METHODS

Isolation and Submerged Culture of Bronchial Epithelial Cells

Bronchial brushings were taken from airway generations 2 - 5 of the left lower lobe and placed into BEC basal medium (BEBM; Lonza, Slough, UK) and transported on ice to the laboratory. Cells were pelleted (400 *g*, 10 mins at 4^{o}C) and re-suspended in 1.5 ml of pre-warmed BEC growth medium (BEGM; BEBM plus BEGM singlequots [Lonza]), supplemented with 500 mg primorcin (Invivogen, Toulouse, France). Cells were seeded onto the base of bovine collagen coated (Stemcell, Cambridge, UK; 100 µg/ml) T25 flasks positioned vertically and incubated at 37^{o}C and 5% CO_2 in air for 21 days with medium changed every 2 - 3 days. Cells were then passaged into bovine collagen coated T25 flasks, followed by T75 and T125 flasks positioned horizontally and grown to 80% confluence with medium changed every 2-3 days. Cells were used at passages 4-5.

Culture of Bronchial Epithelial Cells at Air Liquid Interface

Once a sufficient number of cells were obtained, 2.5 x 10^4 cells were seeded onto the apical surface of Corning 6.5 mm transwell inserts (Sigma-Aldrich, Gillingham, UK) coated with human placental collagen (Sigma-Aldrich; 100 µg/ml) in 200 µl of BEC ALI medium (Gibco 50% v/v Dulbecco Modified Eagle Medium [Fisher Scientific, Loughborough, UK] supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin [Sigma-Aldrich] and 50% BEBM supplemented with 15 ng/ml retinoic acid, 500 mg primorcin, and BEGM singlequots excluding triiodothyronine, retinoic acid, and gentamicin/amphotericin-B) with 600 µl added to the basolateral chamber. Cells were cultured at 37^{o}C in a humidified incubator containing 5% CO_2 in air until 100% confluent with medium changed every 2-3 days. Once confluent the

medium was removed from the apical surface and the cells were exposed to air for 28 days. Every 2-3 days the basolateral medium was changed and once per week 200 µl of phosphate buffered saline (PBS) was applied to the apical surface to wash off excess mucous. The PBS removed was used to analyse mucin-5AC (MUC5AC) by enzyme-linked immunosorbent assay (ELISA) as per the method detailed below. MUC5AC is produced by goblet cells and is therefore an indicator of goblet cell differentiation.

Culture of calu-3 cells at air liquid interface

Calu-3 cells were seeded at a density of 3 x 10^4 cells onto the apical surface of 6.5 mm transwell insert in 200 µl Dulbecco's Modified Eagle medium: nutrient mixture F-12 (DMEM/F12) supplemented with 10% foetal calf serum, 1% MEM non-essential amino acid solution (Gibco), and 1% Penicillin/Streptomycin (Sigma-Aldrich) with 600 µl added to the basolateral chamber. The cells were incubated at 37^{o}C in a humidified incubator containing 5% CO_2 in air. After 3 to 5 days the cultures were exposed to air. The medium on the basolateral side was replaced every 3 to 4 days.

Trans-Epithelial Electrical Resistance

Trans-epithelial electrical resistance (TEER) was measured using a 4 mm chopstick electrode attached to the EVOM2 epithelial volt/ohm meter (World Precision Instruments, Hitchin, UK). Electrodes were sterilised in methanol and equilibrated in PBS prior to taking resistance measurements. The electrodes were placed into the culture system (one in the apical compartment and one in the basolateral compartment) and the resistance across the transwell membrane was measured. A blank measurement was taken using an insert without

cells and subtracted from each reading. The resistance (Ω) reading was multiplied by the surface area of the transwell membrane (0.33 cm^2) to provide a final value (Ω/cm^2).

E-cig vapour extract preparation

ECVE was prepared as previously described [2]. The e-cig used was a VIP® 1100mAh battery with a V5/CE5 clearomiser containing USA tobacco flavour e-liquid with a nicotine strength of 24 mg. This brand was chosen for consistency with our previous work.Briefly e-cig vapour was bubbled through BEC ALI medium for use with primary BECs or DMEM/F12 for use with calu-3 cells using a Watson-Marlow 520R peristaltic pump (Watson- Marlow Ltd, Falmouth, UK). Vapour was generated by manual activation of the heating coil at a flow rate of 238 mL/min. The ECVE was filtered using a syringe driven 0.22 µm filter and then adjusted to the desired optical density (OD) using culture medium.

Cigarette smoke extract preparation

CSE was prepared as previously described [2]. Briefly, one 3R4F Kentucky research cigarette (University of Kentucky, Kentucky, USA) was bubbled through BEC ALI medium for use with primary BECs or DMEM/F12 for use with calu-3 cells using a Watson-Marlow 520R peristaltic pump. The CSE was filtered using a syringe driven 0.22 µm filter and then adjusted to the desired OD using culture medium.

Western Blot

Cell lysates diluted in sample buffer [62.5 mM Tris, 10% glycerol, 1% SDS, 1% β-mercaptoethanol, and 0.01% bromphenol blue, pH 6.8], were electrophoresed on SDS polyacrylamide gels (10%) and transferred to Hy-bond ECL membranes (Whatman International Ltd, Maidstone, UK). Membranes were incubated with blocking buffer [5% dried milk in Tris buffered saline containing 0.1% Tween 20 (TBS/Tween 20)] for 1 h at room temperature and then incubated with primary antibodies (diluted in blocking buffer) at 4 °C overnight (rabbit anti-phospho-p38 MAPK [Thr180/Tyr182]; Cell Signalling, Hitchin, UK and rabbit anti-β-actin, Abcam, Cambridge, UK). After washing in TBS/Tween 20, the membranes were incubated for 60 min with a peroxidase-conjugated secondary antibody (diluted in wash buffer) (horseradish peroxidase-conjugated goat anti-rabbit, Cell Signalling), washed again, and the antibody labelled proteins were visualized by enhanced chemiluminescence (Amersham Biosciences, Chalfont St. Giles, UK). Densitometric analysis was performed by normalising band density to that for β-actin using Quantity One v4.6.1 software (Bio-Rad, Hemel Hempstead, UK).

MUC5AC ELISA

Supernatants from the apical surface of ALI cultures were diluted in PBS and incubated at room temperature for 2 h onto Immulon 2 HB 96 well plates (Fisher Scientific). The plates were washed in PBS then blocked by incubation for 1h at room temperature with 1% bovine serum albumin in PBS. The plates were washed in PBS and then incubated for 2 h at room temperature with 0.5 µg / ml of monoclonal antibody 45M1 [18] raised against MUC5AC (Fisher Scientific). The plates were washed and then incubated with streptavidin-horse radish peroxidase (R&D systems) for 20 mins at room temperature. The plates were washed and then incubated with substrate solution (R&D systems). The reaction was stopped by the

addition of 1M sulphuric acid. Optical density was determined using a microplate reader set to 450nM.

Embedding and Processing of Transwell Membranes

Cells were washed with PBS followed by fixation in Bouin's solution (Sigma-Aldrich) overnight at room temperature. The following day the base of the transwell insert was carefully excised using a biopsy punch and the membranes transferred to 70% industrial denatured alcohol (IDA) (Genta Medical, York, UK) and incubated at room temperature for 30 min. A further three washes in 90%, 95% and 100% IDA for 30 min at each concentration was performed, with a final wash in fresh 100% IDA performed for an additional 30 min. Following the alcohol washes, the membranes were cleared in xylene (Sigma-Aldrich) twice for 30 min each time, then transferred to molten wax (Leica Microsystems, London, UK) and placed in an oven at 60°C for 1 h. The molten wax was replaced with fresh molten wax and samples incubated for 1 h in the oven. All samples were then placed in labelled cassettes, embedded in paraffin and sectioned using a microtome at 4 μm then mounted onto a glass slide.

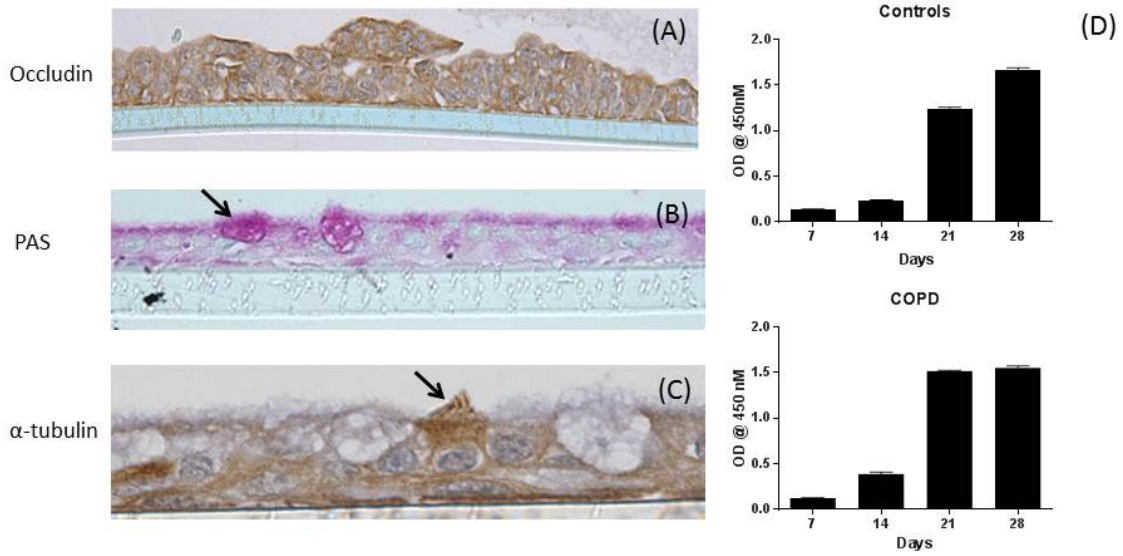
Immunohistochemistry and histology

All sections were de-waxed in xylene and rehydrated in graded alcohols. Heat induced epitope retrieval was performed in citrate buffer pH6 for 20 min at 800 W using a domestic microwave oven. Sections underwent endogenous blockade using 1.5% normal horse serum for 30 min at room temperature. Sections were then incubated in rabbit monoclonal anti-human occludin (Sigma-Aldrich catalogue number SAB4200593; diluted 1:800) or mouse monoclonal anti-alpha tubulin (clone TU-01 Abcam catalogue number ab7750; diluted 1:2000) primary antibodies for 2 h at room temperature. Following this, the immune reaction was detected

by incubating for 30 min at room temperature with a ready-to-use peroxidase-labelled secondary reagent (ImmPRESS™ MP-7401 anti-rabbit, VectorLabs, Peterborough, UK). The slides were washed in TBS-tween (0.05%) then incubated in DAB chromogen (VectorLabs). Slides were counterstained in Mayer's haematoxylin and cover slipped in DPX. To determine the number of mucus-producing cells, 4 μm sections of paraffin-embedded ALI cultures were stained using the Periodic Acid Schiff (PAS) Plus kit (BD Biosciences. Oxford, UK) according to the manufacturer's protocol, dried overnight at room temperature and cover slipped in DPX. Digital micrographs were obtained using a Nikon Eclipse 80i microscope (Nikon UK Ltd, Kingston Upon Thames, UK) equipped with a QI imaging digital camera and Image Pro Plus 6.0 software (Media Cybernetics, Marlow, UK).

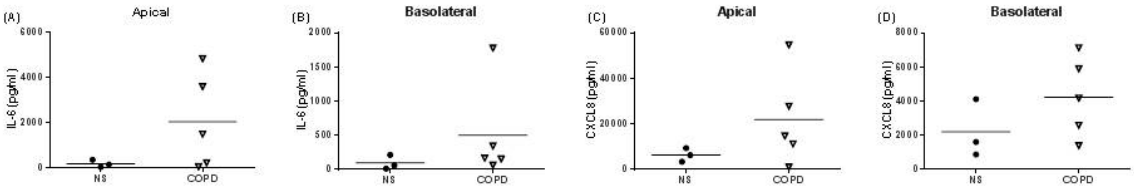
Supplementary Figure 1. Characterisation of BEC differentiation at ALI. After 28 days at ALI, cell cultures were embedded and assessed for tight junction formation by occludin expression (A), goblet cell differentiation by PAS staining (B) and cilia formation by α-tubulin staining (C). In addition, goblet cell differentiation from 7 – 28 days was assessed by MUC5AC release by ELISA (D).

Supplemental figure 1



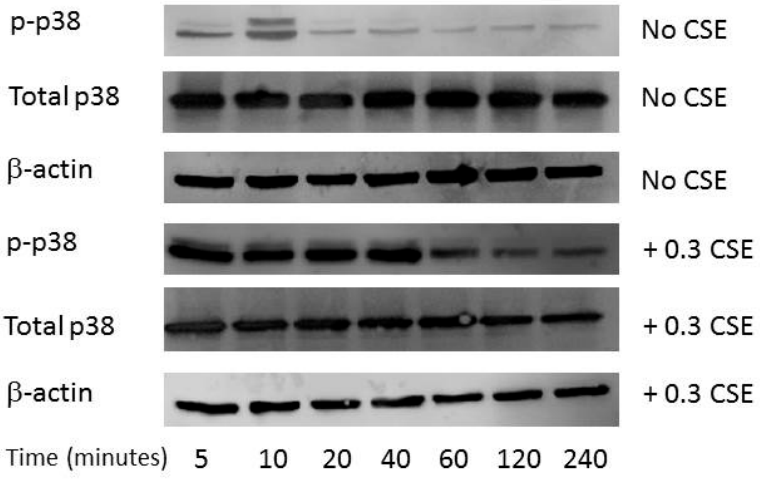
Supplementary Figure 2. A comparison of basal cytokine release between control and COPD BECs. Cells were untreated for 24 h and IL-6 (A and B) and CXCL8 (C and D) were analysed in the apical (A and C) and basolateral (B and D) supernatants.

Supplemental figure 2



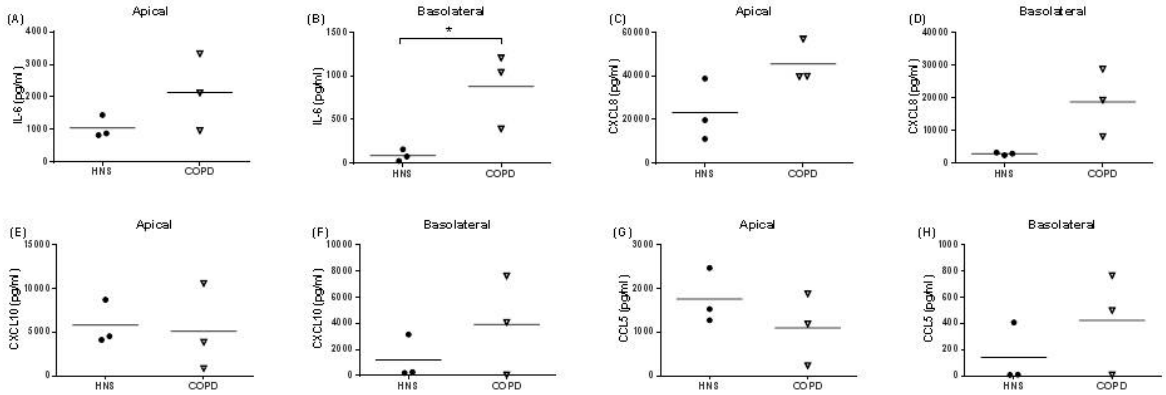
Supplementary Figure 3. A comparison of poly I:C stimulated cytokine release between control and COPD BECs. Cells were stimulated with poly I:C (10 μg/ml) for 24 h and IL-6 (A and B), CXCL8 (C and D), CXCL10 (E and F) and CCL5 (G and H) were analysed in the apical (A, C, E and G) and basolateral (B, D, F and H) supernatants. * = significant difference between controls and COPD patient where $p<0.05$).

Supplemental figure 3



Supplementary Figure 4. The effect of CSE on p38 MAPK activation in calu-3 cells. Calu-3 cells were exposed to CSE (0.3 OD) for 5 – 240 mins before cell lysates were analysed for phosphorylated p38 MAPK (p-p38) by western blot. P-p38 levels were normalised to the loading control β-actin.

Supplemental figure 4



Supplementary Figure 5. The effect of ECVE and CSE on LDH release from poly I:C stimulated cells. Primary BECs were pre-treated with ECVE (0.01 – 0.1 OD) or CSE (0.1 – 0.3 OD) for 1 h prior to poly I:C (10 µg/ml) stimulation for 24 h. Supernatants were analysed for LDH release. ** = significant difference compared to poly I:C stimulated cells where $p<0.01$. UNT = untreated cells.

Supplemental figure 5