Supplemental Material

Epithelial tethering of MUC5AC-rich mucus impairs mucociliary transport in asthma

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Supplemental Methods

Human tissue sections and human bronchial epithelial (HBE) cells. Postmortem lung tissues containing bronchi were obtained from 7 individuals with fatal asthma. Bronchial tissues from 3 lung transplant donors and 1 patient undergoing lung resection for metastatic carcinoma were processed identically and used as controls. HBE cells were isolated from lungs not used for transplantation (total of 9 individuals for all studies) and cultured on 6.5 or 12 mm Transwell inserts (Corning, Corning, NY) at air-liquid interface as described previously (1). After 21 days in culture, cells were maintained in medium with or without IL-13 (10 ng/mL, Peprotech, Rocky Hill, NJ) for the remainder of the culture period. After 28 days, cultures were washed by applying 100 μL phosphate buffered saline (PBS; Life Technologies, Carlsbad, CA) to the apical surface of the cultures for 15-30 min; after aspirating the apical medium, cultures were continued for 3 more days to standardize the duration of mucus accumulation as previously described (2, 3).

Preparation of lung sections and HBE cell cultures for immunofluorescence. Lung tissues were fixed with 10% neutral buffered formalin, processed in an automated tissue processor (Tissue-Tek VIP, Sakura Finetek USA, Torrance, CA) and embedded in paraffin. Cell culture inserts were prepared by aspirating culture medium from the basolateral compartment and adding Carnoy's solution, a non-aqueous fixative that preserves the mucus gel layer (4, 5), to both the basolateral (1 ml) and apical compartments (0.5 ml). After 30 min fixation at room temperature, inserts were washed twice in absolute methanol and twice in absolute ethanol. Some specimens (consisting of the filter and the attached cells and gel) were excised from the supports, cleared in xylenes and infiltrated and embedded on edge in paraffin while others were processed as whole mounts. Paraffin embedded microscopic sections (5 μm) of human bronchial tissue and HBE cell

cultures were de-waxed in xylene and rehydrated through a graduated ethanol series and water. Whole mount preparations of HBE cell cultures were also taken through a graduated ethanol series and water. For cytospin analysis, we removed accumulated mucus from the apical surface of HBE cultures with 10 mM dithiothrietol (DTT; Thermo Scientific, Fremont, CA, USA) in PBS for 10 min. The epithelial surface of the cultures was then washed thrice in PBS before incubation with 0.25 % trypsin-EDTA (UCSF Cell Culture Facility) for 10-15 min at 37 °C. The cell suspension was neutralized, passed through a 100 μm filter (Falcon), resuspended in paraformaldehyde (PFA; Electron Microscopy Sciences, Fort Washington, PA) for 30 min at room temperature, and deposited on microscope slides by cytospin.

Immunostaining. Nonspecific background staining was blocked by incubating in 5% normal goat serum in phosphate buffered saline with 0.05% Tween 20 for 30 min at room temperature prior to primary antibody incubation. The primary antibodies used were: mouse monoclonal anti-MUC5AC (clone 45-M1; Thermo Scientific; 1:150) (6), rabbit polyclonal anti-MUC5B (H-300; sc-20119, Santa Cruz Biotechnology, Inc., Dallas, TX, USA; 1:150) (7), and rabbit polyclonal anti-axonemal dynein intermediate chain 1 (HPA021843, Sigma-Aldrich, St. Louis, MO; 1:200) (8). After overnight incubation at 4 °C with 2 or more primary antibodies, slides were washed and incubated with appropriate secondary antibodies (AlexaFluor 488 goat anti-mouse, AlexaFluor 568 goat anti-rabbit, and AlexaFluor 405 goat anti-mouse; Life Technologies). All secondary antibodies were used at a 1:200 dilution and incubation was performed at room temperature for 2-3 h. 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. In some experiments, we used the CellMask Deep Red plasma membrane stain (649/666 nm; C10046; Thermo Scientific) to identify apical epithelial cell membranes; staining was performed on live cells for 15 min at 37 °C as per the manufacturer's instructions. Slides were washed with PBS

and post-fixed in 2% PFA and mounted with Fluoromount-G (Southern Biotech, Birmingham, AL).

Microscopy. Images were acquired using a Yokagawa CSU22 spinning-disk confocal microscope connected to a Nikon Ti-E (Nikon Imaging Center, UCSF). Slides were placed on the microscope stage and images were acquired using appropriate lasers and a 10× or 40× dry objective. A brightfield image was captured together with the fluorescent images to help differentiate between intracellular and extracellular staining. Identical acquisition settings were used throughout acquisition. For acquisition of images of sections to be used for quantification of immunostaining, fields of view along the epithelium were randomly selected using the bright field image (but blinded to the fluorescent staining). For acquisition of images of whole mount preparations, multiple fields were selected using random sets of x,y-coordinates and z-stack images were captured at an interplane distance of 0.5 μm. Images were exported as 16-bit files and processed using Fiji (9).

Image quantification. MUC5AC- and MUC5B-stained pixels within epithelial cells and within the extracellular gel were identified using the automated thresholding function in the Fiji plug-in JACOP (10). To count the number of cells in a field of view, images were separated into individual colors, thresholded and enumerated using the Analyze Particles plug-in. To assess the co-localization of MUC5AC with MUC5B, Manders' co-localization coefficients (11) were calculated using the JACOP plug-in (9). To measure the continuity of extracellular mucus with underlying mucous cells, we selected an image in the x-y plane from within the extracellular gel (approximately 10 μm from the membrane) of a z-stack of an IL-13 treated culture and randomly positioned a grid upon it using the grid plugin in Fiji. We then assessed whether each point in the grid was in contact with either MUC5AC or MUC5B and followed the point from the gel into

the epithelium along the z axis, thus assessing each randomly selected point in 3D. A point was defined as continuous if it was in contact with the either of the mucins from the gel through the to the epithelium; otherwise the point was defined as discontinuous. Measurements of cilia height were made from brightfield images acquired from sections of HBE cultures as previously described (12).

Mucin gene expression analysis. Mucin mRNAs were quantified by real time RT-PCR (qRT-PCR) as previously described (13). Briefly, HBE cell cultures were incubated with pre-warmed PBS to remove the airway surface liquid then lysed and homogenised in Qiazol. cDNA was prepared and qRT-PCR was performed using SYBR green chemistry. Each sample was run in triplicate and the median value of the technical replicates was used for analysis. Mucin mRNA levels were normalized to *GAPDH* levels using the ΔCt method.

Quantification of secreted and cell-associated mucins from human airway epithelial cells. To harvest apical secretions, pre-warmed PBS was applied to the apical surface of the HBE cell cultures and returned to the incubator for 30 min. We subsequently harvested the airway surface liquid, centrifuged to remove cellular debris (300 × *g*, 5 min), and then mixed secretions 1:1 with guanidinium buffer containing protease inhibitors. To harvest cell-associated mucins, HBE cells were lysed by applying 100 µL guanidinium buffer containing protease inhibitors to the apical side of the insert following removal of the apically accumulated mucin; the plate was then placed on an orbital shaker overnight at 4 °C. Lysates were then centrifuged and mixed with an equal volume of PBS in preparation for immunoblotting. 2-10 µL of serial dilutions of secreted and cell-associated samples in guanidinium buffer containing protease inhibitors were applied to a nitrocellulose membrane and allowed to dry. The membrane was then incubated in blocking buffer (4 % (w/v) milk solids in Tris-buffered saline with 0.05% Tween 20 [TBST]), washed

briefly in TBST, and incubated overnight with mouse anti-MUC5AC antibody (clone 45M1) or rabbit anti-MUC5B antiserum (H-300) (7) diluted 1:200 in blocking buffer. After washing in TBST cells were incubated with HRP-conjugated goat anti-mouse IgG (ab97040, Abcam, Cambridge, MA; for MUC5AC) or anti-rabbit IgG H&L (ab97080, Abcam; for MUC5B). The HRP-conjugated secondary antibodies were detected through oxidation of the chromogenic substrate 3, 3'-diaminobenzadine (DAB, 2%, Sigma-Aldrich) using 1% hydrogen peroxide. After stopping the reaction with distilled water and drying the blot, immunoblot intensities were quantified using the gel function in Fiji (9). A calibration curve was constructed using dilutions of a control sample.

Measurement of mucus transport. Mucociliary transport was measured using fluorescent microspheres with methods based on those used previously (2, 14-16). 2-μm fluorescent microspheres (FluoSpheres; Life Technologies) were used as they have been previously shown to be too large to enter mucus gels (2, 14-16). A 10 μL suspension of 2-μm green fluorescent microspheres (505/515) was added to the apical compartment of each HBE culture and allowed to sediment and disperse over a 15 min interval. A minimal volume of PBS was used to deliver the fluorescent microspheres in to minimize the effect on the ASL volume. We then transferred cultures to an optical cell dish (MatTek, Ashland, MA, USA) and placed them on the stage of a spinning disc confocal microscope under a dry 10× objective. Temperature was maintained at 37 °C and perfluorocarbon (Sigma-Aldrich) was added to the apical surface to prevent evaporation (16). Particle transport of the 2-μm fluorescent microspheres was imaged in the plane of the gel by recording sequential images every 1 s over a period of 10-15 s. We positioned cultures centrally on the microscope stage and moved to randomly generated x,y-coordinates. We analyzed images using the particle tracker within the Mosaic plugin (17). Briefly, particles in

consecutive images were identified by the plug-in and linked. We then calculated the speed of the fluorescent microspheres in the xy-plane. Images showing tracings of fluorescent microsphere movement were generated using the MTrackJ (18) plugin in Fiji (9).

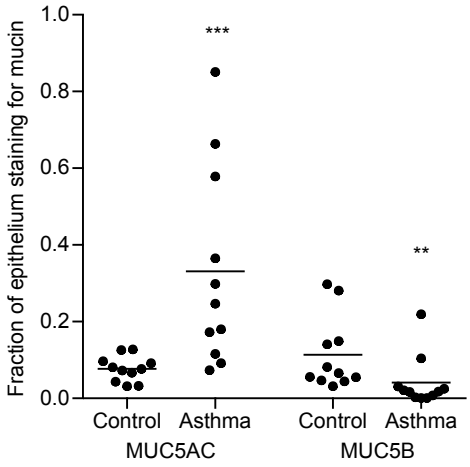
Ciliary beat frequency measurements. To measure ciliary beat frequency we transferred HBE cell cultures to an optical cell dish (MatTek, Ashland, MA, USA) and placed them on the stage of a spinning disc confocal microscope under a dry 10× objective. Temperature was maintained at 37 °C and perfluorocarbon (Sigma-Aldrich) was added to the apical surface to prevent evaporation (16). High-speed videos of the cultures were captured. For each culture, 3 regions of interest were selected randomly and ciliary beat frequency was calculated by measuring pixel intensity over time with Fiji (9) as previously described (19, 20).

Mucus solids concentration analysis. We measured the solids concentration of mucus using pre-weighed 10 mm diameter Kimwipe (Kimtech, Roswell, GA) meshes using methods described previously (21). We sterilized each mesh in 70% ethanol, then dried and applied the meshes to the apical surface (1 h) of an HBE cell culture. The mesh was subsequently removed from the culture and transferred to a microbalance (model No. 27; Cahn Electronic Balance, Cerritos, CA). Multiple measurements of mesh weight were made between ~30 and 120 s after removal of the mesh from the culture and a linear regression line was fitted to these points and extrapolated to time 0 to determine the initial 'wet' weight (22). Meshes were then dried in a 60 °C oven overnight and the 'dry' weight recorded. After subtraction of the mesh weight, we calculated the percent solids by dividing dry weight by wet weight. To simulate mucus we used solutions of low melting agarose (Type VII; Sigma-Aldrich) in PBS as previously described (12).

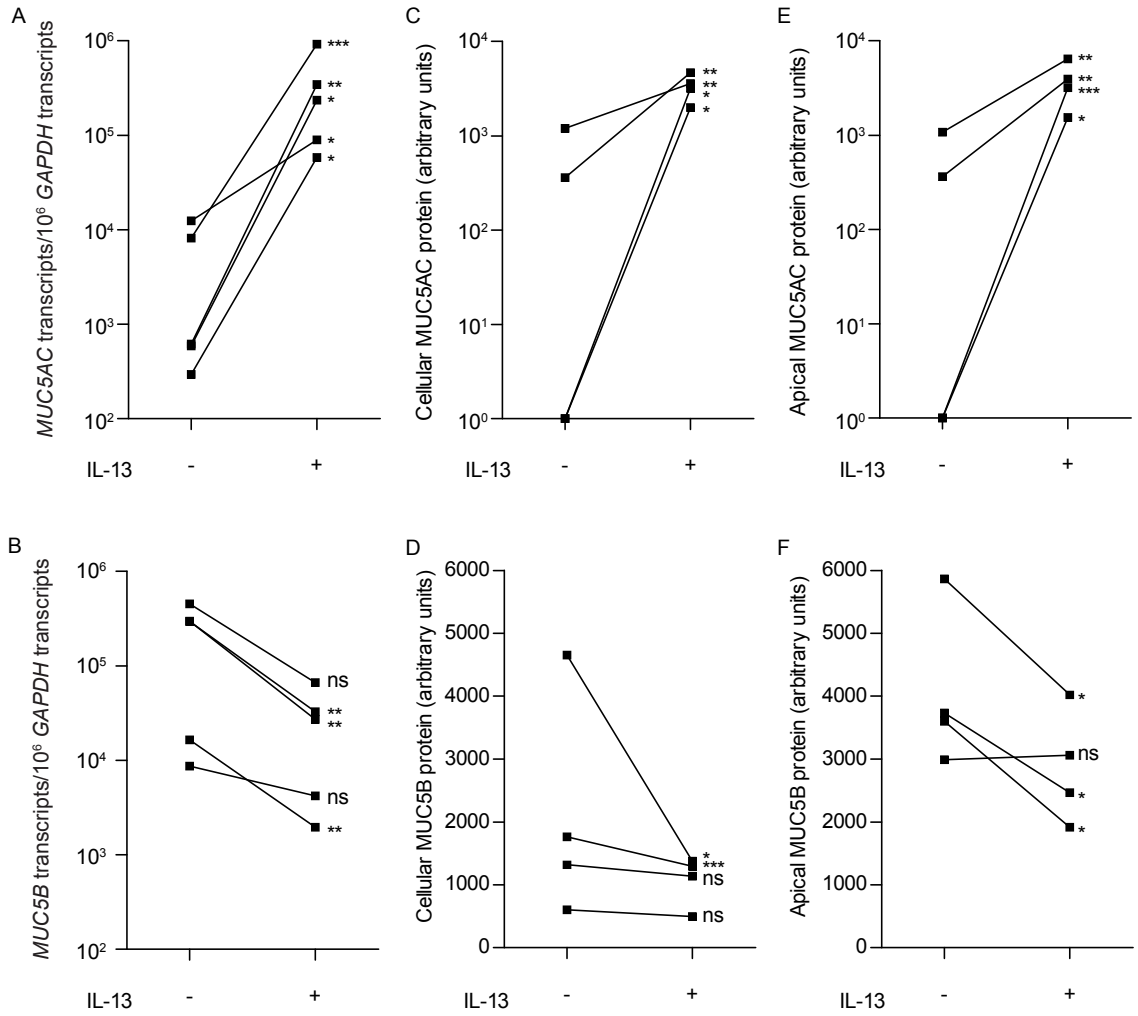
Assessment of mucus adherence. To assess adherence of the extracellular mucus, we compared unwashed HBE cell cultures to washed cultures. Washing was performed by applying PBS to the

apical surface of the cells, incubating at 37 °C for 15 min, and then removing the apical liquid. We then fixed the washed and unwashed cultures in Carnoy's solution and stained for mucins as described above. Each image was inspected by a blinded observer to determine whether MUC5AC and MUC5B were present or absent in the extracellular gel or within epithelial cells. Quantitation of mucin staining was performed using Fiji as described above. Peak intensity of mucin staining from whole mount images was calculated using the time series analyzer plugin (23) in Fiji.

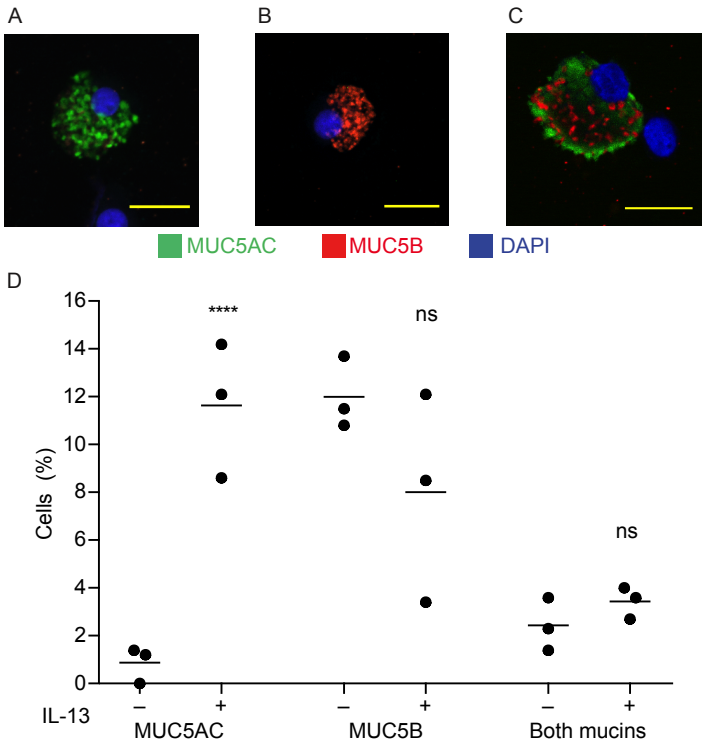
Mucus transplantation. To measure transport in the absence of mucus, the mucus gel layer was removed by washing with a DTT-containing solution as previously described (14, 24). We added 40 µl of PBS with 10 mM DTT (Thermo Scientific) to the apical surface of each culture for 10 min, aspirated the apical solution, and washed three times with 40 µl PBS without DTT. Fluospheres were then added and mucus transport measured in the absence of the endogenous mucus layer. After measuring transport in the absence of mucus, cultures were washed twice with 40 µl PBS without DTT and received mucus transplants. For mucus transplantation, mucus from multiple untreated donor HBE cell cultures was harvested by adding 40 µl of PBS without DTT for 10-15 min, collected, and pooled. 40 µl aliquots of the pooled mucus were then applied to recipient HBE cell cultures. After 15 min, fluospheres were added for measurement of transport following mucus transplantation.



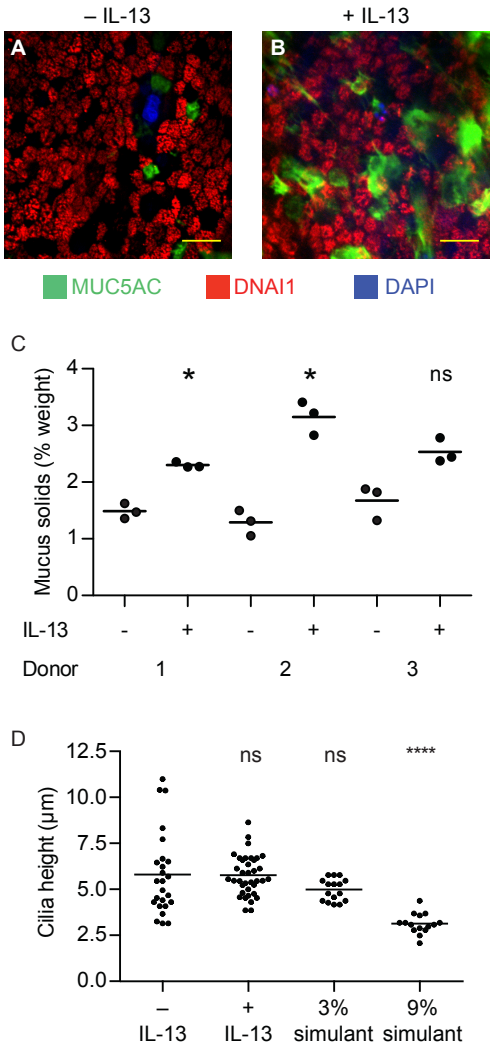
Supplemental Figure 1. Epithelial content of MUC5AC is increased and MUC5B is decreased in fatal asthma. Area of MUC5AC and MUC5B staining was measured in 2-5 fields each from 4 individuals without airway disease (unfilled) and 3 individuals with fatal asthma (filled). Values represent mean. **, $p < 0.01$; ***, $p < 0.001$ by Mann-Whitney *U*-test.



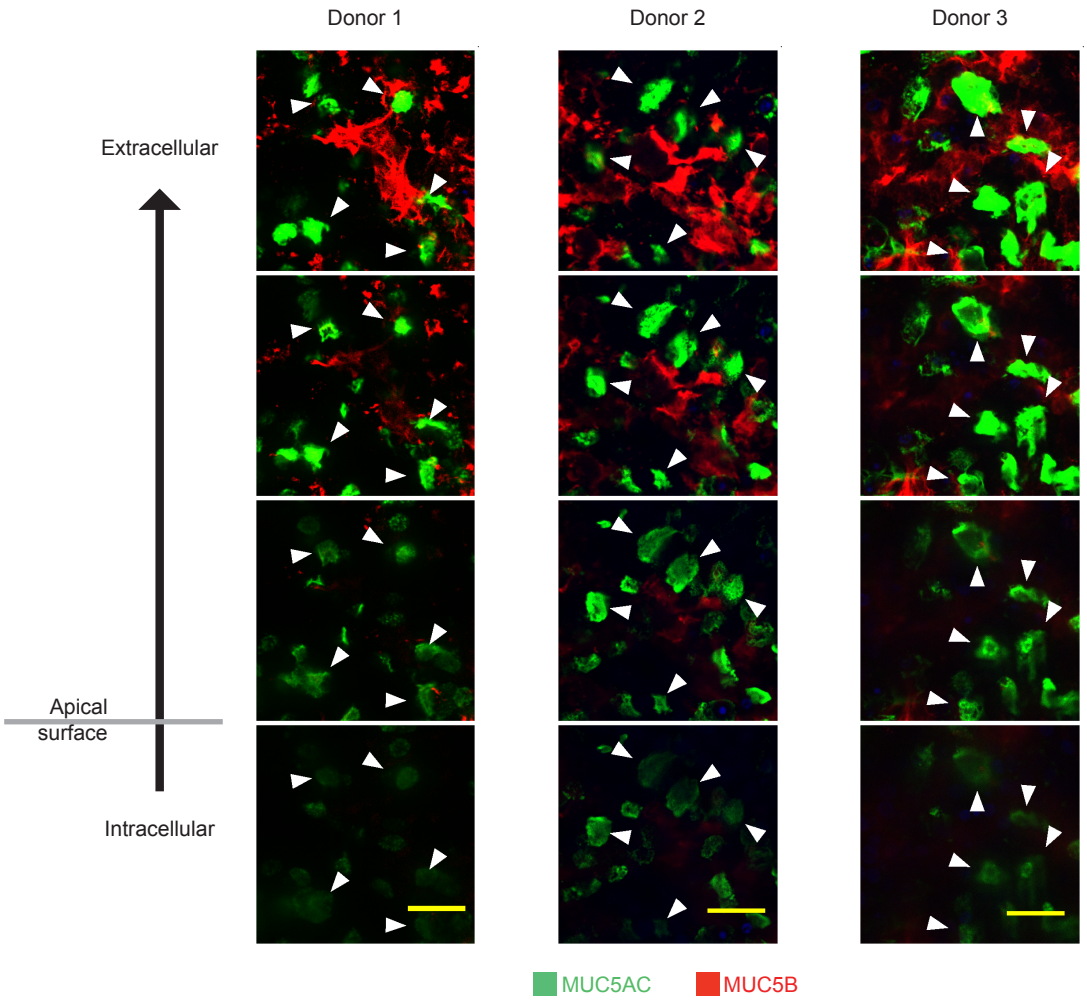
Supplemental Figure 2. IL-13 stimulation of HBE cell cultures increases MUC5AC and decreases MUC5B. (**A, B**) *MUC5AC* and *MUC5B* mRNA levels in bronchial epithelial cells from 5 human donors after culture in the absence (-) or presence (+) of IL-13. Each point represents the mean value for triplicate cultures from a single donor. (**C-F**) MUC5AC and MUC5B protein levels in cell lysates (C, D) and apical washings (E, F) from triplicate cultures from 4 individuals. Each point represents the mean value for triplicate cultures from a single donor. *, $p < 0.05$; ; **, $p < 0.01$; ***, $p < 0.001$; ns, not significant for IL-13-stimulated versus unstimulated from the same individual by Student's *t*-test.



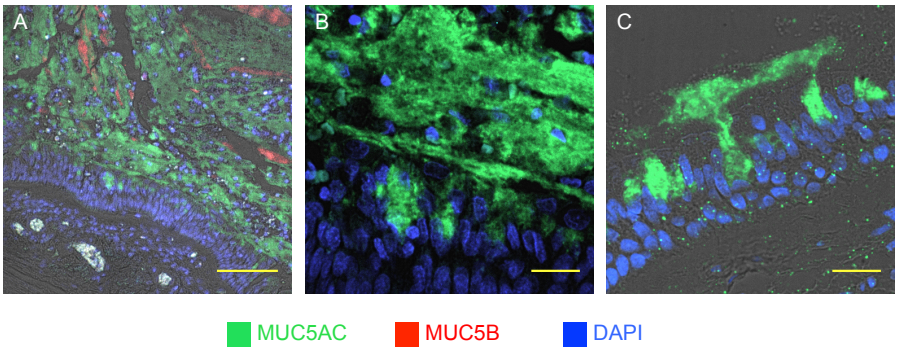
Supplemental Figure 3. IL-13 stimulation of HBE cells alters cellular mucin content. (**A-C**) Immunohistochemical staining of mucins from dissociated HBE cells. Scale bar = 20 µm. (**D**) Percentage of dissociated HBE cells staining for MUC5AC, MUC5B, or both MUC5AC and MUC5B were calculated from 3 fields each from cytospins prepared from unstimulated and IL-13-stimulated cultures from the same donor. A total of 243 unstimulated cells and 487 IL-13-stimulated cells were counted. ****, $p < 0.0001$; ns, not significant for IL-13-stimulated versus unstimulated cultures by Student's *t*-test.



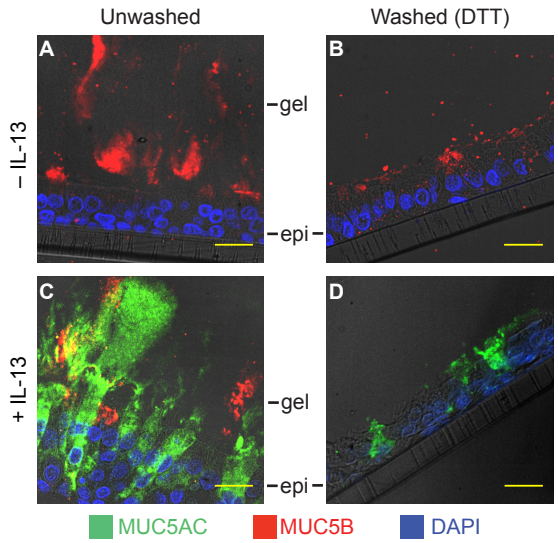
Supplemental Figure 4. IL-13 stimulation does not prevent ciliary differentiation or collapse the cilia. (**A, B**) Ciliated cells are present in both unstimulated and IL-13-stimulated HBE cell cultures. Sections were stained for MUC5AC and the ciliated cell marker axonemal dynein AI1 (DNAI1). Scale bar = 20 µm. (**C**) IL-13 stimulation results in a modest increase in mucus solids concentration. Each point represents the solids concentration (dry weight/wet weight) for one of three cultures generated from cells from each of three individuals (1, 2, and 3). *, $p < 0.05$; ns, not significant compared to unstimulated cultures by Student's *t*-test. (**D**) Cilia height is not altered by IL-13 stimulation. Cilia height was measured in unwashed unstimulated cultures (– IL-13), unwashed IL-13-stimulated cultures (+ IL-13). Cilia height was measured from sections made from HBE cell cultures prepared using cells from 3 individuals (3 fields per individual). To validate that the fixation method used was able to detect periciliary layer collapse, we replaced the mucus gels with agarose gels simulating 3% mucus solids (similar to IL-13-stimulated cultures) or 9% mucus solids (similar to that reported in CF (12)) prior to Carnoy's fixation. ****, $p < 0.0001$; ns, not significant compared to unstimulated cultures by ANOVA with Tukey-Kramer post-test.



Supplemental Figure 5. MUC5AC-domains in the extracellular gel are continuous with MUC5AC-containing mucous cells in the epithelium. Selected images from z-stacks of whole mount preparations of unwashed IL-13-stimulated HBE cell cultures from 3 different donors (1-3). Each image represents an individual section from the z-stack; successive images are 4 μm apart. Arrowheads indicate continuous regions of MUC5AC staining that extend from within mucous cells (bottom) into the extracellular apical gel (top).



Supplemental Figure 6. Regions of fatal asthma mucus plugs close to the airway epithelium predominantly contain MUC5AC and not MUC5B. (**A**) Low power view of a mucus plug from an individual with fatal asthma. (**B**) Higher magnification view of a region from the same mucus plug. (**C**) Mucus plug from another individual with fatal asthma. Scale bars represent 100 μm (A) or 20 μm (B, C). Together with the two images from Fig. 1C and D (which were from two different individuals), these images represent all of the four individuals with mucous plugs from our set of seven fatal asthma cases.



Supplemental Figure 7. Washing with a DTT-containing solution removes mucus. (**A-D**) Immunohistochemical staining of sections from unstimulated or IL-13-stimulated cultures immediately immersed in non-aqueous fixative (unwashed) or fixed after washing the apical surface in PBS with 10 mM DTT to remove adherent mucus. Labels: epi, epithelium; gel, extracellular mucus gel. Scale bars represent 20 μm.

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