Supporting Information

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SI Materials and Methods

Muc5ac-GFP cDNA Cloning and Transfection. Muc5ac genomic regions corresponding to exons 1–31 (19,200 bp) and exons 31–49 (9,714 bp) were available through GenBank under accession numbers AJ511870 and AJ511871. Computer analysis predicted the location of all 49 exons, which allowed generation of a Muc5ac cDNA template and primer design that included the Kozack consensus sequence/start codon and the stop codon. mRNAs were extracted from C57BL/6J mouse lungs and reverse transcribed. Muc5ac cDNA was cloned into three fragments [5′ end, variable number of tandem repeats (VNTR), and 3′ end], using high-fidelity polymerases (Herculase DNA polymerase; Stratagene). Clone assembly to generate the full-length cDNA was achieved via unique restriction sites and ligation. GFP tagging was performed internally, immediately 5′ of the VNTR, by use of enzyme digestion and ligation. Mutagenesis was performed to introduce glycine and alanine residues at each end of the GFP insert to allow proper protein folding. Full sequencing of the GFP-tagged cDNA confirmed that no random mutations occurred during cloning and verified proper insert direction. The Muc5ac-GFP fusion protein was cloned into the expression cassette consisting of the rat CCSP promoter (1) in a pTG1 plasmid (2) [University of North Carolina (UNC) Animal Model Core] that was successfully used for another model (3). TG1-rCCSP-Muc5ac/GFP (15 kb) was used to transfect 293T cells via lipofectamine as described by the manufacturer (Invitrogen), and positive cells were identified 48 h after transfection via fluorescence microscopy.

Generation of Transgenic Animals and Real-Time RT-PCR. All mouse studies were approved by the UNC Institutional Animal Care and Use Committee. Transgenic mice with airway-specific overexpression of Muc5ac-GFP were generated by microinjection of fertilized C57BL/6 oocytes with the expression cassette pTG1-rCCSP-Muc5ac/GFP. Founder animals were bred with C57BL/6 mice, producing independent lines of Muc5ac-GFP transgenic mice. Transgene-positive animals were identified by PCR for GFP expression. All studies reported in this article were conducted with a single transgenic mouse line that showed the highest level of transgene expression. WT littermates served as controls. We monitored animal weight from birth to adult stage and detected no significant differences between transgenic and WT littermate controls. Mice were housed in a pathogen-free animal facility and had free access to chow and water. Total lung mRNAs were harvested from whole lung homogenates, including trachea. For proximal vs. distal airway analyses (which excluded the trachea), each lung lobe was dissected by a median cross-section, separating proximal from distal regions before homogenization. Real-time PCR analysis from reverse-transcribed RNA (RNeasy kit; Qiagen) was conducted using a Roche LightCycler. Specific primers were designed to detect endogenous and exogenous Muc5ac simultaneously (shown in Fig. S1) and specific primers were designed to detect Muc5b (Fwd-CTGGCACCTGCTCTGTGCA and Rev-CACTGCTTTGAGGCAGTTCT). mRNA primers (18S) were used for expression normalization. The fold increase of mucin mRNA expression was determined by crossing-point analysis and standard curves (LightCycler Software version 3.5; Roche).

Bronchoalveolar Lavage and Inflammatory Cell Counts. Bronchoalveolar lavage (BAL) was performed to collect fluid and quantify mucin, lung inflammatory cells, and virus titers as previously described (4). Briefly, mice were euthanized with CO_2 and tracheas cannulated with a blunt 20-gauge needle via an incision. Whole lungs were lavaged once with 30 $\mu L/g^{-1}$ body weight of PBS. Collected BAL samples were processed dependent on the proposed experiment, to detect either protein expression or inflammatory cell counts. For cell counts, BAL cells were pelleted by centrifugation at $1,000 \times g$ for 5 min at 4 °C, cells resuspended in 100 μL of PBS, and total cells counted with a hemocytometer. Cytospin slides of 30,000–60,000 cells per slide were obtained (StatSpin CytoFuge 2), air dried, and stained with modified Giemsa for differential cell counts (Newcomer Supply) of at least 200 cells per slide.

Antibodies and Western Blotting. Western blotting and immunohistochemistry for GFP and CCSP expression used a goat polyclonal anti-GFP antibody (Rockland), a rabbit polyclonal anti-GFP antibody (Abcam), and a goat anti-CCSP antibody (Santa Cruz). Rabbit polyclonal antibodies to Muc5b (UNC222) (5) and Muc5ac (UNC294) were generated by immunizing rabbits with synthetic peptides corresponding to unique peptide sequences (CQPQCQWTKWIDVDY and HYITFDGQRYSFNGD, respectively). Antibody specificity was demonstrated by peptide BLAST and Western blotting, as shown in Fig. 3. To assess the expression of Muc5ac-GFP in transgenic animals, BAL was used to harvest lung secretions. Briefly, equal amounts of bronchoalveolar lavage fluid (BALF) samples (40 μL) were loaded into an agarose gel (0.8%) under native or reducing conditions (10 mM DTT for 5 min at 70 °C). BALF mucins were resolved by electrophoresis (80 V, 90 min) and transferred by vacuum blotting (VacuGene XL; GE Heathcare) to nitrocellulose membranes. Blots were probed with Muc5ac, Muc5b, and/or GFP antibodies. Despite pooling and concentrating BALF from several WT animals, the endogenous Muc5ac signal was weak with an elevated background, limiting studies in WT mice with the available biological tools. IRDye secondary antibody fluorescence was detected with a Li-Cor Odyssey Infrared Imaging System. Detection of sialic acid residues used the biotinylated anti-lectins, maackia amurensis (MAA) and elderberry bark (SNA) for α2,3- and, α2,6-linked sialic acids (Vector Laboratories), respectively, a goat anti-biotin antibody (Vector Laboratories) and a donkey anti-goat antibody labeled with IRDye (Licor).

HPLC and Refractometry/Light Scattering and Density Gradient. Pooled mouse BALF was run through a size-exclusion S1000 column, collecting the void into 0.5-mL aliquots (as described in ref. 6). A light-scattering detector and a reflective index detector connected to a Dawn program and a MALLS system were used to monitor the constituents of each fraction, thus providing data on molecular weight (absolute molecular size and mass distribution) and radius of gyration/compactness. Fractions obtained by gel permeation chromatography were subjected to slot-blotting and either stained with alcian blue periodic acid–Schiff (AB-PAS) to determine glycoprotein-rich fractions or immunoblotted. For CsCl density gradient experiments, CsCl was added to pooled BALF samples and placed into 10-mL ultracentrifugation tubes (as described in ref. 7). Centrifugation was performed for 48 h, tubes were reopened, and ~1-mL aliquots were carefully collected from the top to the bottom of the tube without disturbing the layered solution. In this technique, sedimentation depends on the surface-to-mass ratio. Fractions were subjected to AB-PAS staining and immunodetection.

Tissue Histology. Lungs fixed in 10% (vol/vol) neutral buffered formalin and embedded in paraffin were prepared as histological sections. After deparaffinization, sections were stained with hematoxylin and eosin (H&E) or AB-PAS or blocked for 1 h in 3% BSA, before

immunohistochemistry was performed. Primary antibody (goat anti-GFP) was applied overnight, followed by a secondary antibody (anti-goat Alexa Fluor 488). Slides were mounted with an antifade, DAPI medium (Vectashield; Vector Laboratories). Fluorescence was detected with a Leica SP2 laser scanning confocal microscope, using 20X/0.4 air, or an AchroPlan ×63/1.4 oil-immersion objective lens. For studies of mucus layer morphology, lungs were fixed with 1% osmium tetroxide in perfluorocarbon (OsO_4/PFC; FC-72) (8) and embedded in epon resin, and sections were prepared for electron microscopy. For frozen sections, lung tissue specimens were embedded in OCT (Cryo Embedding Medium) and rapidly frozen on dry ice before performing cryosections with a microtome. For fresh tissue preparation, tracheas were embedded in a low-melting temperature agarose, sectioned with a vibratome, and transferred to histology slides.

Ovalbumin Sensitization. Airway mucus metaplasia was induced in mice via ovalbumin (OVA) sensitization and challenge, using a procedure detailed previously (9). Briefly, OVA was injected i.p. into each mouse on days 0, 7, and 14. On days 21 and 24, 50 μL of 2.0% OVA in PBS was instilled by aspiration into tracheas of isoflurane-anesthetized mice. The instillation procedure was completed in <20 s; recovery from the procedure was 100%. Experimental procedures were performed 2–3 d following the second OVA instillation.

Mucociliary Clearance Measurement. To measure mucociliary transport in the trachea (as described in ref. 10), mice were anesthetized with a mixture of ketamine/xylazine (90 and 10 mg/kg body weight, respectively) and placed on a heated pad, and a rectal temperature probe was inserted to monitor body temperature. The trachea was surgically exposed (not opened) and a drop of water-equilibrated mineral oil was placed on the exterior of the trachea to prevent desiccation. Then a 15-μL suspension of 3 μm fluorescent beads (Polyscience) (prepared by adding 10 μL stock beads to 2 μL egg white and 99 μL distilled water) was injected into the mouse's nose. Sufficient beads were aspirated for visualization through the exposed trachea. The mouse was positioned with the head elevated ~10° and tracheal transport of fluorescent beads was recorded using a Leica MZ 16FA fluorescent dissecting microscope and a digital camera interfaced to a DVD recorder. The rate of MCC was determined by measuring the time required for fluorescent beads to move a measured distance by an investigator blinded to the animal's genotype. Usually, 5–10 particles were tracked for each animal and the average was taken as the rate of MCC. After MCC was measured on the anesthetized mouse, the mouse was killed by exsanguination (aorta sectioning) and the MCC measurements were repeated. Results are presented as an average of live and dead animals.

Viral Infection and Titer. The influenza virus A/PR/8/34 (H1N1) (Reading) has been extensively characterized as in refs. 11 and 12. In brief, as assessed by sequencing and sequence alignment, this A/PR/8/34 (H1N1) (Reading) exhibited a lineage consistent with the Mount Sinai lineage and was shown biochemically to preferentially bind α2,3-sialic acid. This PR8 virus was propagated on Madin–Darby Canine Kidney (MDCK) cells. Mice were briefly anesthetized with isoflurane and inoculated with 700 pfu of influenza virus via the intranasal route in 20 μL of PBS. Mice were weighed daily and assessed for visual signs of clinical disease, including inactivity, ruffled fur, labored breathing, and huddling behavior. At specific times after inoculation (day 2 and day 4), mice were euthanized, the lungs harvested, and the right lower lobes homogenized for virus titers, and the left lobe was fixed for histology studies. Virus titers were determined by standard plaque assay on MDCK cells, as previously described (13). These experiments were performed in three litters in separate experiments at three designated time points. A total number of 18 mice were inoculated for in vivo measurement of infection as determined by viral titers.

For inflammatory response analysis, a total of 10 mice were inoculated in independent experiments. Inoculation occurred as described above. Mice were euthanized and lungs were lavaged at day 2 p.i. For BALF cell counts, see *Bronchoalveolar Lavage and Inflammatory Cell Counts*.

To determine whether BALF or purified mucins neutralized influenza virus infection, virus was incubated directly with BALF or purified Muc5ac and plaque assays were performed. WT and Muc5ac-transgenic (Muc5ac-Tg) BALFs were collected from 10 different animals for each group and pooled, reaching a total protein concentration of 1 mg/mL. BALF pools were UV treated for sterilization. Purified Muc5ac was obtained from BALF pooled from 10 Muc5ac-Tg animals. The BALF pool was treated with 6 M guanidine to dissociate small globular proteins and Muc5ac was purified by a CsCl density gradient, detecting Muc5ac-rich fractions by slot blot. Briefly, influenza virus stocks were diluted to 10^4 plaque-forming units (pfu)/mL in PBS. Diluted virus (100 μL) was mixed with 100 μL of PBS alone, PBS + BSA (2 mg/mL), WT BALF (1 mg/mL), Muc5ac-Tg BALF (1 mg/mL), or purified Muc5ac (2 mg/mL) and incubated at room temperature for 20 min. Each mixture was then serially diluted (10-fold dilutions) in serum-free DMEM and applied to MDCK cell monolayers for 1 h at 37 °C. The inoculum was then removed, and cells were treated with 2% agar-based overlay medium. Three days following overlay, MDCK cells were fixed and stained with crystal violet to visualize and quantitate plaques.

The same technique was applied for BALF treated with neuraminidase. BALFs were treated for 1 h at 37 °C with neuraminidase (150 milliunits/mL). Neuraminidase was deactivated by heat at 60 °C for 5 min before incubation with the virus. Treated and untreated BALFs were incubated at room temperature for 20 min with the virus and applied in serial dilutions to MDCK cells (as described above).

Statistical Analyses. Results are expressed as means ± SEM. Statistical analyses were performed using *t* tests or GraphPad Prism 4.0 for survival curves. A P value of <0.05 was considered as the minimum acceptable probability for the difference between the means.

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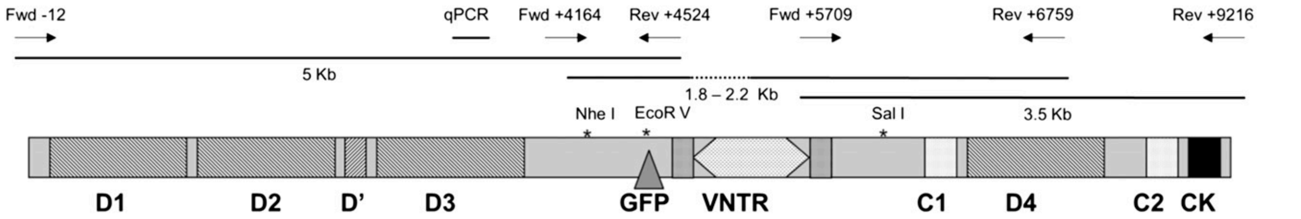

Fwd -12:	5'-TTGCTCCACAGCATGGGTGT-3' (start codon)	Rev +4524:	3'-AACCTCCTCAGTCCCTGTTACC-5'
Fwd +4164:	5'-TCTGGTCTCCCTGGATGGATGTTA-3'	Rev +6759:	3'-TAGCCAGGATTGTCAGGTGG-5'
Fwd +5709:	5'-CTCAGGGTCCACCATGCCTT-3'	Rev +9216:	3'-TTGGGCCTTTCCAGTAAACT-5' (stop codon)
qPCR Fwd:	5'-ACCACTTTCTCCTTCTCCACA-3'	qPCR Rev:	3'-ATGGATGTTAGCCGTCCTG-5'

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Fig. S1. (Continued)

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8901 TCTGCGCTGC GATGATGGCT CCAGTCAGAC CTTCAGCTAC ACCCAGGTTG AGAAGTGTGG CTGCCTGGGC CAGCAGTGCC ATGCTTTAGG GGACACAAGC
9001 CATGCAGAGT CCTCAGAACA AGAGTTCAAG TCCAAGGAAA GTGAGGAACA TGGCCAGCAG TTGGCATTCA GGGTCAGCGA GGACATGCTT GGGCCTTTCC
9101 AGTAAACT

Fig. S1. Muc5ac-GFP cDNA cloning strategy and sequence. Shown are primer map and sequences used to clone Muc5ac cDNA. Primers (shown as arrows) were designed from the reconstructed Muc5ac cDNA sequence. Lines below primers characterize the three clones obtained by high-fidelity PCR. Dotted line shows a region with high variability. Unique restriction sites (NheI, SalI, and EcoRV) were used for clone assembly. Muc5ac cDNA sequence that was cloned and overexpressed in mice is shown with start and stop codons in red, GFP tag in green, and primer location in blue. Quantitative PCR primers are underlined.

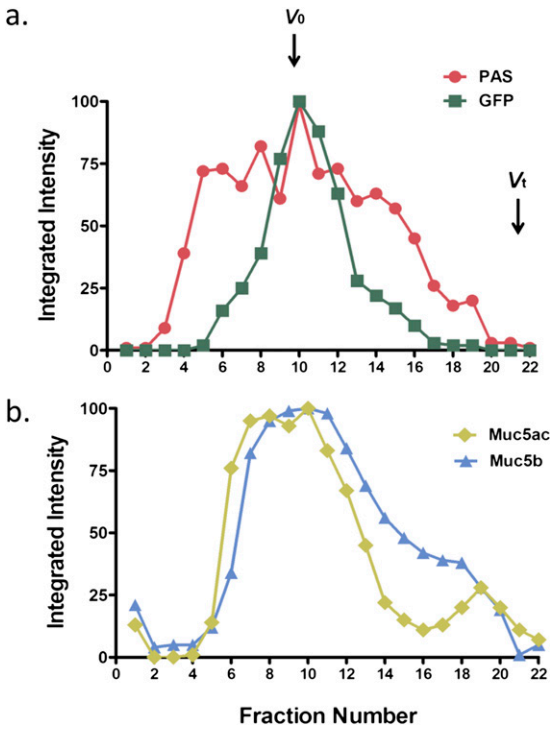

Fig. S2. HPLC/light scattering analysis of Muc5ac-Tg BALF. BALF samples from Muc5ac-Tg animals were pooled, denatured in 6 M GuHCl, and passed through a size-exclusion S1000 column. The void volume or V_o (i.e., large excluded polymers) was analyzed via light scattering and reflective index detectors connected to a Dawn program and a MALLS system to determine the average molecular weight ($M_r = 5.1 \times 10^7$ g/mol) and radius of gyration ($R_g = 228$ nm) of the V_o peak. The value for each parameter is consistent with mucin constituents. The V_o volume was collected in 0.5-mL aliquots that were subjected to slot-blotting. (*A*) Membranes were either stained with periodic acid–Schiff (PAS) to distinguish glycoprotein-rich fractions or probed with GFP. Integrated intensity is shown as a graph, revealing that GFP and PAS coelute in the V_o volume. (*B*) The V_o fractions were also analyzed by slot blot, using Muc5ac (UNC294) and Muc5b antibody. Integrated intensity is shown as a graph. The results show that Muc5ac, dominated by the transgenic form, coelutes with endogenous Muc5b in the V_o (void) volume.

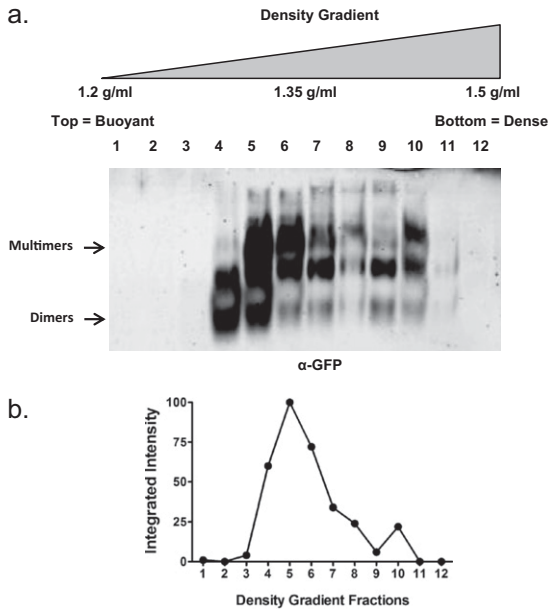

Fig. S3. Isopycnic density-gradient centrifugation, in CsCl/4 M guanidinium chloride, of Muc5ac-Tg BALF to confirm transgene multimerization and glycosylation. Equilibrium density gradient centrifugation was used to separate molecules with high buoyancy (top of the gradient) from dense material (bottom of the gradient) contained in pooled Muc5ac-Tg BALF. In this technique, sedimentation depends on the surface-to-mass ratio. Mucins possess a large carbohydrate backbone, which causes migration of these large glycoproteins through the gradient. Following centrifugation, 1-mL aliquots were carefully collected from the top to the bottom of the tube without disturbing the layered solution. (*A*) Fractions (1–12) were subjected to Western blotting to detect GFP-rich fractions, with fraction 4 dominated by dimers and tetramers, whereas other fractions (5–10) were dominated by denser high-range multimers. (*B*) Integrated intensity of slot-blot GFP staining is shown as a graph, revealing the strongest signal halfway through the gradient.

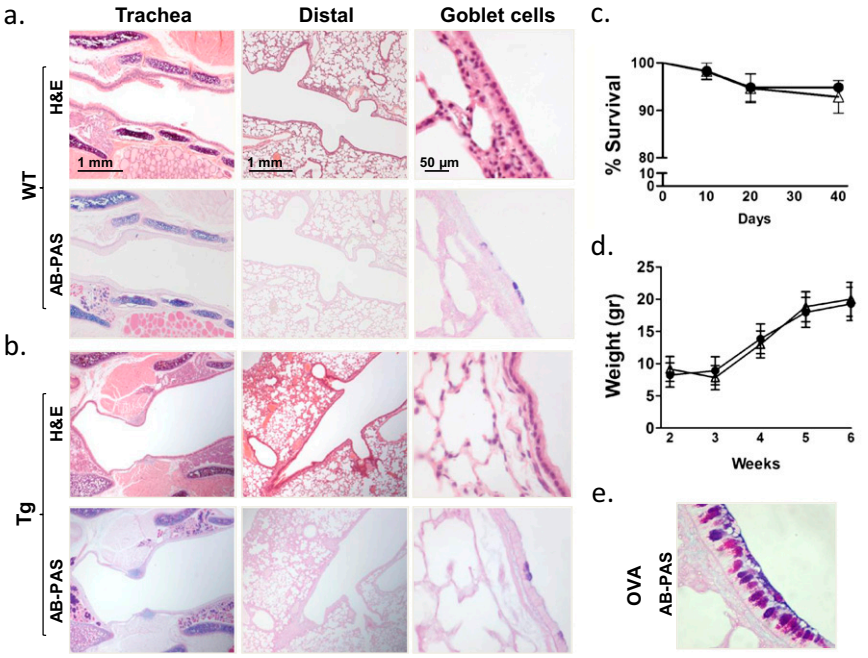

Fig. S4. Lung histology, survival, and growth curves of Muc5ac-Tg mice. Lung histology of 21-d-old WT (*A*) and Muc5ac-Tg (*B*) littermates with H&E and AB-PAS sections shown is representative of $n = 32$ Muc5ac-Tg mice and $n = 30$ WT mice. Survival (*C*) and growth curves (*D*) for transgenic (Δ, Tg; $n = 56$) and wild-type littermate controls (●, WT; $n = 52$) were compared and showed no difference. (*E*) Mucosubstance staining is compared in ovalbumin-synthetized (OVA) mice.

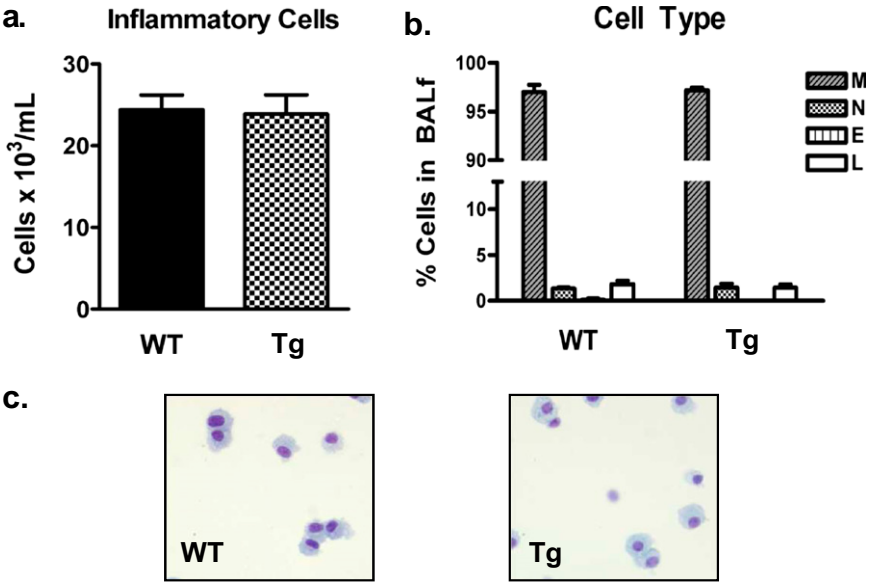

Fig. S5. Inflammation study on BALF from Muc5ac-Tg vs. WT mice. BALF was centrifuged and cells were totaled and stained with Newcomer's differential stain to identify inflammatory cells. (*A*) Total inflammatory cell count was determined for transgenic and wild-type littermate controls ($n = 5$ per group). (*B*) Percentage of inflammatory cell type was calculated in each animal (E, eosinophils; L, lymphocytes; M, macrophages; N, neutrophils) ($n = 5$ per group). (*C*) No macrophage activation was detected, as reflected in size measurements.

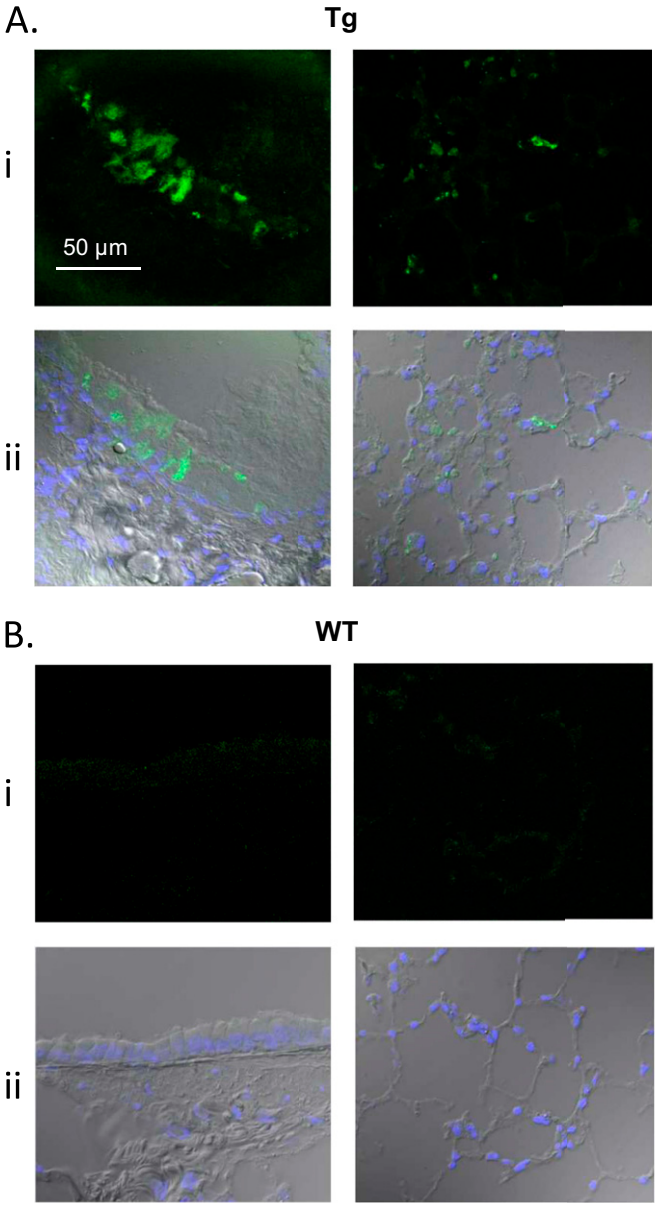

Fig. S6. Native GFP fluorescence from frozen Muc5ac-Tg and WT lung sections. Shown is confocal imaging of native GFP signal captured from Muc5ac-Tg (*A*, *i*) and WT (*B*, *i*) frozen sections, stained with DAPI for nuclear labeling. Cross-sections of mouse airways and parenchyma are shown. Overlaid GFP/DIC/DAPI images are displayed for Muc5ac-Tg (*A*, *ii*) and WT (*B*, *ii*) mice.

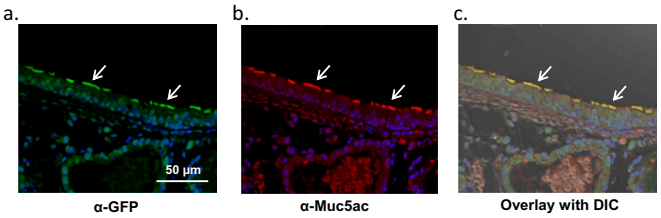

Fig. S7. Muc5ac-/GFP–positive layer lining the epithelial surfaces of Muc5ac-Tg mouse trachea. Longitudinal sections of Muc5ac-Tg trachea were stained with (*A*) a goat anti-GFP and (*B*) the rabbit anti-Muc5ac antibody (UNC294), which revealed an irregular mucus blanket coating the epithelial surfaces of Muc5ac-Tg animals. *C* shows the overlay of signals with DIC.

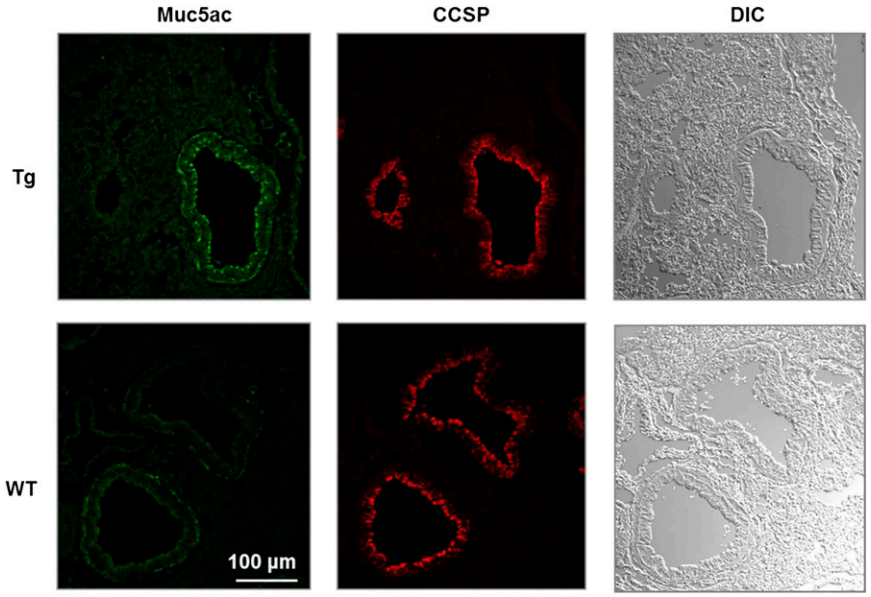

Fig. S8. Immunohistochemical detection of a Muc5ac layer lining the large airways of Muc5ac-Tg but not WT mice. Cross-sections of fixed WT and Muc5ac-Tg lungs were stained with the rabbit anti-Muc5ac and a goat anti-CCSP antibody. CCSP labeled all airways. Whereas Muc5ac was not detected in WT mice, a positive Muc5ac signal was detected lining the large airways of Muc5ac-Tg animals.

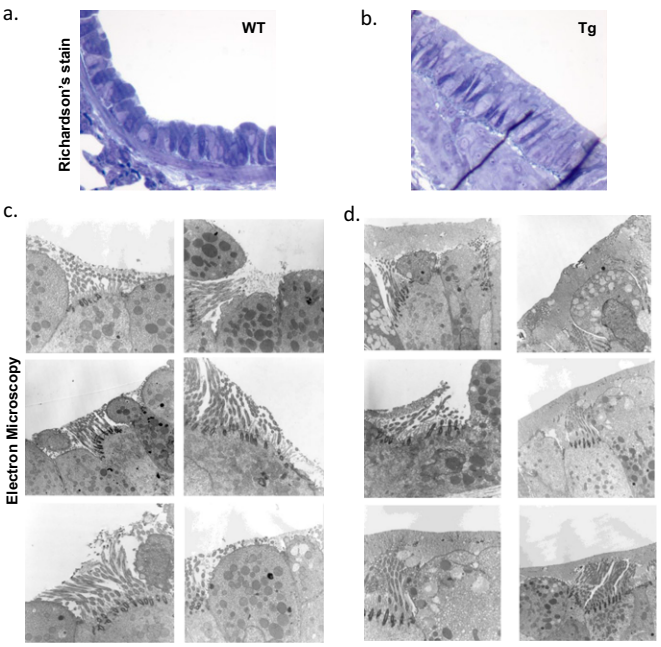

Fig. S9. Electron microscopy images illustrating the mucus layers observed in Muc5ac-Tg vs. WT animals. Lungs were fixed with perfluorocarbon-OsO4, sectioned at the midsection of the lobes. (*A* and *B*) Richardson's stain of WT (*A*) and Muc5ac-Tg (*B*) intermediate-size airways. (*C* and *D*) Electron microscopy images of selected airways that showed sporadic mucus patches in WT mice (*C*) and revealed the presence of a "thicker" mucus layer in Muc5ac-Tg mice (*D*). Images are representative of three animals from each genotype.

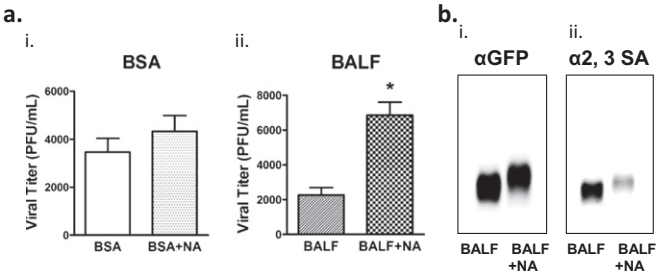

Fig. S10. Neuraminidase treatment of Muc5ac-Tg BALF restores PR8 infectivity in vitro. BSA or Muc5ac-Tg BALF was treated with neuraminidase (150 milliunits/mL) for 1 h to remove the terminal sialic acids. Neuraminidase was deactivated by heat (60 °C for 5 min) before incubation with PR8 virus (10^4 pfu/mL solution). (*A*) Neuraminidase treatment showed no effect with BSA control (*i*). However, treatment of BALF (~1 mg/mL) with neuraminidase restored PR8 infectivity (*ii*). Values are means ± SEM, $*P < 0.005$ ($n = 3$). These results suggest that sialic acids are, at least in part, responsible for the decreased infection observed with Muc5ac-Tg BALF. (*B*) Confirmation of effectiveness neuraminidase treatment via mucin agarose gel on reduced Muc5ac-Tg BALF. GFP signal (*i*) showed a delayed migration of Muc5ac-GFP in treated BALF compared with untreated BALF, which concurs with the removal of negative charges associated with sialic acids. α2,3 lectin-binding signal (*ii*) was greatly reduced in treated BALF.