PUB* mice. *A*, The spleens of Spi-C transgenic *PUB* mice contain fewer cells than *PUB* littermates. Total spleen cell counts were performed for groups of *PUB* and Eμ–Spi-C$^+$ *PUB* mice. The result shown represents the mean and SD of six mice per group. *B*, An Spi-C transgene increases mean CD23 expression on B220$^+$ cells. Splenocytes were prepared from adult *PUB* mice or Eμ–Spi-C$^+$ *PUB* mice, stained with anti-B220 and anti-CD23 Abs, and analyzed by flow cytometry. The results shown represent the mean and SD of CD23 mean fluorescence gated on B220$^+$ lymphocytes for six mice per group. *C*, Expression of an Spi-C transgene increases the frequency of CD23$^+$ B220$^+$ cells in the spleens of *PUB* mice. Splenocytes were prepared and analyzed as described for *B*. The results shown represent the mean and SD of the frequency of B220$^+$ CD23$^+$ cells for six mice per group. *D*, Spleens of Spi-C transgenic *PUB* mice contain an increased frequency of IgM$^+$ CD23$^+$ FO B cells compared with that of *PUB* littermates. Splenocytes were prepared and stained with anti-B220, anti-CD93, anti-IgM, and anti-CD23 Abs and analyzed by flow cytometry. The indicated results are gated on B220$^+$ CD93$^-$ lymphocytes. *Right lower ovals* in each panel represent FO B cells, and *left upper ovals* represent MZ B cells. *E*, Quantitation of the result shown in *D*. The results shown are the mean and SD of six mice per group. The frequency of FO B cells in WT spleen are shown for reference (*left bar*). $*p < 0.05$; $**p < 0.001$; $***p < 0.001$.

transcript levels were reduced in sorted *PUB* FO-I cells (Fig. 6*A*, *top panel*) and FO-II/MZ-P B cells (Fig. 6*A*, *middle panel*) relative to that in WT B cells. Expression of the Spi-C transgene in Eμ-Spic$^+$ *PUB* FO-I or FO-II/MZ-P B cells increased *Mef2c* transcript levels (Fig. 6*A*, *top panel* and *middle panel*). Next, we measured levels of *Fcer2a* transcripts, encoding the cell surface marker CD23. *Fcer2a* transcripts were reduced by 15-fold in PUB FO-I B cells relative to WT FO-B cells (Fig. 6*A*, *top panel*) and were reduced by 41-fold in PUB FO-II/MZ-P B cells relative to WT FO-II/MZ-P B cells (Fig. 6*A*, *middle pane*l). Importantly, *Fcer2a* transcript levels were substantially rescued by expression of the Spi-C transgene in Eμ-Spic$^+$ *PUB* FO-I or FO-II/MZ-P B cells (Fig. 6*A*, *top panel* and *middle panel*). This result suggested that *Fcer2a* might be a direct target gene of PU.1, Spi-B, and Spi-C in FO B cells. For evidence that these results are not trivially explained by a reduced frequency of CD23-expressing cells within the FO-I or FO-II/MZ-P B cell populations, we repeated the experiment by sorting B220$^+$ CD93$^-$ IgM$^+$ CD23$^+$ FO B cells from WT, *PUB*, and Eμ–Spi-C$^+$ *PUB* mouse spleen and performed RT-qPCR. As shown in the *bottom panel* of Fig. 6*A*, expression of both *Mef2c* and *Fcer2a* transcripts was reduced in *PUB* CD23$^+$ FO B cells and restored in Eμ–Spi-C$^+$ *PUB* CD23$^+$ FO B cells (Fig. 6*A*). Taken together, these experiments demonstrate that *Fcer2a* transcript levels are reduced in the absence of PU.1 and/or Spi-B and are restored by ectopic expression of Spi-C.

To determine if PU.1, Spi-B, and Spi-C might directly regulate *Fcer2a*, we examined the previously characterized promoter region of the human gene (41) using phylogenetic comparison of human and mouse DNA sequences coupled with prediction of transcription factor binding sites, as described in *Materials and Methods*. This analysis predicted two conserved Mef2c binding sites and an Ets binding site in the mouse *Fcer2a* promoter (Fig. 6*B*). Notably, CD23 expression is reduced in B cells from *Mef2c* KO mice (20, 40). Therefore, CD23 expression might be regulated by PU.1, Spi-B, and Spi-C as well as Mef2c. To determine if the predicted Ets site in the *Fcer2a* promoter was functional, we amplified the *Fcer2a* promoter region from C57BL/6 genomic DNA by PCR and ligated it into the luciferase reporter vector pGL3-basic. Site-directed mutagenesis was used to mutate the GGAA core of the Ets binding site to a GGAC, previously shown to impair PU.1 binding (31) (Fig. 6*C*). Transient transfection analysis was performed using the B cell lymphoma line WEHI-279, which is considered a model for FO B cells because it expresses IgM, IgD, and CD23 (27). WEHI-279 cells express PU.1 and Spi-B but not Spi-C (see later). The *Fcer2a* promoter was active in WEHI-279 cells, and mutation of the predicted Ets binding site significantly reduced activity of the promoter (Fig. 6*D*). Overall, these results show that PU.1 and Spi-B are essential for maintaining steady-state *Fcer2a* transcript levels in FO B cells and suggest that PU.1, Spi-B, and Spi-C can interact with a binding site in the *Fcer2a* promoter region.

Spi-C directly activates Fcer2a *transcription*

To determine the consequences of Spi-C expression in WEHI-279 cells, we constructed a retroviral vector encoding 3XFLAG-tagged Spi-C (Fig. 7*A*). Retrovirus was generated by transient transfection and used to infect WEHI-279 cells. Infected WEHI-279 cells were enriched by cell sorting. RT-PCR analysis showed

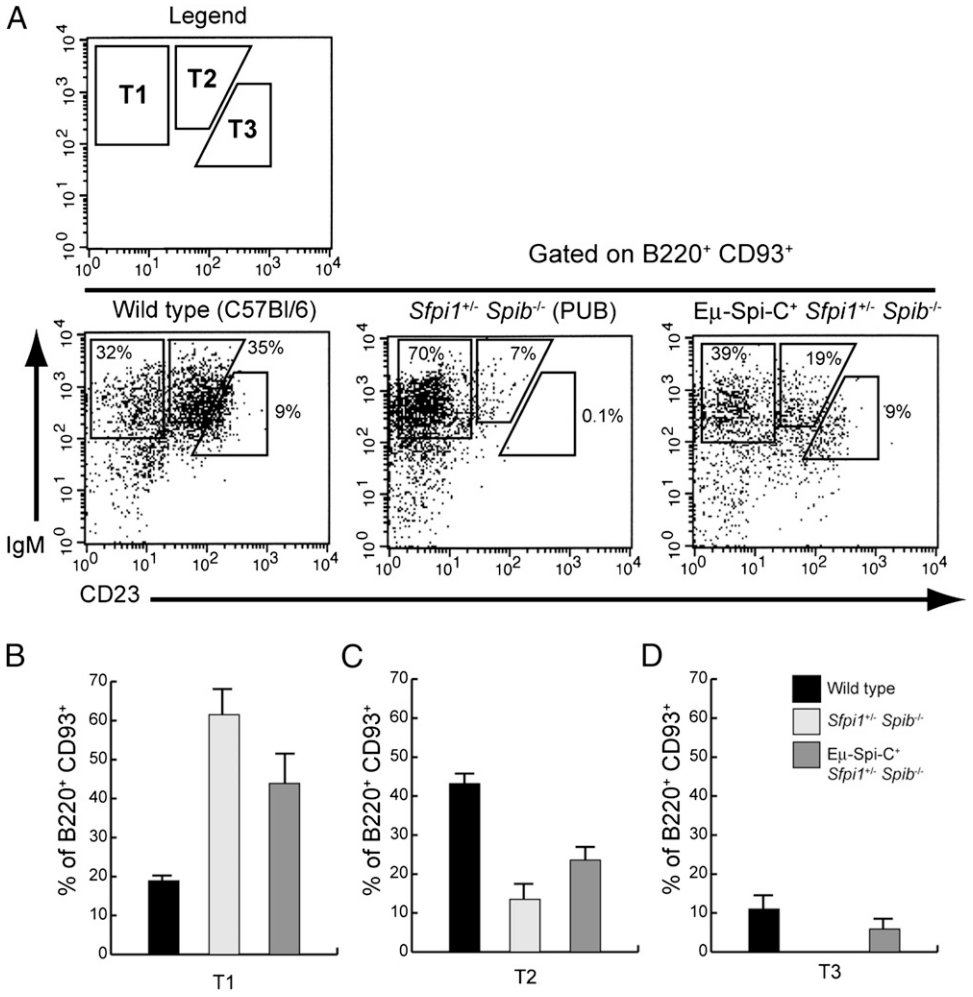

FIGURE 5. CD23$^+$ transitional B cells are reduced in frequency in spleens of *PUB* mice but "rescued" in Eμ–Spi-C transgenic *PUB* mice. *A*, CD23$^+$ transitional B cells are reduced in frequency in spleens of *PUB* mice compared with that in WT, and expression of an Spi-C transgene increases the frequency of these cells. Splenocytes from mice of the indicated genotypes were prepared and stained with anti-B220, anti-CD93, anti-IgM, and anti-CD23 Abs and analyzed by flow cytometry. The results shown are gated on B220$^+$ CD93$^+$ transitional cells representing ~10% of splenic lymphocytes. A key is shown in the *upper left panel*. T1 indicates IgM$^+$ CD23$^-$ T1 cells, T2 indicates IgM$^+$ CD23$^+$ T2 cells, and T3 indicates IgM^{low} CD23$^+$ T3 cells. *B*, Quantitation of results in *A* for T1 cells. *C*, Quantitation of results shown in *A* for T2 cells. *D*, Quantitation of results shown in *A* for T3 cells. The results shown for *B–D* represent mean and SD for groups of five mice. Genotypes are indicated in the key in *D*.

that uninfected WEHI-279 cells expressed *Sfpi1* and *Spib* transcripts but not *Spic* transcripts (Fig. 7*B*). Postinfection and cell sorting, WEHI-279 cells expressed GFP (Fig. 7*C*) and 3XFLAG-tagged Spi-C as measured by RT-PCR (Fig. 7*B*, *lower panel*) and immunoblot analysis (Fig. 7*D*). Notably, WEHI-279 cells infected with 3XFLAG-tagged Spi-C expressed dramatically higher levels of endogenous CD23 cell surface protein (Fig. 7*E*). RT-qPCR analysis showed that *Fcer2a* mRNA transcript levels were increased more than 6-fold by proviral expression of FLAG-tagged Spi-C (Fig. 7*F*), whereas endogenous *Lyn* and *Mef2c* transcripts were not significantly affected. Taken together, these results suggest that Spi-C directly activates *Fcer2a* transcription in WEHI-279 cells.

To determine if Spi-C protein interacts directly with the *Fcer2a* promoter region, we performed ChIP analysis of 3XFLAG-tagged Spi-C binding sites in WEHI-279 cells. Chromatin was prepared from WEHI-279 cells expressing 3XFLAG-tagged Spi-C as well as from uninfected control cells and immunoprecipitated with either anti-FLAG or control Abs. qPCR was used to determine the relative amount of immunoprecipitated DNA from regulatory regions including the *IgH* intronic enhancer (*Eμ*), which has previously been shown to interact with Spi-C (29), a region of the *Mef2c* intronic enhancer previously shown to interact with PU.1 (*Mef2c C+D*) (39), and the *Fcer2a* promoter region (*Fcer2a-p*). Negative controls included the proximal promoter regions of the erythrocyte-specific gene erythropoietin receptor (*EpoR-p*), the promoter of the T cell-specific *CD3g* gene (*CD3g-p*), and the ubiquitously expressed *Hprt* and *Gapdh* genes. ChIP analysis confirmed that Spi-C was enriched at the *Eμ* enhancer, *Mef2c* enhancer, and *Fcer2a* promoter, but not significantly enriched at control promoter regions (Fig. 7*G*). Therefore, Spi-C directly associates with the *Fcer2a* promoter region in WEHI-279 cells.

Discussion

The long-term goal of these experiments is to identify transcription factors that control differentiation of immature B cells into FO and MZ B cells. Analysis of peripheral B cell subsets in *PUB* mice revealed an ~9-fold reduction in the frequency of CD23$^+$ FO B cells compared with that in WT mice. In contrast, PUB mice had an ~2-fold increase in the frequency of MZ B cells. This phenotype could be genetically complemented by transgenic expression of Spi-C, as frequencies of CD23$^+$ B cells were partially restored in Eμ–Spi-C$^+$ *PUB* mice. We found that the *Fcer2a* gene,

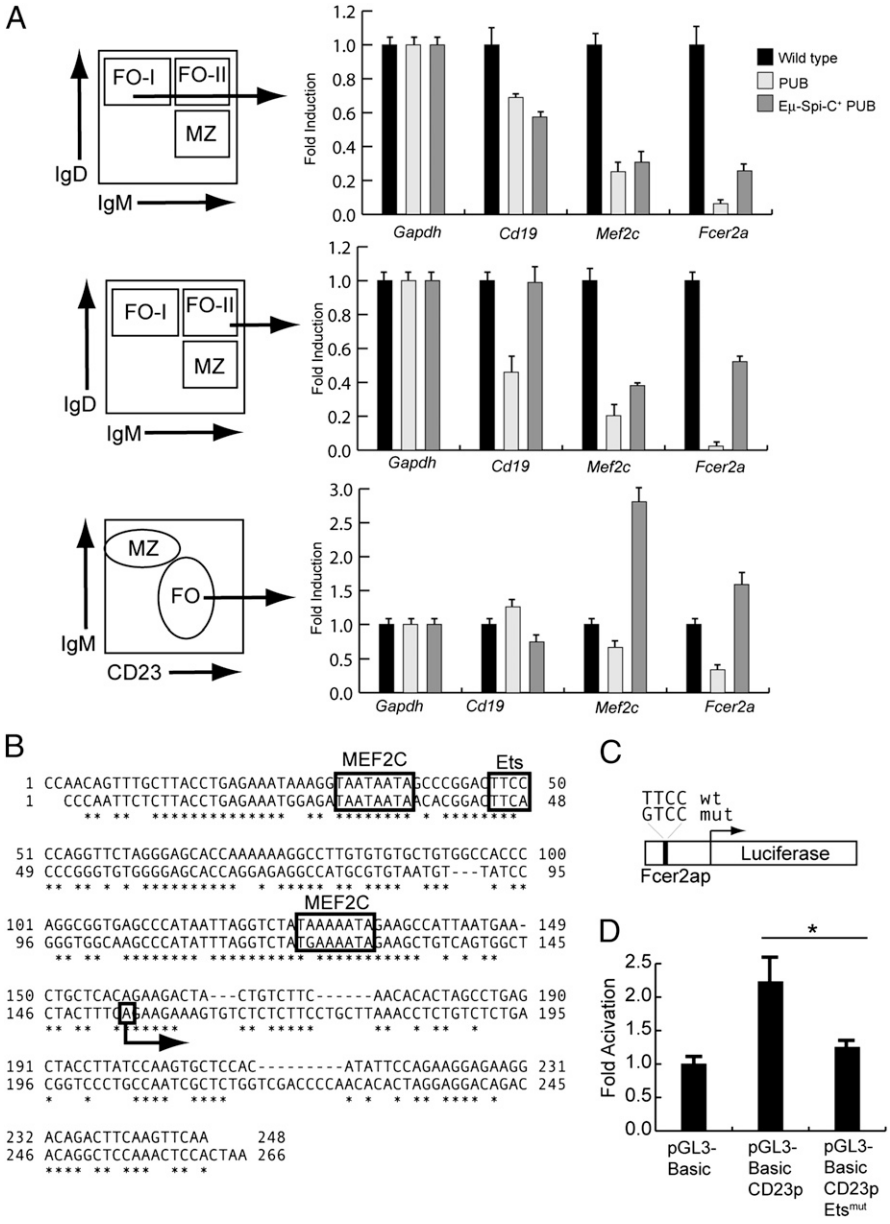

FIGURE 6. CD23 expression is activated by PU.1, Spi-B, and Spi-C. *A*, Regulation of gene expression by PU.1, Spi-B, and Spi-C in FO B cells. Cell sorting was used to enrich $B220^+$ $CD93^-$ IgD^{hi} IgM^{lo} FO-I B cells (*upper panels*), $B220^+$ $CD93^-$ IgD^{hi} IgM^{hi} FO-II/MZ-P B cells (*middle panels*), or $B220^+$ $CD93^-$ IgM^+ $CD23^+$ FO B cells (*lower panels*) from the spleens of WT, PUB, or Eμ–Spi-C^+ PUB mice. RNA was prepared from each sample and RT-qPCR used to quantify steady-state mRNA transcript levels for genes indicated on the *x*-axis, normalized to *Gapdh* transcript levels. The *y*-axis represents fold change in mRNA transcript levels relative to WT levels. Error bars show SD of triplicate analyses. *B*, Schematic showing annotated region of *Fcer2a* promoter. Murine DNA sequence is shown on top with human DNA sequence beneath. Asterisks indicate mouse–human DNA sequence identity. Predicted Mef2c and Ets transcription factor binding sites are indicated by boxes. Box with arrow represents a known TSS in the human *Fcer2a* gene. *C*, Schematic of luciferase reporter vector. The *Fcer2a* promoter shown in *B* was cloned by PCR and ligated into pGL3-basic. The predicted Ets binding site (wt) was mutated (mut) using site-directed mutagenesis. *D*, Mutation of the predicted Ets binding site reduces promoter activity in WEHI-279 B cells. WEHI-279 B cells were transfected with the plasmids indicated on the *x*-axis. The *y*-axis indicates fold-induction of luciferase activity relative to pGL3-basic. Luciferase activity was normalized by transfection with a constant amount of *Renilla* luciferase expression vector. Error bars represent SD of the mean from a representative triplicate experiment. $*p < 0.05$.

encoding the cell surface protein CD23, is activated by PU.1, Spi-B, and Spi-C in FO B cells. In summary, these results show that FO B cell differentiation is regulated by the Ets transcription factors PU.1, Spi-B, and Spi-C.

Splenic B cells from *PUB* mice are unresponsive to CD40 or anti-IgM stimulation but notably proliferate almost normally in response to LPS (20). MZ B cells were shown to account for the majority of splenic B cell proliferation in response to LPS (42). It was previously noted that IgD^{hi} cells were reduced in frequency and that overall IgM levels were increased in *PUB* splenic B cell populations, consistent with a decrease in FO to MZ B cell ratios (20). The results presented in this study support the hypothesis that B cell function is impaired in *PUB* mice at least in part because of a decrease in FO to MZ B cell ratios.

PU.1, Spi-B, and Spi-C are multifunctional transcription factors that are expressed in several cell types in the immune system. PU.1 is expressed in myeloid cells as well as in B cells and is required for both myeloid and B cell development (16, 43). Spi-B is expressed in B cells, T cells, and plasmacytoid dendritic cells and is required for the development of plasmacytoid dendritic cells (44–46). Therefore, it is possible that mutation of PU.1 or Spi-B could cause both cell-autonomous and non-cell-autonomous defects to B cell development. In the Eμ–Spi-C transgenic mouse, transcription of Spi-C cDNA is activated by the *IgH* intronic enhancer, which has been shown to activate high levels of transcription exclusively in B cells and immature T cells (24, 47). Therefore, the observation that the Eμ–Spi-C transgene rescues $CD23^+$ FO B cell development in *PUB* mice is most compatible with a cell-autonomous requirement for Ets transcription factors in the development of $CD23^+$ FO B cells.

There are several possible mechanisms by which PU.1, Spi-B, and Spi-C might regulate FO B cell development. One possibility is that these transcription factors regulate BCR signal strength that in turn regulates the FO versus MZ B cell decision (4). Pillai and Cariappa (4) have suggested that BCR signal strength is a primary determinant of FO versus MZ B cell differentiation. In general, strong BCR signaling drives differentiation of transitional B cells toward the FO B cell fate, whereas weak BCR signaling favors differentiation of transitional B cells into MZ B cells (4). Mutations in genes encoding proteins important for BCR signaling

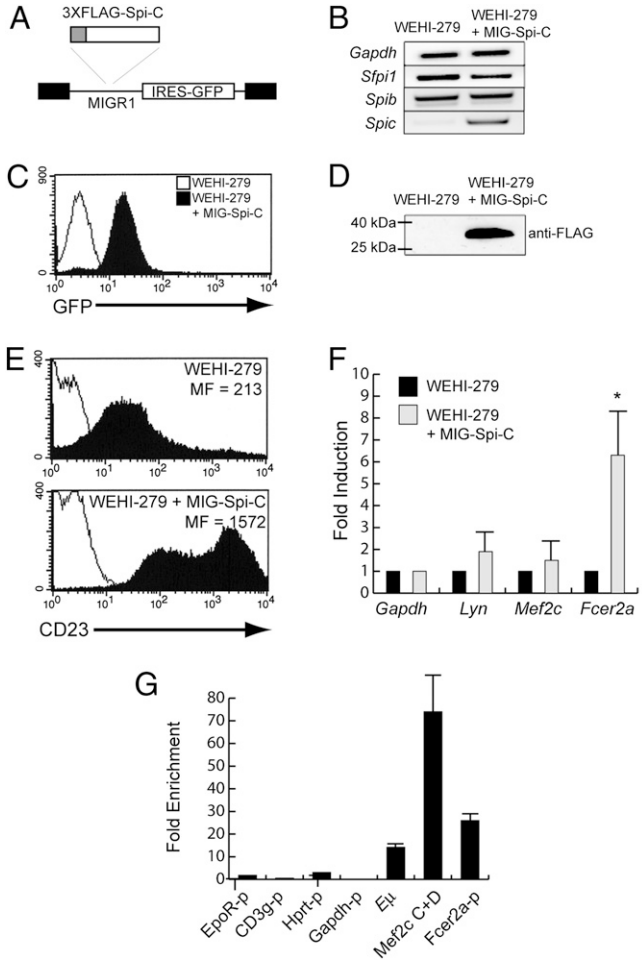

FIGURE 7. Spi-C directly activates *Fcer2a* transcription. *A*, Schematic of retroviral vector encoding 3XFLAG-tagged Spi-C and GFP. *B*, WEHI-279 B cells express PU.1 and Spi-B but not Spi-C. RT-PCR was performed to determine the presence of *Sfpi1*, *Spib*, and *Spic* transcripts in uninfected (*left column*) or MIG–3XFLAG–Spi-C–infected (*right column*) WEHI-279 B cells. *Gapdh* transcript levels were measured as a control. *C*, Green fluorescence in WEHI-279 B cells postinfection and cell sorting (filled histogram) compared with that of uninfected WEHI-279 B cells (open histogram). *D*, Expression of FLAG-tagged Spi-C in infected WEHI-279 cells. The presence of FLAG-tagged Spi-C protein was determined in lysates from uninfected WEHI-279 cells (*left column*) or MIG–3XFLAG–Spi-C–infected WEHI-279 cells (*right column*) by immunoblotting with anti-FLAG Ab. *E*, Ectopic expression of Spi-C induces CD23 expression. Panels show cell surface expression of CD23 in uninfected WEHI-279 cells (*upper panel*) or MIG–3XFLAG–Spi-C–infected WEHI-279 cells (*lower panel*). MF indicates mean fluorescence. *F*, Increased *Fcer2a* transcript levels in WEHI-279 cells as a consequence of Spi-C expression. RT-qPCR analysis was performed on RNA prepared from uninfected WEHI-279 cells (black bars) or MIG–3XFLAG–Spi-C–infected WEHI-279 cells (gray bars) for genes indicated on the *x*-axis. The *y*-axis represents changes in mRNA transcript levels relative to WT levels. *G*, Interaction of Spi-C with target genes in WEHI-279 B cells. ChIP was performed on chromatin prepared from MIG–3XFLAG–Spi-C–infected WEHI-279 B cells using preimmune mouse IgG or anti-FLAG Abs. qPCR was used to measure the amount of immunoprecipitated DNA after purification. Primers were designed to recognize the promoter region of the erythropoietin receptor gene (*EpoR-p*), the promoter region of the *CD3g* gene (*Cd3g-p*), the promoter region of the *Hprt* gene (*Hprt-p*), the promoter region of the *Gapdh* gene (*Gapdh-p*), the *IgH* intronic enhancer (*Eμ*), the intronic enhancer of the *Mef2c* gene (*Mef2c* sites C+D), or the promoter of the *Fcer2a* gene (*Fcer2a-p*). The *y*-axis shows results expressed as fold enrichment of specific regions immunoprecipitated with anti-FLAG Ab compared with that of preimmune mouse IgG. $*p < 0.05$.

generally cause reductions in the frequency of FO B cells and increases in the frequency of MZ B cells (4). Weak BCR signaling combined with signaling through the Notch pathway reinforces the MZ B cell fate (13). BCR signaling was previously shown to be impaired in *PUB* mice (20), and PU.1 and Spi-B were subsequently shown to regulate several genes involved in modulation of BCR signaling (48, 49). BCR signaling is also modulated by ectopic expression of Spi-C in B cells (24). Therefore, control of BCR signal strength by PU.1, Spi-B, and Spi-C could be involved in regulating FO B cell development.

A second, not mutually exclusive mechanism for how PU.1, Spi-B, and Spi-C regulate FO B cell development is that these transcription factors might regulate genes encoding determinants of FO versus MZ identity. Our results show that PU.1, Spi-B, and Spi-C regulate expression of CD23, the low-affinity IgE Fc receptor FcεRII, which is a developmental stage-specific marker of FO B cell development (5). A previously published genome-wide comparison of gene expression between WT and $Sfpi1^{-/-}$ $Spib^{-/-}$ cultured pro-B cells performed by our laboratory revealed changes in several genes involved in FO and MZ differentiation (50). Genes whose transcript levels were significantly decreased in cultured $Sfpi1^{-/-}$ $Spib^{-/-}$ pro-B cells included Btk (2.5-fold). Mutation of Btk in either mice or humans causes reduced frequencies of FO B cells while largely sparing MZ B cells (10, 51). Genes significantly increased in cultured $Sfpi1^{-/-}$ $Spib^{-/-}$ pro-B cells included CD22 (22-fold), CD9 (7-fold), CD1d (2.7-fold), and HES1 (2-fold). CD22, CD9, and CD1d are expressed at high levels on the surfaces of MZ B cells and are important for MZ B cell development (37, 52, 53). HES1 is a known target gene of Notch signaling, which is known to be essential for MZ B cell

development (12, 13). These results suggest a bias in gene expression of cultured *Sfpi1*$^{-/-}$ *Spib*$^{-/-}$ pro-B cells toward MZ rather than FO B cell differentiation. Future studies will be designed to determine which of these downstream target genes are sufficient to explain the phenotype of *PUB* mice.

Although various studies have described the importance of proteins involved in cell signaling for FO B cell differentiation, few transcription factors have been described that are important for FO versus MZ lineage fate decisions. Notably, one transcription factor previously identified as important for FO B cell differentiation was Fli-1, a member of the extended Ets protein family that includes PU.1, Spi-B, and Spi-C (15). Our experiments establish that PU.1 and Spi-B are important for FO B cell differentiation. Although our experiments show that FO B cell differentiation was most significantly impaired in *PUB* mice, most of the described phenotypes appeared to be present but milder in either *Sfpi1*$^{+/-}$ or *Spib*$^{-/-}$ mice. In summary, these results suggest that PU.1, Spi-B, and Spi-C act on target genes involved in FO B cell differentiation.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Weller, S., M. C. Braun, B. K. Tan, A. Rosenwald, C. Cordier, M. E. Conley, A. Plebani, D. S. Kumararatne, D. Bonnet, O. Tournilhac, et al. 2004. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood* 104: 3647–3654.
2. Weill, J. C., S. Weller, and C. A. Reynaud. 2009. Human marginal zone B cells. *Annu. Rev. Immunol.* 27: 267–285.
3. Schmidlin, H., S. A. Diehl, and B. Blom. 2009. New insights into the regulation of human B-cell differentiation. *Trends Immunol.* 30: 277–285.
4. Pillai, S., and A. Cariappa. 2009. The follicular versus marginal zone B lymphocyte cell fate decision. *Nat. Rev. Immunol.* 9: 767–777.
5. Allman, D., R. C. Lindsley, W. DeMuth, K. Rudd, S. A. Shinton, and R. R. Hardy. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J. Immunol.* 167: 6834–6840.
6. Loder, F., B. Mutschler, R. J. Ray, C. J. Paige, P. Sideras, R. Torres, M. C. Lamers, and R. Carsetti. 1999. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med.* 190: 75–89.
7. Srivastava, B., R. C. Lindsley, N. Nikbakht, and D. Allman. 2005. Models for peripheral B cell development and homeostasis. *Semin. Immunol.* 17: 175–182.
8. Thomas, M. D., B. Srivastava, and D. Allman. 2006. Regulation of peripheral B cell maturation. *Cell. Immunol.* 239: 92–102.
9. Merrell, K. T., R. J. Benschop, S. B. Gauld, K. Aviszus, D. Decote-Ricardo, L. J. Wysocki, and J. C. Cambier. 2006. Identification of anergic B cells within a wild-type repertoire. *Immunity* 25: 953–962.
10. Hardy, R. R., K. Hayakawa, D. R. Parks, and L. A. Herzenberg. 1983. Demonstration of B-cell maturation in X-linked immunodeficient mice by simultaneous three-colour immunofluorescence. *Nature* 306: 270–272.
11. Hikida, M., S. Johmura, A. Hashimoto, M. Takezaki, and T. Kurosaki. 2003. Coupling between B cell receptor and phospholipase C-gamma2 is essential for mature B cell development. *J. Exp. Med.* 198: 581–589.
12. Witt, C. M., W. J. Won, V. Hurez, and C. A. Klug. 2003. Notch2 haploinsufficiency results in diminished B1 B cells and a severe reduction in marginal zone B cells. *J. Immunol.* 171: 2783–2788.
13. Saito, T., S. Chiba, M. Ichikawa, A. Kunisato, T. Asai, K. Shimizu, T. Yamaguchi, G. Yamamoto, S. Seo, K. Kumano, et al. 2003. Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* 18: 675–685.
14. Wang, D., S. A. John, J. L. Clements, D. H. Percy, K. P. Barton, and L. A. Garrett-Sinha. 2005. Ets-1 deficiency leads to altered B cell differentiation, hyperresponsiveness to TLR9 and autoimmune disease. *Int. Immunol.* 17: 1179–1191.
15. Zhang, X. K., O. Moussa, A. LaRue, S. Bradshaw, I. Molano, D. D. Spyropoulos, G. S. Gilkeson, and D. K. Watson. 2008. The transcription factor Fli-1 modulates marginal zone and follicular B cell development in mice. *J. Immunol.* 181: 1644–1654.
16. Iwasaki, H., C. Somoza, H. Shigematsu, E. A. Duprez, J. Iwasaki-Arai, S. Mizuno, Y. Arinobu, K. Geary, P. Zhang, T. Dayaram, et al. 2005. Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* 106: 1590–1600.
17. Polli, M., A. Dakic, A. Light, L. Wu, D. M. Tarlinton, and S. L. Nutt. 2005. The development of functional B lymphocytes in conditional PU.1 knock-out mice. *Blood* 106: 2083–2090.
18. Su, G. H., H. M. Chen, N. Muthusamy, L. A. Garrett-Sinha, D. Baunoch, D. G. Tenen, and M. C. Simon. 1997. Defective B cell receptor-mediated responses in mice lacking the Ets protein, Spi-B. *EMBO J.* 16: 7118–7129.
19. Bartholdy, B., C. Du Roure, A. Bordon, D. Emslie, L. M. Corcoran, and P. Matthias. 2006. The Ets factor Spi-B is a direct critical target of the coactivator OBF-1. *Proc. Natl. Acad. Sci. USA* 103: 11665–11670.
20. Garrett-Sinha, L. A., G. H. Su, S. Rao, S. Kabak, Z. Hao, M. R. Clark, and M. C. Simon. 1999. PU.1 and Spi-B are required for normal B cell receptor-mediated signal transduction. *Immunity* 10: 399–408.
21. Carlsson, R., A. Hjalmarsson, D. Liberg, C. Persson, and T. Leanderson. 2002. Genomic structure of mouse SPI-C and genomic structure and expression pattern of human SPI-C. *Gene* 299: 271–278.
22. Bemark, M., A. Mårtensson, D. Liberg, and T. Leanderson. 1999. Spi-C, a novel Ets protein that is temporally regulated during B lymphocyte development. *J. Biol. Chem.* 274: 10259–10267.
23. Hashimoto, S., H. Nishizumi, R. Hayashi, A. Tsuboi, F. Nagawa, T. Takemori, and H. Sakano. 1999. Prf, a novel Ets family protein that binds to the PU.1 binding motif, is specifically expressed in restricted stages of B cell development. *Int. Immunol.* 11: 1423–1429.
24. Zhu, X., B. L. Schweitzer, E. J. Romer, C. E. Sulentic, and R. P. DeKoter. 2008. Transgenic expression of Spi-C impairs B-cell development and function by affecting genes associated with BCR signaling. *Eur. J. Immunol.* 38: 2587–2599.
25. Kohyama, M., W. Ise, B. T. Edelson, P. R. Wilker, K. Hildner, C. Mejia, W. A. Frazier, T. L. Murphy, and K. M. Murphy. 2009. Role for Spi-C in the development of red pulp macrophages and splenic iron homeostasis. *Nature* 457: 318–321.
26. Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh. 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* 265: 1573–1577.
27. Waldschmidt, T. J., D. H. Conrad, and R. G. Lynch. 1988. The expression of B cell surface receptors. I. The ontogeny and distribution of the murine B cell IgE Fc receptor. *J. Immunol.* 140: 2148–2154.
28. Zhang, X., C. Guo, Y. Chen, H. P. Shulha, M. P. Schnetz, T. LaFramboise, C. F. Bartels, S. Markowitz, Z. Weng, P. C. Scacheri, and Z. Wang. 2008. Epitope tagging of endogenous proteins for genome-wide ChIP-chip studies. *Nat. Methods* 5: 163–165.
29. Schweitzer, B. L., K. J. Huang, M. B. Kamath, A. V. Emelyanov, B. K. Birshtein, and R. P. DeKoter. 2006. Spi-C has opposing effects to PU.1 on gene expression in progenitor B cells. *J. Immunol.* 177: 2195–2207.
30. Suter, U., R. Bastos, and H. Hofstetter. 1987. Molecular structure of the gene and the 5′-flanking region of the human lymphocyte immunoglobulin E receptor. *Nucleic Acids Res.* 15: 7295–7308.
31. Houston, I. B., M. B. Kamath, B. L. Schweitzer, T. M. Chlon, and R. P. DeKoter. 2007. Reduction in PU.1 activity results in a block to B-cell development, abnormal myeloid proliferation, and neonatal lethality. *Exp. Hematol.* 35: 1056–1068.
32. Schmittgen, T. D., and K. J. Livak. 2008. Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* 3: 1101–1108.
33. Allman, D., and S. Pillai. 2008. Peripheral B cell subsets. *Curr. Opin. Immunol.* 20: 149–157.
34. Cariappa, A., C. Boboila, S. T. Moran, H. Liu, H. N. Shi, and S. Pillai. 2007. The recirculating B cell pool contains two functionally distinct, long-lived, post-transitional, follicular B cell populations. *J. Immunol.* 179: 2270–2281.
35. Jin, H., and T. R. Malek. 2006. Redundant and unique regulation of activated mouse B lymphocytes by IL-4 and IL-21. *J. Leukoc. Biol.* 80: 1416–1423.
36. Oliver, A. M., F. Martin, and J. F. Kearney. 1999. IgMhighCD21high lymphocytes enriched in the splenic marginal zone generate effector cells more rapidly than the bulk of follicular B cells. *J. Immunol.* 162: 7198–7207.
37. Sonoda, K. H., and J. Stein-Streilein. 2002. CD1d on antigen-transporting APC and splenic marginal zone B cells promotes NKT cell-dependent tolerance. *Eur. J. Immunol.* 32: 848–857.
38. Carlsson, R., C. Persson, and T. Leanderson. 2003. SPI-C, a PU-box binding ETS protein expressed temporarily during B-cell development and in macrophages, contains an acidic transactivation domain located to the N-terminus. *Mol. Immunol.* 39: 1035–1043.
39. Stehling-Sun, S., J. Dade, S. L. Nutt, R. P. DeKoter, and F. D. Camargo. 2009. Regulation of lymphoid versus myeloid fate 'choice' by the transcription factor Mef2c. *Nat. Immunol.* 10: 289–296.
40. Wilker, P. R., M. Kohyama, M. M. Sandau, J. C. Albring, O. Nakagawa, J. J. Schwarz, and K. M. Murphy. 2008. Transcription factor Mef2c is required for B cell proliferation and survival after antigen receptor stimulation. *Nat. Immunol.* 9: 603–612.
41. Visan, I., M. Goller, I. Berberich, C. Kneitz, and H. P. Tony. 2003. Pax-5 is a key regulator of the B cell-restricted expression of the CD23a isoform. *Eur. J. Immunol.* 33: 1163–1173.

42. Oliver, A. M., F. Martin, G. L. Gartland, R. H. Carter, and J. F. Kearney. 1997. Marginal zone B cells exhibit unique activation, proliferative and immunoglobulin secretory responses. *Eur. J. Immunol.* 27: 2366–2374.
43. Klemsz, M. J., S. R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki. 1990. The macrophage and B cell-specific transcription factor PU.1 is related to the ets oncogene. *Cell* 61: 113–124.
44. Su, G. H., H. S. Ip, B. S. Cobb, M. M. Lu, H. M. Chen, and M. C. Simon. 1996. The Ets protein Spi-B is expressed exclusively in B cells and T cells during development. *J. Exp. Med.* 184: 203–214.
45. Schotte, R., M. C. Rissoan, N. Bendriss-Vermare, J. M. Bridon, T. Duhen, K. Weijer, F. Brière, and H. Spits. 2003. The transcription factor Spi-B is expressed in plasmacytoid DC precursors and inhibits T-, B-, and NK-cell development. *Blood* 101: 1015–1023.
46. Schotte, R., M. Nagasawa, K. Weijer, H. Spits, and B. Blom. 2004. The ETS transcription factor Spi-B is required for human plasmacytoid dendritic cell development. *J. Exp. Med.* 200: 1503–1509.
47. Bodrug, S. E., B. J. Warner, M. L. Bath, G. J. Lindeman, A. W. Harris, and J. M. Adams. 1994. Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the myc gene. *EMBO J.* 13: 2124–2130.
48. Garrett-Sinha, L. A., P. Hou, D. Wang, B. Grabiner, E. Araujo, S. Rao, T. J. Yun, E. A. Clark, M. C. Simon, and M. R. Clark. 2005. Spi-1 and Spi-B control the expression of the Grap2 gene in B cells. *Gene* 353: 134–146.
49. Rao, S., L. A. Garrett-Sinha, J. Yoon, and M. C. Simon. 1999. The Ets factors PU.1 and Spi-B regulate the transcription in vivo of P2Y10, a lymphoid restricted heptahelical receptor. *J. Biol. Chem.* 274: 34245–34252.
50. Schweitzer, B. L., and R. P. DeKoter. 2004. Analysis of gene expression and Ig transcription in PU.1/Spi-B-deficient progenitor B cell lines. *J. Immunol.* 172: 144–154.
51. Hoek, K. L., G. Carlesso, E. S. Clark, and W. N. Khan. 2009. Absence of mature peripheral B cell populations in mice with concomitant defects in B cell receptor and BAFF-R signaling. *J. Immunol.* 183: 5630–5643.
52. Samardzic, T., D. Marinkovic, C. P. Danzer, J. Gerlach, L. Nitschke, and T. Wirth. 2002. Reduction of marginal zone B cells in CD22-deficient mice. *Eur. J. Immunol.* 32: 561–567.
53. Won, W. J., and J. F. Kearney. 2002. CD9 is a unique marker for marginal zone B cells, B1 cells, and plasma cells in mice. *J. Immunol.* 168: 5605–5611.