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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

Rodney P. DeKoter,*,† Marc Geadah,*,† Sonam Khoosal,*,† Li S. Xu,*,† Gobi Thillainadesan,‡,§,‖ Joseph Torchia,‡,§,‖ Shu Shien Chin,‖ and Lee Ann Garrett-Sinha‖

Splenetic 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 Fcεr2α 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.

H uman 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; Mez2c, 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.

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generate lymphoid progenitors (16). Despite this early requirement, deletion of \textit{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 (\textit{Sfpi1}"\textsuperscript{−/−}, \textit{Spib}"\textsuperscript{−/−}, \textit{PUB}"\textsuperscript{−/−}) (20). Peripheral B cell differentiation has not been examined in detail in \textit{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 (\textit{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 \textit{PUB} mice. In contrast, \textit{PUB} mice had an increase in the frequency of MZ B cells. Transgenic expression of Spi-C in \textit{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

\textit{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). C57BL/6, C57BL/6 X C3H, 129/Ola, \textit{Spib}"\textsuperscript{−/−}, and \textit{Fcer2a}"\textsuperscript{−/−} mice were previously generated and characterized, and genotyping was performed with PCR as described (18, 20, 24, 26). To generate Eμ–Spi-C" PUB mice, Eμ–Spi-C transgenic mice were bred with PUB females to generate Eμ–Spi-C" PUB 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.

\textit{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 in continuous passage in DMEM (4.5 g/l glucose; Lonza, Shawinginag, Quebec, Canada) supplemented with 10% FBS (BioWest, Miami, FL), penicillin/streptomycin/ \textit{t}-glutamine stock, and 5 mM HEPES buffer (Sigma-Aldrich). WEHI-279 cells were expanded in culture for 1 wk, analyzed for GFP expression, and GFP" cells sorted as described above.

\textit{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 polybreine (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.

\textit{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). Flow cytometry, mouse splenocytes were washed, and hypotonic lysis with ammonium chloride buffer was performed, followed by washing with allopurinol-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 (B384), FITC-conjugated anti-CD21 (eBioD9), and FITC-conjugated anti-CD1d (1B1), or biotin-conjugated anti-IL-21R (eBioA49). All Abs were purchased from BioLegend (San Diego, CA). Analysis of stained cells was done using a BD FACS Calibur (San Jose, CA) system, and sorting was performed using a FACS Aria 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 FACS Aria II system.

\textit{Immunofluorescence staining}

Splenic sections were embedded in Tissue-Tek OCT compound (Sakura-Finetec, Torrance, CA) and frozen on dry ice. Splenic 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 Vectashield 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. Diameter of follicles and MZ were measured using SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI).

\textit{Transient transfection analysis}

WEHI-279 cells in early log phase growth were washed three times in serum-free DMEM with 4.5 g/l glucose (Lonza). Triplicate sets of cells were infected with fluorescing (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. Diameter of follicles and MZ were measured using SPOT Advanced imaging software (Diagnostic Instruments, Sterling Heights, MI).

\textit{Reverse transcription-quantitativePCR}

Total RNA was isolated from sorted FO B cells or WEHI-279 B cells using RNA-Bea 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 glycerine, then cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS) containing ProteaseInhibitor 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 Dynamic Beads (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–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–HCl), and twice with Tri–EDTA buffer at pH 8. Immunocomplexes were eluted with elution buffer (1% SDS, 0.1 M NaHCO₃). 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 splen 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 FcεRI (CD23), whereas MZ B cells do not express CD23 (5). B220⁺ CD93⁻ IgM⁺ CD23⁺ cells represent FO cells, whereas B220⁺ CD93⁻ IgM⁺ 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. 1A, 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. 1A, 1B). 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. 1B). The reduction in CD23⁺ FO B cell frequencies in PUB mice was accompanied by an increase in the frequency of B220⁺ CD93⁻ IgM⁺ CD23⁻ cells, suggesting an increase in MZ B cell frequencies (Fig. 1A, 1C). The mean CD23 staining intensity (Fig. 1D, 1E) and frequency of B220⁺ CD23⁺ cells (Fig. 1D, 1F) 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 Spib. These results suggest that PU.1 and Spib 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 (IgDhi IgM⁻) (34). As shown in Fig. 2A and 2B, IgDhi IgM⁻ FO B cells were significantly reduced in frequency in the spleens of PUB mice compared with that in WT mice. In addition, IgDhi IgM⁺ B cells from PUB spleen expressed very low levels of CD23 compared with that in WT IgDhi IgM⁺ B cells (data not shown). IgDlo IgM⁺ B cells, which represent MZ B cells, were significantly increased in frequency in PUB mice compared with that in WT mice (Fig. 2A, right panel, Fig. 2C). 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-21Rhi B cells compared with that in WT mice (60% compared with 92%; Fig. 2D). 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.

In contrast with reduced frequencies of FO B cells in PUB mice, the relative frequency of IgM⁺ CD23⁻ B cells was significantly increased in PUB mice compared with that in WT mice (Fig. 1A, 1C). Furthermore, the frequencies of IgDhi IgM⁺ B cells and IL-21Rhi B cells were increased in PUB mice compared with those in WT mice (Fig. 2C, 2D). 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⁺ cells, previously shown to represent MZ B cells (36). The frequency of B220⁺ CD93⁻ IgM⁺hi CD21⁺ MZ B cells in the spleens of adult PUB mice was approximately double that of WT mice (Fig. 3A, 3B). MZ B cells express high levels of CD1d on their cell surfaces (37). The frequency of B220⁺ IgM⁺hi CD1d⁺ 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. 3B). 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 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. 3C). B cell follicle diameters were decreased in PUB compared with that in WT mice (Fig. 3C). Ratios of MZ to follicle diameters were significantly increased in PUB mice compared with those in WT mice (Fig. 3D). Sfpi1⁻/⁻ or Spib⁻/⁻ mice had intermediate phenotypes that were not significantly different from those of WT mice.
mice (Fig. 3C, 3D). 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. 3C, 3D). The spleens of Eμ–Spi-C+ PUB mice were ∼50% of the size of PUB littermates (Fig. 4A) 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. 4B) and frequencies of B220+ CD23+ cells (Fig. 4C) were substantially increased in Eμ–Spi-C+ PUB mice compared with that in PUB mice. The frequency of splenic B220+ CD93+ IgMlo CD23- or B220+ CD93- IgMhi CD23hi was significantly reduced by expression of the Spi-C transgene (Fig. 4D and data not shown). Expression of the Spi-C transgene did not increase the frequency of IgDlo IgMhi B cells in PUB mice; however, CD23 expression promoted by Spi-C was located within the IgDhi IgMlo and IgDlo IgMhi 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, IgMlo CD21+ T1 cells represented (on average) 19% of B220+ CD93+ splenocytes, and IgMlo CD23+ T2 cells represented 43% and IgMhi 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
13%, and T3 cells 0.1% (Fig. 5). Notably, even though overall transitional cell numbers were reduced, in Etu–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 transitional cell numbers were reduced, in Etu–Spi-C+ mice.

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− IgDhi IgMlo cells (representing FO-I B cells) or B220+ CD93− IgDlo IgMhi cells (representing FO-II/MZ-P B cells) (34) from spleens of WT, PUB, and Eta–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, 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 (Me2c) is a MADS-box transcription factor that is important for B cell differentiation and function (39, 40). We recently showed that Me2c is directly activated by PU.1 in B cells (39). As would be predicted by this observation, Me2c

FIGURE 2. Reduction in the frequency of IgDhi IgMlo FO B cells in the spleens of PUB mice. A, The frequency of IgDhi IgMlo “folllicular-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 IgDhi IgMlo cells (panels in A, upper left quadrants) for seven mice per group. C, Quantitation of the frequency of IgDhi IgMlo B cells (panels in A, lower right quadrants) for seven mice per group. D, IL-21Rhi 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-21Rhi and IL-21Rlo 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 IgMlo 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 IgMlo CD21hi FO B cells, and the upper right box indicates IgMhi CD21+ MZ B cells. The results shown are gated on B220+ CD93− lymphocytes. B, The frequency of IgMlo CD21hi 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, Spib−/− 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.
The indicated results are gated on B220 + CD93 that of PUB littermates. Total spleen cell counts were performed for groups of PUB and Equ–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 Equ–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, Splenocytes of Spi-C transgenic PUB mice contain an increased frequency of IgM+ CD23+ FO B cells compared with that of PUB littersmates. 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.

**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 Equ–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 Equ–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, Splenocytes of Spi-C transgenic PUB mice contain an increased frequency of IgM+ CD23+ FO B cells compared with that of PUB littersmates. 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.

transcription factor binding sites, as described in Materials and Methods. This analysis predicted two conserved Met2c binding sites and an Ets binding site in the mouse Fcer2a promoter (Fig. 6B). Notably, CD23 expression is reduced in B cells from Met2c KO mice (20, 40). Therefore, CD23 expression might be regulated by PU.1, Spi-B, and Spi-C as well as Met2c. 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. 6C). 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. 6D). Overall, these results show that PU.1 and Spi-B and Spi-C are essential for maintaining steady-state Fcer2a transcription 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. 7A). 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
that uninfected WEHI-279 cells expressed Spi1 and Spi2 transcripts but not Spi3 transcripts (Fig. 7B). Postinfection and cell sorting, WEHI-279 cells expressed GFP (Fig. 7C) and 3XFLAG-tagged Spi-C as measured by RT-PCR (Fig. 7B, lower panel) and immunoblot analysis (Fig. 7D). Notably, WEHI-279 cells infected with 3XFLAG-tagged Spi-C expressed dramatically higher levels of endogenous CD23 cell surface protein (Fig. 7E). RT-qPCR analysis showed that Fcε2a mRNA transcript levels were increased more than 6-fold by proviral expression of FLAG-tagged Spi-C (Fig. 7F), whereas endogenous Lyn and Mef2c transcripts were not significantly affected. Taken together, these results suggest that Spi-C directly activates Fcε2a transcription in WEHI-279 cells.

To determine if Spi-C protein interacts directly with the Fcε2a promoter region, we performed ChIP analysis of 3XFLAG-tagged Spi-C binding sites in WEHI-279 cells. Chromatin was prepared from uninfected control cells and immunoprecipitated with anti-FLAG or control Abs. qPCR was used to determine the relative amount of immunoprecipitated DNA from regulatory regions including the IgH intronic enhancer (Ep), 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 Fcε2a promoter region (Fcε2a-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 Ep enhancer, Mef2c enhancer, and Fcε2a promoter, but not significantly enriched at control promoter regions (Fig. 7G). Therefore, Spi-C directly associates with the Fcε2a 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 Fcε2a gene,
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.

Splenoc 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 IgDhi 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). Pilail 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

![Image](http://www.jimmunol.org/)

**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+ IgDhi IgMlo FO-I B cells (upper panels), B220+ CD93+ IgDlo IgMhi 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 indicated using site-directed mutagenesis. D, Mutation of the predicted Ets binding site mute 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.
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 *Fcer2a*, 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*2/2*Spib*2/2 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*2/2*Spib*2/2 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*2/2*Spib*2/2 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.

**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 y-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 (*E*), 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.
development (12, 13). These results suggest a bias in gene expression of cultured $Sfp1^{−/−}$ $Spi-B^{−/−}$ 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 $Sfp1^{−/−}$ or $Spi-B^{−/−}$ 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

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