SUPPLEMENTARY INFORMATION

Cell culure

Airway samples were obtained with the use of a protected specimen brush, hence ensuring that samples obtained from lower airways were free of contamination from organisms from the upper airway. However, other groups report that samples obtained in this way contain viable bacteria when obtained from the airways of patients with COPD.[1] The airways of patients with COPD are therefore less likely than healthy subjects to be sterile, but the pathogens themselves are unlikely to have persistent effects on the cells when cell culture is established. It is more likely that any differences in the cell cultures can be attributed to intrinsic phenotype, rather than to their site of retrieval. These findings are in line with observations from other groups reporting functional differences between epithelial cells from smokers, with and without COPD.[2]

Airway epithelial cells are obtained either from brushings,[2-3] from surgical specimens,[4] or using the explant cell culture technique from endobronchial biopsies or from human inferior turbinate tissue.[5] Each of these methods have their own individual merits. Although the yield of cells is lower from bronchial brushings as compared to that obtained from surgical specimens, the former method facilitates the study of bronchial epithelial cells from those with milder disease. In fact, the regulation of mRNA of DUOX1 and DUOX2, which are important in the production of hydrogen peroxide generating enzymes, differ in their expression in those with mild/moderate COPD [6] as opposed to those with more severe disease.[7] This observation suggests that the pathophysiology of COPD may change as the disease progresses.

Using microarray data acquisition, recent research comparing paired nasal and bronchial epithelial cells (also acquired by brushings from each anatomical site) from never smokers and current smokers, identified 119 genes whose expression was similarly affected by cigarette smoking in both bronchial and nasal epithelium.[8] These particular genes were primarily related to detoxification, oxidative stress and wound healing. findings suggest that there are stable epigenetic changes which may account, in part, for differences in airway epithelial cells induced by cigarette smoking. This would be an interesting area for future

research. Keeping in mind that the use of LPS alone has obvious limitation, repeating these experiments using live pathogens, and so more closely modelling the *in vivo* situation, would serve to extend our observations.

Although it could be argued that the process of cell culturing may change the epithelial phenotype as the reviewer suggests through the multiple cycles of proliferation involved in achieving ALI cultures, recent research suggests this is unlikely to be the case, at least for primary nasal epithelial cells.[17] In this particular publication, differences in ciliary beat frequency were observed in subjects exposed to cigarette smoke in comparison to their non-smoking counterparts. This consistent finding across 20 subjects suggests that, at least for phenotypic and physiological endpoints, that the process of cell culture did not alter the cells themselves. Therefore, it may in fact be that cell culture *in vitro* facilitates, or selects for, only certain populations of progenitor cells, and so their progeny emerge as the cultures mature under ALI conditions. This aspect, if valid, could in fact be considered beneficial in terms of the study of airway epithelial cells.

TUNEL Assay

Cells were initially fixed with 4% paraformaldehyde in PBS followed by permeabilization step with 0.25% Triton X-100. After removing the media and washing with PBS, a sufficient volume of fixative was added to completely cover the coverslips. The cells were then incubated for 15 minutes at room temperature and the fixative was removed. Then a permeabilisation reagent (0.25% Triton X-100 in PBS) was added to completely cover the coverslips. The samples were then incubated for 20 minutes at room temperature and subsequently washed twice with deionized water.

DNase I was used to generate a positive control by inducing strand breaks in the DNA and so induce a positive TUNEL reaction. For the positive control, coverslips were washed with deionized water and 100 μL of the DNase I solution was added and incubated for 30 minutes. After this period, the coverslips were washed once with deionized water and the TdT reaction followed. The TdT reaction protocol uses 100 μL of the TdT reaction cocktail per coverslip. For each coverslip, 100 μL of the TdT reaction cocktail was needed. This included 94 μL of the TdT reaction buffer, 2 μL of EdUTP, 4 μL of TdT to make a final volume of 100 μL. Initially 100 μL of the TdT reaction buffer was added to each coverslip and incubated for 10

minutes at room temperature. Then, after removing the TdT reaction buffer, 100 μL of the TdT reaction cocktail was added to each coverslip and incubated overnight at room temperature. The coverslips were then washed twice with 3% BSA in PBS for 2 minutes each.

Prior to imaging, the Click-iT reaction buffer additive was prepared and added to the coverslips. For each coverslip, 100 μL of the Click-iT reaction cocktail was added to each coverslip which was made up of 97.5 uL of the Click-iT reaction buffer and 2.5 μL of the Click-iT reaction buffer additive. This was incubated for 30 minutes at room temperature and protected from the light. Then the Click-iT reaction cocktail was removed and each coverslip washed with 3% BSA in 1X PBS for 5 minutes. Coverslips were mounted onto glass coverslips with mounting media with DAPI and imaged using a LEICA fluorescence microscope.

Immunofluoresence

Selected cultures were fixed in 4% PFA and washed three times in PBS. Cells were exposed to a 1:250 dilution of rabbit anti-E-Cadherin primary antibody overnight at 4°C. After washing, cells were exposed to a 1:500 dilution of goat anti-rabbit IgG Alexofluor 568 at 4 degrees in the dark for 1 hour. Cells were then washed thrice in PBS, and the membrane was cut out using a scalpel and mounted on a microscope slide using Vectashield with DAPI (Vector Laboratories, Peterborough, UK). Negative control slides were also prepared by omitting the primary antibodies to demonstrate specificity. Images were captured and visualized using LAS AF (Leica) acquisition software.

Selected Transwells from the same individual with COPD were fixed in 4% PFA and washed three times in PBS. Cells were exposed to a 1:200 dilution of rabbit anti-MUC5AC primary antibody, and mouse anti-acetylated alpha tubulin antibody overnight at 4 degrees. After washing, cells were exposed to a 1:250 dilution of goat anti-mouse IgG Alexofluor 488 and goat anti-rabbit Alexofluor 568 at 4 degrees in the dark for 1 hour. Cells were then washed thrice in PBS, and the membrane was cut out using a scapel and mounted on a microscope slide using Vectashield with DAPI (Vector Laboratories, Peterborough, UK). Negative control slides were also prepared by omitting the primary antibodies to demonstrate specificity. Images were captured and visualized using LAS AF (Leica) acquisition software.

FACS

Expression of TLR-4 was determined by FACS analysis. In brief, cells were detached from wells by incubating with cell dissociation fluid (Sigma-Aldrich) in PBS at 37 ºC. For the analysis of intracellular TLR-4, cells were permeabilised and fixed by incubating them with fixation and permeabilization buffer (eBioscience, USA) as per supplier's instructions, and then washed twice with staining solution containing 1.0% BSA and 0.02% sodium azide in PBS. Cells, adjusted to 1 x 10^5 cells per 100 μl in PBS/1% BSA, were subsequently stained in darkness for 30 min at 4 ºC with phycoerthrin (PE)-conjugated anti-TLR-4 monoclonal antibodies (eBioscience, USA). For experiments measuring phospho-p38, a mouse monoclonal antibody (Cell Signalling, UK) was used, and a FITC Conjugated Goat antibody to mouse as a secondary (Cell Signalling, UK). A minimum of 10,000 events were initially gated using a forward and side scatter dot-plot, where only a single population of cells was evident (data not shown).

Cells, again adjusted to 1 x 10^5 cells per 100 μl , were suspended in binding buffer and incubated with the fluorochrome-conjugated Annexin V (Av) for 15 minutes. Subsequently, after washing, cells were re-suspended in fresh binding buffer and stained with PI. In order to set gates and establish appropriate compensation settings, cells were stained with PI alone, Av alone, and both PI and Av. In order to obtain positive control samples for apoptosis and secondary necrosis respectively, cells were treated with 2 μM staurosporin and 0.1% Triton-X respectively for 8 h.

Western blots

For Western blotting, cells were lysed in radioimmunoprecipitation assay buffer (Sigma-Aldrich, UK). The protein concentration was determined by bovine serum albumin protein assay kit according to manufacturer's instructions (Fisher Scientific, UK). Equal amounts of protein (15 μg/lane) were separated in a 12% SDS gel, and transferred to a polyvinylidene difluoride sheet by electroelution with a constant voltage of 100 V for 90 minutes at room temperature. After blocking with 0.1% Tween 20 supplemented with PBS (T-PBS) containing skim milk overnight, the sheet was incubated with a 1:1,000 dilution anti-

phospho-p38 specific antibody (Abcam, UK) at 4 degrees overnight. The sheet was then washed three times with T-PBS, and incubated with goat HRP conjugated anti-rabbit antibody (1:3,000 dilution; Abcam, UK) for 1 h at room temperature. After washing with T-PBS three times, immunoreactive protein bands were revealed with an enhanced chemiluminescence western blot analysis system. After being "stripped" using a Western Blot Stripping Buffer (Thermoscientific, UK), membranes were re-probed with a polyclonal antibody against total p38 MAPK. Experiments were repeated using a rabbit primary antibody to total and phospho ERK1 + 2 (both 1:1,000 dilution, Abcam, UK), and for total and phospho JNK1 (1 μg/ml and 1:200 respectively; Abcam, UK).

To determine the effects of CSE on caspase 3 activation in PBEC cultures, separate cultures from each of the three patient groups were treated with 50 μg/ml PA LPS, either with or without pre-treatment with CSE for 24 h. For the detection of cleaved caspase-3, both full length and cleaved caspase-3, a 1:1,000 dilution of primary antibody was used in each case (Cell Signalling, UK). A 1:1,000 dilution of primary antibody was used for the detection of phospho-NF-κB (Ser536) and IκB-α (Cell Signalling, UK).

Nuclear Extract Preparation

After media was aspirated from the Transwells, cells were washed with 1 ml of ice-cold PBS/Phosphatase. Cells were then removed from the Transwell inserts by gentle scraping with a cell lifter and transferred to a pre-chilled 15 ml conical tube. The cell suspension was centrifuged for 5 min at 500 rpm in a pre-cooled centrifuge at 4°C. The supernatant was discarded and the cell pellet kept on ice. The cell pellet was resuspended in 500 μL of hypotonic buffer by pipetting up and down several times. The sample was then incubated for 15 min on ice after transferring to a pre-chilled microcentrifuge tube. 25 μL of detergent was added and vortexed for 10 seconds. Then, the suspension was centriguged for 30 seconds at 14,000 g in a microcentrifuge pre-cooled to 4°C. The supernatant (cytoplasmic fraction) was transferred into a pre-chilled microcentrifuge tube and the pellet used for nuclear fraction collection.

To prepare the nuclear extracts, the nuclear pellet was resuspended in 50 μL of complete lysis buffer and pipetted several times. The sample was then vortexed for 10 seconds at the highest setting and incubated on ice on a rocking platform set at 150 rpm. Finally the sample was vortexed for 30 seconds at the highest setting and centrifuged for 10 minutes at 14,000 g in a pre-cooled microcentrifuge tube. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube and stored at -80°C. Protein concentrations were determined using BCA protein assay kit (Fisher Scientific, UK).

SUPPLEMENTARY FIGURES

Figure S1. **Confirmation of (a) the presence of functional intercellular tight junctions (immunocytochemistry), (b) differentiation with goblet cells and cilia (Confocal) and (c) the cobblestone appearance of the Transwell cultures (Direct light microscopy).** Well differentiated PBEC cultures were (A) stained again E-Cadherin (red) demonstrating the presence of tight junctions and (B) stained against MUC5AC (green) and ß-tubulin (red) demonstrating the presence of goblet cells and cilia respectively on a laser confocal microscope (Leica TCS/NT). A cobblestone appearance with visible overlying mucous was evident on direct light microscopy (C).

Figure S2. **Effect of PA LPS in the presence or absence of CSE on caspase-3 activation in control PBEC cultures measured using western blotting**. Lanes 1-4 represent treatment of PBECs with 0, 12.5, 25 and 30 μg/ml LPS (4 h) respectively. Lanes 5-8 represent 5%, 10%, 30% and 50% CSE for 4 h. Full length caspase is 35 kDa and the large fragment of caspase-3 resulting from cleavage 17 kDa. Image representative of seperate western blots.

Figure S3. **Effect of PA LPS on PBEC tyrosine phosphorylation of p38 kinase in the presence or absence of CSE measured using Western Blotting and FACS.** (a) For the western blot, lanes 1-4 represent treatment of PBECs with 0, 10, 20, 50 μg/ml PA LPS (4 h) respectively. Lanes 5-8 represent equivalent concentrations and exposure times but include pre-treatment with CSE (24 h). Top two lanes are from a COPD patient, middle two lanes from a SWAO, and lower two lanes from a NS subject (b) Densitometry data for COPD subjects are displayed as median ± IQR (n=4 for each group).*$p<0.05$.(c) FACS histograms represents COPD PBECs treated with PA LPS 50 μg/ml (24 h) either with (purple histogram) or without (black histogram) CSE pre-treatment (24 h). Cells were fixed, permeabilised and stained with a PE conjugated antibody against phospho-p38 or equivalent isotype-matched control. (d) Mean fluorescence Intensity is shown (n=5 for each group). Data are displayed as median ± IQR. *$p<0.05$.

Figure S4. **Effect of PA LPS on PBEC tyrosine phosphorylation of JNK1 in the presence or absence of CSE measured using Western blotting. (a)** Lanes 1-4 represent treatment of PBECs with 0, 10, 20, 50 μg/ml LPS (4 h) respectively. Lanes 5-8 represent equivalent concentrations and exposure times but include pre-treatment with CSE (24 h). Top two lanes are from a COPD patient, middle two lanes from a SWAO, and lower two lanes from a NS subject (b) Densitometry data for COPD subjects are displayed as median ± IQR (n=4 for each group). *$p<0.05$.

Figure S5. **Effect of LPS on PBEC tyrosine phosphorylation of ERK kinase in the presence or absence of CSE measured using Western blotting**. (a) Lanes 1-4 represent treatment of PBECs with 0, 10, 20, 50 μg/ml PA LPS (4 h) respectively. Lanes 5-8 represent equivalent concentrations and exposure times but include pre-treatment with CSE for 24 h. Top two lanes are from a COPD patient, middle two lanes from a SWAO, and lower two lanes from a NS subject (b) Densitometry data for COPD subjects are displayed as median ± IQR (n=4 for each group).*$p<0.05$.

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