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Nfkb1 Activation by the E26 Transformation-Specific Transcription Factors PU.1 and Spi-B Promotes Toll-Like Receptor-Mediated Splenic B Cell Proliferation

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Generation of antibodies against T-independent and T-dependent antigens requires Toll-like receptor (TLR) engagement on B cells for efficient responses. However, the regulation of TLR expression and responses in B cells is not well understood. PU.1 and Spi-B (encoded by *Sfpi1* and *Spib*, respectively) are transcription factors of the E26 transformation-specific (ETS) family and are important for B cell development and function. It was found that B cells from mice knocked out for Spi-B and heterozygous for PU.1 ($Sfpi1^{+/-}$ $Spib^{-/-}$ [*PUB*] mice) proliferated poorly in response to TLR ligands compared to wild-type (WT) B cells. The NF-κB family member p50 (encoded by *Nfkb1*) is required for lipopolysaccharide (LPS) responsiveness in mice. *PUB* B cells expressed reduced *Nfkb1* mRNA transcripts and p50 protein. The *Nfkb1* promoter was regulated directly by PU.1 and Spi-B, as shown by reporter assays and chromatin immunoprecipitation analysis. Occupancy of the *Nfkb1* promoter by PU.1 was reduced in *PUB* B cells compared to that in WT B cells. Finally, infection of *PUB* B cells with a retroviral vector encoding p50 substantially restored proliferation in response to LPS. We conclude that *Nfkb1* transcriptional activation by PU.1 and Spi-B promotes TLR-mediated B cell proliferation.

Toll-like receptors (TLRs) expressed by B cells recognize conserved microbial products. Engagement of TLR ligands by B cells is required for thymus-independent responses that are sufficient to promote class switch recombination, proliferation, and antigen presentation (1, 2). Generation of optimal T-dependent antibody responses also requires TLR signaling in B cells (3, 4). For example, efficient antibody responses to protein antigens after immunization with synthetic nanoparticles required engagement of TLRs on B cells (5); therefore, identification of factors controlling TLR expression and responses in B cells has important implications for the generation of neutralizing antibody responses.

Murine B cells express and respond to TLR1, TLR2, TLR4, TLR6, TLR7/8, and TLR9 ligands (6–8), resulting in NF-κB activation through MyD88 or TRIF (TIR domain-containing adapter inducing beta interferon)-dependent pathways (9). NF-κB activates genes involved in cytokine synthesis, antibody secretion, and cell proliferation (10). The NF-κB family includes p105, which is processed into p50 (encoded by *Nfkb1*), p100 that is processed into p52 (encoded by *Nfkb2*), RelA (p65), Rel (c-Rel), and RelB, which all interact with DNA as hetero- or homodimers (11). Full TLR signaling in B cells may require major histocompatibility complex class II (MHC-II) interaction with Bruton's tyrosine kinase (Btk) and CD40 (12). However, many aspects of gene regulation involving TLR expression and signal transduction in B cells remain unclear.

PU.1 and Spi-B are E26 transformation-specific (ETS) family transcription factors encoded by *Sfpi1* and *Spib*, respectively, and are important in B cell development and function (13). PU.1 is expressed in most hematopoietic cell types, directly regulating many genes involved in cellular communication (14). Spi-B is expressed in B cells, in plasmacytoid dendritic cells, and, at lower levels, in T cells (15). PU.1 and Spi-B share 67% amino acid homology in their DNA binding domain and can bind an identical consensus sequence containing the core motif 5′-GGAA-3′ (16–18). Both transcription factors are expressed in B cells, regulate common target genes, and are functionally redundant (19, 20). $Sfpi1^{-/-}$ mice die during embryogenesis and produce no B cells (21). In contrast, viable $Spib^{-/-}$ mice have fewer B cells, which are defective in B cell receptor (BCR) signaling and are unable to generate antibody responses to T-dependent antigens (22). $Sfpi1^{+/-}$ $Spib^{-/-}$ (*PUB*) mice have greater impairment in B cell numbers and BCR signaling than $Spib^{-/-}$ or $Sfpi1^{+/-}$ mice (22, 23) and also have reduced follicular (FO) and increased marginal zone (MZ) B cell frequencies than wild-type (WT) mice (13). Therefore, PU.1 and Spi-B are critically important for B cell development and BCR signaling, but it remains unclear what these transcription factors regulate downstream to explain this phenotype.

In this study, it was determined whether PU.1 and Spi-B regulate innate immune responses in B cells. Impairment in TLR-mediated proliferation in *PUB* B cells was observed. Gene and protein expression analysis, luciferase reporter assays, and chromatin im-

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munoprecipitation (ChIP) experiments demonstrated that PU.1 and Spi-B directly activate *Nfkb1* encoding p50. Infection of *PUB* B cells with a retroviral vector encoding p50 significantly increased proliferation in response to lipopolysaccharide (LPS). Therefore, decreased p50 expression is sufficient to explain many aspects of the *PUB* B cell phenotype. Our results suggest that PU.1 and Spi-B are important transcriptional regulators of TLR responses in B cells.

MATERIALS AND METHODS

Generation and breeding of mice. Mice were housed at Western University's Health Sciences animal facility (London, Ontario, Canada) and monitored under an approved animal use subcommittee protocol in accord with Western University Council on Animal Care. C57BL/6 (WT) mice were purchased from Charles River Laboratories (Pointe-Claire, Quebec, Canada). *PUB* mice were generated by mating male and female *PUB* mice, and genotyping was performed by PCR as previously described (22, 23). Experiments were performed on mice 6 to 16 weeks of age.

B cell enrichment and proliferation analysis. Red blood cells (RBCs) were removed from spleen cell suspensions by hypotonic lysis with ammonium chloride solution. B cells were enriched by negative selection using biotin-conjugated anti-CD43 (S7) antibody (Ab), streptavidin (SA) microbeads, and LD depletion columns and a VarioMACS separation unit (Miltenyi Biotec, Germany). B cells (2×10^5/well) were plated in 96-well flat-bottom plates and stimulated with LPS (10 μg/ml) (List Biological Laboratories, Campbell, CA), anti-IgM Ab [50 μg/ml affinity pure $F(ab')_2$ fragment, goat anti-mouse IgM, μ-chain specific] (Jackson ImmunoResearch Laboratories, Inc., Jackson Grove, PA), Pam3CSK4 (1 μg/ml), heat-killed *Listeria monocytogenes* (HKLM) (10^8 cells/ml), poly(I·C) of low or high molecular weight (LMW or HMW, respectively [10 μg/ml]), ST-FLA (10 μg/ml). FSL1 (1 μg/ml), ODN1826 (5 μM) (InvivoGen, San Diego, CA), interleukin-2 (IL-2 [10 ng/ml]), IL-4 (10 ng/ml), IL-5 (10 ng/ml), B cell activating factor (BAFF) (100 ng/ml) (Peprotech, NJ), or LEAF purified anti-mouse CD40 (IC10 [10 μg/ml]) (BioLegend, San Diego, CA) in complete Dulbecco's modified Eagle's medium (DMEM). Proliferation was assessed after 72 h of incubation at 37°C with a TACS MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] cell proliferation assay (Trevigen, Gaithersburg, MD) used according to the manufacturer's instructions. For [^{3}H]thymidine incorporation assays, [^{3}H]thymidine (1 mCi/ml/well) was added after 72 h of stimulation, followed by scintillation counting 18 h later.

Flow cytometry. Antibodies purchased from eBioscience (San Diego, CA) or BioLegend (San Diego, CA) included allophycocyanin (APC)-conjugated anti-B220 (RA3-6B2), anti-MHC-II (I-A/I-E [M5.144.15.2]), anti-CD40 (3/23), BAFF receptor (BAFF-R) (eBio7H22-E16), phycoerythrin (PE)-conjugated anti-CD19 (1D3), IgG isotype control (eBio299Arm), anti-CD69 (H1.2F3), anti-CD281/TLR1 (eBioTR23), anti-CD282/TLR2 (T2.5), IgG2a κ isotype control (eBM2a), anti-CD14 (Sa14-2), anti-CD180/RP105 (RP/14), fluorescein isothiocyanate (FITC)-conjugated anti-CD21/CD35 (eBio8D9), Alexa Fluor 488-conjugated anti-CD1d (1B1), biotin-conjugated anti-CD25 (7D4), anti-CD5 (53-7.3), or SA-conjugated PE. For proliferation analyses, cells were stained with the proliferation dye eFluor 450 (eBioscience). Antibody-stained cell analysis and sorting were performed using the FACSCalibur and FACSAriaIII systems, respectively (BD Biosciences, San Jose, CA). Sorted cells were determined to be of >98% purity. Data analysis was performed using FlowJo software (FlowJo LLC, Ashland, OR).

RT-qPCR. For reverse transcription-quantitative PCR (RT-qPCR), RNA was isolated using TRIzol reagent (Life Technologies, Inc., Burlington, Ontario, Canada). cDNA was synthesized using an iScript cDNA synthesis kit (Bio-Rad, Mississauga, Ontario, Canada), and qPCR was performed with a Rotor-Gene 6000 instrument (Corbett Life Sciences, Valencia, CA). Relative mRNA transcript levels were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β-2-microglobulin (β2m) and compared between samples using the comparative threshold cycle method. Calculations were performed using REST 2009 software (24). Primer sequences are listed in Table S1 in the supplemental material.

Immunoblot analysis. Lysates were prepared using the Laemmli method and applied to 8% to 10% SDS–polyacrylamide gels for electrophoresis. Proteins were transferred to nitrocellulose membranes using a Trans-Blot semidry system (Bio-Rad), and membranes were blocked for 1 h in 5% milk in Tris-buffered saline–Tween (TBST). Membranes were probed with polyclonal anti-MyD88 (eBioscience), anti-Btk (B3H5) (Cell Signaling Technology, Whitby, Ontario, Canada), anti-Nfkb1 (Poly6197) (BioLegend), or anti-β-actin (I-19) (Santa Cruz Biotechnology) Ab and diluted to concentrations recommended by their manufacturers in 1% milk–TBST overnight at 4°C. A secondary horseradish peroxidase-conjugated anti-goat or anti-mouse Ab was incubated in 1% milk–TBST. Membranes were washed and visualized with SuperSignal West Pico reagent (Thermo-Fisher Scientific, Waltham, MA).

Bioinformatic analysis. A transcription start site (TSS) was previously described for the human *NFKB1* promoter (25). Human, mouse, rat, macaque, dog, cow, and Tasmanian devil DNA sequences were obtained from the Ensembl database and aligned using MacVector (Accelrys, San Diego, CA). Predicted transcription factor binding sites were analyzed using MatInspector (Genomatix, Ann Arbor, MI), with scores of >0.9 selected for further analysis.

Plasmids and cloning. The *Nfkb1* promoter was amplified from C57BL/6 genomic DNA by PCR using LA *Taq* (TaKaRa Clontech Laboratories, Inc., Mountain View, CA), purified, and cloned into pSC-A vector using a PCR cloning kit (Agilent Technologies, La Jolla, CA). A 468-bp *Nfkb1* promoter fragment was PCR amplified using a forward primer containing a HindIII site and subcloned. The promoter was HindIII digested from pSC-A and ligated into the HindIII site of the pGL3-basic (Promega) luciferase reporter vector. Predicted ETS binding sites were mutated using the QuikChange Lightning site-directed mutagenesis kit according to the manufacturer's instructions (Agilent Technologies). The murine stem cell virus-internal ribosomal entry site-green fluorescent protein (MSCV-IRES-EGFP) retroviral vector MIGR1 has been described previously (26). The MSCV-P50-IRES-EGFP vector MIG-P50 was generated by cloning p50-FLAG from the p50 cFlag pcDNA3 vector (plasmid 20018) purchased from Addgene, Cambridge, MA) by XhoI and EcoRI digestion. P50-FLAG was cloned into the psC-A vector, excised with EcoRI, and ligated into the MIGR1 vector. MIG–c-Rel (plasmid 26984) was obtained from Addgene (Cambridge, MA) (27). All constructs were verified by DNA sequencing. Transfection-quality plasmid DNA was prepared by growth in *Escherichia coli* DH5α cells and purified with the Qiagen plasmid Maxiprep kit. All restriction enzymes were purchased from New England BioLabs (Ipswich, MA). The primer sequences used for cloning are listed in Table S1 in the supplemental material.

Generation of retrovirus. MIG-P50, MIG–c-Rel, and MIGR1 retroviral vectors were generated by transient transfection using polyethyleneimine (PEI) (PEIpro; Polyplus-transfection, Illkirch, France) into Platinum-E (Plat-E) packaging cells (28). Plat-E cells were maintained in complete DMEM (1.5 g/ml glucose) with blasticidin (10 μg/ml) and puromycin (1 μg/ml). Cells were passaged in antibiotic-free medium 72 h prior to transfection and then plated at 3×10^6 cells per 100-mm-diameter culture dish. A 2:1 PEI/DNA ratio (20 μl PEIpro and 10 μg plasmid DNA) was used for transfections, with a final working concentration for PEI of 0.5 μg/ml. Transfections were performed using DMEM without fetal bovine serum (FBS) or antibiotics for 4 h at 37°C. Cells were washed with fresh complete DMEM following transfection and incubated for 48 h at 37°C. Virus-containing supernatants were collected following 48 h, and virus production was confirmed by green fluorescence and FLAG-tagged protein using flow cytometry and immunoblotting, respectively.

Retroviral infection of primary B cells. B cells were isolated from spleens by magnetic enrichment as mentioned above and stained with the cell proliferation dye eFluor 450 (10 μM; eBioscience). B cells were stimulated for 24 h with LPS, IL-2 (10 ng/ml), IL-4 (10 ng/ml), and IL-5 (10 ng/ml). Next, cells were infected by spinoculation with virus by centrifu-

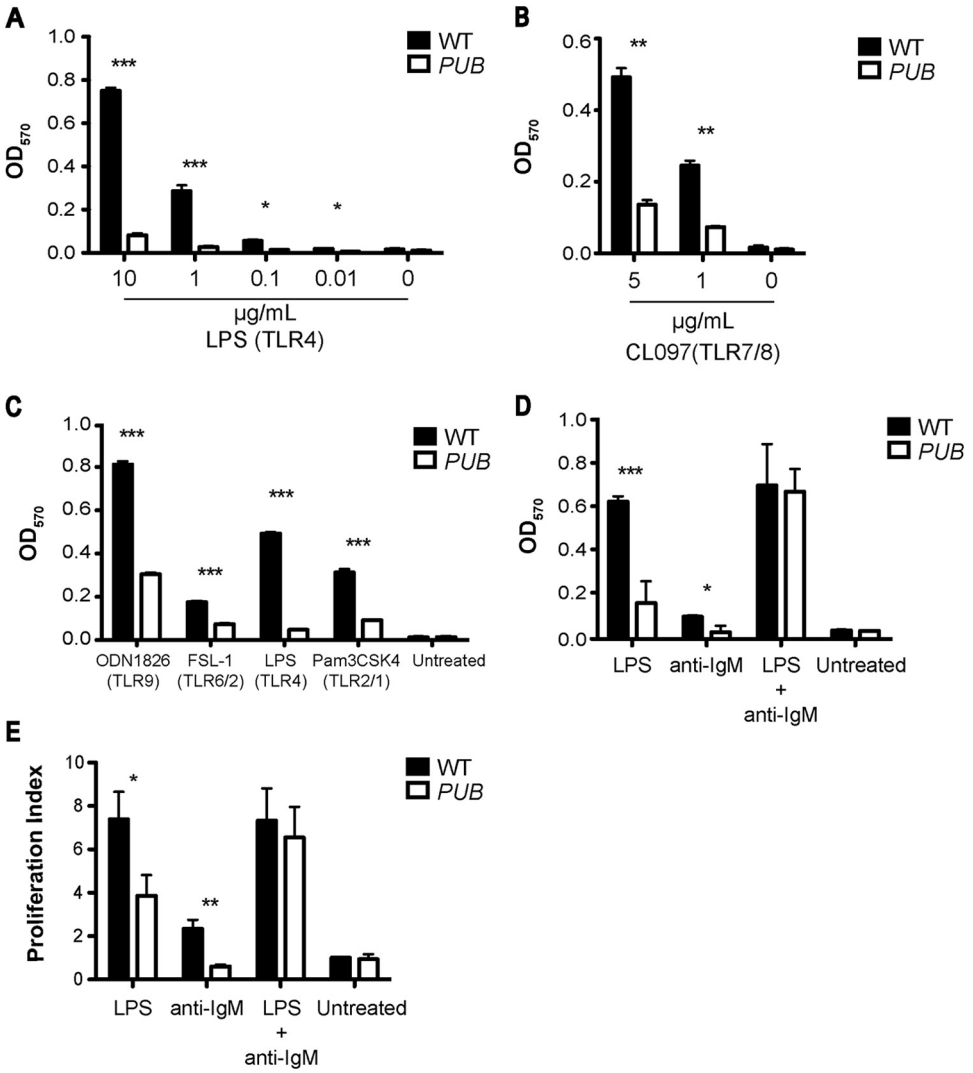


FIG 1 Impaired TLR-mediated proliferation in *PUB* (*Sfpi1*$^{+/-}$ *Spib*$^{-/-}$) B cells. (A and B) B cells from *PUB* mice responded poorly compared to WT cells stimulated with different concentrations of LPS (A) and CL097 (B). The *y* axis indicates the optical density at 570 nm (OD_{570}), and the *x* axis indicates the concentrations of the agonists. (C) B cells from *PUB* mice responded poorly compared to WT B cells stimulated with the indicated TLR9, TLR6/2, TLR4, and TLR2/1 ligands. (D) Impaired *PUB* B cell proliferation in response to anti-IgM stimulation. (E) Quantitation of four independent experiments as done in panel D. Proliferation indices were calculated by normalizing the OD_{570} of treatments to that of the WT untreated cells. For panels A to E, B cell proliferation was assessed by MTT proliferation assays following 72 h of stimulation. Data show the means ± standard deviations (SD) from triplicate wells for panels A to D and are representative of at least two independent experiments. Values in panel E are shown as means ± standard errors of the means (SEM) ($n = 4$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

gation at 800 × *g* for 2 h at 30°C in the presence of Polybrene at a final concentration of 4 μg/ml. Infection frequencies were detected by flow cytometric analysis for green fluorescence expression. Proliferation of infected cells was assessed by the dilution of eFluor 450 using flow cytometry.

Transient transfection by electroporation. WEHI-279 cells in mid- to early log-phase growth were washed three times with serum-free DMEM (4.5 g/liter) (Lonza). Cells were incubated for 10 min at room temperature with 10 μg of each luciferase reporter plasmid and 1 μg of pRL-TK (Promega). Cell-DNA mixtures were electroporated at 220 V and 950 mF using 4-mm gap cuvettes and a GenePulser II with a capacitance extender (Bio-Rad), incubated at room temperature for 10 min, and then transferred to 6-well culture plates in complete DMEM and incubated at 37°C for 24 h. A dual-luciferase reporter assay system (Promega) was performed on cell lysates. Light production was measured using a Lumat LB 9507 tube luminometer (Berthold Technologies, Oak Ridge, TN).

ChIP-seq analysis. ChIP sequencing (ChIP-seq) experiments were performed using mouse WEHI-279 cells. Cells were spin infected with the MIGR1 vector for expression of 3×FLAG-PU.1 or 3×FLAG-Spi-B. Immunoprecipitation was performed using anti-FLAG microbeads (M2) (Sigma-Aldrich, St. Louis, MO). Purified chromatin was sequenced using an Illumina HiSeq instrument. Peak finding and data analysis were performed using Galaxy Suite (29–31). Reads were aligned to the reference genome (NCBI37/mm9), using BOWTIE (32). Peaks were called using MACS version 1.0.1 (33) with default parameters, a *P* value cutoff for peak detection of 1e−05, and an effective genome size of 2.7e+9 bp. Functional analysis of *cis*-regulatory regions bound by PU.1 and Spi-B were identified using GREAT V.2.0.2 (34).

ChIP experiments. Chromatin was prepared from enriched B cells as previously described (13). In brief, cells were cross-linked and lysed followed by sonication. Sonicated chromatin was incubated overnight at 4°C with anti-PU.1 or control mouse anti-IgG Ab (Santa Cruz Biotechnology) conjugated to protein G DynaBeads (Life Technologies, Inc.). Magnetic

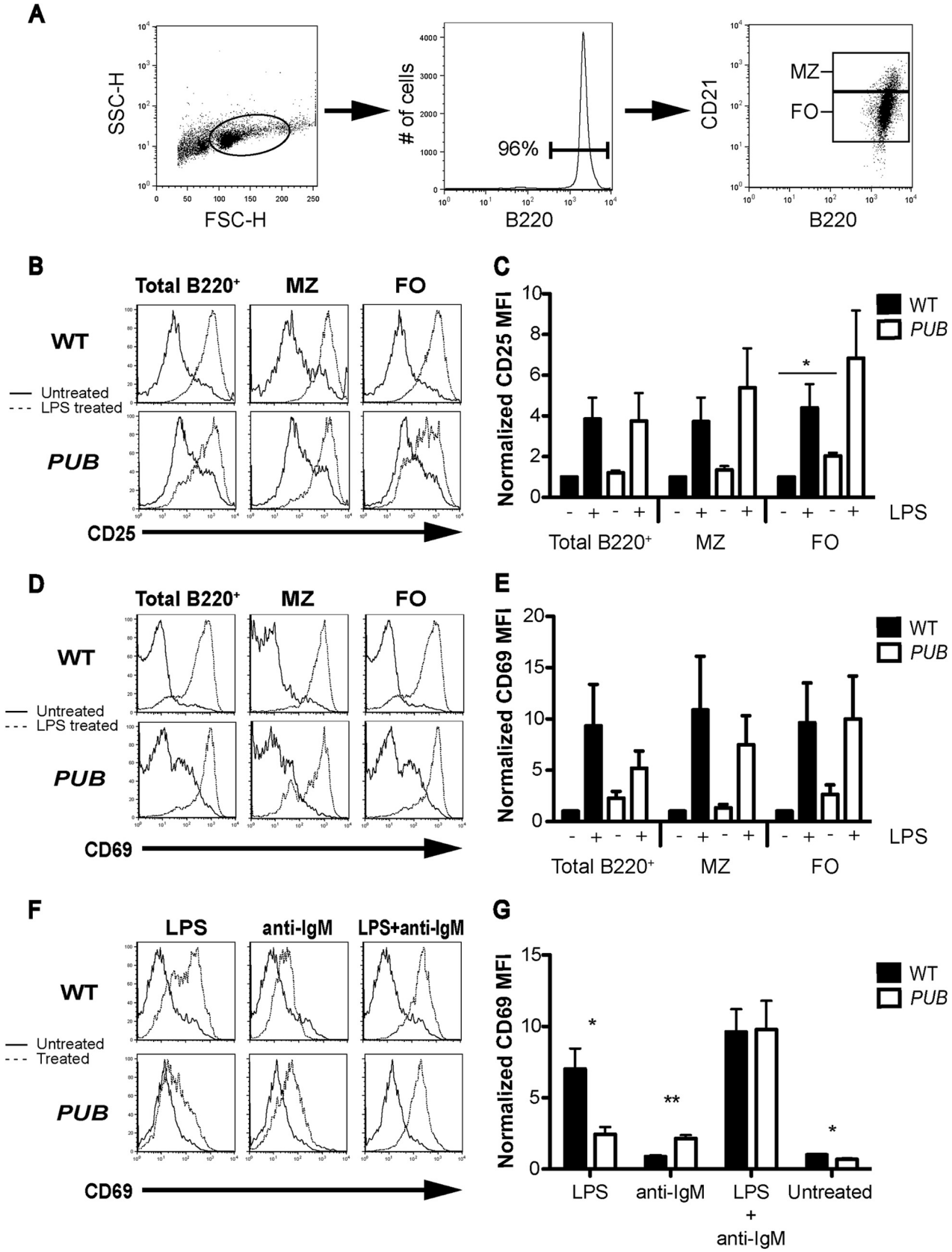


FIG 2 Splenic *PUB* B cells are activated following LPS stimulation. (A) Flow cytometry gating strategy on total B220$^+$, MZ (CD21^{hi} B220$^+$), or FO (CD21^{int} B220$^+$) B cell phenotypes. (B) Histograms show increased CD25 expression following 24 h of LPS stimulation in WT and *PUB* B cells. (C) Quantitation of data shown in panel B. (D) Histograms show increased CD69 expression following 24 h of LPS stimulation in WT and *PUB* mice. (E) Quantitation of data shown in panel D. (F) CD69 expression is lower in *PUB* total B cells than WT total B cells at 72 h poststimulation with LPS. CD69 expression is also shown for stimulation with anti-IgM and LPS plus anti-IgM. (G) Quantitation of data shown in panel F. Values in panels C, E, and G show the normalized CD25 or CD69 MFIs ± SEM from five independent experiments. Normalized MFI was calculated by dividing MFI values by the WT untreated MFI for each given subset. *, $P < 0.05$; **, $P < 0.01$.

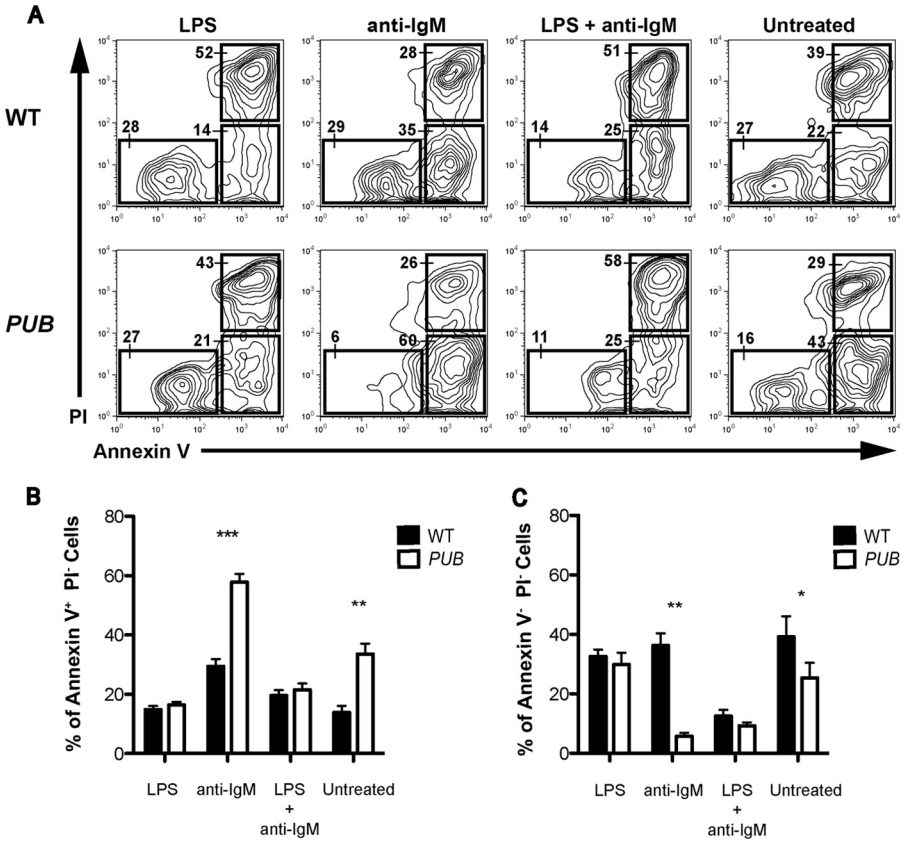


FIG 3 Apoptosis in *PUB* B cells is not elevated following LPS stimulation. (A) Apoptosis was analyzed by flow cytometry using annexin V and PI staining in enriched B cells that were untreated or treated with the indicated stimuli for 72 h. (B) Quantitation of apoptotic (annexin V^{hi} PI^{-}) cells. (C) Quantitation of live (annexin V^{-} PI^{-}) cells. Data in panels B and C show the mean frequencies ± SEM from four independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

bead complexes were enriched using a MagneSphere Technology magnetic separation stand (Promega) and washed. Immunocomplexes were eluted, and cross-linked chromatin was reversed overnight at 65°C. DNA was purified using a QIAquick PCR purification kit (Qiagen). Enrichment was measured using qPCR of immunoprecipitated DNA using primers indicated in Table S1 in the supplemental material.

ELISA analysis. Serum was collected and quantified using a Quantikine enzyme-linked immunosorbent assay (ELISA) mouse BAFF/BLyS/TNFSF13B immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Plates were analyzed using an Eon microplate spectrophotometer with Gen5 software (BioTek, Winooski, VT).

Statistical analysis. Statistical significance was determined using Student's *t* test unless otherwise indicated. *P* values of ≤0.05 were considered significant. Statistical analysis was performed using Prism 5.0 (GraphPad Software, La Jolla, CA).

ChIP-seq data accession number. ChIP-sequencing data are available from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession no. GSE58128.

RESULTS

***PUB* mice exhibit impaired TLR-mediated B cell proliferation.** *PUB* B cells were previously reported to have reduced proliferation in response to anti-IgM or lipopolysaccharide (LPS) (22). To determine if impaired proliferation was limited to LPS (TLR4 ligand), splenic B cells were enriched by CD43 depletion (see Fig. S1A in the supplemental material) and cultured with various TLR ligands. Compared to WT B cells, *PUB* B cells proliferated poorly at all tested concentrations of LPS (Fig. 1A) or CL097 (TLR7/8 ligand) (Fig. 1B) and proliferated poorly in response to Pam3CSK4 (TLR2/1), FSL1 (TLR6/2), ODN1826 (TLR9) (Fig. 1C), or anti-IgM (Fig. 1D and E) stimulation. Both WT and *PUB* B cells failed to respond to flagellin (TLR5) and poly(I·C) LMW or HMW (TLR3) (see Fig. S1B) but proliferated nearly equally well in response to stimulation with anti-CD40 or LPS plus anti-CD40 (see Fig. S1C and D), which indicated there was no general proliferation impairment in *PUB* B cells. Anti-CD40 stimulation did not restore responsiveness of *PUB* B cells to anti-IgM (see Fig. S1C and D). Interestingly, although *PUB* B cells proliferated poorly with stimulation with anti-IgM or LPS alone, stimulation with LPS plus anti-IgM resulted in equivalent WT and *PUB* proliferation (Fig. 1F to G). Similar results were obtained using the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) proliferation assays and [^{3}H]thymidine incorporation assays (see Fig. S1D). Overall, these results suggested that TLR-mediated B cell proliferation requires PU.1 and Spi-B.

Altered splenic B cell composition could explain proliferative defects in *PUB* B cells. However, *PUB* mouse spleens have increased ratios of MZ to FO B cell frequencies (13), and MZ B cells were shown to have greater TLR-mediated proliferative potential than FO B cells (35). Former studies reported reduced B cell frequencies in *PUB* spleens (22), which was confirmed by our study (see Fig. S2A and B in the supplemental material). *PUB* mouse

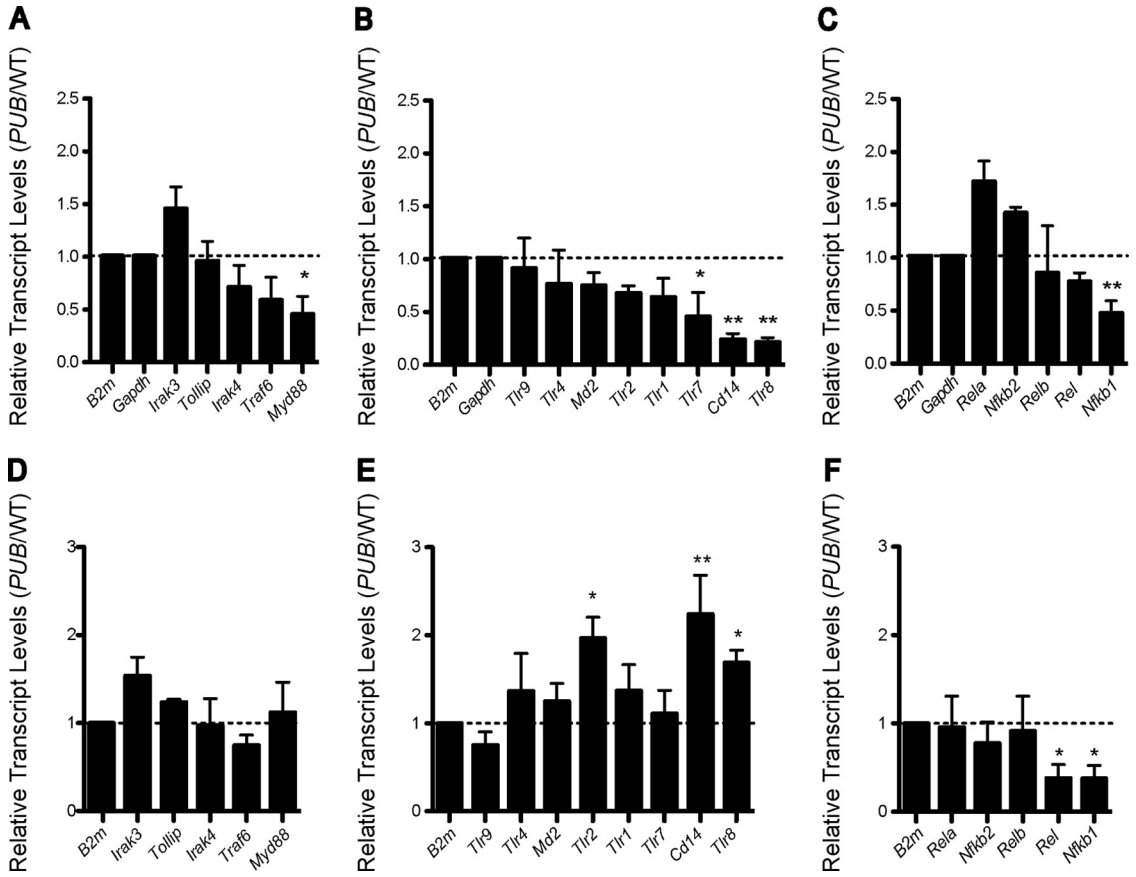


FIG 4 Measurement of steady-state and LPS-stimulated transcript levels of TLR signaling genes. (A to C) Steady-state transcript levels of genes related to downstream TLR signaling (A), TLRs and related receptors (B), and NF-κB subunits (C) were measured in MZ B cells of *PUB* and WT mice. (D to F) Transcript levels in total B cells following 16 h of LPS stimulation of genes related to downstream TLR signaling (D), TLRs and related receptors (E), and NF-κB subunits (F). Analysis was performed on RNA prepared from MZ B cells enriched by cell sorting or total enriched B cells. RT-qPCR was used to determine relative mRNA transcript levels in *PUB* B cells compared to WT B cells after being normalized to *Gapdh* or the β2m gene. The *y* axis indicates the mean fold changes ± SEM relative to WT levels for three individual mice. Values of 1 (dashed lines) indicate there was no difference in transcript levels between *PUB* and WT B cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

spleens contained elevated frequencies of $CD11b^+$ $GR1^+$ myeloid cells, but the absolute numbers of $CD11b^+$ $GR1^+$ splenocytes were slightly reduced compared to those in WT mice (see Fig. S2C and D). Flow cytometric analysis of $CD19^+$ $CD1d^{hi}$ $CD5^+$ B regulatory cells (36) revealed no significant difference in frequencies between *PUB* and WT mouse spleens (see Fig. S2E). Furthermore, spleens from *PUB* mice contained fewer cells than those from WT mice (see Fig. S2F). Elevated B cell activating factor (BAFF) is common in B cell lymphopenia (37) and conceivably could impede TLR-mediated proliferation. Steady-state levels of serum BAFF were found to be elevated in *PUB* mice compared to those in WT mice (see Fig. S3A in the supplemental material). BAFF receptor (BAFF-R) expression was reduced on *PUB* B cells (see Fig. S3B), consistent with what was expected from higher BAFF levels (37). To determine if increased BAFF could impair *PUB* B cell proliferation, splenic B cells were LPS stimulated with and without recombinant BAFF. Additional BAFF increased proliferation in both untreated and LPS-treated groups in WT and *PUB* B cells (see Fig. S3C). Therefore, impaired TLR-mediated *PUB* B cell proliferation was not explained by altered B cell composition or elevated BAFF levels.

***PUB* B cells are activated following stimulation.** We wanted to determine the mechanism of impaired TLR-mediated B cell proliferation in *PUB* mice. To determine whether *PUB* B cells were capable of activation by TLR ligands, mean fluorescence intensities (MFIs) of CD25 or CD69 activation markers were measured on total $B220^+$ cells or B cells with the MZ ($CD21^{hi}$ $B220^+$) or FO ($CD21^{int}$ $B220^+$) phenotype after stimulation (Fig. 2A). All WT and *PUB* B cell subsets upregulated CD25 following 24 h of LPS stimulation compared to untreated cells (Fig. 2B and C). Untreated FO phenotype *PUB* B cells expressed more CD25 than WT cells (Fig. 2C). Next, CD69 expression was measured 24 h after LPS stimulation (Fig. 2D and E). Both WT and *PUB* B cells increased CD69 expression following LPS stimulation for all subsets (Fig. 2E). CD69 was also measured 72 h poststimulation in total $B220^+$ cells. *PUB* B cells failed to upregulate CD69 to WT levels following 72 h of LPS stimulation (Fig. 2F to G). In contrast, *PUB* B cells expressed higher levels of CD69 following stimulation with anti-IgM, and stimulation with LPS plus anti-IgM resulted in equivalent expression between WT and *PUB* B cells (Fig. 2F and G). In summary, *PUB* B cells were activated following LPS stimulation, but CD69 upregulation was reduced compared to that of the WT when measured at 72 h.

Apoptosis in *PUB* splenic B cells is not increased following LPS stimulation. *PUB* B cells were previously reported to exhibit increased steady-state levels of apoptosis (22). Therefore, im-

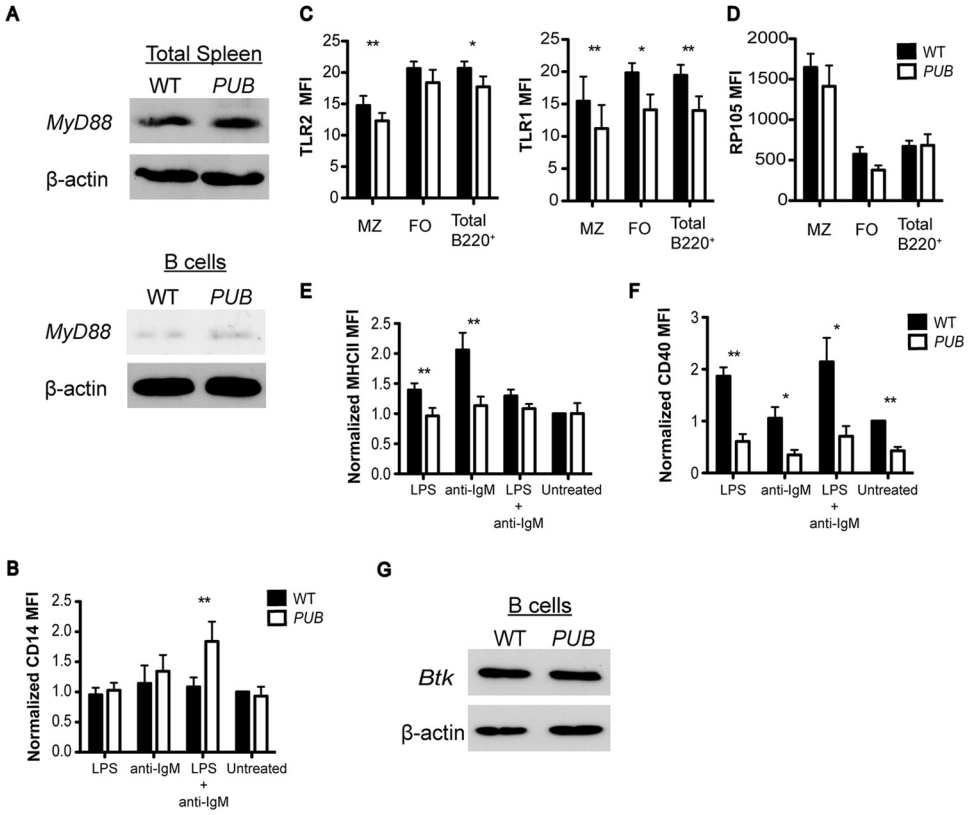
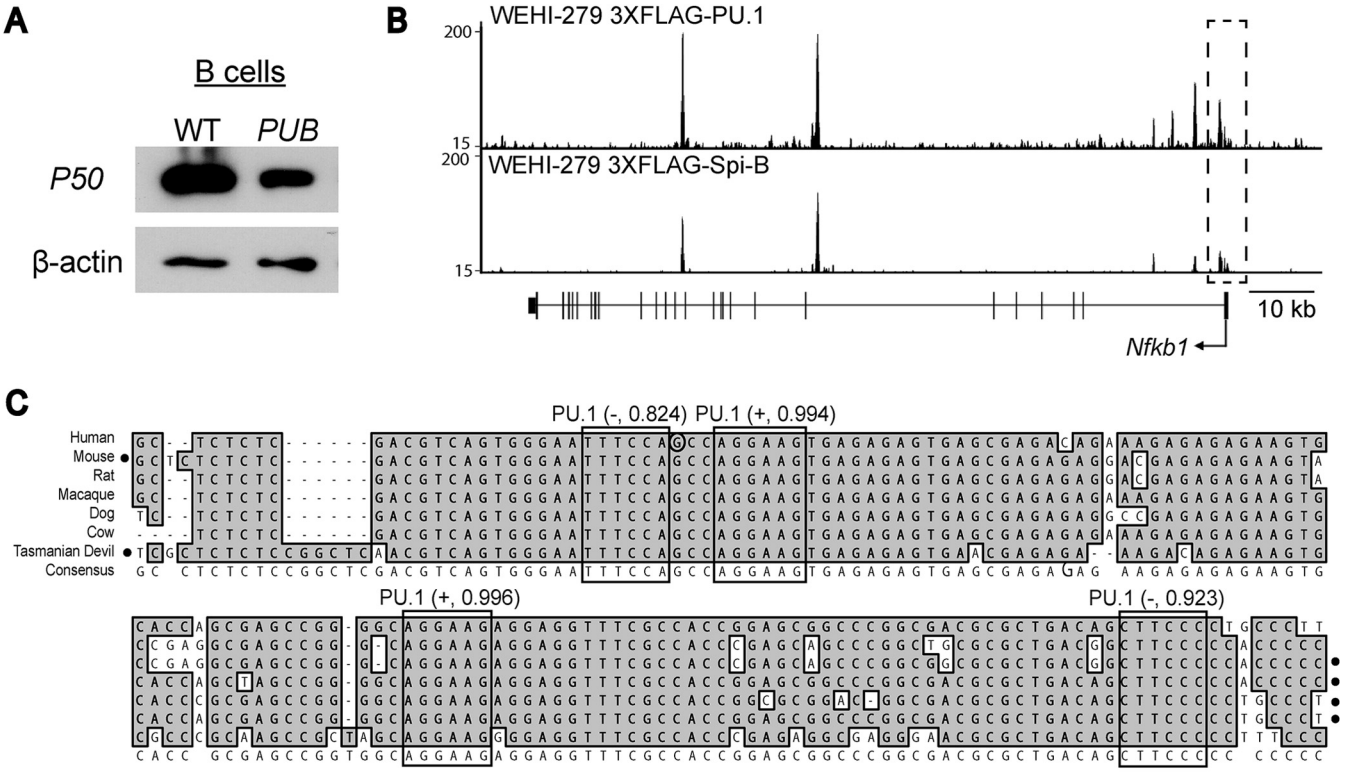
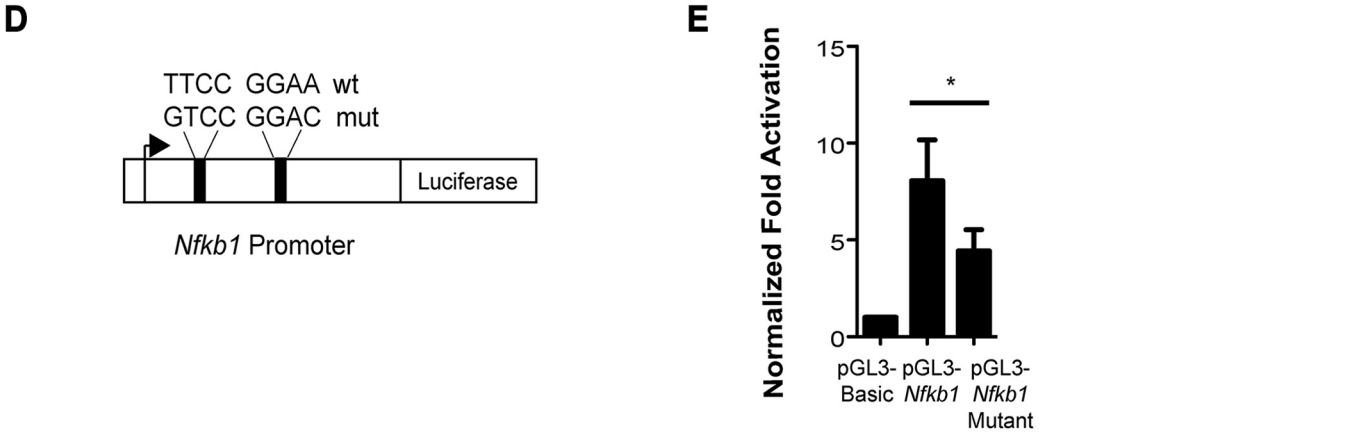


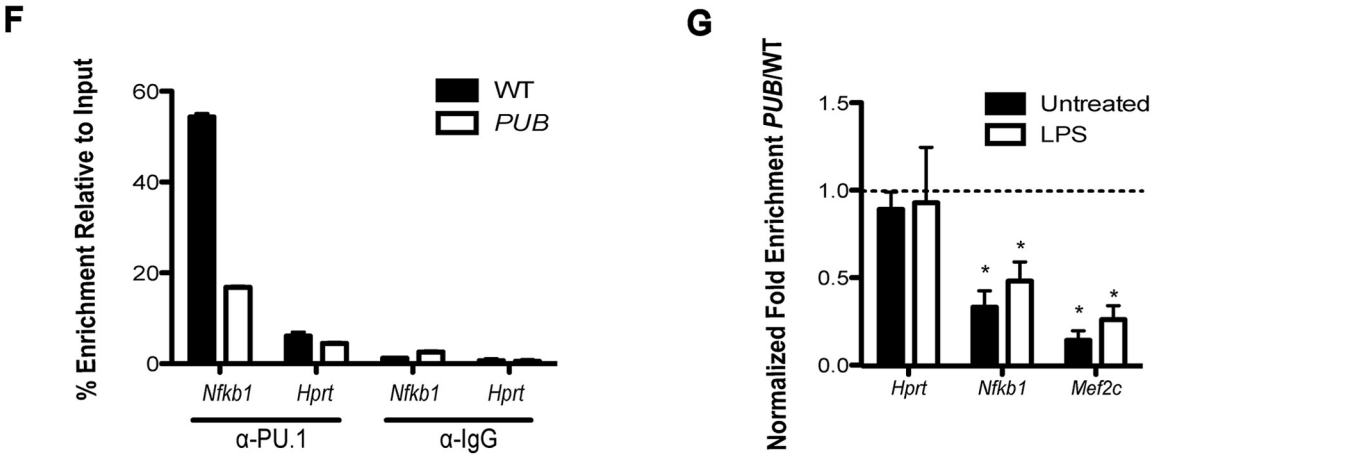
FIG 5 *PUB* B cells have decreased basal surface expression of TLR1 and TLR2 and cannot upregulate MHC-II and CD40 expression following stimulation. (A) Immunoblotting for MyD88 and β-actin was performed using total spleen cells (top panel) or enriched B cell lysates (lower panel) from WT and *PUB* mice. (B) CD14 expression is increased in *PUB* B cells following LPS–anti-IgM stimulation but not increased when stimulated with LPS or anti-IgM alone. (C) *PUB* B cells have decreased TLR1 and TLR2 expression. TLR1 and TLR2 MFI was measured for gated MZ ($CD21^{hi}$ $B220^{+}$), FO ($CD21^{int}$ $B220^{hi}$), and total $B220^{+}$ cells using flow cytometry. Data show means ± SEM ($n = 5$). (D) RP105 surface levels are equivalent between WT and *PUB* B cells. RP105 MFI was measured for MZ, FO, and total $B220^{+}$ cells using flow cytometry. Data show means ± SEM ($n = 3$). *PUB* B cells fail to upregulate MHC-II (E) or CD40 (F) expression following stimulation with LPS, anti-IgM, or LPS plus anti-IgM. (G) Immunoblotting for Btk and β-actin was performed using enriched B cell lysates from WT and *PUB* mice. Immunoblotting experiments are shown as representative of two individual mice. For panels B, E, and F, enriched B cells were stimulated for 72 h before analysis of CD14, MHC-II, and CD40 expression by flow cytometry. Values represent MFIs of total B cells expressing the indicated markers normalized to those of untreated WT B cells. Normalized MFIs were calculated by dividing MFI values by the WT untreated MFI. Data show the means ± SEM from four independent experiments. *, $P < 0.05$; **, $P < 0.01$.

paired TLR-mediated *PUB* B cell proliferation could be due to increased apoptotic cell death. To test this hypothesis, B cells were stained for annexin V and propidium iodide (PI) after 72 h of incubation with or without LPS and/or anti-IgM (Fig. 3A). Under the culture conditions used, a mean of 36% of WT B cells were low/negative for annexin V staining after anti-IgM stimulation, while a mean of 6% of *PUB* B cells were low/negative for annexin V staining after anti-IgM stimulation (Fig. 3A and C). Therefore, few *PUB* B cells survived anti-IgM stimulation, in accord with the proliferation results shown in Fig. 1E. The frequencies of apoptotic (annexin V^{hi} PI^{-}) *PUB* B cells untreated or anti-IgM stimulated were significantly higher than those in WT controls, and the frequencies of live (annexin V^{-} PI^{-}) *PUB* B cells untreated or anti-IgM stimulated were significantly decreased compared to those in the WT controls. However, the frequencies of apoptotic cells between WT and *PUB* B cells treated with LPS or LPS plus anti-IgM were similar (Fig. 3B). No significant difference was observed between live WT and *PUB* B cells stimulated with LPS or LPS plus anti-IgM (Fig. 3C). Therefore, these results suggest that reduced proliferation of *PUB* B cells relative to WT cells in response to LPS was not due to increased apoptosis.

PUB mice have altered transcript and protein levels of genes involved in TLR signaling. MZ B cells are more readily activated and proliferative than FO B cells after LPS stimulation (35, 38). Despite increased frequencies of MZ B cells in *PUB* mice (13), impaired proliferation occurred in splenic *PUB* B cells for all MyD88-dependent TLR ligands compared to that in WT B cells (Fig. 1B and C). Therefore, components of the MyD88-dependent signaling pathway might be regulated by PU.1 and Spi-B. To test this hypothesis, RNA was prepared from sorted MZ B cells from WT and *PUB* mice. Steady-state levels of mRNA transcripts encoding components of the MyD88-dependent signaling were measured by RT-qPCR analysis. Transcript levels of downstream signaling components (Fig. 4A), TLRs and related receptors (Fig. 4B), and NF-κB/Rel subunits (Fig. 4C) were examined. Significant reductions in *Myd88*, *Cd14*, *Tlr8*, *Tlr7*, and *Nfkb1* transcripts were measured in *PUB* MZ B cells (Fig. 4A to C). Next, enriched total B cells were stimulated with LPS for 16 h and gene expression profiles were assessed by RT-qPCR. Transcript levels of downstream TLR signaling genes were not significantly different in *PUB* B cells from those in WT B cells (Fig. 4D). Although steady-state transcript levels of TLR and related receptors were reduced in *PUB* MZ







B cells, transcript levels of *Tlr2*, *Cd14*, and *Tlr8* were higher in total LPS-stimulated *PUB* B cells (Fig. 4E). Finally, LPS-stimulated *PUB* B cells expressed lower transcript levels of *Rel* and *Nfkb1* (Fig. 4F).

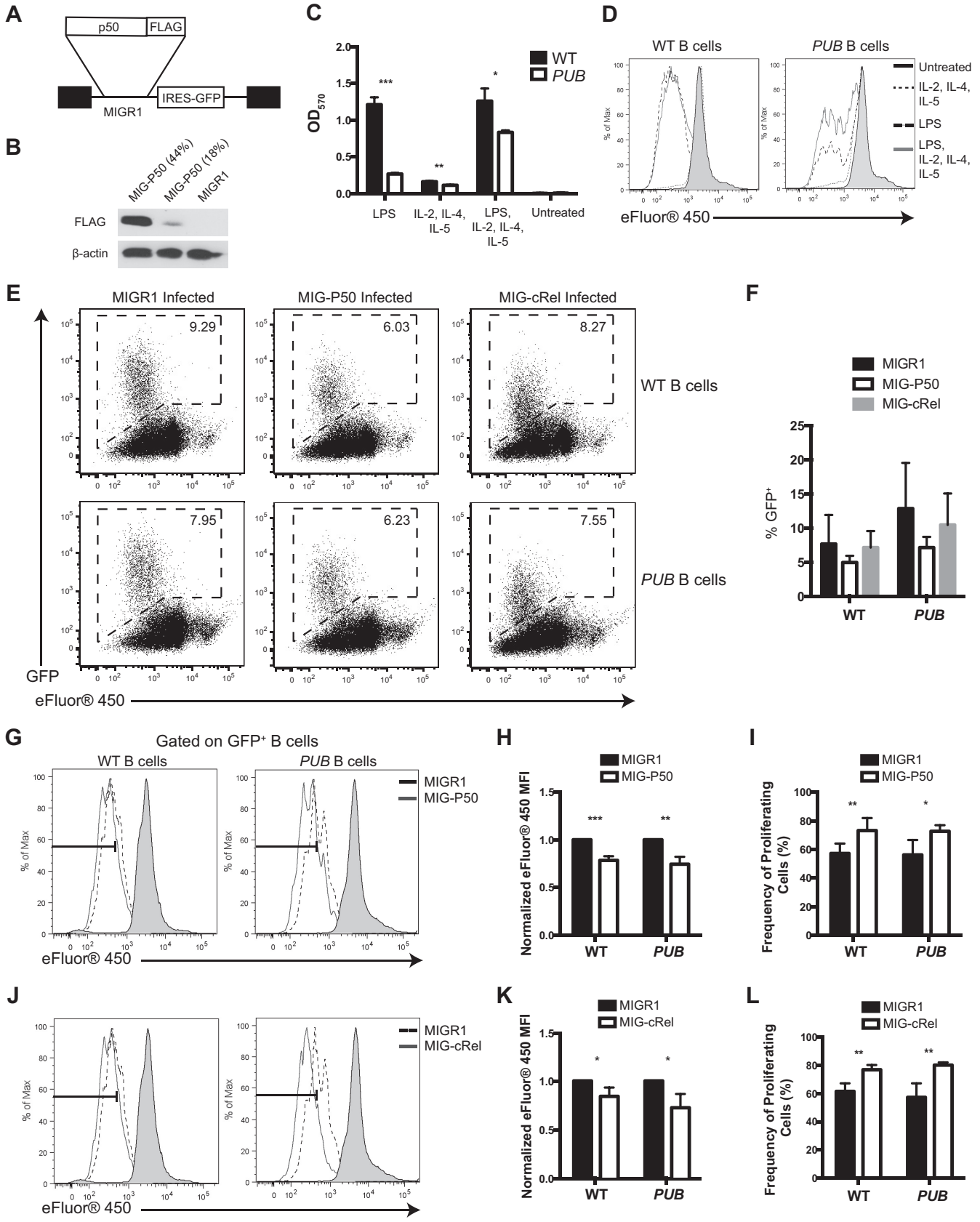
MyD88 is the adapter protein utilized by all TLRs to which B cells responded in our hands (Fig. 1). However, immunoblotting analysis showed MyD88 levels were unchanged between *PUB* and WT enriched B cells (Fig. 5A). Despite the presence of elevated *Cd14* transcripts in LPS-stimulated *PUB* B cells, surface CD14 expression was equal to WT expression 72 h post-LPS or anti-IgM stimulation and was elevated when stimulated with LPS plus anti-IgM (Fig. 5B). Therefore, our results suggest that MyD88 and CD14 did not account for defective TLR responses in *PUB* B cells.

Next, surface expression of TLRs and related receptors was examined by flow cytometry. Reduced TLR2 and TLR1 expression was observed on freshly isolated MZ, FO, and total B cells of *PUB* mice (Fig. 5C). LPS can also activate B cells through binding RP105 (39), but the levels between WT and *PUB* B cells were equal (Fig. 5D). TLR signaling in B cells has been reported to involve MHC-II, CD40, and Btk interaction (12), and PU.1 activates the *Btk* promoter (40). PU.1 also directly regulates the transcriptional coactivator CIITA, which regulates MHC-II genes (41). Since PU.1 transcriptionally regulates genes involved with TLR signaling, TLR-mediated B cell proliferation might be regulated by PU.1 and/or Spi-B through these auxiliary pathways and interactions. Decreased gene transcription in *PUB* mice could result in decreased protein and consequently fewer protein interactions required for TLR signaling. To test this hypothesis, protein levels of MHC-II, CD40, and Btk were measured. *PUB* B cells failed to upregulate surface MHC-II (Fig. 5E) or CD40 (Fig. 5F) following 72 h of LPS or anti-IgM stimulation. Immunoblotting analysis showed equal levels of Btk protein expression between *PUB* and WT B cells (Fig. 5G). Overall, decreased TLR responsiveness in *PUB* B cells was accompanied by reduced TLR1 and TLR2 expression and a failure to upregulate MHC-II or CD40. However, these observations were not sufficient to explain the *PUB* phenotype.

Direct *Nfkb1* activation by PU.1 and Spi-B. Steady-state levels of *Rel* and *Nfkb1* mRNA transcripts were expressed at reduced levels in *PUB* B cells stimulated with LPS compared to those in WT B cells (Fig. 4F). It was previously shown that *Rel* is regulated by PU.1 and/or Spi-B in B cells (42). *Nfkb1* encodes p50 and is required for murine B cell proliferation in response to LPS (43). Immunoblotting analysis confirmed there were lower p50 levels in splenic *PUB* B cells (Fig. 6A). We therefore investigated the role of reduced *Nfkb1* in *PUB* B cell proliferation.

PU.1 and Spi-B can interchangeably bind sites in target genes (18). To determine locations of binding similarity between PU.1 and Spi-B, genome-wide ChIP-sequencing (ChIP-seq) was performed using anti-FLAG Ab on WEHI-279 B cell lymphoma cells overexpressing either 3×FLAG-tagged PU.1 or Spi-B. Both PU.1 and Spi-B were enriched at the *Nfkb1* promoter (Fig. 6B). To identify the specific binding site(s), we aligned the murine *Nfkb1* promoter sequences from multiple species, using a published transcription start site (TSS) for reference (25). At the promoter, a 213-bp region was found to be 87% conserved between multiple species, with the exception of the 67% conservation of the Tasmanian devil sequence (Fig. 6C). Potential PU.1/Spi-B transcription factor binding sites were predicted using MatInspector software. Three conserved binding sites with a matrix similarity score of >0.9 were predicted among all species, and a lower-scoring fourth site was identified near the TSS (Fig. 6C). Next, luciferase assays were used to determine the contributions of PU.1 and Spi-B to *Nfkb1* transcriptional activation. The conserved *Nfkb1* mouse promoter region was cloned and tested by transient transfection in WEHI-279 cells. Site-directed mutagenesis was performed on the predicted PU.1/Spi-B binding sites of the two closest sites of the TSS of the promoter, by replacing the GGAA (TTCC on complement strand) binding site with GGAC (GTCC) (Fig. 6D), which was previously reported to abolish PU.1 binding (13, 20). Luciferase reporter assays showed that the *Nfkb1* promoter was active in WEHI-279 cells, and mutation of PU.1/Spi-B sites significantly reduced transcriptional activation (Fig. 6E). Next, we determined if PU.1 interacts directly with the *Nfkb1* promoter in mouse splenic B cells as was suggested by reanalysis of published ChIP-seq data (44). ChIP analysis in mouse splenic B cells confirmed the PU.1 interaction with the *Nfkb1* promoter (Fig. 6B). To determine if PU.1 interaction with the *Nfkb1* promoter was reduced in *PUB* B cells, chromatin was prepared from freshly isolated and LPS-stimulated B cells and immunoprecipitated with anti-PU.1 or control Abs. WT and *PUB* B cells were LPS stimulated for 16 h, as this time point provided increased p50 expression upon LPS exposure (45). Relative amounts of immunoprecipitated DNA were determined by qPCR from the *Nfkb1* promoter region, the positive-control *Mef2c* gene enhancer (13), and the negative-control *Hprt* gene. ChIP analysis confirmed there was less PU.1 occupancy at the *Nfkb1* promoter of both freshly isolated and LPS-treated *PUB* B cells, whereas no significant difference occurred at *Hprt* (Fig. 6F to G). Together, these data indicate that both PU.1 and Spi-B directly

FIG 6 *Nfkb1* is directly activated by PU.1 and Spi-B. (A) *PUB* B cells express decreased p50 protein levels. Immunoblotting for p50 and β-actin was performed using enriched B cell lysates from WT and *PUB* mice. (B) Chromatin immunoprecipitation-sequencing (ChIP-seq) analysis of the PU.1 and Spi-B interaction with the *Nfkb1* gene. Murine WEHI-279 B cell lymphoma cells expressing 3×FLAG-tagged PU.1 (top) or Spi-B (bottom) were analyzed using anti-FLAG ChIP-seq. Peaks located within and near the *Nfkb1* gene are shown, and the dashed box represents PU.1 and Spi-B binding at the *Nfkb1* promoter. (C) Schematic showing alignment of the annotated *Nfkb1* promoter between multiple species. Gray text represents conserved nucleotides, and boxes indicate predicted PU.1 binding sites with similarity score and strand location (+ or −) listed. The circled nucleotide represents a known TSS in the human *Nfkb1* promoters. Dots represent TSS upstream or downstream of the annotated sequence. (D) Luciferase reporter vector schematic with an arrow representing the murine *Nfkb1* TSS. The *Nfkb1* promoter sequence was cloned by PCR and ligated into pGL3-basic. Predicted ETS binding sites (wt) were mutated (mut) using site-directed mutagenesis. (E) Mutated ETS binding sites reduced promoter activity in WEHI-279 B cells. Cells were transfected with the plasmids indicated on the *x* axis. The *y* axis indicates fold induction of luciferase activity relative to that of pGL3-basic. Luciferase activity was normalized by transfection with the *Renilla* luciferase expression vector. Data show means ± SEM ($n = 5$). (F) The interaction of PU.1 with the *Nfkb1* promoter is reduced in *PUB* B cells. ChIP data show the percentage of enrichment relative to input for PU.1 interaction with *Nfkb1* and *Hprt* promoters and are representative of three independent experiments. (G) Mean fold enrichment of PU.1 binding in *PUB* B cells normalized to WT B cells for the indicated genes. Values of 1 (dashed lines) indicate no difference in PU.1 binding between *PUB* and WT B cells compared to IgG. ChIP was performed on chromatin prepared from freshly isolated or LPS-stimulated (16 h) splenic WT and *PUB* B cells using anti-PU.1 and control IgG Abs. qPCR was used to measure the amount of immunoprecipitated DNA after purification. Primers were designed to recognize promoters of *Nfkb1*, *Hprt*, and the *Mef2c* enhancer. Data show the means ± SEM from three independent experiments. *, $P < 0.05$.



activate *Nfkb1* transcription in mice, suggesting that reduced p50 expression in *PUB* B cells is the result of reduced PU.1 and Spi-B.

Forced expression of p50 or c-Rel in *PUB* B cells increases TLR-mediated proliferation. PU.1 and Spi-B have been previously reported to directly regulate *Rel* transcription (42). The *Rel* gene encodes the NF-κB family member c-Rel, which is required for survival and proliferation of B cells (42, 46). Lower transcript levels of both *Rel* and *Nfkb1* were detected in LPS-stimulated *PUB* B cells (Fig. 4F). Therefore, it is conceivable that forced expression of c-Rel or p50 could increase TLR-mediated proliferation in *PUB* B cells.

To determine if the *PUB* phenotype could be complemented by forced p50 or c-Rel expression, a retroviral vector was constructed encoding FLAG-tagged p50 (MIG-P50) (Fig. 7A). A previously described retroviral vector containing c-Rel in the MIGR1 vector was used to generate MIG–c-Rel virus (27). FLAG-tagged p50 protein was confirmed to be expressed in retroviral packaging cells transfected with MIG-P50 by immunoblotting (Fig. 7B). For efficient retroviral integration, B cells needed to be in a state of division. Since *PUB* B cells proliferated poorly in response to LPS alone (Fig. 3A and C), WT or *PUB* B cells were stimulated with LPS alone or LPS and IL-2 plus IL-4 plus IL-5 (LPS+IL-2+4+5), conditions previously reported to improve survival (47). LPS+IL-2+4+5 had minimal effects on proliferation/survival in unstimulated or LPS-stimulated WT B cells measured using the MTT assay (Fig. 7C). However, the addition of IL-2+4+5 increased the frequency of *PUB* B cells that proliferated/survived compared to LPS alone (Fig. 7C). To differentiate between a proliferation and survival effect of IL-2+4+5 on *PUB* B cells, cells were stained with the proliferation dye eFluor 450 prior to stimulation, and the dilution of eFluor 450 was assessed 72 h later by flow cytometry. WT and *PUB* B cells responded minimally to IL-2+4+5 alone, based on the dilution of eFluor 450 (Fig. 7D). In WT B cells, addition of IL-2+4+5 did not affect LPS-stimulated dilution of eFluor 450 dye. In contrast, the frequency of cells that diluted eFluor 450 dye was dramatically increased in *PUB* B cells stimulated with LPS+IL-2+4+5 compared to LPS alone (Fig. 7D). These results suggested that IL-2+4+5 increased survival in LPS-stimulated *PUB* B cells.

Next, to determine whether increased expression of p50 or c-Rel could increase proliferation in infected WT or *PUB* cells, B cells were stimulated with LPS and IL-2+4+5 for 24 h and then infected with MIGR1 (control), MIG-P50, or MIG–c-Rel retrovirus. GFP expression was used as a marker for p50 or c-Rel infection, and a GFP^+ population was gated for proliferation analysis (Fig. 7E). WT and *PUB* B cells were infected with MIGR1, MIG-P50, and MIG–c-Rel retroviruses with similar frequencies (Fig. 7F). Next, proliferation was assessed for gated GFP^+ p50-infected cells using eFluor 450 staining and flow cytometry 48 h postinfection. All cells that expressed GFP had also diluted eFluor 450, compared to GFP-negative cells, which was expected since retroviral infection requires cell division (Fig. 7E). For both WT and *PUB* GFP^+ B cells infected with MIG-P50, there was an increase in the frequency of cells that had diluted eFluor 450 compared to cells infected with MIGR1, suggesting one or more extra rounds of cell division (Fig. 7G). This result was statistically significant based on five independent experiments and was expressed either as a decrease in the eFluor 450 mean fluorescence intensity (MFI) gated on GFP^+ cells (Fig. 7H) or as the relative frequency of cells diluting eFluor 450 dye past the gate shown in Fig. 7G (Fig. 7I). Finally, proliferation was assessed on MIG–c-Rel-infected B cells (gated GFP^+ cells) by eFluor 450 staining using flow cytometry 48 h postinfection (Fig. 7J). Similar to MIG-P50 infection, MIG–c-Rel infection in WT and *PUB* B cells resulted in a greater degree of proliferation than that in MIGR1-infected cells (Fig. 7K). In addition, a greater frequency of B cells proliferated when infected with MIG–c-Rel compared to MIGR1 (Fig. 7L). Overall, these results demonstrated that infection with retroviral vectors encoding p50 or c-Rel expression was capable of increasing TLR-mediated proliferation in WT and *PUB* B cells.

DISCUSSION

The goal of this study was to understand how TLR-initiated signaling in B cells is regulated. B cells from *PUB* mice proliferated poorly in response to TLR ligands. LPS-stimulated *PUB* B cells were activated and did not have increased apoptosis relative to WT cells. The mechanism for the *PUB* phenotype revealed reduction in steady-state and LPS-stimulated levels of *Nfkb1* transcripts and accordingly lower p50 protein levels. Mutation of two of the three ETS binding sites within the cloned *Nfkb1* promoter reduced its

FIG 7 Forced expression of p50 or c-Rel increases proliferation in *PUB* B cells. (A) Schematic of the retroviral vector encoding FLAG-tagged p50 and GFP. (B) p50 expression in transfected Plat-E cells as demonstrated by immunoblot analysis performed using anti-FLAG and anti-β-actin antibodies. Cell lysates were generated from Plat-E lysates transfected with MIGR1 and MIG-P50 at high (44%) and low (18%) transfection efficiencies. (C) Proliferation of WT and *PUB* B cells in response to LPS and IL-2+4+5. Proliferation was assessed 72 h poststimulation using an MTT proliferation assay. Data show the means ± SD from triplicate measurements and are representative of 2 individual experiments. (D) *PUB* B cells have enhanced survival when stimulated with IL-2+4+5 in combination with LPS compared to LPS or IL-2+4+5 alone. The shaded histogram represents untreated B cells. Proliferation was assessed by the dilution of the proliferation dye eFluor 450 using flow cytometry 72 h poststimulation. The data shown are representative of three independent experiments. (E) MIGR1-, MIG-P50-, and MIG–c-Rel-infected B cells were identified by GFP expression (dashed gate) using flow cytometry. B cells were labeled with proliferation dye eFluor 450 and stimulated with LPS and IL-2+4+5 for 24 h prior to infection with virus. (F) Similar infection frequencies between MIGR1-, MIG-P50-, and MIG–c-Rel-infected WT and *PUB* B cells based on the gated population in panel E. (G) GFP^+ B cells were gated and assessed for dilution of eFluor 450 to compare proliferation between MIG-P50 (solid line)- and MIGR1 (dashed line)-infected B cells. The shaded histogram represents unstimulated control B cells. (H) WT and *PUB* B cells infected with MIG-P50 proliferate and have increased dilution of eFluor 450 compared to MIGR1-infected cells. Results are expressed as the mean fluorescence intensity (MFI) of gated GFP^+ cells. (I) Increased frequency of proliferating cells observed in MIG-P50-infected cells compared to MIGR1-infected cells. Results are expressed as the mean frequency of cells that diluted eFluor 450 past the gate shown in panel G. (J) GFP^+ B cells were gated and assessed for dilution of eFluor 450 to compare proliferation between MIG–c-Rel (solid lines)- and MIGR1 (dashed lines)-infected B cells. The shaded histogram represents unstimulated control B cells. (K) WT and *PUB* B cells infected with MIG–c-Rel proliferate more than MIGR1-infected cells, based on dilution of eFluor 450. (L) Increased frequency of proliferating cells observed in MIG–c-Rel-infected cells compared to that in MIGR1-infected cells. Results are expressed as the mean frequency of cells that diluted eFluor 450 past the gate shown in panel J. For panels H and I and K and L, data are a quantitation of results from five individual experiments as performed in panels G and J, respectively. Data show the means ± SD. Normalized MFIs in panels H and K were calculated by dividing MFI values of MIG-P50/MIG–c-Rel infected cells by MFI values of MIGR1-infected cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

activation, suggesting that PU.1 and Spi-B regulate its transcription. PU.1 and Spi-B interacted with the *Nfkb1* promoter, as shown by ChIP analysis, and PU.1 occupancy at the *Nfkb1* promoter was decreased in *PUB* B cells. Proliferation of *PUB* B cells was increased upon retroviral complementation with p50. In summary, these results suggest that activation of *Nfkb1* by PU.1 and/or Spi-B promotes TLR-mediated splenic B cell proliferation.

PU.1 and Spi-B are thought to be able to bind identical DNA binding sites (18). This idea was supported by our ChIP-seq results showing that PU.1 and Spi-B directly bind to the *Nfkb1* promoter. Multiple TLR signaling genes have been characterized as direct PU.1 targets, including *Tlr4*, *Tlr9*, *Btk*, and *Rel* (42, 48–50). It is likely that altered protein levels of these genes may contribute to the *PUB* B cell phenotype in addition to reduced p50. Notably, transcript levels of *Rel* were reduced in *PUB* B cells stimulated with LPS. The *Rel* gene encodes the NF-κB family member c-Rel, which is important for survival and proliferation in B cells (42, 46). Reduced c-Rel expression impairs B cell proliferation, as $Rel^{+/-}$ and $Rel^{-/-}$ B cells proliferate poorly in response to LPS or anti-IgM (46). Therefore, it is likely that impaired proliferation of *PUB* B cells in response to LPS or anti-IgM is explained by reduced p50 and c-Rel (42). Proliferation in $Rel^{-/-}$ B cells is reported to be more impaired than that in $Nfkb1^{-/-}$ B cells in response to either LPS or anti-IgM (46, 47, 51). In addition, $Rel^{+/-}$ B cells have reduced proliferation in response to LPS or anti-IgM compared to WT B cells, but there is no difference in levels of proliferation between $Nfkb1^{+/-}$ and WT B cells for the same mitogens (43, 52). LPS-stimulated $Nfkb1^{-/-}$ B cells fail to proliferate, anti-IgM stimulation results in only a small reduction in proliferation, and anti-CD40/CD40L stimulation results in normal proliferation (22, 38, 43). Deletion of both NF-κB1 and c-Rel ($Nfkb1^{-/-}$ $Rel^{-/-}$) in B cells results in a further impaired response to LPS or anti-IgM compared to $Nfkb1^{-/-}$ or $Rel^{-/-}$ B cells. Future studies will need to be done to determine whether restoration of both p50 and c-Rel together can further improve proliferation responses in *PUB* B cells.

PUB B cells were unable to increase MHC-II or CD40 expression upon LPS or anti-IgM stimulation. The impairment of MHC-II and CD40 upregulation upon anti-IgM expression could be explained by increased apoptosis in *PUB* B cells. CD40 was functional in *PUB* B cells since anti-CD40 stimulation appeared normal despite decreased levels of CD40 on *PUB* B cells, furthermore, stimulation with LPS plus anti-CD40 was able to rescue defective LPS-mediated proliferation. Failure to upregulate MHC-II and CD40 upon stimulation could result in decreased protein interaction necessary for TLR activation, which could be a consequence of reduced p50 signaling. *PUB* B cells were activated by LPS, as measured by CD25 and CD69, but CD69 was not upregulated as highly as WT B cells. The *Cd69* gene promoter has multiple PU.1 binding sites and is transcriptionally regulated by NF-κB complexes (53, 54). Therefore, reduced MHC-II, CD40, and CD69 upregulation upon LPS stimulation may be a consequence of reduced p50 expression.

Although proliferation in *PUB* B cells was impaired upon TLR or IgM stimulation alone, the combination of LPS plus anti-IgM synergistically restored proliferation and activation to WT levels in *PUB* B cells. The molecular basis of this observation is unclear, but a comparable finding was made using $Nfkb1^{-/-}$, $Rel^{-/-}$, or $Nfkb1^{-/-}$ $Rel^{-/-}$ B cells, where LPS synergized with anti-IgM when inducing proliferation (51). Furthermore, LPS and IL-2+4+5 were used to maximize cell survival to allow B cells into early log-phase growth for viral infection. The combination IL-2+4+5 was previously reported to not induce proliferation on its own but synergized with proliferative responses to mitogens in WT, $Rel^{-/-}$, and $Nfkb1^{-/-}$ B cells (47). Similarly, *PUB* B cells did not proliferate highly in response to the combination IL-2+4+5 alone, but the proliferation response was increased in response to LPS and IL-2+4+5. This synergistic proliferation observed in *PUB* B cells is likely due to multiple or alternate signaling pathways becoming activated that are not regulated by PU.1 and Spi-B. For example, LPS can also activate phosphoinositol 3-kinase (PI3K) (55) or mitogen-activated protein kinase (MAPK) pathways (56). Therefore, there are likely alternative pathways that can compensate in the event of the loss of PU.1 and Spi-B function. Further analysis of the involvement of PU.1 and Spi-B in the activation of these pathways through TLR ligands is required.

PUB mice have reduced frequencies of B cells with an increased ratio of MZ to FO B cells, indicating PU.1 and Spi-B may regulate peripheral B cell differentiation (13). Differentiation of both FO and MZ B cells requires canonical NF-κB signaling. Weak BCR signaling promotes MZ B cell development, whereas strong BCR signaling drives FO B cell development (57). $Nfkb1^{-/-}$ mice have MZ B cells, although in decreased numbers (58), and MZ B cells accumulate over time in spleens of these mice (59). Therefore, increased MZ B cell and decreased FO B cell frequencies in *PUB* mouse spleens might be caused by a combination of altered NF-κB signaling, weakened BCR signaling, and increased apoptosis upon BCR stimulation.

In summary, our experiments establish the importance of PU.1 and Spi-B for innate immune responses in B cells. Decreased p50 and *Nfkb1* gene activation in *PUB* mice is associated with poor TLR-mediated B cell proliferation. Understanding TLR regulation in B cells has implications for generation of antibody responses, since effective thymus-independent responses and production of antibodies in vaccinations are dependent on direct TLR triggering on B cells (5). Further analysis of mice lacking Spi-B and with reduced PU.1 is expected to provide additional insight into other genes involved in TLR signaling and antibody-forming responses.

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