Supporting Information

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SI Materials and Methods

Generation of the Conditional Foxp1 Functional KO Mouse Strain. Homology arms and the target region containing *Foxp1* exons 10–12 were generated by PCR by using a BAC template (RP23-209N16). The resulting targeting vector contained a neomycin selection cassette flanked by *frt* sites and exon 10–12 target region flanked by *lox*p sites. A BsrGI restriction site was added at the 5′ end of the target region for Southern blot validation. Before electroporation into E14K embryonic stem cells, the targeting vector was linearized via NotI restriction. Clones resistant to selection with G418 were screened for correct integration before injection of a correctly targeted clone into blastocysts derived from C57BL/6 mice to generate chimeric mice and subsequently mice with the recombinant allele. Genotyping of *Foxp1* loci was done by PCR by using a common forward primer 5′-CTG CAC AGC AGG GTA GTT AGT G-3′ and reverse primers 5′-CTG CTC TAC TGC GTT CTT CCT C-3′ (WT), 5′-GCT CTA CTG CGT TCT TTG TAC ATT T-3′ (flox), or 5′-ATG CTA GGC GGT ACT AAA TAG AAC-3′ (del).

To generate the floxed (flox) allele, recombinant mice were crossed to *flp* deleter mice [B6;SJL-Tg(ACTFLPe)9205Dym/J]. Subsequently, mice were crossed to the Cre-deleter mice [B6.C-Tg(CMV-cre)1Cgn/J] or, for conditional deletion of *Foxp1* in B cells, to CD19-Cre mice [B6.129P2(C)-Cd19tm1(cre)Cgn/J] (20). Experimental mice were on a 129/Sv-C57BL/6 mixed background and were used for experiments at the age of 6–12 wk.

In vivo immunization. Mice were immunized i.p. with 25 μg of the T-independent type II antigen TNP-Ficoll (Biosearch Technologies) or 200 μg NP-OVA, absorbed in 800 μg alum (Sigma) for T-dependent immunizations. Blood was drawn from tail veins at various time points and used to quantify antigen- and isotype-specific antibodies by ELISA as described previously (53).

Measurement of serum immunoglobulins. Ig isotypes were analyzed by ELISA performed on serially diluted serum samples by using anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM antibodies (Southern Biotechnology Associates) as described previously (53).

Immunohistochemistry. Slides were deparaffinized in xylene and rehydrated by alcohol washes of decreasing concentration (100%, 96%, 70%). After heat-induced antigen retrieval (10 mM citrate buffer, pH 6), unspecific protein and peroxidase binding was blocked with 3% hydrogen peroxide and 3% normal goat serum (Abcam). Immunohistochemistry was performed with a Dako Autostainer (DAKO) by using antibodies against activated caspase 3 (1:200; no. 9661; Cell Signaling) and CD45R/B220 (1:50; no. 550286; BD Bioscience). For antibody detection, the Dako Envision-HRP rabbit-labeled polymer (DAKO) was used for activated caspase 3 and a biotinylated secondary rabbit anti-rat antibody followed by incubation with streptavidin peroxidase was used for B220/CD45R. Antibody binding was visualized by diaminobenzidine (BS04-500; Immunologic). Counterstaining was done by using hematoxylin, and slides were dehydrated by alcohol washes of increasing concentration (70%, 96%, 100%) and xylene and coverslipped by using Pertex mounting medium (Histolab).

Slides were scanned with a Leica AT2 scanning system, and immunohistochemistry was evaluated with Leica Imagescope by a German board-certified pathologist counting activated caspase 3-positive cells. Numbers were normalized to area.

Nonidet P-40 Buffer. Nonidet P-40 lysis buffer contained 50 mM Tris, pH 7.5, 100 mM NaCl, 5% (vol/vol) glycerol, 0.2% (vol/vol) Nonidet-P40, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM Na_3VO_4, and protease inhibitors.

Western Blot Antibodies. Foxp1 (no. 2005; Cell Signaling Technology) or JC-12 was used at 1:1,000 in 5% BSA Tris-buffered saline solution with Tween-20 (TBS-T). β-Actin (Sigma) was used at 1:1,000 in 5% BSA TBS-T. Bcl-xl (no. 2762; Cell Signaling Technology) was used at 1:1,000 in 5% BSA TBS-T.

Flow Cytometry. Single-cell suspensions of spleen or BM were treated with lysis buffer to remove red blood cells. Single-cell suspensions of spleen, BM, or peritoneal cavity or cultured cells were stained in FACS buffer (PBS solution, 2% FCS). After blocking Fc receptors using anti-CD16/32 antibodies, cells were stained with the appropriate combination of the following antibodies (indicating antigen and clone): B220 (RA3-6B2), CD43 (S7 or eBioR2/60), CD3 (17A2), CD24 (M1/69), BP1 (6C3), CD5 (53-7.3), B and T cell activation antigen (GL7), IgG1 (A85.1), CD138 (281.2), CD86 (GL1), CD62L (MEL-14), MHCII (114.15.2), CD95 (Jo2), CD19 (1D3), IgM (II/41), IgD (11-26), CD21 (7G6), and CD23 (B3B4). Cells were acquired on a BD FACSCanto system and analyzed with FlowJo (TreeStar).

Oligonucleotide Pull-Down Assay. Oligonucleotide pull-down assays were performed with biotinylated dsDNA probes according to sequences in the Bcl-xl promoter. Forward and reverse oligonucleotides were annealed by heating to 85 °C for 20 min and cooling down to room temperature. dsDNA oligonucleotides (2 nmol) were added to washed Strep-Tactin Superflow resins (IBA) followed by rotor incubation at 4 °C for 2 h. Cells were lysed in Nonidet P-40 lysis buffer. Oligonucleotide sequences for pull-down assay were as follows. Foxp1 bs-1, 5′-biotin-GCA CCC CAC GCA CAA AGA CGG GGT GA-3′ and 5′-TCA CCC CGT CTT TGT GCG TGG GGT GC-3′; Foxp1 bs-2, 5′-biotin-GGA GAC CCC CCA CAA ACA CCG CTA GTT G-3′ and 5′-CAA CTA GCG GTG TTT GTG GGG GGT CTC C-3′; and Foxp1 bs-3, 5′-biotin-TTC TTT CTT TCT ATT TAA GTA CCA GCC C-3′ and 5′-GGG CTG GTA CTT AAA TAG AAA GAA AGA A-3′. Equal amounts of cell lysates were added to resin and rotated at 4 °C overnight. Eluate was analyzed via Western blotting. Input samples were diluted 1:10 in lysis buffer.

Pro-B Cell Culture and Transduction. Pro-B cells were cultured in IMDM (Invitrogen) with GlutaMAX containing 20% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 50 μM β-mercaptoethanol in the presence of 10 ng/mL IL-7. MSCV-based retroviral transductions with Cre-ER^{T2}-Puro and ER^{T2}-Puro (EV) were performed as described previously (51). For nuclear translocation of estrogen receptor fusion proteins, 4-hydroxytamoxifen (4-OHT) was added at a concentration of 500 nM. Twenty-four and 48 h after tamoxifen-induced Cre activation, protein samples were harvested and analyzed for Bcl-xl expression by immunoblot performed as described previously (51).

EdU/BrdU Labeling and Treatment with Anti–IL-7R Antibody. For in vitro proliferation, splenic B cells were stimulated by using IgM or control for 16 h. Then, EdU was added at 10 μM for 1 h and the staining was performed as advised by the manufacturer (Thermo Fisher Scientific). For in vivo proliferation detection, mice were fed 0.8 mg/mL BrdU (Sigma-Aldrich) in drinking water for 14 d. B220 expression and BrdU incorporation was assessed via the APC BrdU Flow Kit (BD Biosciences). Block of

B lymphopoiesis in BM was achieved by injecting mice i.p. with 0.25 mg anti–IL-7R monoclonal antibody (A7R34) every second day (27).

BCR Repertoire Analysis. RNA of MACS-purified splenic B cells from littermate controls ($n = 5$) and *Foxp1*$^{flox/del;CD19}$ ($n = 5$) mice was isolated by using a Qiagen RNeasy Kit. Library preparation was performed by using the SMARTer Mouse BCR H/K/L Profiling Kit from Clontech according to instructions and sequenced on an Illumina MiSeq sequencer. Reads were aligned with MiXCR and analyzed in R3.3 with the tcR package (53, 54).

In Vitro Differentiation of B-1 Cells. Total peritoneal cavity cells (1×10^5 cells per well) were cultured in a flat-bottom 96-well plate in complete B cell medium supplemented with combinations of 10 μg/mL of LPS (Sigma), 0.5 μg/mL CD40L (R&D Systems), and recombinant IL-4 (rIL-4) (1 ng/mL; Peprotech) and analyzed for B220, CD138, and IgM expression at day 3 of culture by flow cytometry.

B Cell Differentiation Assay. Purified B cells at a concentration of 2×10^6 cells per milliliter were stimulated in complete B cell medium supplemented with combinations of 10 μg/mL of LPS (Sigma), 0.5 μg/mL CD40L (R&D Systems), and rIL-4 (1 ng/mL; Peprotech). Survival of cells was analyzed at day 3 of culture by flow cytometry. Supernatant of cell cultures was analyzed for antibody secretion after 4 d of culture. For in vitro GC assays, purified B cells (2×10^4 cells per well) were cultured in a flat-bottom 24-well plate in the presence of feeder cells (10^5 per well) expressing CD40L and BAFF (40LB feeder cells) that had been irradiated with 120 Gy as previously described (26). rIL-4 (1 ng/mL; Peprotech) and anti-IgM $F(ab)_2$ (10 μg/mL; Jackson ImmunoResearch) was added to the primary culture for 4 d.

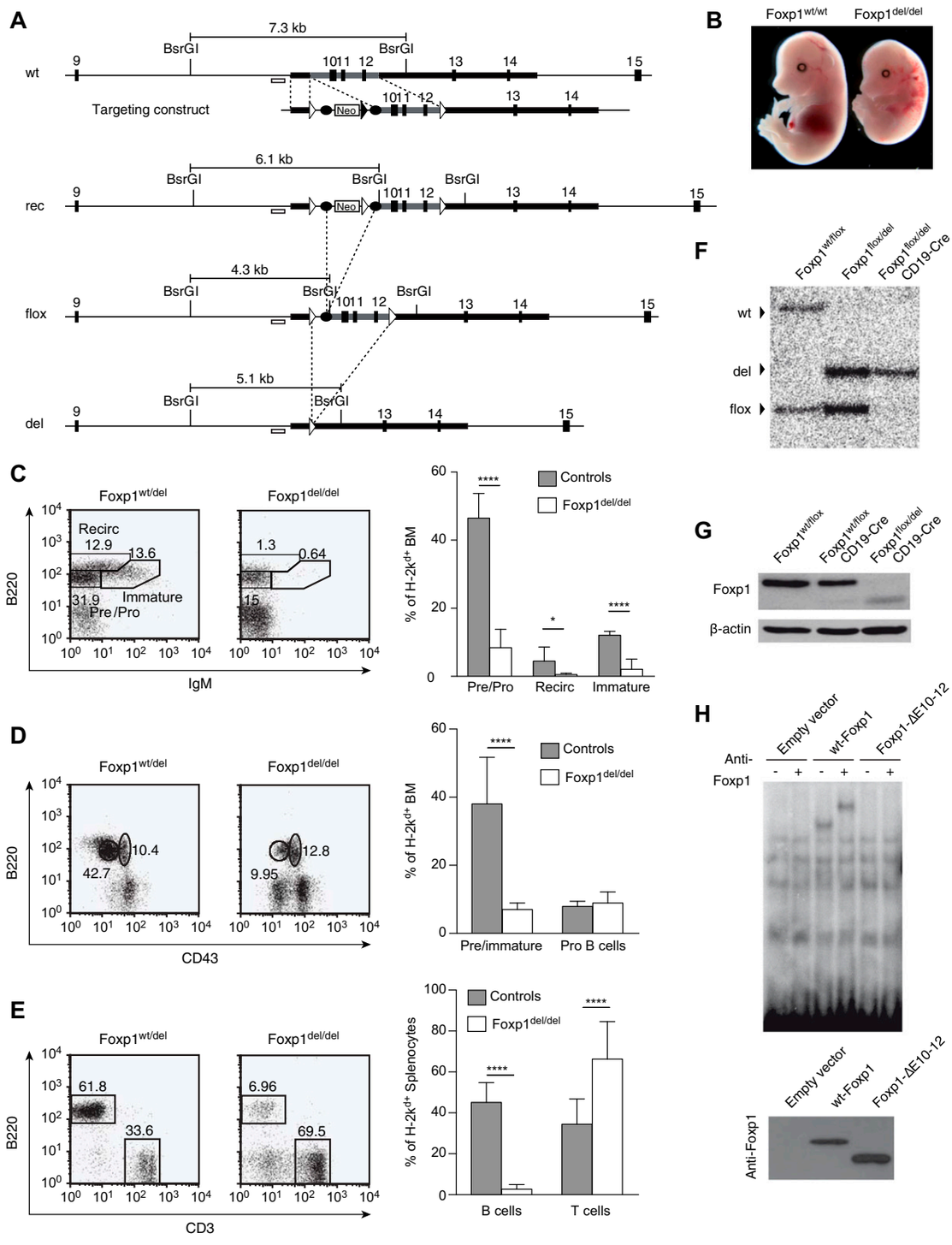

Fig. S1. (*A*) Targeting strategy. Shown is the relevant genomic *Foxp1* region with exons represented by numbered black boxes. Homology arms are shown as bold black lines, the targeted region that is flanked by *lox*P sites (open triangles) as a bold gray line, and the neomycin selection cassette (Neo) as an open box flanked by *frt* sites (closed circles). Homologous recombination of the targeting construct with the WT locus in ES cells first resulted in the recombinant locus (rec). Removal of the neomycin cassette was achieved by crossing the recombinant allele to *flp* deleter mice, leading to the floxed locus (flox). Crossing of the flox allele to a Cre deleter mouse strain led to deletion of the targeted region (del). Restriction sites for BsrGI are depicted, as are the fragment sizes that are expected in Southern blot analysis using the probe as indicated (open box). (*B*) Representative images of *Foxp1*$^{wt/wt}$ and *Foxp1*$^{del/del}$ embryos at developmental stage E15.5. (*C* and *D*) Flow cytometry of BM from fetal liver chimera. *Rag2*-deficient mice (H-2K^{d}) were reconstituted with *Foxp1*$^{wt/del}$ (control) or *Foxp1*$^{del/del}$ fetal liver cells (H-2K^{b}) and analyzed 10 wk after transfer. BM was analyzed for immature (B220^{lo}IgM^{+}) B cells (*C*) and percentages of pro-B cells (B220^{lo}CD43^{+}) and a mixed population of pre-B and immature B cells (B220^{lo}CD43^{-}) (*D*). Cells were pregated for H-2 K^{b+} donor cells. (*Right*) Summary of control ($n = 5$) vs. Foxp1$^{del/del}$ ($n = 7$) chimera. (*E*) Flow cytometry of splenocytes from fetal liver chimera stained for B cells (B220^{+}) and T cells (CD3^{+}). Numbers in dot plots indicate the percentages of viable lymphocytes. (*Right*) Summary of control ($n = 9$) vs. Foxp1$^{del/del}$ ($n = 10$) chimera. (*F*) Southern blotting of BsrGI-digested genomic DNA from purified B cells from *Foxp1*$^{wt/flox}$, *Foxp1*$^{flox/del}$, and *Foxp1*$^{flox/del;CD19}$ mice. In presence of the CD19 Cre allele the *Foxp1* flox allele (4.3 kb) is converted to the del allele (5.1 kb). (*G*) Western blotting of Foxp1 expression in purified B cells from *Foxp1*$^{wt/flox}$, *Foxp1*$^{wt/flox;CD19}$, and *Foxp1*$^{flox/del;CD19}$ mice. β-Actin was used as a loading control. The JC-12 antibody, provided by Alison Banham, Nuffield Division of Clinical Laboratory Sciences, Radcliffe Department of Medicine, University of Oxford, John Radcliffe Hospital, Oxford, was used for detection of Foxp1. (*H*) For electromobility shift assays, in vitro-translated WT Foxp1 or Foxp1-ΔE10-12 proteins were incubated with Foxp1-binding probes in the absence or presence of Foxp1 (JC12) supershifting antibody. Western blot confirming the expression of Foxp1 after overexpression of Foxp1 WT, Foxp1-ΔE10-12, or an empty vector control is shown below. *$P < 0.05$ and ****$P < 0.0001$.

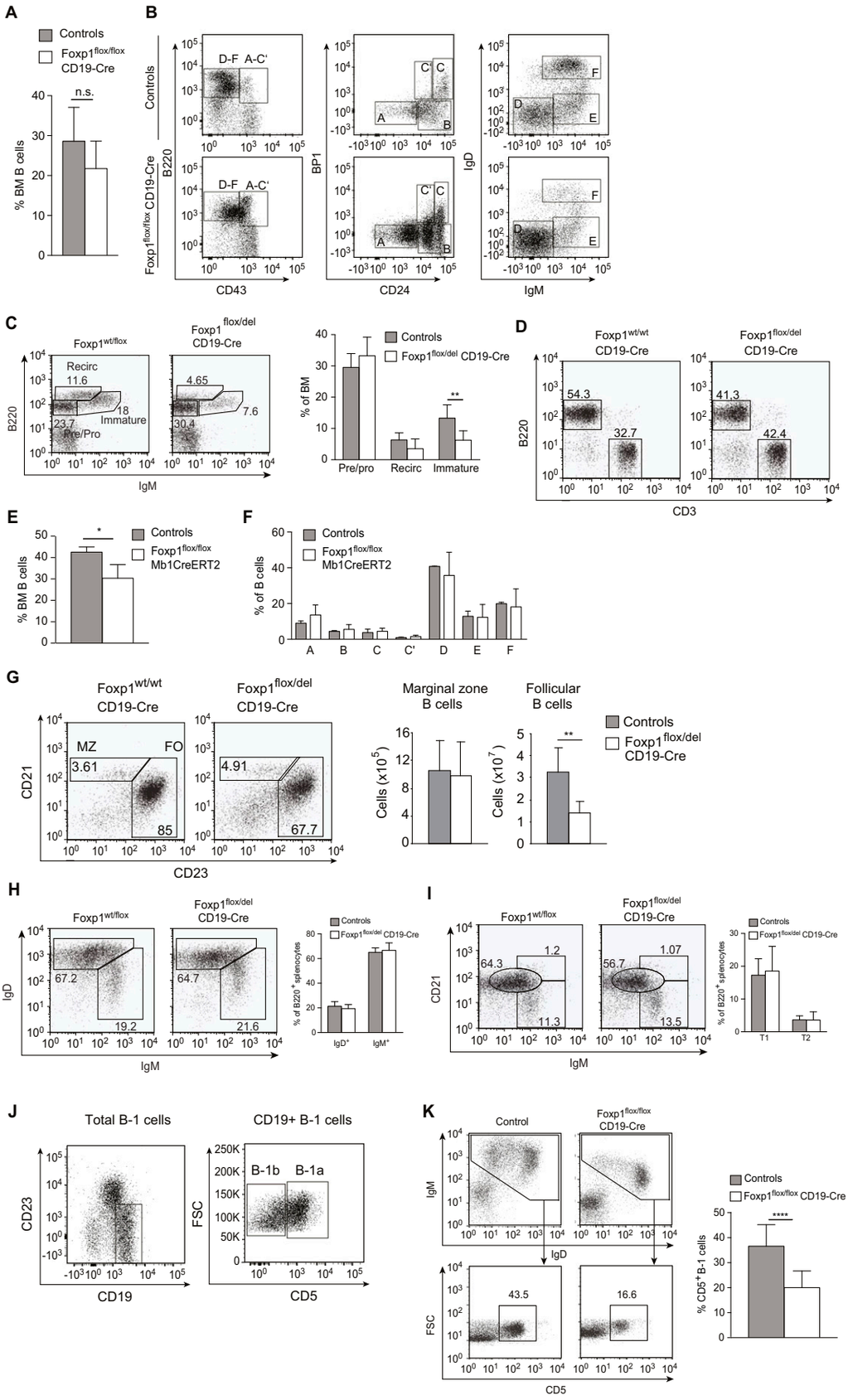

Fig. S2. Flow cytometry of B cell development in the BM of *Foxp1*$^{wt/flox}$ (control) and *Foxp1*$^{flox/del;CD19}$ mice. (*A*) Percentages of B220-expressing cells. (*B*) B cells (B220^{+}) were subdivided into early (CD43^{+}) and late (CD43^{-}) progenitors. Early progenitors were further subdivided on the basis of CD24 and BP-1 expression levels into Hardy populations A–C. Late progenitors were separated according to IgM and IgD receptor expression into Hardy populations D–F. (*C*) BM cells were distinguished into pro-B/pre-B cells (B220^{lo}IgM^{-}), immature B cells (B220^{lo}IgM^{+}), and recirculating B cells (B220^{hi}IgM^{+}). Bar chart summarizes results for $n = 8$. (*D*) Representative blots of splenic *Foxp1*$^{wt/wt;CD19}$ or *Foxp1*$^{flox/del;CD19}$ cells after staining for B220 and CD3. (*E*) *Foxp1*$^{flox/flox;Mb1CreERT2}$ and control mice were treated for five consecutive days with tamoxifen. On day 7, BM was analyzed for the frequency of B cells ($n = 3$, respectively). (*F*) BM B cell fractions according to Hardy et al. (23) were analyzed in *Foxp1*$^{flox/flox;Mb1CreERT2}$ and control mice after acute Foxp1 deletion ($n = 3$, respectively). (*G*) Splenocytes from *Foxp1*$^{wt/wt;CD19}$ mice and *Foxp1*$^{flox/del;CD19}$ mice were gated for B220^{+} and analyzed for follicular mature (CD21^{lo}CD23^{hi}) and MZ B cells (CD21^{hi}CD23^{lo}). Bar graphs show quantification of total cell numbers ($n \geq 9$ mice per group). (*H*) Splenocytes from *Foxp1*$^{wt/flox;CD19}$ and *Foxp1*$^{flox/del;CD19}$ mice were gated for B220^{+} and analyzed for immature (IgM^{+}IgD^{-}) and mature B cells (IgM^{-}IgD^{+}). Bar graph represents a summary of nine biological replicates. (*I*) Flow cytometry of splenic B cells from *Foxp1*$^{wt/flox;CD19}$ mice and *Foxp1*$^{flox/del;CD19}$ mice pregated for B220^{+} and analyzed for transitional type 1 (T1; B220^{+}CD21^{lo}CD23^{lo}IgM^{hi}) and type 2 B cells (T2; B220^{+}CD21^{hi}CD23^{hi}IgM^{hi}). (*Right*) Summary of seven mice per group is shown in the bar graph. (*J*) Analysis of cells from the peritoneal cavity of *Foxp1*$^{wt/wt;CD19}$ mice and *Foxp1*$^{flox/del;CD19}$ mice. Cells were gated on IgM^{+}IgD^{+} (*Left*) and subsequently analyzed for the B-1 B cell marker CD5 (*Right*). (*K*) Exemplary gating strategy for flow cytometric analysis of peritoneal B-1 cells from *Foxp1*$^{wt/flox;CD19}$ mice and *Foxp1*$^{flox/del;CD19}$ mice is shown: cells were pre-gated for IgM and/or IgD expression and analyzed for CD5 (*Left*). The analysis of ≥15 mice per group is quantified in the bar graph (*Right*). $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$. n.s., not significant.

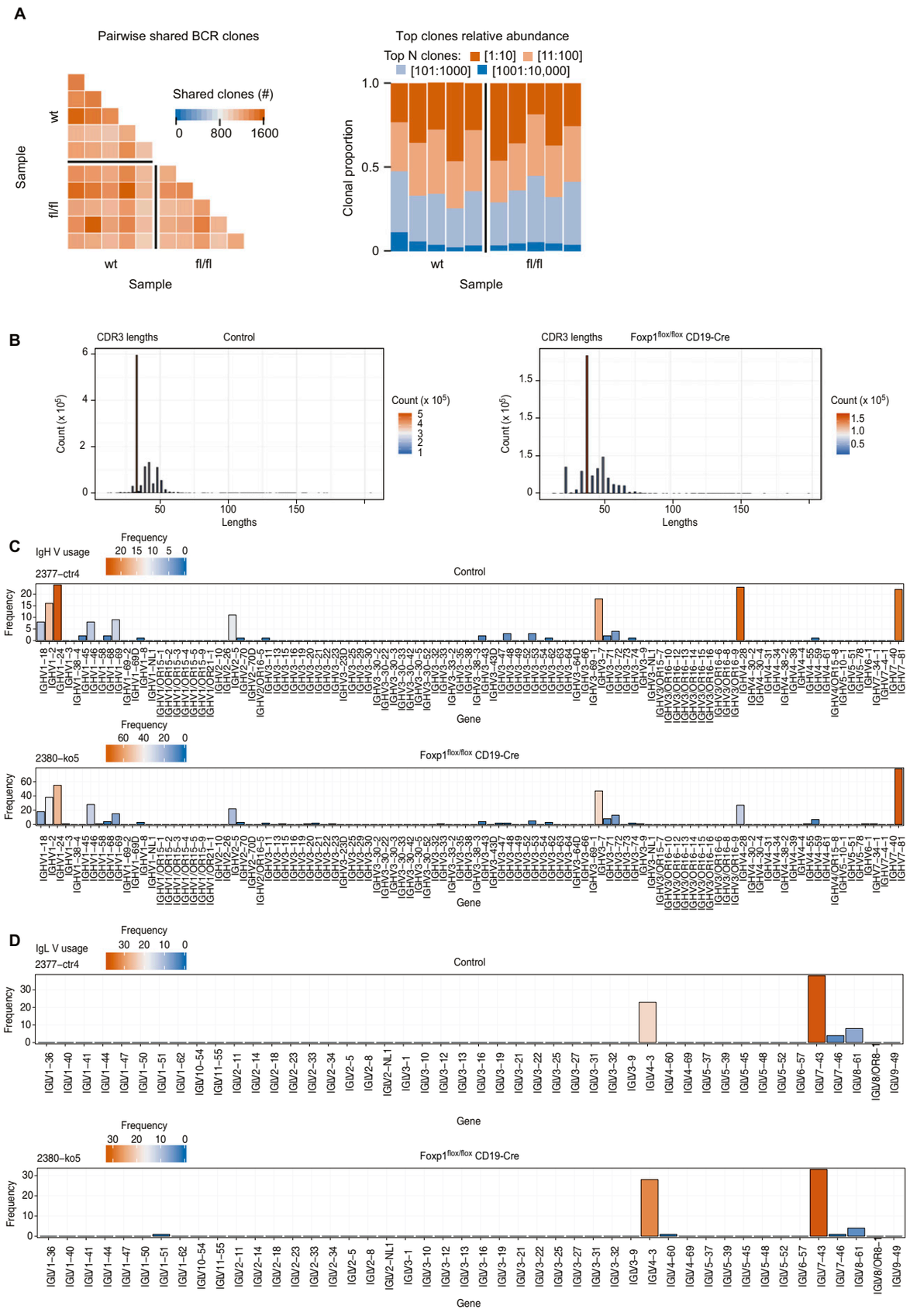

Fig. S3. (*A*) Number of pairwise shared clones with identical CD3R amino acid sequence within BCR repertoires of *Foxp1*$^{flox/flox;CD19}$ and control animals ($n = 5$, respectively; *Left*). Relative abundance of the top N clones within BCR repertoires of *Foxp1*$^{flox/flox;CD19}$ and control animals ($n = 5$ for each genotype; *Right*). (*B*) Representative graphs of the CDR3 length distributions from control (*Left*) and *Foxp1*$^{flox/flox;CD19Cre}$ mice (*Right*) as determined by BCR sequencing. (*C* and *D*) Representative histograms showing the frequency of V gene usage for IgH (*C*) and IgL (*D*) rearrangements of B cells from a control and a *Foxp1*$^{flox/flox;CD19Cre}$ mouse.

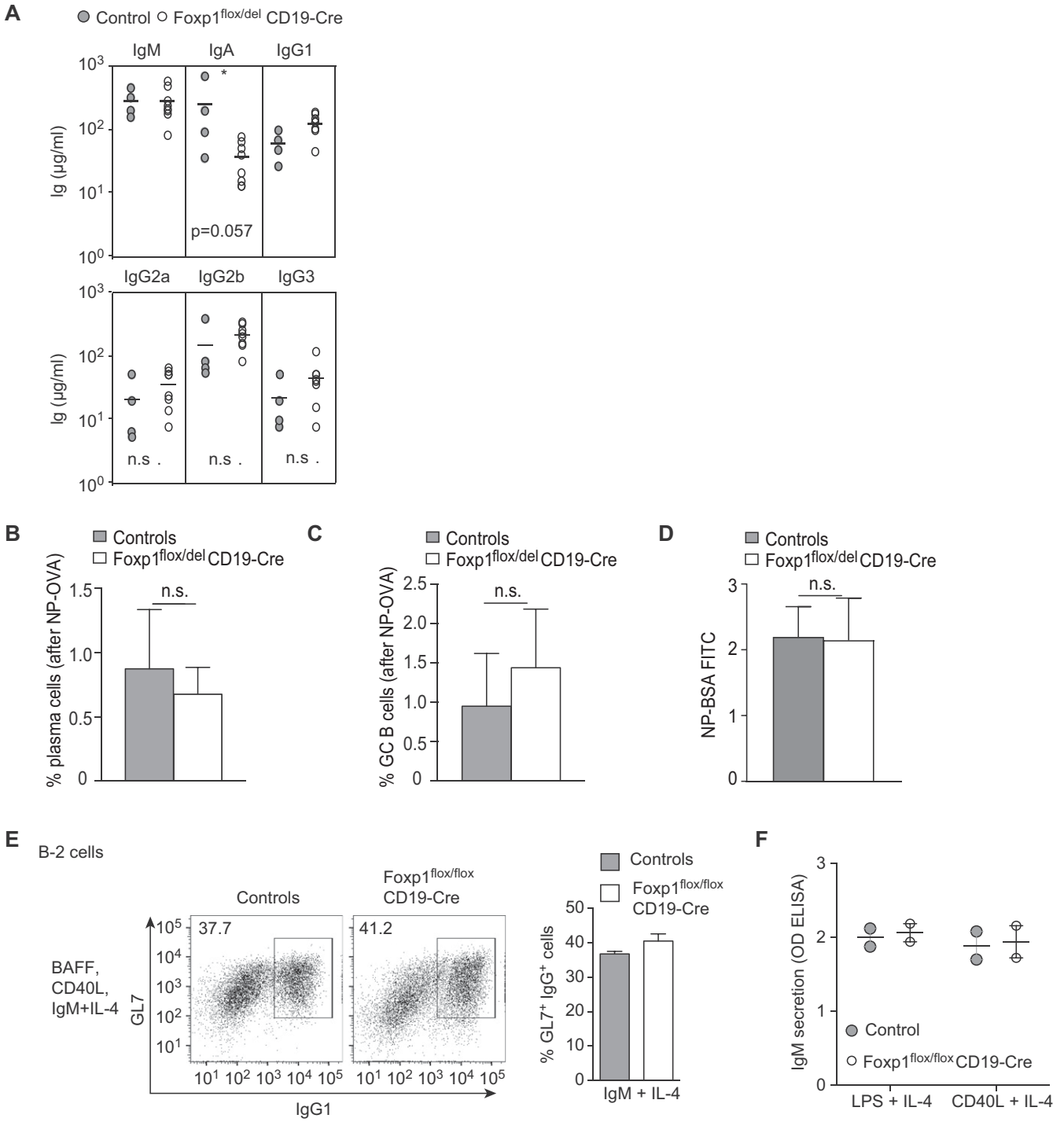

Fig. S4. (*A*) Concentrations of basal Ig isotypes in the sera of nonimmunized control *Foxp1*$^{wt/flox}$ mice and *Foxp1*$^{flox/del;CD19}$ mice measured by ELISA. Means are indicated as black lines. (*B–D*) Splenic B cells were analyzed for GC differentiation via expression of PNA and Fas/CD95 (*B*) for plasma cell formation via expression of CD38 and CD138^{hi} (*C*) or for NP-BSA binding (*D*) after immunization with NP-OVA of control ($n = 10$) and *Foxp1*$^{flox/del;CD19}$ mice ($n = 4$). (*E*) Flow cytometric analysis after in vitro stimulation of isolated splenic B cells of control and *Foxp1*$^{flox/flox;CD19}$ mice for formation of GC-like cells and class switch recombination. Representative FACS blot for GL-7 and IgG1 expression is shown. (*Right*) Graph indicates a summary of two independent experiments with four biological replicates for the percentage of GL-7– and IgG1-expressing cells. (*F*) Supernatants of isolated splenic B cells cultured with LPS or CD40L and IL-4 were analyzed for IgM secretion by ELISA. Results of two independent experiments are shown of pooled supernatants from two biological replicates, respectively. *$P < 0.05$. n.s., not significant.

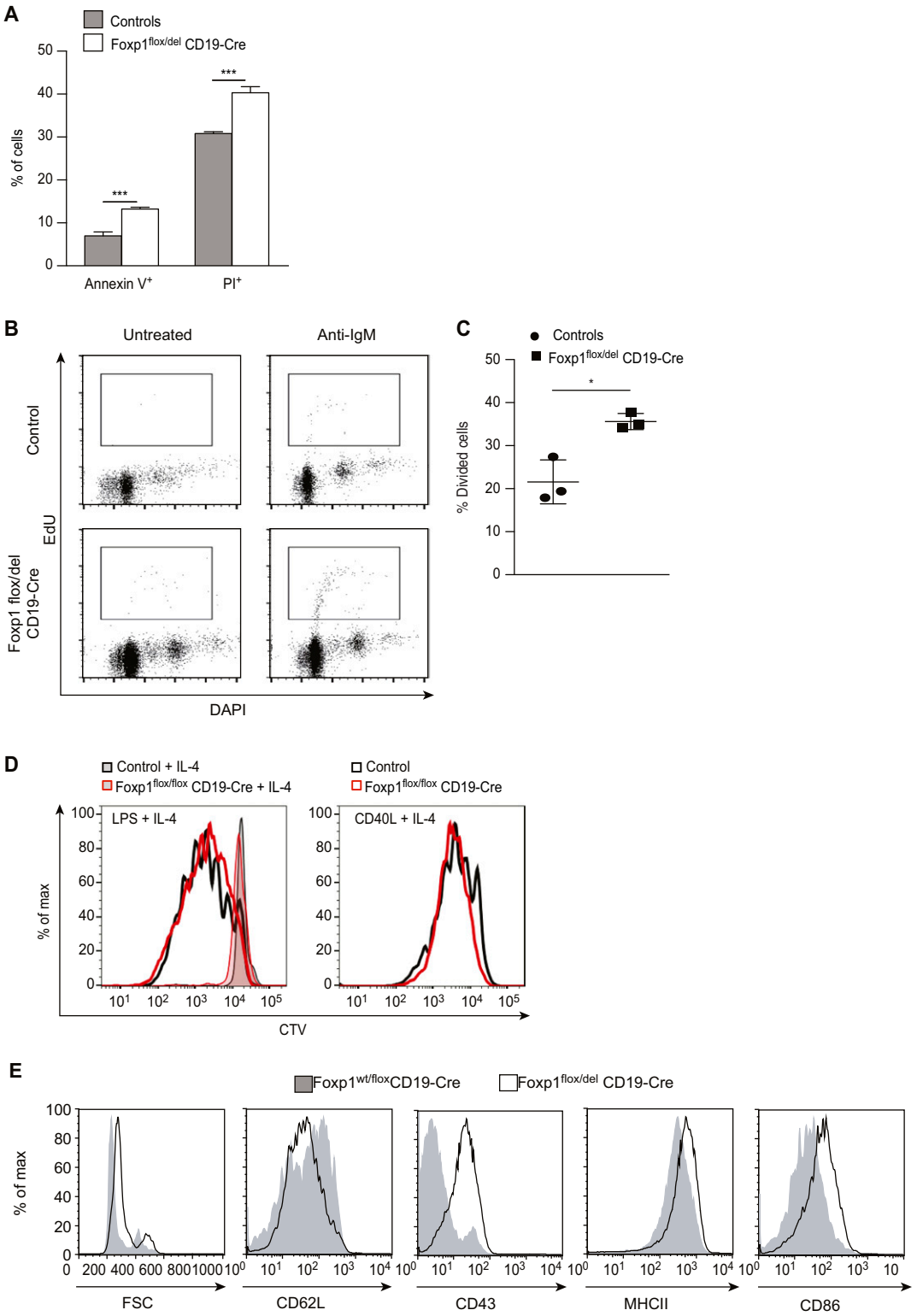

Fig. S5. (*A*) Analysis of apoptotic vs. necrotic cell death in B cells of *Foxp1*$^{wt/wt;CD19}$ control mice and *Foxp1*$^{flox/del;CD19}$ mice ($n = 3$ per group). Apoptotic cells were defined as Annexin V$^+$ and propidium iodide$^-$, necrotic cells as Annexin V$^+$ and propidium iodide$^+$. (*B*) Proliferation of splenic B cells purified from Foxp1$^{flox/wt}$ mice and *Foxp1*$^{flox/del;CD19}$ mice after stimulation with anti-IgM (10 μg/mL) for 16 h. EdU was added for the last 1 h and staining performed as described in *Materials and Methods*. Representative FACS plots in the presence and absence of IgM stimulation are shown. (*C*) Proliferation of splenic B cells purified from *Foxp1*$^{wt/wt;CD19}$ mice and *Foxp1*$^{flox/del;CD19}$ mice was measured by using CFSE (Sigma) and stimulated with anti-IgM (10 μg/mL). Division was tracked by assessing CFSE fluorescence in 1.5×10^4 viable B cells by flow cytometry 48 h after stimulation. Panel shows percentage of divided cells from control and *Foxp1*$^{flox/del;CD19}$ mice as ratio means ± SD ($n = 3$). (*D*) Splenic B cells from *Foxp1*$^{flox/del;CD19}$ mice or littermate control mice were stained with cell trace violet (CTV) and stimulated with IL-4 alone or in combination with LPS or CD40. After 72 h, proliferation was determined by CTV dilution. (*E*) Flow cytometry of splenic B cells from *Foxp1*$^{wt/flox;CD19}$ mice and *Foxp1*$^{flox/del;CD19}$ mice pregated for B220$^+$ and analyzed for cell size (FSC) or expression of CD62L, CD86, CD43, and MHCII (representative example of four individual experiments). *$P < 0.05$ and ***$P < 0.001$.

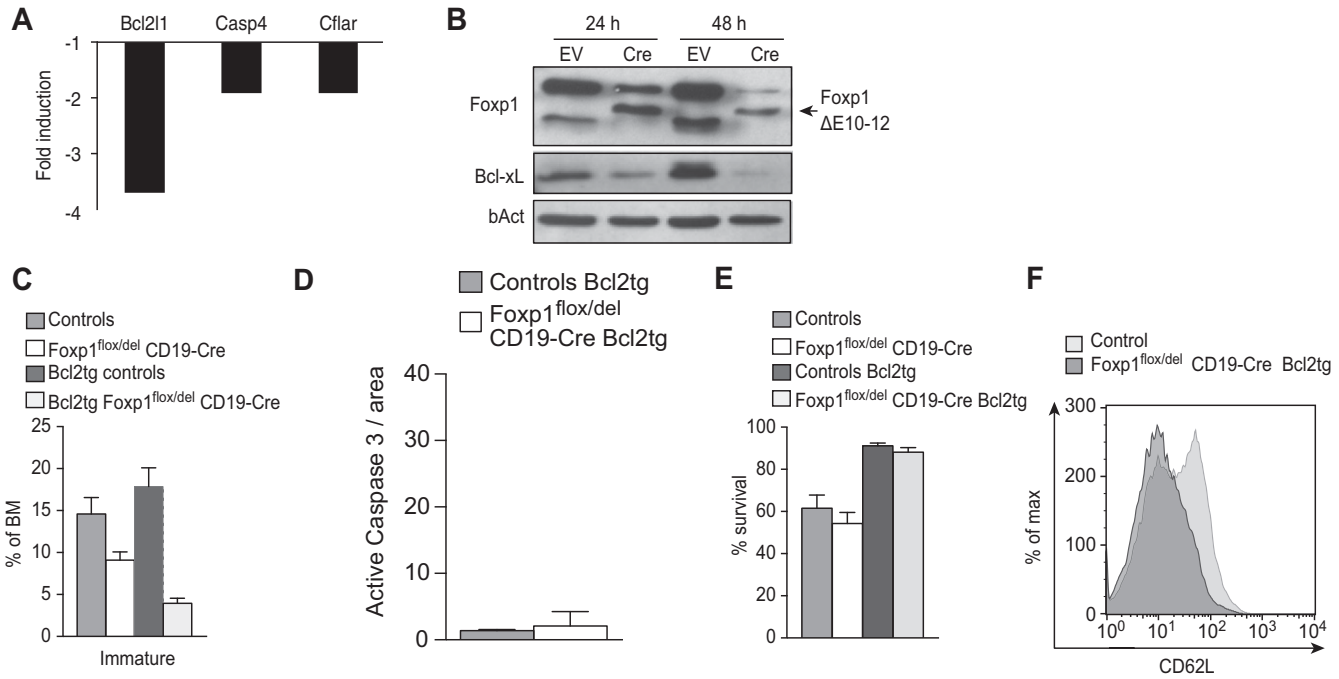

Fig. S6. (*A*) Quantitative RT-PCR of cDNA obtained from purified splenic B cells from *Foxp1*$^{flox/del;CD19}$ mice or *Foxp1*$^{wt/flox}$ control mice was performed, and the fold regulation of the depicted genes was calculated. (*B*) Immunoblot for Foxp1 (antibody no. 2005; Cell Signaling Technology), Bcl-xl, and β-actin 24 h or 48 h after 4-OHT–mediated Cre activation. Deletion of Foxp1 is shown in cultured pro-B cells of *Foxp1*$^{flox/flox}$ mice. (*C*) Flow cytometry of B cell development in the BM of *Foxp1*$^{wt/flox}$ (control), *Foxp1*$^{flox/del;CD19}$ mice, and *Foxp1*$^{wt/flox;Bcl2tg}$ control and *Foxp1*$^{flox/del;CD19;Bcl2tg}$ mice. BM cells were distinguished into pro-B/pre-B cells (B220^{lo}IgM^{-}), immature B cells (B220^{lo}IgM^{+}), and recirculating B cells (B220^{hi}IgM^{+}). Bar chart summarizes results for $n = 3$ for each group. (*D*) Activated caspase 3-positive cells were counted and numbers were normalized to the area of the analyzed histology sections. Data are shown for two biological replicates for each genotype. (*E*) Isolated B cells from WT, *Foxp1*$^{flox/del;CD19}$, *Foxp1*$^{wt/flox;Bcl2tg}$ control, and *Foxp1*$^{flox/del;CD19;Bcl2tg}$ mice were cultured for 24 h and analyzed for viability by Annexin V and PI staining. Percentage of living cells is indicated ($n = 3$ per group). (*F*) FACS blot of CD62L reduction in B cells derived from *Foxp1*$^{flox/del;CD19;Bcl2tg}$ mice compared with controls. Representative example for six mice per group analyzed.