Irgm1 Coordinately Regulates Autoimmunity and Host Defense at Select Mucosal Surfaces

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SUPPLEMENTAL INFORMATION

Supplementary Methods

Histology and IHC. Tissues were fixed in 10% neutral buffered formalin, trimmed, processed for paraffin, embedding, sectioned (5 µm), and stained with H&E. The slides were scanned using an Aperio slide scanner (Leica Biosystems, IL) and images were captured using Aperio's ImageScope. For anti-CD3, -Pax5, -IgM, -IgA, -IgG, -Ki67, -CXCL13, and -TNFSF13B antibodies, formalin-fixed, paraffin-embedded tissues were deparaffinized and rehydrated through graded alcohols. Antigen retrieval was performed using the Decloaking Chamber™ NxGen, at 110ºC for 15 minutes with citrate buffer, pH 6.0 or Rodent Decloaker (CD3) (Biocare Medical). Endogenous peroxidase was quenched with 3% hydrogen peroxide. For anti-CD4, anti-CD8a, and PNA, frozen sections were briefly fixed using Shandon Rapid-Fixx (Thermo Scientific). Endogenous peroxidase was quenched with 0.3% hydrogen peroxide. Non-specific binding was blocked using 10% normal horse serum (Pax5), 10% normal goat serum (IgM, IgA, IgG), or 5% normal rabbit serum (CD4, CD8a) (Jackson ImmunoResearch), followed by the avidin/biotin blocking kit (Vector Laboratories), or Rodent Block M (CD3, Ki67) (Biocare) or 2.5% normal horse serum (CXCL13, TNFSF13B)(Vector). Primary antibodies used were: rabbit polyclonal anti-CD3 (abcam ab5690); goat polyclonal Pax5 (Santa Cruz sc-1974); biotinylated goat anti-mouse IgM (Vector BA-2020); biotinylated goat anti-mouse IgA (KPL 16-18-01); biotinylated goat anti-mouse IgG (Vector BA-9200); rabbit monoclonal anti-Ki67 (SP6, Biocare CRM325); goat polyclonal anti-CXCL13 (R&D Systems AF470); rabbit polyclonal anti-TNFSF13B (Boster Bio PA1541); rat monoclonal anti-CD4 (BD Biosciences 550280); rat monoclonal anti-CD8a (BD Biosciences 550281); and biotinylated peanut agglutinin (PNA, Vector B-1075). Negative control slides were incubated with concentration-matched isotype control antibodies. Anti-Pax5 was incubated with biotinylated horse anti-goat secondary antibody (Vector), and anti-CD4 and -CD8a were incubated with biotinylated rabbit anti-rat secondary antibody (Vector). The antigen-antibody complex was labeled using Rabbit-on-Rodent HRP polymer, anti-CD3 (Biocare); Super Sensitive HRP label, anti-Pax5, anti-CD4, anti-

CD8a (Biogenex), Vectastain Elite ABC label RTU, anti-IgM, anti-IgA, anti-IgG, PNA (Vector), ImmPRESS HRP anti-goat IgG, anti-CXCL13 (Vector), ImmPRESS HRP anti-rabbit IgG, anti-TNFSF13B (Vector), followed by 3,3-diaminobenzidine (Dako). Slides were then counterstained with hematoxylin, dehydrated, and coverslipped. All incubations were carried out at room temperature. Anti-Pax5, anti-Ki67, and anti-CXCL13 were stained using the Biocare IntelliPATH automated stainer.

ELISPOT. Goat anti-mouse Igκ and Igλ (Southern Biotech; 2 μg/ml) was diluted in carbonate coating buffer and 75 μl/well was added to ELISpot Multiscreen 96-well Filtration Plate (Millipore) which was incubated at 4°C overnight. Wells were washed 5x with PBS and blocked with 0.5%BSA/PBS at room temp for 1-2 hours. After removal of blocking buffer, 200 μl of the RPMI media (RPMI 1640+glutamine+10% FBS+55uM 2-ME) was added for equilibration and left on until cells were added. Spleen, bone marrow, or perfused lung were homogenized to a single cell suspension at 9.0×10^6 cells/ml. Once cells were prepared, 100 ul of RPMI was added to each well and 50 ul of cell suspension (4.5×10^5 cells) was added to the top well and diluted 3-fold down the plate for a final volume of 100 μl/well. After incubation (3h, 5% CO_2, 37°C), the plate was flooded with dH_2O to remove all cells and culture media, 200 μl of blocking buffer was added to each well and the plate was incubated at RT for 15-30 min, or overnight at 4°C. After removing blocking buffer, alkaline phosphatase conjugated goat anti-mouse IgG, IgM, or IgA (Southern Biotech) was added at 1:10,000 dilution (50 μl/well) and the plate was incubated at RT for 1 hour. The plate was then washed with PBS, rinsed with dH2O, developed with 50 μl/well BCIP-NBT (Sigma) for 30 min and then flooded with dH2O. Spots were quantified with an optical glass binocular magnifier (Optivisor) and additionally by ZellNet. B1-8γ1 and H33- Lγ1 B cell hybridomas were used as controls to verify specific IgM- and IgG-secreting cells (1).

PC-specific ELISPOT. As reported (2), plates were coated with 100µL of 5µg/mL PC-BSA (Biosearch Tech) in PBS solution overnight at 4°C. The next day, the solution was flicked off and 200µl 1% gelatin in PBS was added and plates were incubated 6-10 hrs at 37°C. Perfused mouse lungs were processed as above, plates were washed with 1x RPMI, and 5x106 cells were added into the top well of each plate. Cells were diluted ½ down the plate in RPMI+2% ultra low IgG FCS+100µM 2ME, and incubated for 5 days (37°C, 5% CO2). After incubation, the plate was dipped in distilled deionized water + 0.05% Tween for 2 washes, followed by PBS + 0.05% Tween and set at RT for 10 minutes. This was repeated 3 times. The plates were then incubated with 50µL of 1:2000 AP-coupled antibody (IgM or IgA) in PBS + 1% gelatin + 0.05% Tween for 1-2 hours at 37°C. Plates were then dipped with PBS + 0.05% Tween and set at RT for 10 minutes (washes repeated 3 times). Plates were then developed with 50 µl/well BCIP-NBT (Sigma) at 4°C overnight and analyzed as above.

Opsonization assay. Bacteria were labeled with 30 µM Pkh26 (PKH26 Red Fluorescent Cell Linker Kits, Sigma)(3). Mice were i.t. administered $2x10^5$ CFU of labeled *S. pneumoniae*, and 30 minutes later, were euthanized and BALF collected. BAL pellets were stained with anti-IgA-FITC, anti-IgM-AF647 and anti-CD45 eFluor 450. The SSC setting was set to detect bacteria.

References

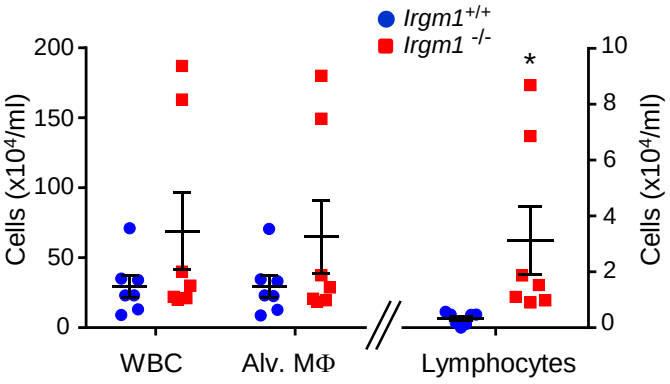
1. Dal Porto, J.M., Haberman, A.M., Shlomchik, M.J., and Kelsoe, G. 1998. Antigen drives very low affinity B cells to become plasmacytes and enter germinal centers. *J Immunol* 161:5373-5381.
2. Patel, P.S., and Kearney, J.F. 2015. Neonatal exposure to pneumococcal phosphorylcholine modulates the development of house dust mite allergy during adult life. *J Immunol* 194:5838-5850.
3. Weber, G.F., Chousterman, B.G., Hilgendorf, I., Robbins, C.S., Theurl, I., Gerhardt, L.M., Iwamoto, Y., Quach, T.D., Ali, M., Chen, J.W., et al. 2014. Pleural innate response activator B cells protect against pneumonia via a GM-CSF-IgM axis. *J Exp Med* 211:1243-1256.

Supplementary Table 1.

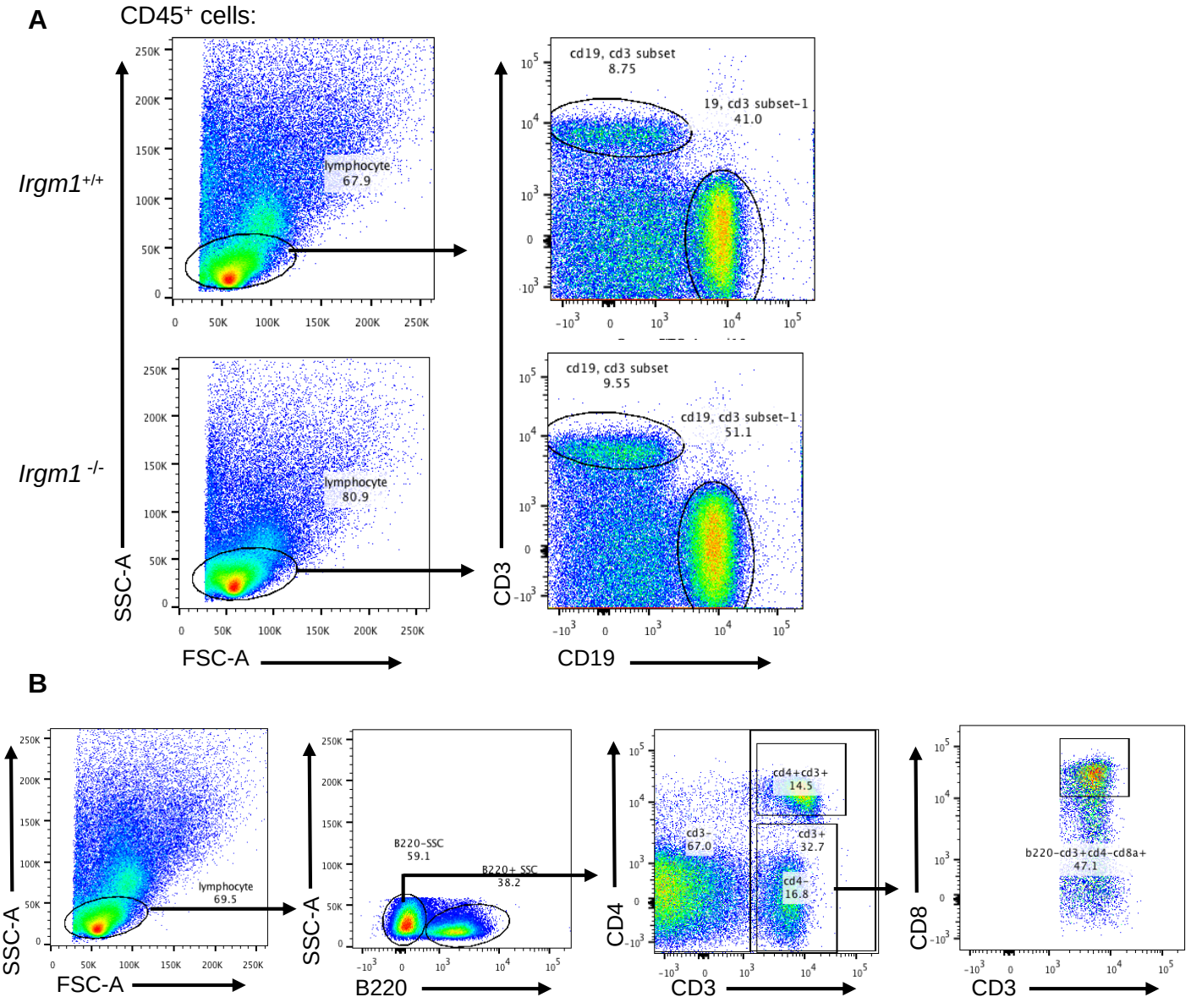
Organs with no overt histopathologic abnormalities in *Irgm1*$^{-/-}$ mice	
Adrenal Glands	Muscle (Thigh)
Brain	Nerve (Sciatic)
Clitoral Glands	Nose
Esophagus	Oral Cavity
Eyes	Ovaries
Femur	Parathyroid Glands
Gall Bladder	Pituitary Gland
Harderian Glands	Preputial Glands
Heart and Aorta	Prostate
Large Intestine	Seminal Vesicles
Small Intestine	Skin
Kidneys	Spinal Cord
Liver	Spleen
Lymph Nodes	Stomach
Lymph Mesenteric	Testes, Epididymis
Lymph Mandibular	Thymus
Mammary Gland	Thyroid Glands
Vagina	Tongue
Zymbal's Glands	Trachea
Uterus	Urinary Bladder

Supplementary Table 2.

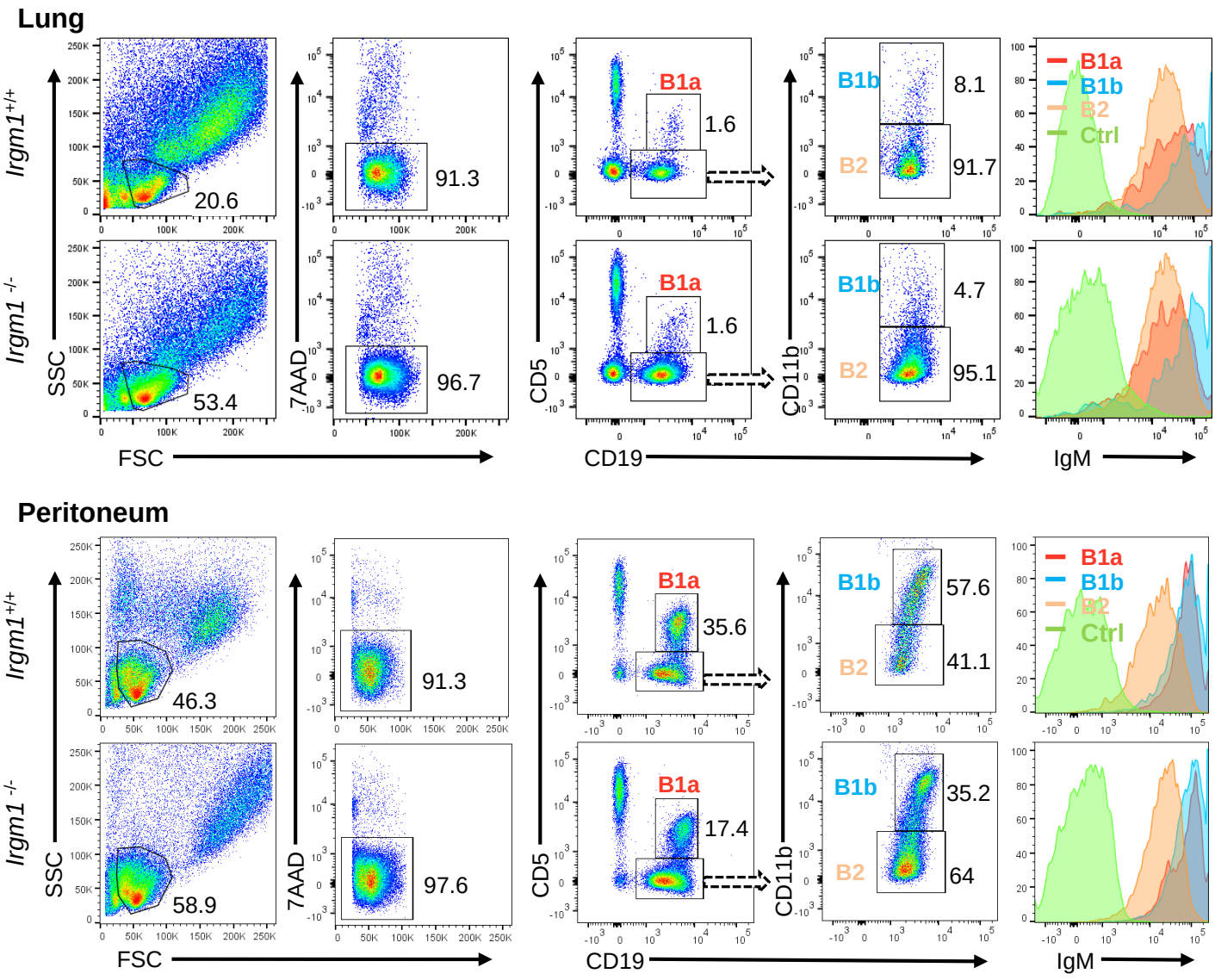
Negative serologic assays in naïve Irgm1$^{-/-}$ mice
Mouse Hepatitis Virus (MHV)
Mouse Rotavirus/Enzootic Diarrhea of Infant Mice (EDIM)
Minute Virus of Mice (MVM)
Mouse Parvovirus (MPV)
Mouse Norovirus (MNV)
Parvovirus NS-1 Protein
Sendai Virus
Pneumonia Virus of Mice (PVM)
Theiler's Encephalomyelitis Virus (GDVII)
Reovirus (REO)
Mycoplasma pulmonis
Lymphocytic Choriomeningitis Virus (LCMV)
Mouse Thymic Virus (MTV or MTLV)
Mouse Cytomegalovirus (MCMV)
Mouse Adenovirus (MAD-1, MAD-2)
Pneumonitis Virus (K Virus)
Hantaan Virus
Polyoma Virus
Ectromelia Virus
Cilia Associated Respiratory (CAR)-Bacillus
Encephalitozoon cuniculi
Lactate Dehydrogenase-Elevating Virus (serum)



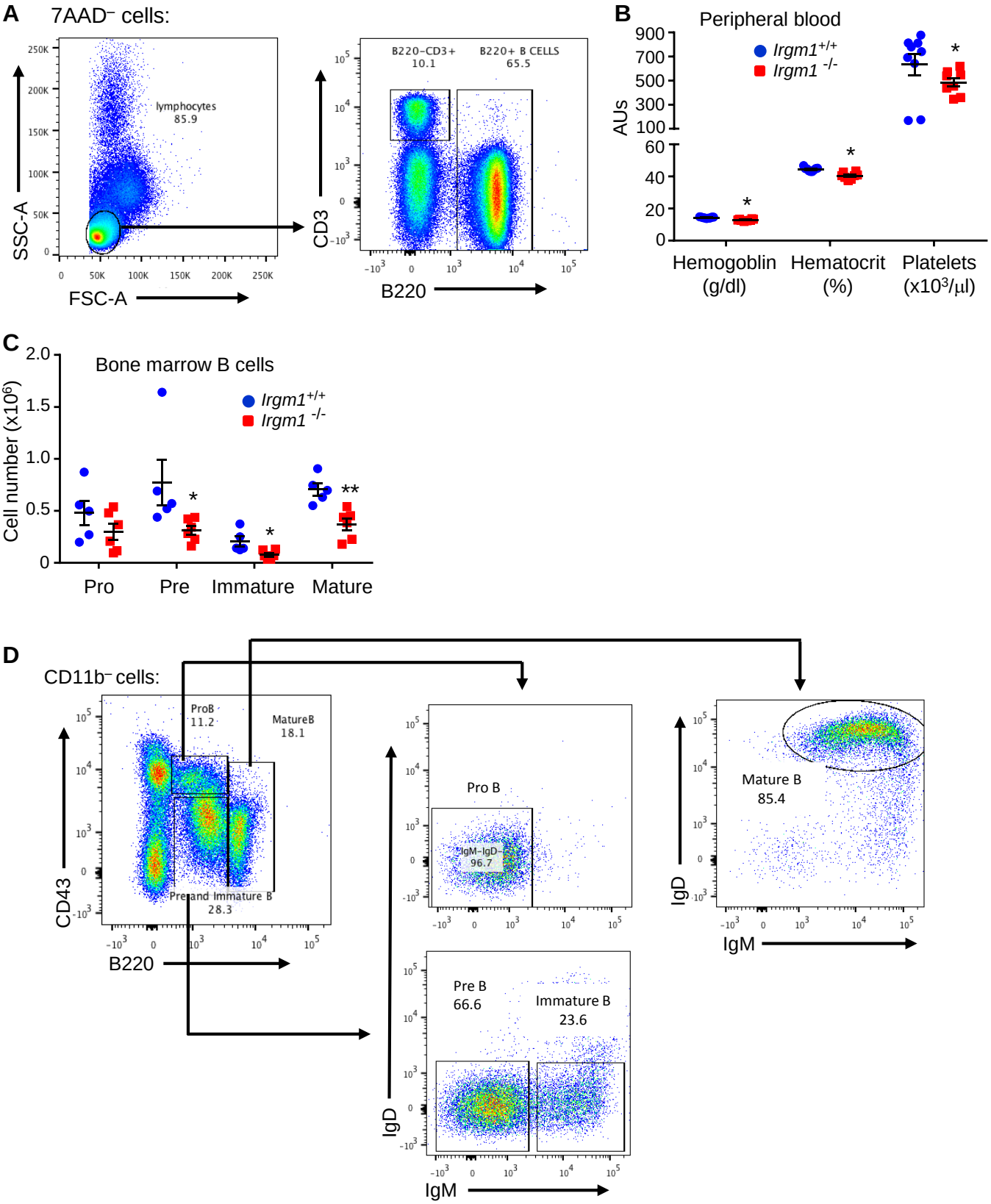
Supplementary Figure 1. Increased lymphocytes in airspace of naive *Irgm1*$^{-/-}$ mice. Total leukocytes (WBC), alveolar macrophages, and lymphocytes were counted in bronchoalveolar lavage fluid of naive *Irgm1*$^{-/-}$ mice and controls (N=7/genotype). Data are mean +/- SEM and are representative of 3 independent experiments. *, P<0.05 by unpaired two-tailed Student's t test.



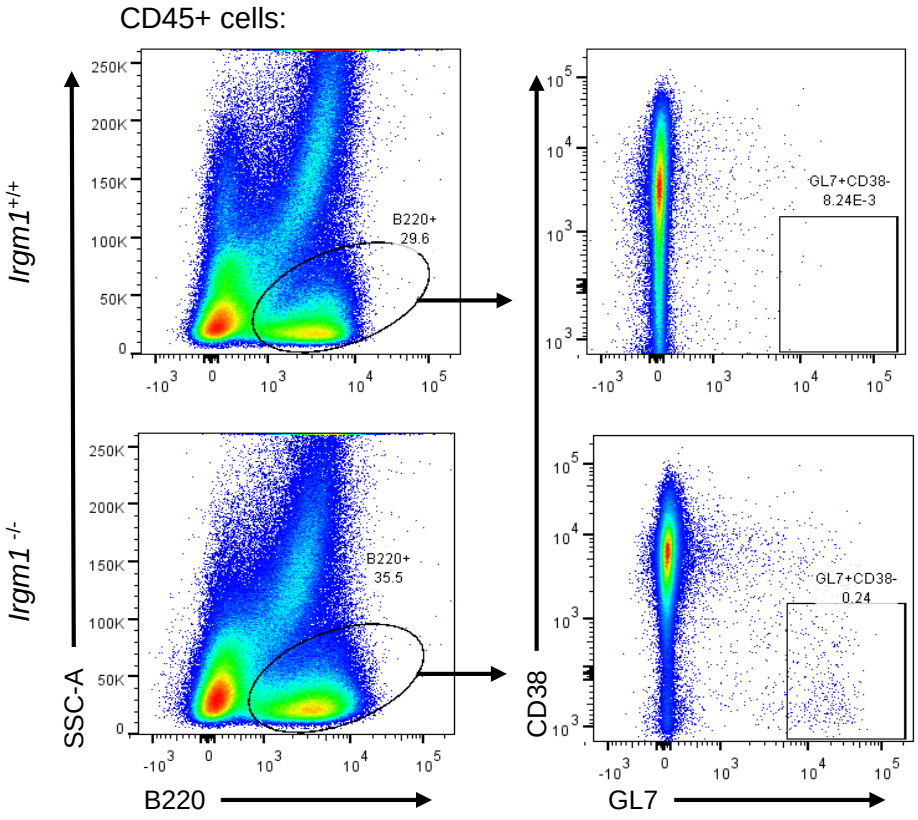
Supplementary Figure 2. Flow cytometry gating strategy for B cells and T cells. (**A**) B cells were CD19^{+}CD3^{-} and were also confirmed to be B220^{+} (data not shown). (**B**) T cells were B220^{-}CD19^{-}CD3^{+} and were further phenotyped by anti-CD4 and -CD8 antibodies.



Supplementary Figure 3. Flow cytometry gating strategy for B cell subsets. After specification as $SSC^{lo}FSC^{lo}7AAD^{-}$, B cells were identified in the lung and peritoneum as follows: B1a: $CD19^{+}CD5^{+}CD11b^{+}IgM^{hi}$; B1b: $CD19^{+}CD5^{-}CD11b^{+}IgM^{hi}$; B2: $CD19^{+}CD5^{-}CD11b^{-}IgM^{lo}$. B1a and B1b cells were also confirmed as $CD23^{lo}$ in the peritoneum (data not shown).

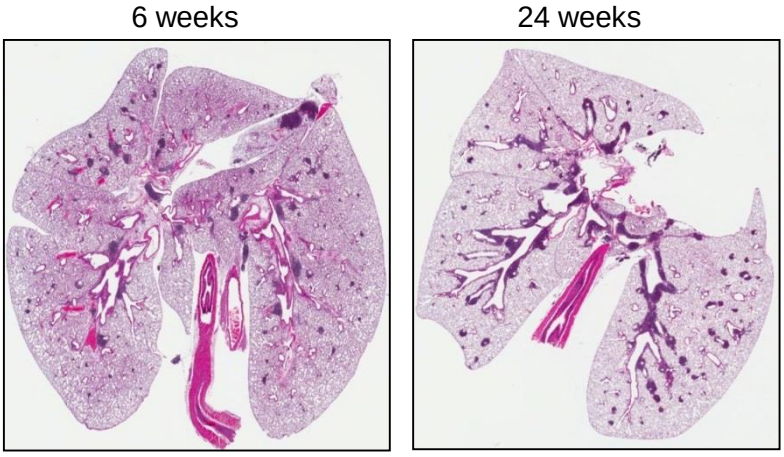


Supplementary Figure 4. Identification of blood cells in circulation and bone marrow. (**A**) In peripheral blood, B cells were identified by flow cytometry as $CD3^{-}B220^{+}$ and T cells as $CD3^{+}B220^{-}$. (**B**) Erythrocyte and platelet indices were quantified in blood of naive *Irgm1*$^{-/-}$ mice and controls (N=8-10/genotype) using a HEMAVET 1700 hematology analyzer (Drew Scientific, Inc.). (**C-D**) B cell subpopulations were quantified in bone marrow of naive *Irgm1*$^{-/-}$ mice and controls (N=5-6/genotype) (**C**), using the gating strategy shown in ***D***. Data are mean +/- SEM and are representative of at least 2 independent experiments. *, P<0.05; **, P<0.01 by unpaired two-tailed Student's t test.

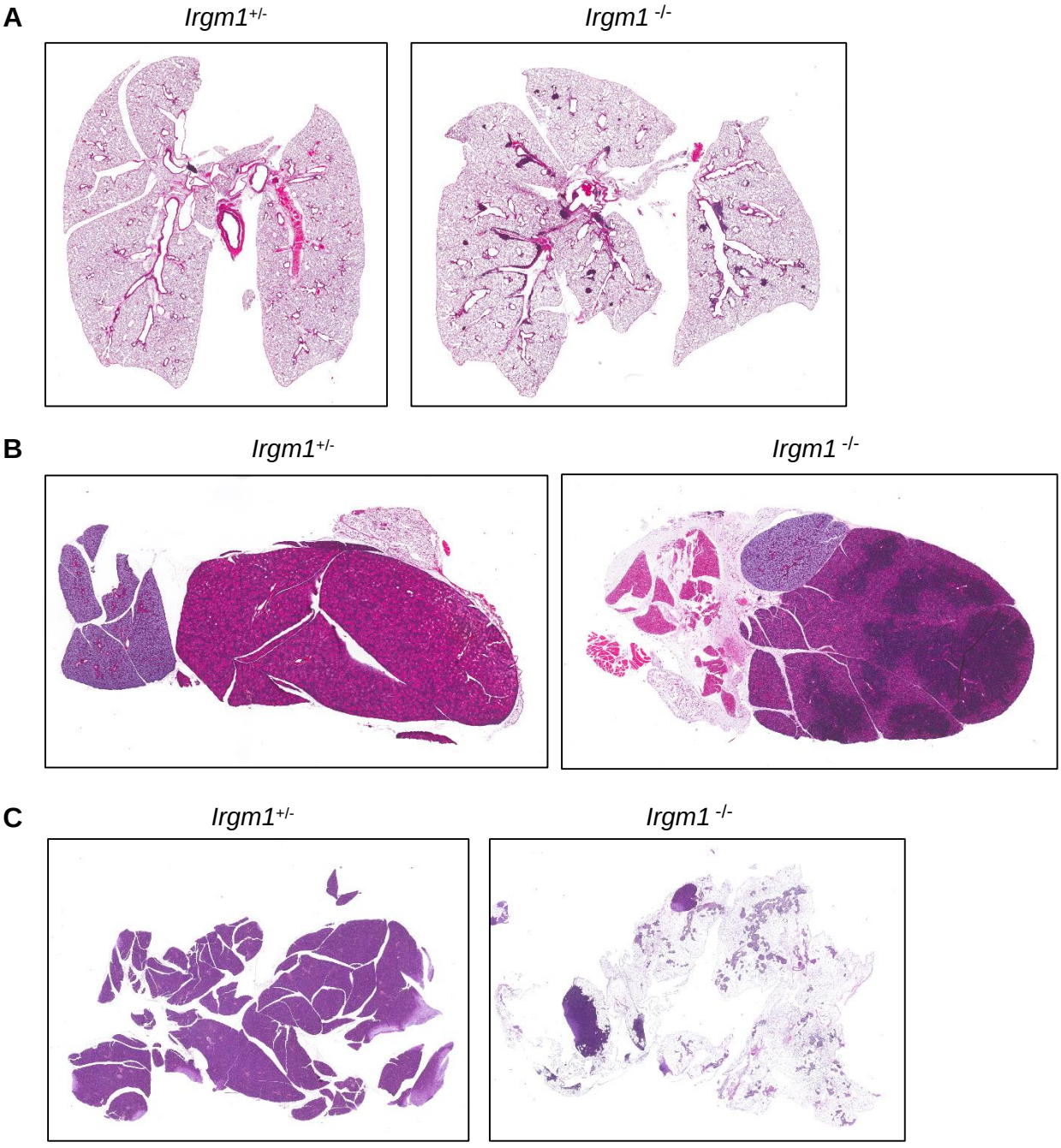


Supplementary Figure 5. Flow cytometry gating strategy for germinal center B cells. Germinal center B cells were identified as $CD45^{+}B220^{+}GL7^{+}CD38^{-}$.

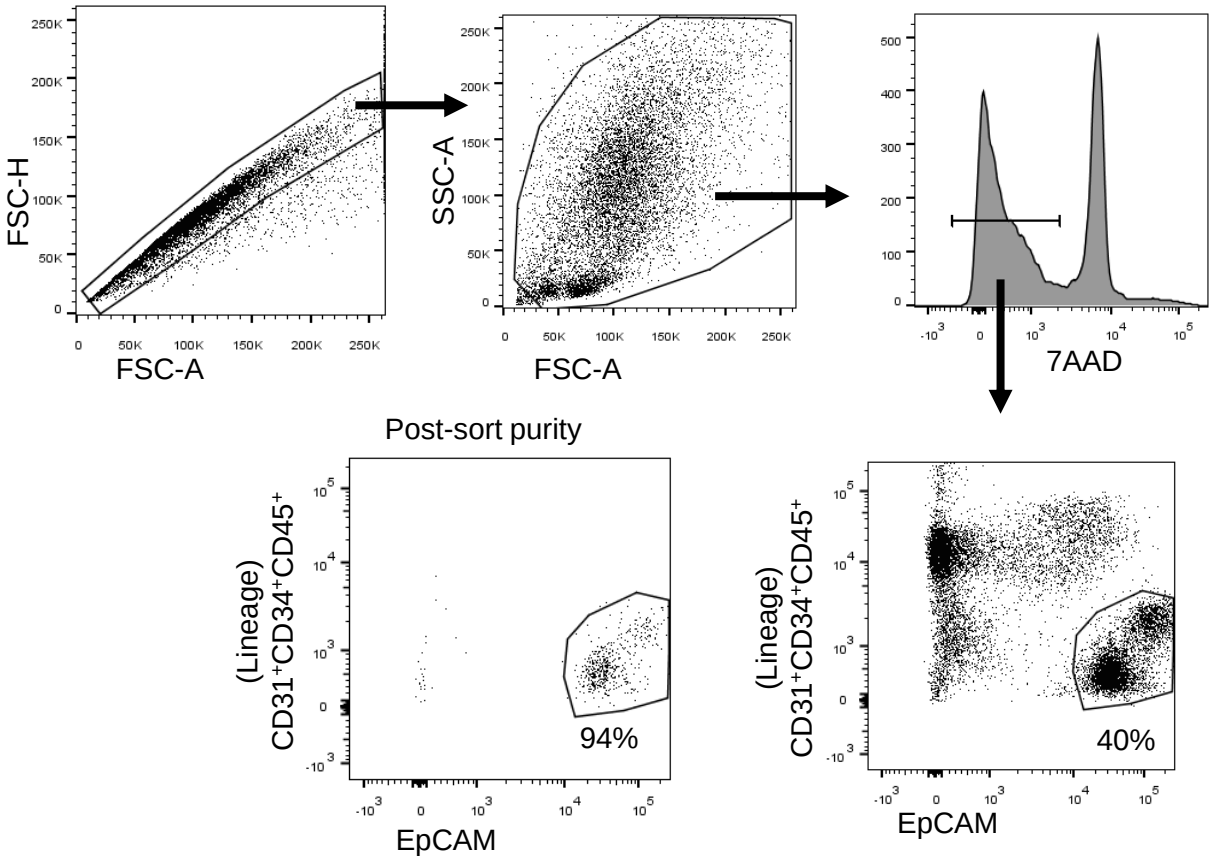
Supplementary Figure 6. Clinical signs of exocrine deficiency in naive *Irgm1*$^{-/-}$ mice. (**A**) Serum amylase was quantified using an Olympus AU400e (Beckman Coulter, Inc., Irving, TX). N=5-7/genotype. **, P<0.01 by unpaired two-tailed Student's t test. (**B**) *Irgm1*$^{-/-}$ mice displayed a high frequency of periorbital hair loss, as shown. Clinical findings are representative of >10 mice.



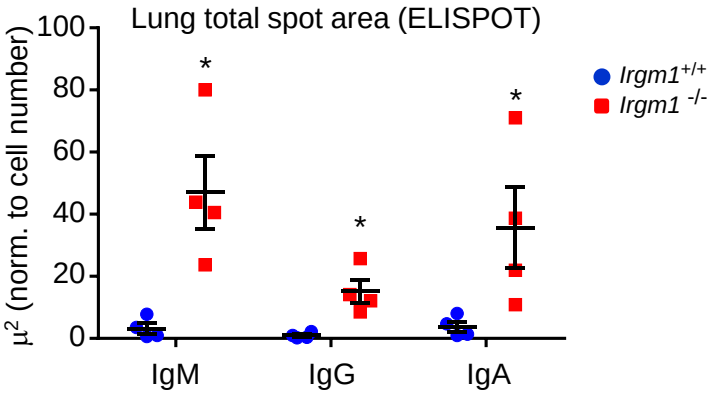
Supplementary Figure 7. Age-dependent progression of lymphocytic infiltrates in lungs of *Irgm1*$^{-/-}$ mice. Representative hematoxylin and eosin-stained lung sections are shown from 6 and 24 week-old *Irgm1*$^{-/-}$ mice, demonstrating marked progression of the peribronchovascular lymphocytic aggregates with age. Findings are representative of at least 4 mice/age. Original magnification, 1.1x.



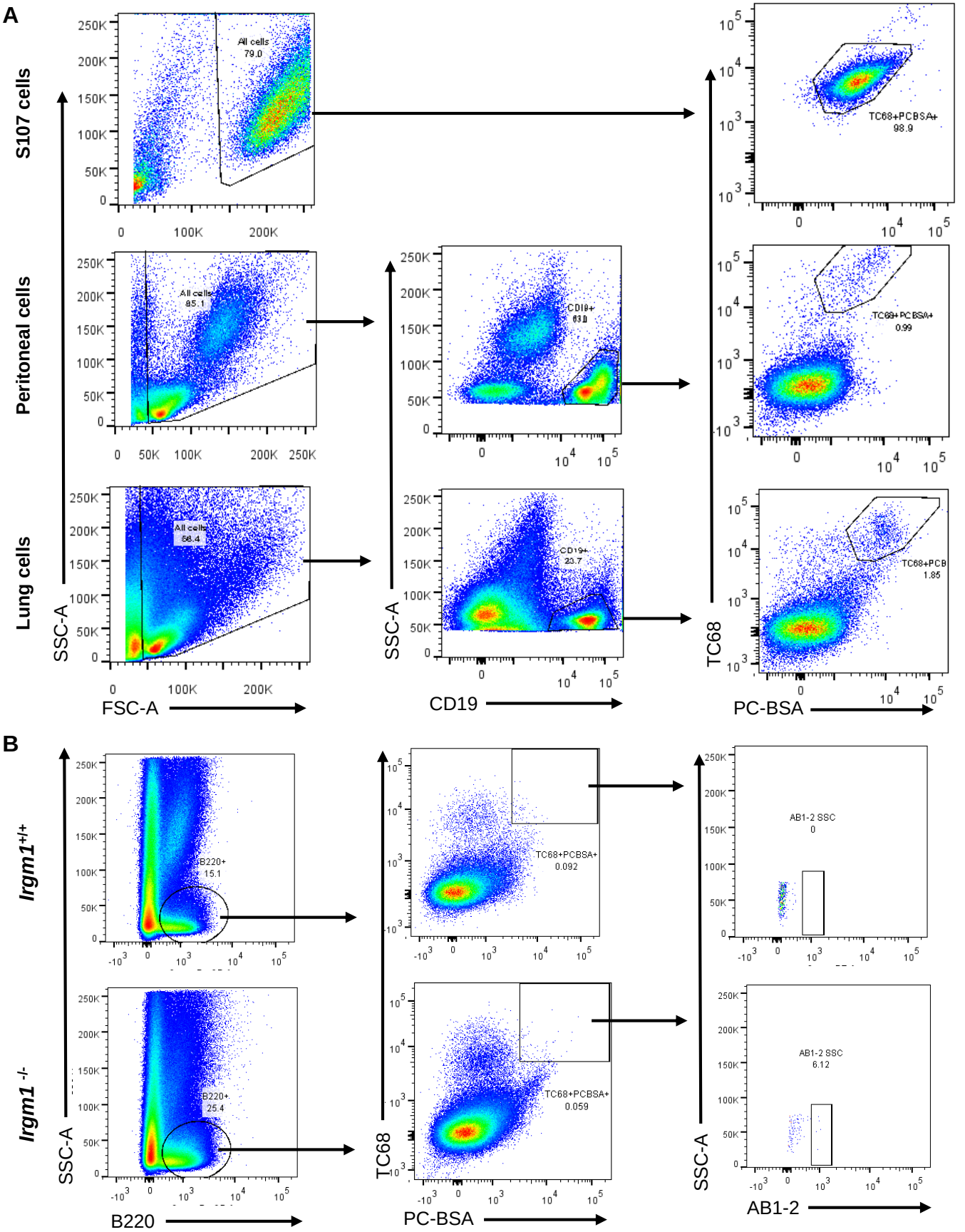
Supplementary Figure 8. Histopathology in germ-free naive *Irgm1*$^{-/-}$ mice. (**A-C**) Hematoxylin and eosin-stained sections of (**A**) lung (original magnification, 1.1x), (**B**) submandibular salivary gland (original magnification, 2.7x), and (**C**) pancreas (original magnification, 2.1x) are shown for germ-free *Irgm1*$^{-/-}$ mice and *Irgm1*$^{+/-}$ controls. Findings are representative of 4 *Irgm1*$^{-/-}$ and two *Irgm1*$^{+/-}$ mice surveyed.



Supplementary Figure 9. Flow cytometry gating strategy for lung epithelial cells. Epithelial cells were sorted from lungs using the fluorescence-activated cell sorting strategy shown, and were identified as CD45$^-$ CD31$^-$CD34$^-$EpCAM$^+$. Post-sort cell purity was >92-94%.



Supplementary Figure 10. ELISPOT of immunoglobulins in lung. Total spot area for IgM, IgG, and IgA was normalized to cell number in lung preparations as a metric of total antibody production. N=4/genotype. Data are mean +/- SEM and are representative of 2 independent experiments. *, P<0.05 by unpaired two-tailed Student's t test.



Supplementary Figure 11. Flow cytometry gating strategy for PC-reactive T15 idiotype B cells. (**A**) Peritoneal lavage and lung digest cells were gated by scatter properties, anti-CD19 antibody, TC68 antibody, and reactivity to PC-BSA, as shown. S107 myeloma cells, which secrete PC-reactive IgA κ-chain antibody of the T15 idiotype, serve as a positive control. (**B**) Lung digest cells from *Irgm1*$^{+/+}$ and *Irgm1*$^{-/-}$ mice were gated as shown.