

Supporting Information

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SI Methods

Cells, Antibodies, and Mice. Madin–Darby canine kidney (MDCK) type II cell line was a gift from Keith Mostov (University of California School of Medicine, San Francisco, CA). MDCK-rat FcRn (Fc receptor for IgG), MDCK-vector, and MDCK-rat FcRn-GFP cells have been previously described (1). Cells were grown in DMEM complete medium supplemented with 10 mM Hepes, 10% FCS, 1% L-glutamine, nonessential amino acids (Invitrogen), and 1% penicillin/streptomycin (Invitrogen Life Technologies). When necessary, media were also complemented with 400 µg/mL of G418 (Invitrogen). Cells were grown in 5% CO₂ at 37 °C. mAb anti-EEA1 was obtained from BD Biosciences. Goat anti-mouse polymeric IgA receptor (pIgR) was from R&D Systems. Mouse anti-canine LAMP-2 was from AbD Serotec, and anti-β-tubulin antibody was obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the National Institute of Child Health and Human Development and maintained by the University of Iowa. ZO-1-specific antibody was obtained from Invitrogen. Alexa Fluor-conjugated 488, 555, and Alexa Fluor 633 goat anti-mouse or rabbit antibodies were purchased from Molecular Probes. Affinity-purified rabbit IgG against mouse FcRn was previously described (2). HRP-conjugated donkey anti-rabbit or rabbit anti-mouse antibody was purchased from Pierce Biotechnology. Affinity-purified mouse IgG and chicken IgY were obtained from Rockland Immunochemicals. Sulfo-NHS-LC-biotin was from Pierce.

Male inbred C57BL/6 mice aged 6 to 8 wk were purchased from Charles River Laboratories. The FcRn-KO mice in a C57BL/6 background were from Jackson Laboratory. All mice were housed in specific pathogen-free animal resources facility. All animal studies were reviewed and approved by the institutional animal care and use committee at the University of Maryland.

Virus and mAb. Influenza A virus (strain A/Puerto Rico/8/1934 H1N1) was a gift from Peter Palese (Mount Sinai School of Medicine, New York, NY). Influenza PR8 virus was grown in 10- to 11-d-old embryonated chicken eggs. Purification of the virus was performed by differential centrifugation and sedimentation through a sucrose gradient. PR8 HA-specific hybridoma Y8 (IgG2a) and H36-4 (IgG2a) were from Coriell Institute for Medical Research, and the NP-specific hybridoma HB-65 (IgG2a) was from the American Type Culture Collection. These mAbs have been described previously (3). Purification of mAb from cell culture medium was done by affinity chromatography using Protein A (Pierce), dialyzed against PBS solution and stored in PBS solution at –80 °C. Influenza virus (2 mg/mL) was biotinylated by using 150 µM sulfo-NHS-SS-biotin (Pierce) according to manufacturer's instructions, and free biotin was completely removed by desalting column (Pierce).

SDS/PAGE, Western Blot, and Avidin Blot. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories). Then, proteins or biotin-labeled proteins were resolved on a 12% SDS/PAGE gel under reducing conditions. Proteins were transferred onto a nitrocellulose membrane (Schleicher and Schuell). The membranes were blocked with 5% nonfat powdered milk, probed separately with anti-FcRn, anti-pIgR, or β-tubulin Ab for 1 h, and followed by incubating with HRP-conjugated rabbit anti-mouse IgG, HRP-conjugated donkey anti-rabbit IgG or HRP-avidin, respectively. All blocking, incubating, and washing

were performed in PBS solution with 0.05% Tween 20. Proteins were visualized by the ECL method (Pierce).

TCID₅₀ Assay and Hemagglutination Inhibition Assay. TCID₅₀ was determined in MDCK cells. Samples were serially diluted 10-fold in Opti-MEM I (Invitrogen). MDCK cells were plated 1 d before PR8 infection in 96-well plates. MDCK confluent monolayers were then infected with the diluted virus for 1 h at 37 °C. Infected cells were subsequently washed and incubated with fresh medium supplemented with 1 µg/mL TPCK-trypsin (Sigma) for 72 h. Supernatant were collected and the endpoint viral titer was determined by a hemagglutination assay.

The antiviral activity of PR8 HA specific mAb was measured by standard hemagglutination inhibition (HI) assay with minor modifications. Approximately four hemagglutination units of PR8 viruses were incubated at pH 5.0 or pH 7.4 Opti-MEM for 2 h at 4 °C. Spin desalting columns were used to exchange the buffer to neutral pH (7.4). Y8 or H36 mAbs were serially diluted 10-fold in V-bottom 96-well plates and incubated for 1 h at room temperature with viruses treated at different pH values. Subsequently, 1% chicken red blood cells were added and incubated for 30 min at room temperature. The highest serum dilution that inhibited hemagglutination was considered the HI titer of the mAb.

In Vitro and in Vivo Transcytosis. IgG transcytosis in MDCK monolayers was measured by a modification of previously described methods (1). MDCK cells expressing rat FcRn or FcRn-GFP, and MDCK-vector control, were grown onto 0.4-µm pore size transwell filter inserts (Corning Costar) to form a monolayer exhibiting transepithelial electrical resistances (300 Ω/cm²). Transepithelial electrical resistance was measured by using a volt-ohm meter equipped with planar electrodes (World Precision Instruments). Monolayers were equilibrated in Hanks balanced salt solution. IgG, IgG-biotin, or IgY-biotin (400 µg/mL) were applied to the basolateral compartment in pH 7.4 serum-free DMEM (Invitrogen) supplemented with 10 mM Hepes, 10 mM sodium pyruvate, 1% L-glutamine, 1% nonessential amino acids, and 1% penicillin/streptomycin, and incubated for 2 h at 37 °C. Transported proteins were sampled from the apical chamber and analyzed by SDS/PAGE under reducing conditions. Proteins were visualized by Western blot-ECL or avidin blot-ECL analysis. For in vivo IgG or IgY transport, 200 µg of biotinylated mouse IgG or chicken IgY in 100 µL of PBS solution were injected i.p. into mice. Lung lavages were collected 24 h later. The transported IgG-biotin or IgY-biotin antibody was analyzed by SDS/PAGE and Western blot-ECL or avidin blot-ECL analysis (Pierce).

Intracellular Neutralization of PR8 Virus by Y8 mAb. Y8 IgG (400 µg/mL) or an irrelevant murine IgG2a antibody was applied to the lower compartment when MDCK-FcRn or MDCK-vector cell monolayers become polarized on insert filters. Cells were incubated with IgG antibody for 2 h at 37 °C. Subsequently, PR8 virus (100 pfu/cell) was inoculated into the apical chamber for 1.5 h at 4 °C; then, cells were warmed to 37 °C for an additional 45 min to allow infection. Inserts were completely washed to remove the residual antibody or virus. Cells were incubating for additional 24 h at 37 °C, at which time the apical supernatants were removed. The apical supernatants were tested for virus titers by TCID₅₀.

Nocodazole Treatment. MDCK transfectants (1×10^5) were seeded onto the transwell to allow polarization. Cells were preincubated with or without nocodazole (33 µM) for 2 h; nocodazole was then

removed from the chambers. Y8 mAb (400 $\mu\text{g}/\text{mL}$) was subsequently added into the basolateral chamber to allow transport for 2 h. PR8 virus was added to the apical chamber for 45 min to allow infection. Cells were completely washed to remove the IgG or virus and incubated for an additional 24 h at 37 °C. The amount of PR8 virus in the apical medium was analyzed by a TCID₅₀ assay.

RT-PCR Analysis. For total RNA extraction, cells were pelleted and resuspended in TRIzol reagent (Invitrogen Life Technologies). The influenza PR8 NP gene was amplified by primers (5'-ATCATGGCGTCTCAAGGCAC-3', 5'-TCCTGTATATAGGTCCTC-3') with an One-Step RT-PCR kit (Qiagen). The RNA was also amplified by using GAPDH-specific primers (5'-GGAGAAAGCTGCCAAATATG-3', 5'-TACCAGGAAATGAGCTTGAC-3') as an internal control to monitor the quality of the RNA purification and cDNA synthesis. The PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Immunofluorescence and Confocal Microscopy. Immunofluorescence staining of cells or frozen tissue sections was performed based on methods previously described (2). Briefly, cells were cultivated on coverslips for 24 h and subsequently incubated with Y8 for 2 h at 37 °C. Next, antibody-treated cells were incubated with biotin-labeled virus for 30 min. The cells were rinsed in PBS solution, fixed in 3.7% paraformaldehyde (Sigma) in PBS solution for 30 min at 4 °C, and quenched with 10% glycine for 10 min. After two washings with PBS solution, the coverslips were permeabilized in PBS solution containing 0.2% Triton X-100 for 20 min. The frozen tissues were embedded in optimal cutting temperature media, serially sectioned, fixed in acetone for 5 min at -20 °C, and air-dried for 30 min. Both cells and tissue sections were blocked with 10% normal goat serum for 30 min and stained with affinity-purified primary antibodies in PBS solution with 0.05% Tween 20 with 3% BSA for 1 h, followed by Alexa Fluor 555- or Alexa Fluor 488-conjugated anti-IgG antibodies of the corresponding species in blocking buffer. Biotinylated virus was detected by using streptavidin conjugates labeled with Alexa Fluor 488 (Molecular Probes). After each step, cells were washed at least three times with PBS solution containing 0.05% Tween-20. Coverslips were mounted on slides with ProLong antifade reagent (Molecular Probes) and examined by using a Zeiss LSM 510 confocal fluorescence microscope. The images were processed by using LSM Image Examiner software (Zeiss). Quantitative colocalization measurement was performed by using Zeiss LSM 510 Examiner Software. Pearson correlation coefficient was calculated for describing the colocalization correlation of the intensity distributions between two channels as previously described (2).

Analysis of Intracellular Distribution of Nucleoprotein Protein After PR8 Infection in Presence or Absence of Y8 mAb. MDCK-FcRn cells were cultivated on coverslips for 24 h. Cells were treated with 400 $\mu\text{g}/\text{mL}$ Y8 mAb or isotype-matched IgG for 1 h. Cells were infected with PR8 virus at a multiplicity of infection of 100 pfu/cell at 4 °C for 1.5 h. After they were washed with cold PBS solution three times, cells were shifted to 37 °C in culture medium and collected at 10, 30, 45, 60, 120, or 240 min. Cells were stained with primary anti-EEA1, LMAP-2, and anti-NP mAb. Other staining procedures are the same as the described for immunofluorescence and confocal microscopy. Quantitative colocalization measurements were performed by using Zeiss LSM 510 Examiner Software. Pearson correlation coefficients were calculated for describing the colocalization correlation of intensity distributions between the two channels. In the quantitative experiment with MDCK-FcRn cells, 10 cells per view field were analyzed. $P < 0.05$ was considered as significant.

Passive Protection of WT and FcRn KO Mice Against PR8 Virus by mAb.

Groups of five mice were injected i.p. with 100 μL of PBS solution with 100 μg Y8 or mouse IgG 4 h before challenge to allow distribution and equilibration of antibody to all tissues before virus inoculation. One group of five mice was mock-immunized with PBS solution following the same schedule. Mice were inoculated with 500 pfu PR8 viruses intranasally under an anesthesia induced with 100 μL of 40 mg/mL tribromoethanol (Avertin; Sigma). Mice were kept on their backs under the influence of anesthesia for 45 min to allow infection. Mice were monitored for 10 d for illness and death. Body weight changes were recorded on a daily basis. For virus titration in the lung, viruses were inoculated into MDCK cells and cultured for 3 d, and TCID₅₀ values were measured. Experiments were performed at least three times.

Pathology. To assess pulmonary inflammation after PR8 virus infection, lungs were taken from experimental mice to examine the gross pathologic changes after biopsy. Lungs were also immediately placed in 10% neutral buffered formalin and sent to American HistoLabs, where they were embedded in paraffin and stained with H&E to visualize cellular inflammation. Slides were coded and read "blind."

Statistics. All results are expressed as geometric mean titers with SD of at least three independent experiments. Statistical significance was determined by Student *t* test (two-tailed) and the level of significance was set at $P < 0.05$. For animal survival data, statistical significance was determined by the log-rank test (Kaplan–Meier survival analysis). The analyses were conducted by using GraphPad Prism.

SI Results

Characterization of Influenza PR8-Specific Y8 mAb. Mouse IgG2a Y8 mAb binds to the globular domain of HA. Its cognate epitope is located at the interface of adjacent subunits. For this reason, Y8 mAb can bind influenza virus PR8 HA monomers but not native trimers (4). We further characterized the Y8 mAb in comparison with another HA-specific neutralizing mAb, H36-4, which can bind HA native trimers. Confluent MDCK cells were infected with influenza virus for 1.5 h at 4 °C to allow virus attachment to the cell surface and then at 37 °C for 30 min to permit viral endocytosis. The monolayers were stained with Y8 or H36-4 mAb with or without permeabilizing the cells. As shown in Fig. S1A, H36-4 mAb incubation resulted in a granular appearance of fluorescence staining; in contrast, antibody Y8 remained unreactive with virus particles adsorbed to the cell surface. Consistent with Fig. S1A, H36-4, but not Y8 mAb, can react with surface virus (Fig. S1B). When the infected cells were warmed to 37 °C for 30 min before staining, both the H36-4 and Y8 mAbs reacted with the virus particles in permeabilized cells, as shown by the presence of discrete fluorescent spots in the cytoplasm (Fig. S1B), suggesting the Y8 mAb only recognizes intracellular HA. To further evaluate the difference of binding activity between the Y8 and H36-4 mAb, influenza virus PR8 was incubated with Y8 or H36-4 mAb in pH 5.0 or 7.4 buffer, readjusted to pH 7.4, and followed by HI assay. Treatment of purified influenza virus at pH 5.0 leads to irreversible conformational alterations in HA proteins (3). As shown in Fig. S1C, the H36-4 mAb had potent HI activity at acidic and neutral pH; however, the Y8 mAb had HI activity only at an acidic pH. These results demonstrate that, unlike H36-4 mAb, Y8 mAb can only detect PR8 HA in conformational alterations induced by an acidic pH. Our data are in agreement with previous findings that Y8 binds HA on intact virions in a manner dependent upon conformational changes that accompany acidic activation of viral fusion activity (4). Therefore, the pH sensitivity of Y8 mAb

provides a unique tool to investigate the potential of FcRn-mediated IgG transport to block viral infection in epithelial cells.

Neutralization of Influenza PR8 Virus by Y8 mAb Is Dependent on IgG Transcytosis. Nocodazole, a reversible inhibitor of microtubule polymerization, has been shown to efficiently block IgG transcytosis (5). We showed that preincubation of MDCK-FcRn monolayers with 33 μ m nocodazole abolished the apically directed transport of Y8 IgG in a transcytosis assay (Fig. S3B). Likewise, the virus titers in MDCK-FcRn/IgG cells that were pretreated with nocodazole reached comparable levels as those observed in untreated MDCK-vector cells. Although it was not tested directly, it is expected that MDCK cells will return to a normal cell state following nocodazole removal, thus, the 2-h nocodazole pretreatment should not significantly impact normal viral replication during the subsequent 24-h incubation.

In Vivo Transcytosis of Y8 mAb. By Western blot analysis, FcRn is highly expressed in respiratory epithelial cells (Fig. 4A and Fig. S4). We subsequently tested whether murine FcRn can mediate IgG transport across the airway mucosal barrier in WT vs. FcRn-KO mice. We administrated biotin-IgG into WT (100 μ g) or FcRn-KO (200 μ g) mice i.p. The rationale for injecting twofold more IgG into FcRn-KO mice is both endogenous and injected IgG will exhibit fast clearance in these mice; thus, more IgG is required in FcRn-KO mice to obtain comparable exposure levels. As a specificity control, we also injected 200 μ g of chicken IgY-biotin into the WT mice. Lung bronchoalveolar lavage

(BAL) samples were taken 24 h after each injection and subjected to avidin blot analysis. IgG was detected in the BAL of WT mice (Fig. 4B). The failure to detect IgG in FcRn KO mice and chicken IgY in WT mice is consistent with specific transport of IgG by across alveolar tissue by FcRn in vivo.

Expressions of FcRn and pIgR in Mouse Airway Tissues. Although both IgA and IgG are transcytosed by Fc receptors (6), IgG is the major Ig isotype detected in human bronchoalveolar fluid. We reasoned that the differential expression levels of FcRn and pIgR, which transcytoses IgA through the polarized epithelial cells, might explain this discrepancy in the BAL. We thus examined the levels of mouse FcRn and pIgR expression in the lung and trachea of adult mice by immunofluorescence staining (Fig. S4) and Western blot analysis (Fig. 4A). The liver and intestine were used as controls. FcRn was detected in the epithelial cells of both trachea and lung (Fig. 4A and Fig. S4). However, the pIgR was barely detectable in the lung alveolar epithelial cells, although it was detected in the bronchial and tracheal epithelial cells. As expected, the pIgR was abundant in the epithelial cells of the liver and small intestine. However, mouse FcRn was detected in only the liver (Fig. 4A). These results may explain why large amounts of IgG, but not IgA, appear in the BAL. This observation may have biological significance for antibody-mediated immunity against respiratory infections in lung tissues.

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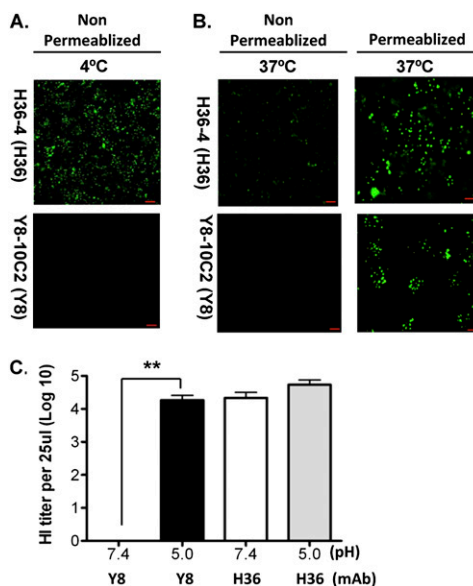


Fig. S1. Characterization of influenza PR8 HA specific monoclonal antibody. (A and B) MDCK cells or their transfectants (1×10^6) were infected with influenza virus PR8 (100 pfu/cell) for 1.5 h at 4 °C (A) or switched to 37 °C (B) for 30 min. Cells were fixed with 3.7% paraformaldehyde for 15 min under either non-permeabilized or permeabilized (0.2% Triton X-100 in PBS solution) conditions as indicated. Cells were incubated with affinity-purified PR8 HA-specific IgG mAb H36-4 (H36) or Y8 (400 μ g/mL), followed by Alexa Fluor 488-conjugated IgG (green). Staining was not observed in the presence of isotype matched IgG. Images were originally obtained at a magnification of 40x. (Scale bars: 5 μ m.) (C) HI titer of H36 and Y8 mAb under different pH conditions. H36 or Y8 mAb (400 μ g/mL) was incubated with PR8 virus at pH 5.0 or pH 7.4 buffer conditions for 1 h, readjusted to pH 7.4, and followed by HI assay with 0.5% chicken red blood cells for 30 min at room temperature. HI titers were expressed as the reciprocal of the highest dilution of the mAb that completely inhibited hemagglutination (** $P < 0.01$).

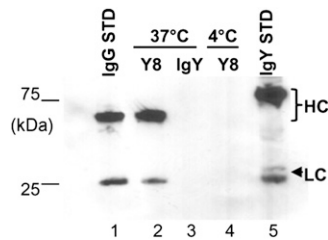


Fig. S2. Transcytosis of Y8 IgG. MDCK-FcRn cells were loaded with biotin-Y8 IgG (lane 2) or biotin-chicken IgY (lane 3) at 37 °C, or biotin-Y8 IgG at 4 °C (lane 4) in basolateral chamber. Medium was collected from the apical side 2 h later and subjected to SDS/PAGE and avidin blot-ECL analysis. IgG or IgY heavy chain (HC) and light chain (LC) are indicated (STD, standard). Intact Y8 IgG, but not chicken IgY, applied to the basolateral side was transported across this MDCK-FcRn monolayer at 37 °C, thus confirming that the observed transcytosis is mediated by FcRn.

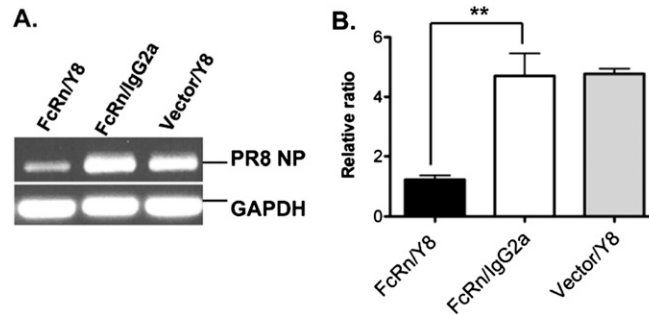


Fig. S3. Detection of the PR8 NP gene. (A) Total RNA was extracted from PR8-infected MDCK-FcRn or MDCK-vector cells by TRIzol reagent during transcytosis. The influenza PR8 NP or GAPDH gene was amplified by one step RT-PCR as described in *Methods*. GAPDH was used as an internal control to monitor the quality of the RNA purification and cDNA synthesis. The PCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide. (B) Densitometry analysis of amplified NP cDNA band intensities normalized to GAPDH (** $P < 0.01$).

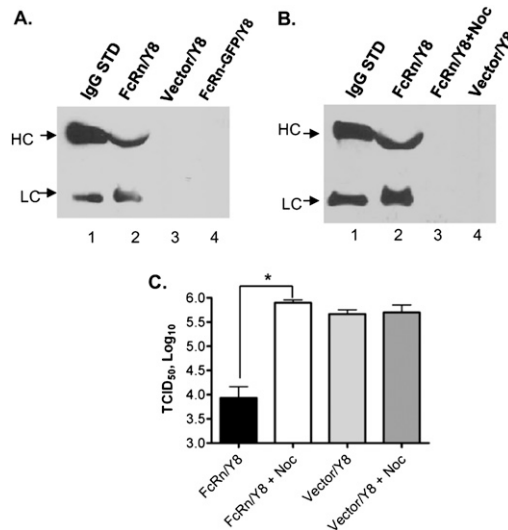


Fig. S4. Neutralization of influenza PR8 virus by Y8 mAb is dependent on IgG transcytosis. MDCK-FcRn, MDCK-FcRn-GFP, and MDCK-vector cells (1×10^5 /well) were grown in a 0.4- μ m 12-well transwell insert and allowed to polarize. (A) The polarized MDCK-FcRn, MDCK-FcRn-GFP, and MDCK-vector cells were loaded with biotin-Y8 IgG (400 μ g/mL) at 37 °C in basolateral chamber. Medium was collected from the apical compartment 2 h later, and subjected to SDS/PAGE electrophoresis and avidin blot-ECL analysis. IgG heavy chain (HC) and light chain (LC) are indicated by arrows to the left of the IgG standard (STD, lane 1). (B) Polarized MDCK-FcRn or MDCK-vector cells were incubated in the absence (lane 2) or presence (lane 3) of nocodazole (33 μ M) for 2 h, and nocodazole was then removed; subsequently, Y8 mAb (400 μ g/mL) was added into the basolateral chamber to allow apically directed transport of Y8 IgG. Medium was collected from the apical compartment 2 h later, and subjected to SDS/PAGE electrophoresis and avidin blot-ECL analysis. IgG heavy chain (HC) and light chain (LC) are indicated by the arrowheads to the left of the IgG standard (STD, lane 1). (C) Polarized MDCK-FcRn or MDCK-vector cells were incubated in the presence or absence of nocodazole (33 μ M) for 2 h, and nocodazole was then removed from the chambers. Y8 mAb (400 μ g/mL) was added into the basolateral chamber for 2 h to allow apically directed transport of Y8 IgG. PR8 virus was added to the apical chamber for 45 min at 4 °C to allow for infection. Cells in both chambers were completely washed to remove the residual IgG or virus. Cells were then incubated for an additional 24 h at 37 °C. The amount of PR8 virus in the apical medium was analyzed by a TCID₅₀ assay (* $P < 0.05$).

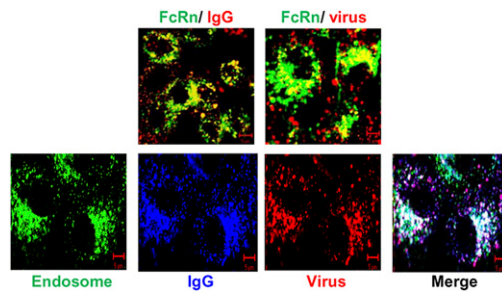


Fig. S5. Colocalization of FcRn, EEA1, IgG, and PR8 virus in MDCK-FcRn cells. MDCK-FcRn cells grown on coverslips were incubated with 200 μ g Y8 Ab for 2 h at 37 $^{\circ}$ C and the monolayers were then rinsed with cold PBS solution to remove residual Ab. Subsequently, the cells were infected with influenza PR8-biotin for 1 h, and then washed, fixed, and permeabilized. The cells were incubated with affinity-purified rabbit anti-FcRn and mAb anti-EEA1, followed by Alexa Fluor 488-, 555-, or 633-conjugated IgG of the corresponding species. Punctuate staining that appears in yellow in the merged images (*Upper*) indicates colocalization of FcRn with the IgG or virus. Similar results were observed from three independent experiments. (Scale bars: 5 μ m.)

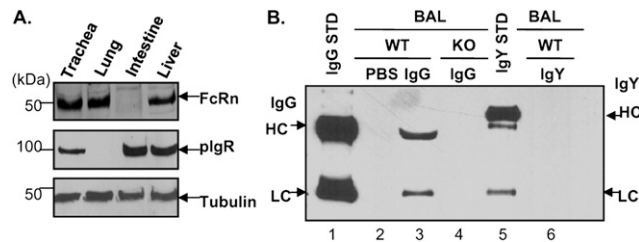


Fig. S6. (A) Western blots analysis of mouse FcRn expression. Epithelial cell lysates (50 μ g) from the trachea, lung, liver, and intestine in FcRn WT and KO mice were subjected to SDS/PAGE. Immunoblotting was performed with the primary antibodies and HRP-conjugated secondary antibodies corresponding to different species. Blots were developed with ECL. (B) IgG transport across the lung mucosal barrier. The 100- μ g (lane 3) or 200- μ g (lane 4) biotin-labeled IgG and 200 μ g chicken IgY (lane 6) were intraperitoneally administered into WT and FcRn KO mice. PBS solution was used as a negative control. Twenty-four hours later, BALs were sampled and subjected to SDS/PAGE. The proteins were blotted with HRP-conjugated avidin and developed with ECL. IgG or IgY heavy chain (HC) and light chain (LC) are indicated by arrows (STD, standard).

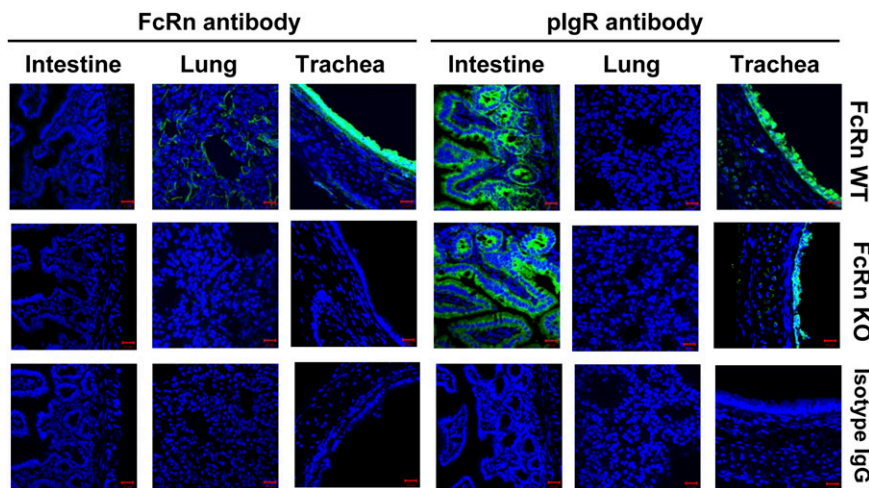


Fig. S7. Immunohistochemical staining of mouse FcRn expression in adult mice. Frozen sections of trachea, lung, and intestine tissues obtained from WT or FcRn KO mice were stained with affinity-purified rabbit anti-FcRn or anti-pIgR antibody (2 μ g/mL) and followed by Alexa Fluor 488-conjugated IgG (green). FcRn or pIgR staining was not observed in the presence of control IgG. The nucleus is stained with DAPI (blue). The data are representative of sections from at least three independent mice. Images were originally obtained at a magnification of 40 \times . (Scale bars: 20 μ m.)

