Supporting information

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SI Materials and Methods

Mice. C57BL/6J (B6), B6.129S6-*Il10*^{tm1Flv}/J, C3H/HeJ, BALB/cJ, NZB/BlNJ, NZBWF1/J, and FVB/NJ mice were obtained from the Jackson Laboratory. OT-II, CB17, and B6.*Rag1*$^{-/-}$ mice were purchased from Taconic. CB17.V_H12 Tg (6-1) mice were provided by Dr. Stephen H. Clarke (University of North Carolina). Mice were bred and housed under specific pathogen-free conditions at our National Institutes of Health animal facility. All animal studies were performed under protocols of LIG-14 and -16E approved by the institutional animal care and use committee of the National Institute of Allergy and Infectious Diseases.

Flow Cytometry: FACS. Single-cell suspensions of spleen and peritoneal cells were prepared and stained with fluorochrome-labeled mAbs using standard procedures. The 2.4G2 mAb (BD Biosciences) was routinely used to block Fc receptors and SYTOX AADvanced Dead Cell Stain (Invitrogen) was used to exclude dead cells. The following antibodies were purchased from BD Biosciences unless otherwise indicated: B220 (RA3-6B2), CD19 (ID3), CD5 (53-7.3), IgM (II/41), IgDa (AMS 9.1), IgDb (217-170), CD23 (B3B4), CD11b (M1/70), CD43 (S7), CD138 (281-2), and CD93 (AA4.1, eBioscience). The anti-PC1 mAb was labeled with allophycocyanin (APC) and used within 6 mo (1). Fluorescein-encapsulated liposomes expressing phosphatidylcholine (PtC liposomes) were generously provided by Steve Clarke (University of North Carolina, Chapel Hill, NC). Cells were analyzed using a FACSCalibur (BD Biosciences), a LSR II (BD Biosciences), or sorted on a FACS Aria (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Inc.).

Adoptive Transfer, Immunization, and ELISA. Pooled peritoneal cells of 15–35 mice were stained and sorted for B-cell subsets. Equal numbers of sort-purified B-1a subsets ($2–5 \times 10^5$) were adoptively transferred into Rag1$^{-/-}$ mice i.p. or i.v. Sera were collected at different times after transfer. For immunization, mice previously reconstituted with B-1a cells were injected i.p. with 20 μg of pneumococcal polysaccharide type 3 (PPS-3) (American Type Culture Collection). At different times following immunization, the mice were bled and sera were assayed for Ag-specific IgM as described (2).

Ig Repertoire Analysis. Total RNA was extracted from sort-purified B-1a subsets using RNeasy Mini kit (Qiagen). cDNA (100 ng) was synthesized using a SuperScript III Reverse Transcriptase kit (Invitrogen) and amplified using Platinum Taq DNA polymerase (Invitrogen) and V_H consensus and Cμ4 primers as reported before (3). The PCR product was extracted from the gel and cloned using the pGEM-T Easy Vector Systems (Promega), and randomly picked clones were subsequently sequenced by the NIAID Sequencing Facility at Rocky Mountain Laboratory.

In Vitro Treatment. Sort-purified B-cell populations were cultured at $0.5–1 \times 10^6$/mL in 24-well plates in the presence and absence of 10 μg/mL of LPS (*Escherichia coli* 055:B5; Sigma-Aldrich) for different periods of time. The cells were stained with anti-CD19 and CD138 Abs and were analyzed by FACS. The concentrations of IgM secreted into the supernatant were measured by ELISA.

Enzyme-Linked Immunosorbent Spot Assay. Enzyme-linked immunosorbent spot (ELISPOT) assays for IL-10–producing cells were performed using a Mouse IL-10 ELISPOT Ready-Set-Go kit (eBioscience) according to the manufacturer's instructions. Sort-purified cells were cultured at $1–2 \times 10^3$/well in 96-well plates with and without LPS (10 μg/mL) and PMA (25 ng/mL; Sigma-Aldrich) for 18 h.

Immunofluorescence Microscopy. Frozen sections of small intestine excised from a region adjacent to the colon were stained with anti-mouse IgA (clone C10-3) followed by FITC-conjugated anti-rat IgG Ab (both from BD Biosciences). Slides were examined using an Olympus IX-81 microscope with a 10× objective and 0.3 numerical aperture at room temperature. Fluorescence images were taken using a Hamamatsu ORCA-AG camera and Slidebook (version 5.0.0.29) acquisition software. Data were processed without deconvolution using Microsoft Power Point.

Antigen Presentation Assay. About 5×10^5 cells/mL of sorted peritoneal B-cell subsets were incubated overnight with 100 μg/mL of $OVA_{323–339}$ peptide (Peptides International Corp.) in a 24-well plate. The cells were washed thoroughly and an aliquot of 5×10^4 B cells was mixed with naïve $CD4^+$ T cells purified from OT-II transgenic mice using a $CD4^+$ T-cell isolation kit (Invitrogen) and labeled with CFSE. The cells were cultured for 3 d with or without TGFβ (2 ng/ml, Peprotech) and anti-IL-10 Ab (10 μg/ml, BD Biosciences) and analyzed by flow cytometry. Some of the cultures were performed using transwell plates to confirm cell-cell contact-dependent T-cell stimulation.

Statistical Analysis. A Student unpaired *t* test or Mann–Whitney test were used to assess statistical significance. $P > 0.05$ was considered statistically insignificant.

1. Abbasi S, et al. (2011) Characterization of monoclonal antibodies to the plasma cell alloantigen ENPP1. *Hybridoma (Larchmt)* 30(1):11–17.
2. Haas KM, Poe JC, Steeber DA, Tedder TF (2005) B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae. *Immunity* 23(1):7–18.
3. Kantor AB, Merrill CE, Herzenberg LA, Hillson JL (1997) An unbiased analysis of V(H)-D-J(H) sequences from B-1a, B-1b, and conventional B cells. *J Immunol* 158(3): 1175–1186.

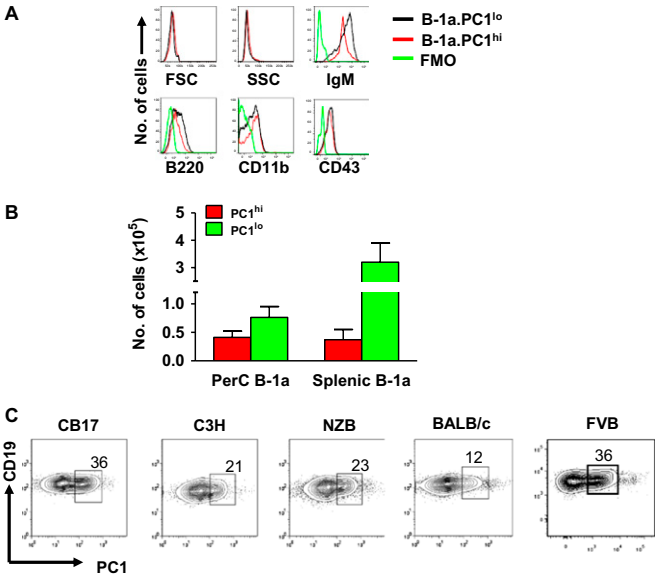

Fig. S1. Characterization of B-1a subsets. (*A*) Overlays show intensities of the indicated parameters. Cells were gated on B-1a.PC1^{lo} and B-1a.PC1^{hi} cells as shown in Fig. 1. FMO, fluorescence minus one control. (*B*) The absolute numbers of splenic B-1a subsets. The same gating scheme as shown in Fig. 1 was used. Data are means ± SEM of five mice per group. PerC, peritoneal cavity. (*C*) Peritoneal B-1a subsets distribution in different strains of mice. Data were gated on CD19^{+}CD5^{+} B-1a cells. The numbers are percentages of cells falling in each gate. Representative data of multiple mice were from at least three independent experiments.

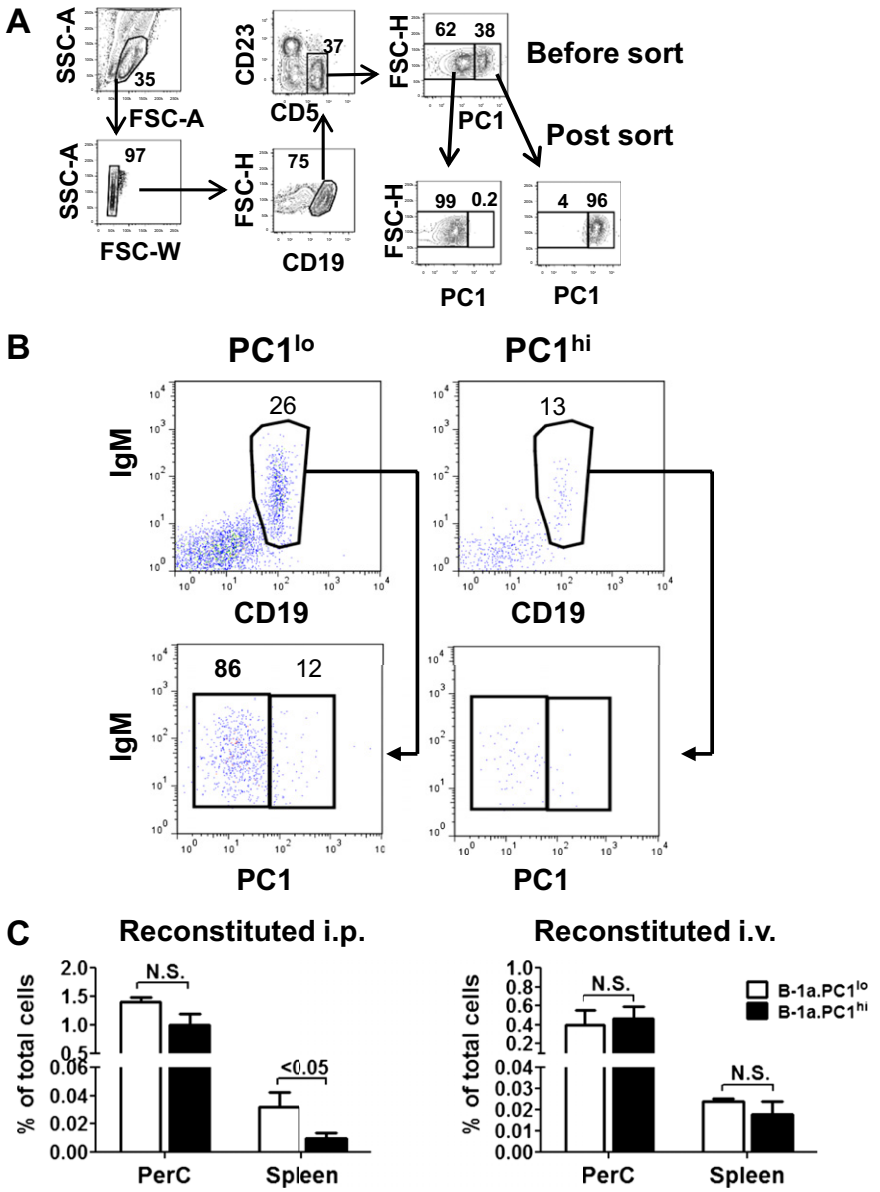

Fig. S2. Migration of B-1a subsets in vivo. (*A*) Gating strategy and postsort analysis show purity of sorted B-1a subsets. (*B*) Equal numbers (3×10^5) of sort-purified B-1a subsets were injected i.p. to 8-wk-old Rag1$^{-/-}$ mice. Mesenteric lymph node (MLN) cells of the recipients were analyzed by FACS at 2 wk following transfer. Cells were gated on lymphocytes. Data represent three independent experiments. Note that PC1^{hi} B-1a cells were scarce in MLN after adoptive transfer. (*C*) Rag1$^{-/-}$ mice were reconstituted i.p. or i.v. with B-1a subsets as in *B* for 2 mo. PerC and spleen cells of the recipients were analyzed by FACS. Error bars are for three mice per group. Data are representative of at least three independent experiments with similar results.

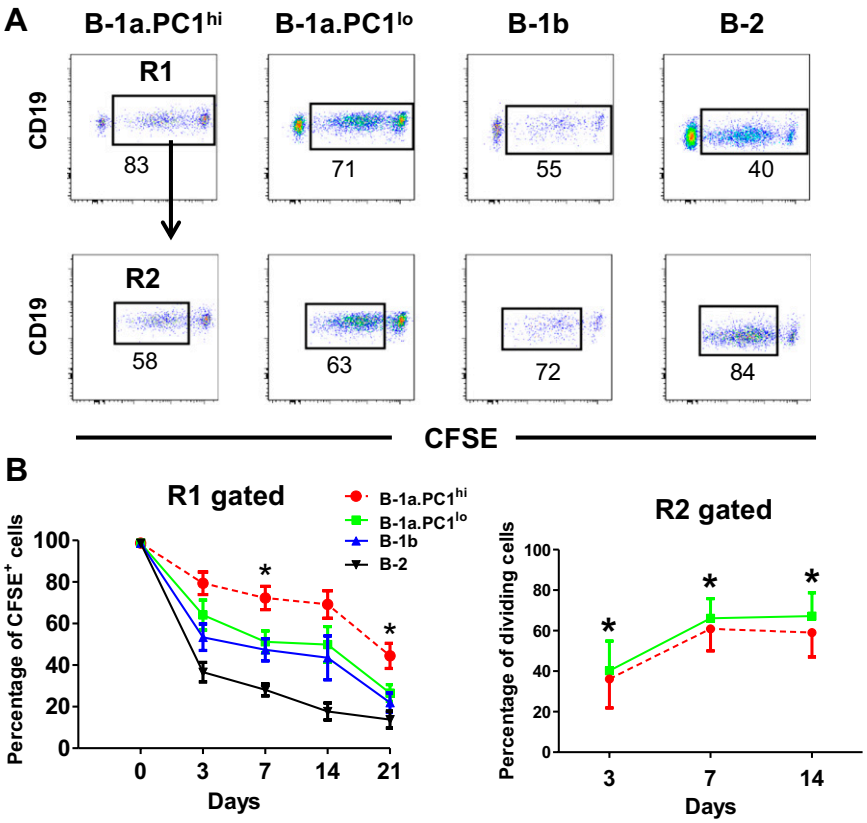

Fig. S3. Turnover rate of B-1a subsets determined by in vivo CFSE labeling. (*A*) Eight-week-old mice were injected i.p. with 50 μg of CFSE (1). Peritoneal washout cells were analyzed by FACS at the indicated times. Top rows show the gating scheme used for quantifying $CFSE^+$ cells (R1) and dividing cells (R2) of peritoneal cells from a mouse injected i.p. with CFSE 3 d previously. Each B-cell subset was defined as shown in Fig. 1*A*. The numbers are percentages of cells falling in each gate. Note that the numbers of R1 in noninjected control mice is 0 and CSFE-injected mice for 2 h is 100% (not shown). (*B*) Summary data are shown (mean ± SEM) for four to six mice per group. The day-0 data represent CSFE injection for 2 h. $*P < 0.05$ (paired *t* test) compared with $PC1^{lo}$ B-1a cells.

1. Lábadi A, Balogh P (2009) Differential preferences in serosal homing and distribution of peritoneal B-cell subsets revealed by in situ CFSE labeling. *Int Immunol* 21(9):1047–1056.

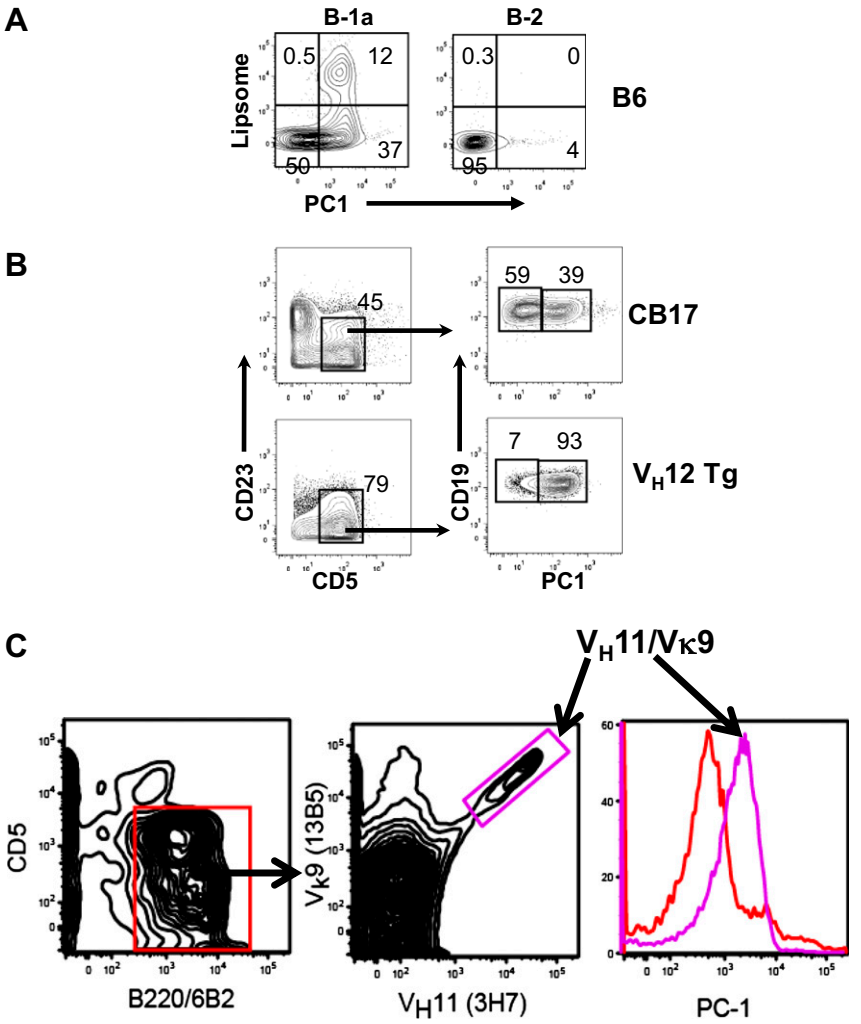

Fig. S4. V_H11 and V_H12 B cells are $PC1^{hi}$. (*A* and *B*) Peritoneal cells from the indicated mice were stained and analyzed by FACS. The numbers are percentages of cells falling in each gate. (*C*) Peritoneal cells of BALB/c mice were stained with antibodies against CD5, B220, V_H11, Vκ9, and PC1 and were analyzed by flow cytometry. Data representative of multiple mice are shown.

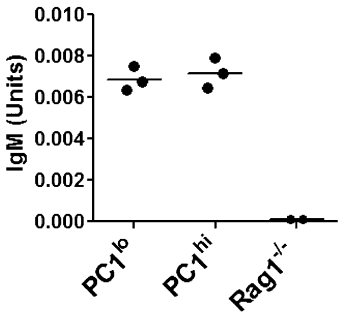

Fig. S5. B-1a subsets secret equivalent amounts of serum IgM when adoptively transferred i.v. into $Rag1^{-/-}$ mice. Sort-purified B-1a subsets were transferred i.v. as described in Fig. S2. Two weeks after transfer, serum levels of IgM in recipients were measured by ELISA.

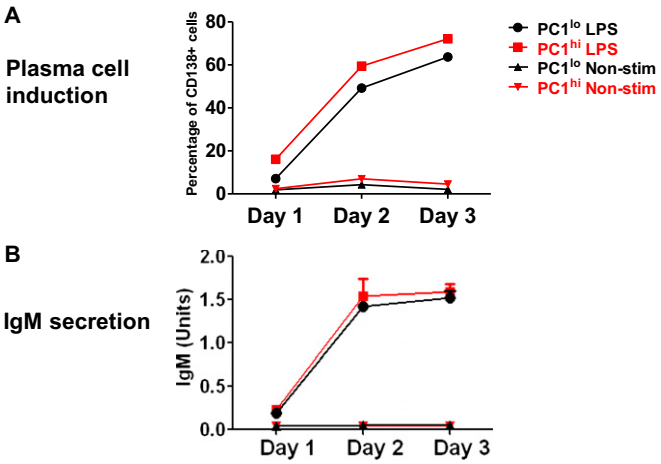

Fig. S6. (*A*) Sort-purified B-1a subsets were cultured with and without LPS for the indicated periods of time. Cells were stained with antibodies recognizing CD19 and CD138 and analyzed by FACS. (*B*) Secreted IgM in supernatants was measured by ELISA. Data are representative of one of three independent experiments with similar results.

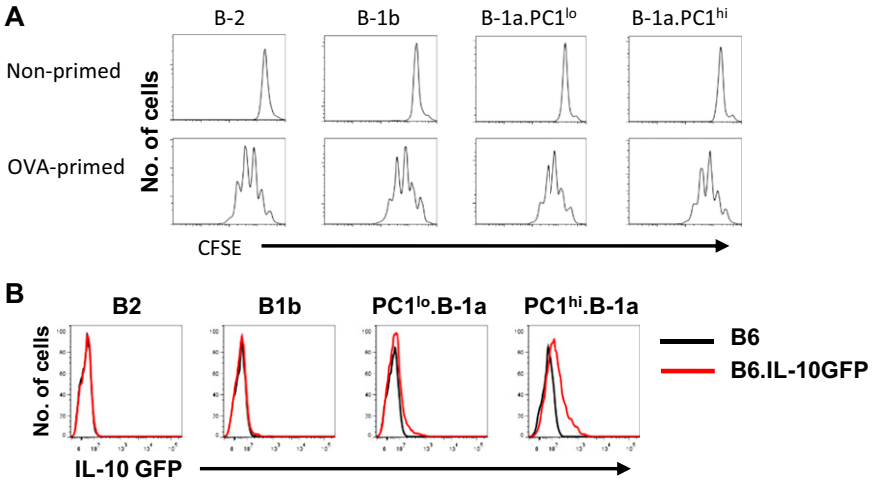

Fig. S7. Regulatory functions of B-1a subsets. (*A*) Stimulation of T-cell proliferation by B-1a subsets. Sort-purified peritoneal B-cell subsets were primed with $OVA_{323–396}$ peptides overnight and then were cocultured with CFSE-labeled $CD4^+$ T cells purified from OT-II transgenic mice for 3 d. The cells were analyzed by FACS. Representative data of three independent experiments are shown. (*B*) IL-10 expression by B-1a subsets assessed by analyzing an IL-10 GFP reporter mouse. Peritoneal cells of B6 and IL-10 GFP reporter mice were stained with antibodies for CD19, CD23, CD5, and PC1 and analyzed by FACS. The overlays are expression of IL10-GFP for each B-cell subpopulation gated as shown in Fig. 1*A*. Data are representative of three independent experiments are shown.

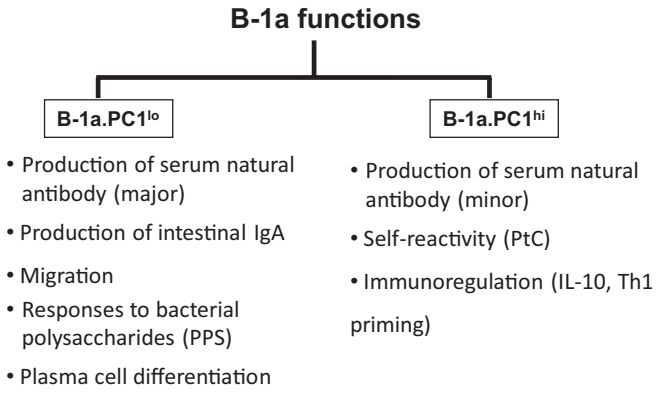

Fig. S8. Segregation of B-1a functions by $PC1^{lo}$ and $PC1^{hi}$ subsets. The classic functions of B-1a are mostly attributed to the $PC1^{lo}$ subset, which represents two-thirds of peritoneal B-1a cells. The $PC1^{hi}$ subset is thought to be enriched for self-reactivity due to the fact that the PtC specificity is detected only in this subset.