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RESEARCH HIGHLIGHT

Cigarette smoke, airway epithelial cells and host defence

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In COPD inflammation driven by exposure to tobacco smoke results in impaired innate immunity in the airway and ultimately to lung injury and remodeling. To understand the biological processes involved in host interactions with cigarette derived toxins submerged epithelial cell culture is widely accepted as a model for primary human airway epithelial cell culture research. Primary nasal and bronchial epithelial cells can also be cultured in models. Air liquid interface (ALI) and submerged culture models have their individual merits, and the decision to use either technique should primarily be determined by the research hypothesis. Cigarette smoke has gaseous and particulate matter, the latter constituent primarily represented in cigarette smoke extract (CSE). Although not ideal in order to facilitate our understanding of the responses of epithelial cells to cigarette smoke, CSE still has scientific merit in airway cell biology research. Using this model, it has been possible to demonstrate differences in levels of tight junction disruption after CSE exposure along with varied vulnerability to the toxic effects of CSE in cell cultures derived from COPD and control study groups. Primary nasal epithelial cells (PNECs) have been used as an alternative to bronchial epithelial cells (PBECs). However, at least in subjects with COPD, PNECs cannot consistently substitute for PBECs. Despite having a constitutional pro-inflammatory phenotype, bronchial epithelial cells retrieved from subjects with COPD have a relatively curtailed inflammatory response to CSE exposure when compared to epithelial cells from their equivalent healthy counterparts. Furthermore, COPD epithelial cells have an increased susceptibility to undergo apoptosis, and have reduced levels of Toll-like receptor-4 expression after CSE exposure, both of which may account for the reduced inflammatory response observed in this group. The use of CSE in both submerged and ALI epithelial cultures has extended our knowledge of cell biology in COPD, and helped to unravel important pathways which may be of relevance in its pathogenesis.

Keywords: COPD; airway inflammation; epithelial cells; cigarette smoke extract; nasal epithelium

Introduction

The bronchial epithelium is more than a mere physical inert barrier to the external environment. It serves as the first line of defence against inhaled pathogens and airborne infections [1-2]. By contributing to the innate immune system it provides an efficient host defence system at the mucosal surface [3].

IL-8 is of particular importance in a number of inflammatory lung diseases[4], and considered a meaningful endpoint to measure in airway epithelial cell research [5-6]. IL-8 is regarded as one of the more important mediators of pathogenesis in airway inflammatory diseases such as pulmonary fibrosis, bronchial asthma and cystic fibrosis by means of recruiting neutrophils [7-8]. IL-6 is induced in lung tissue after cigarette smoke exposure in murine studies [9]. Systemic IL-6 is heightened in COPD subjects with an
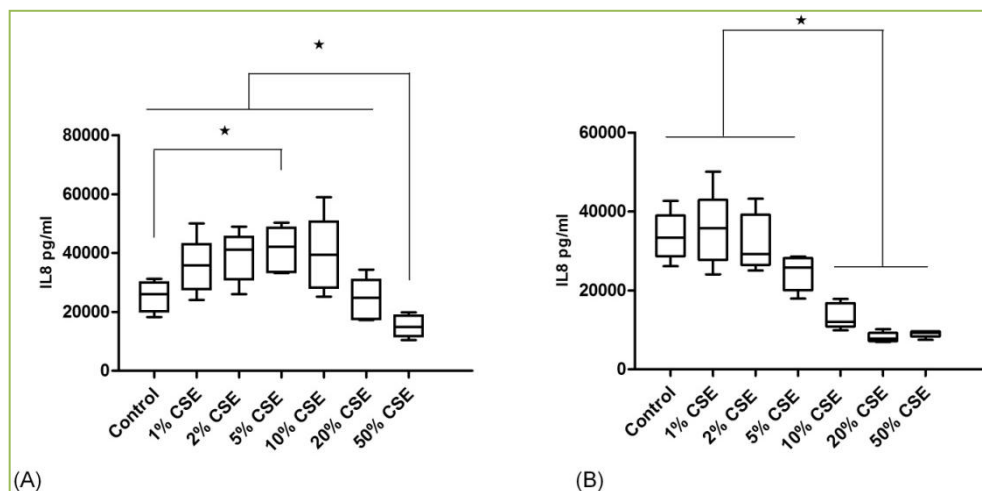
Figure 1. IL-8 dose response from well differentiated cultures after prolonged treatment with CSE. Well differentiated PBEC cultures from (A) healthy subjects and (B) COPD subjects were treated 1%, 2%, 5%,10%, 20% or 50% CSE (or vehicle) for 24 h (n=7 for each group). Supernatants from apical segment were collected and assessed for IL-8 by ELISA. A 24 h exposure to CSE stimulated release of IL-8 up to 5% with cytotoxicity at higher CSE concentrations. Data are displayed as median ± IQR; * (p <0.05, Mann-Whitney test).

inverse relationship to lung function and is associated with impaired exercise metabolism [10]. For COPD subjects, IL-6 contributes to skeletal muscles wasting [11], heightens levels of trabecular bone loss [12] and may be related to further exacerbation risk [13]. IL-6 and IL-8 are therefore relevant measurements for the study of airway epithelial cells and cigarette smoke research [14].

A number of cell culture models, using either epithelial cell lines or primary airway cells from different anatomical sites within the respiratory tract have been selected for airway inflammation studies. All of these models have their individual merits. Aqueous surrogates for volatile cigarette smoke, most commonly cigarette smoke extract (CSE) have become popular substances for chemical stimulation in order to unravel important biological cellular pathways. However, published research findings tend to be inconsistent and often divergent leaving no emerging trend line or consistent message with regard to any inherent inflammatory effects of CSE in cultured epithelial cells.

Strengths and weaknesses of the cigarette smoke extract model

Despite a lack of universal agreement as to the inherent tendency for cigarette smoke to have pro-inflammatory effects on airway epithelial cells, the evidence is more supportive of a pro-inflammatory [14-21] as opposed to an immunosuppressive effect [14, 22-24]. Studies which suggest that CSE has immunosuppressive properties either used a particularly small volume of medium to prepare the stock CSE and subsequently

exposed cells for a prolonged period,[24] or alternatively used the undiluted stock CSE. Only one of these studies used PBECs and treated cells for 4 hours using an undiluted CSE prepared by combusting 2 cigarettes in 50 ml of medium without serum [23]. Studies reporting stimulatory effects of CSE in primary epithelial cells used a relatively dilute CSE for a short period [25]. Therefore, considering all of these observations together, it appears that the more concentrated, or more prolonged CSE exposures, tend to have the greatest likelihood of having immunosuppressive effects, whereas, on the other hand, the more dilute CSE used for relatively brief periods appear to have stimulatory effects [24].

It is clear that caution is required when comparing studies using CSE. Concentrations of CSE vary from as high as 100% CSE for 15 minutes [22] on the one hand to as low as 1% CSE for 24 hours on the other (when those studies using single cigarette to prepare their stock CSE are considered) [24]. CSE has also been applied intermittently at 2 hourly intervals, with the supernatant removed for the duration of exposure to the CSE, and the same supernatant replaced [22]. Other groups also opted to use intermittent exposures to CSE in airway smooth muscle cell culture models [26]. For both of these studies, it was not apparent if a fresh CSE was prepared on each occasion. If multiple cigarettes are used to prepare the CSE, the number of cigarettes can vary from two cigarettes [27] to five [28-29]. Not only is the dilution of the original CSE used in these experiments particularly diverse, it is often documented on the basis of the change
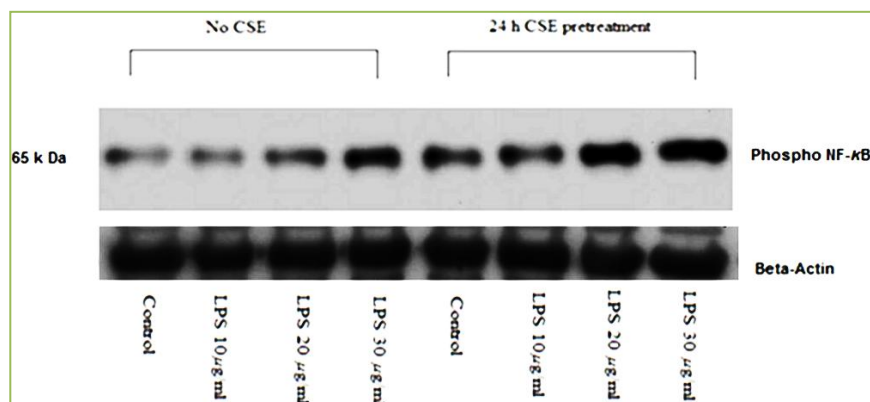
Figure 2. Effect of *Pseudomonas aeruginosa* LPS and 5% CSE on phosho-p65 NF-κB expression in control PBEC cultures measured using Western blotting. Western blot of p65 NF-κB protein expression (with ß-actin controls) in PBECs of healthy non-smokers. Lanes 1-4 represent treatment of Healthy PBECs with 0, 12.5, 25, 50 μg/ml *Pseudomonas aeruginosa* LPS (4 h) respectively. Lanes 5-8 represent equivalent concentrations and exposure times but include pre-treatment with 5% CSE for 24 h. A representative blot is shown (n=3).

in optical density, rather than a percentage dilution of the initial CSE [6, 30-31]. The amount the stock solution is diluted to in order to obtain the final working concentration is not consistently stated [6]. Some investigators opted to test individual components of CSE [32-34]. We have recently demonstrated that acrolein and nicotine, important chemicals in volatile smoke, have pro-inflammatory and immunosuppressive effects respectively [35].

We have demonstrated that the optical density of CSE at 450 nm is constant when multiple 5% CSE preparations are prepared in sequence [36]. While standardising the CSE preparation is important, we have demonstrated that using 25 ml of media to prepare CSE, using a single cigarette, will produce a consistent preparation in terms of optical density [37]. Other groups have reported similar findings [22]. Using optical density as a measure of the "strength" of the extract only assumes greater importance with CSE is prepared using multiple cigarettes [38]. Using our particular CSE preparation we have demonstrated that healthy and COPD PBECs differ in their responses to a range of concentrations of CSE as determined by release of IL-8 (Figure 1). Furthermore, exposing healthy cultures to low concentrations of CSE heightens activation of NF-κB after stimulation with *Pseudomonas aeruginosa* LPS (Figure 2).

CSE has been reported to delay LPS-induced inflammatory responses in primary epithelial cells, but when later time points were considered, levels of IL-8 and GM-CSF trended upwards [22-23]. With this in mind, the outcome of these particular experiments would ultimately be determined by time-point supernatants were collected. An earlier time point would have suggested

that CSE was immunosuppressive, whereas later time points would have indicated pro-inflammatory properties.

The international reference cigarette KY1R3F is most widely used in CSE research [39], but commercial brands have been considered by others to have greater relevance [40]. On occasion, the type of cigarette is not reported [31]. Not all research groups opt to use cigarettes which have a filter [41], which has itself been manually removed by others prior to proceeding with CSE preparation [42]. Perhaps somewhat ironically, the filter itself leads to greater abundance of free radicals that are present in mainstream cigarette smoke [43]. Despite these variances, and in the vast majority of published work, volatile cigarette smoke is combusted through medium without serum [44] or alternatively phosphate buffered saline [42].

CSE has in recent times been criticised and deemed a poor substitute for the prolonged, chronic exposure of tobacco smoke that many smokers are exposed to.[45] The diversity in the methods adopted in the preparation of CSE, highlight the fact that it is difficult to establish a gold standard to which CSE research should aspire. Previous studies using CSE have been guided by the fact that the average smoker smokes in excess of 13 cigarettes per day, and used intermittent CSE exposures at 2 h intervals in an effort to replicate this effect in cell culture models [22]. The response of airway epithelial cells to volatile smoke, at least to a degree, should guide our expectations from CSE research. To add to the shortcomings of CSE, the very nature of the substance, keeping in mind the sheer number if chemicals contained within it, renders it challenging to identify any individual chemical which may be regarded as of greater importance when considering inflammatory responses. Furthermore,
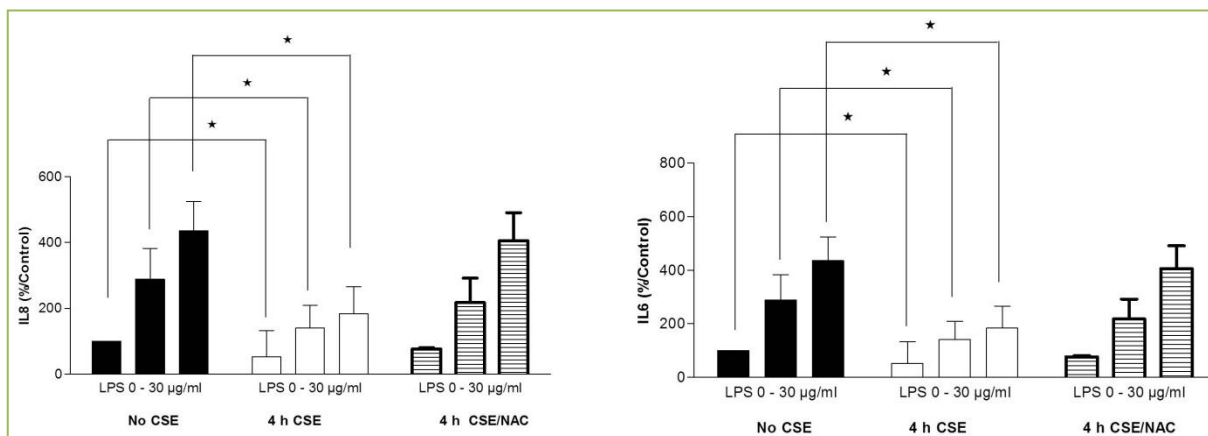
Figure 3. IL-8 and IL-6 response from PNECs after 24h treatment with *Pseudomonas aeruginosa* LPS with or without pre-treatment with CSE ± NAC for 4h. PNEC cells were treated with 5% CSE ± 20 mM N-acetylcysteine (NAC) for 4 h then with increasing concentrations of *Pseudomonas aeruginosa* LPS [0 – 25 μg/ml] for 24 h (n = 3). Supernatants were collected and assessed for IL-6 and IL-8 by ELISA in all cases. Data are displayed as mean ± SEM and * indicates a significant difference ($p < 0.05$). The control IL-8 and IL-6 releases were: 1508 ± 552 and 265 ± 47 pg/ml respectively. Data are displayed as mean ± SEM and * indicates a significant difference ($p < 0.05$; Paired t-test)

it is difficult in understanding the relevance of any particular CSE concentration and duration of exposure. Even if inferences are made on the basis of findings from CSE data, these experiments do not duplicate in an entirely satisfactory manner all of the components that exist in living systems.

It is well documented and accepted that cigarette smoke reduces protective antioxidants.[46] The high concentration of the oxidant molecules present in cigarette smoke contributes to the development of smoke-related lung disease. Oxidative damage is an important process in the pathogenesis of COPD. Heightened levels of markers of free radical damage (such as urinary 8-hydroxy-2-deoxyguanosine and levels of 3-nitrotyrosine in the airway surface fluid) are present in subjects with COPD, which follow a similar pattern to disease severity [47], A single puff of cigarette smoke contains as many as 10^{14} free radicals.[48] Interestingly, the immunosuppressive properties of CSE can be explained, at least in part, by reactive oxidative species. This has been demonstrated by the addition of n-acetylcysteine (NAC) to CSE which mitigates its immunosuppressive properties (Figure 3 and Figure 4). Although the reported effects of CSE, and for acrolein, are diverse in the literature, the ability of NAC influence the effects of CSE in cell culture is more consistent, an effect which is also apparent in dendritic cells and human pulmonary macrophages [49-50].

Nasal vs bronchial epithelial cells for airway epithelial cell culture research

The first publication using nasal epithelial cells as starting material for cell culture was in 1973 using neonates [51]. This was followed by the use of nasal polyp epithelium the following year [52]. Then, in 1983, epithelial cells were obtained from surgical specimens of turbinate tissue [53]. This was followed by two similar reports in 1985 [54-55]. It was a further 6 years later until there was evidence of successfully obtaining adequate cell numbers by the use of simple nasal brushings [56]. Despite some initial criticisms of the technique [57], this latter method is regarded as a feasible and an acceptable technique to obtain adequate numbers of nasal (and bronchial) epithelial cells and to use these cells to successfully perform cell culture in both monolayer and in air-liquid interface (ALI) models [58-59].

The use of primary nasal epithelial cells (PNECs) instead of PBECs for the purposes of cell culture research is more convenient and practical in terms of obtaining epithelial cells for experiments. Devalia el al, by comparing epithelial cells from the nasal and bronchial mucoas acquired from subjects attending for turbinectomy and thoracic surgery, demonstrated striking similarities between the nasal and bronchial cells when their morphology or ciliary activity are determined [60]. These nasal and bronchial cells maintained contact with their underlying tissues for a more prolonged period prior to be used in their experiments, adding strength to their results. It should be pointed out that the samples were not obtained from the same individuals - however, their results are highly unlikely to have changed had the samples been paired. However, using PNEC cells as an alternative to PBEC cells depends on a satisfactory and robust relationship between both cells in terms of inflammatory responses and expression of receptors that
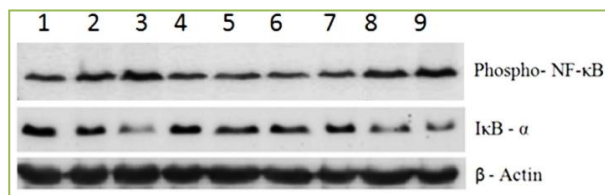
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Figure 4. Effect of *Pseudomonas aeruginosa* LPS, *Pseudomonas aeruginosa* LPS with CSE pre-treatment and *Pseudomonas aeruginosa* LPS with CSE/NAC pre-treatment on phospho-NF-κB expression in healthy PNEC cultures determined using Western blotting. Representative Western blot of phosho-NF-κB and IκB-α protein expression in healthy PNECs (with ß-actin loading controls). Lanes 1-3 represent treatment of PNECs with 0, 10, 30 *µ*g/ml *Pseudomonas aeruginosa* LPS (1 h), lanes 4-6: 0, 10, 30 *µ*g/ml *Pseudomonas aeruginosa* LPS (1 h) with pre-treatment with 5% CSE for 4 h,and lanes 7-9 represent 0, 10, 30 *µ*g/ml *Pseudomonas aeruginosa* LPS (1 h) with pre-treatment with 5% CSE/NAC for 4 h (n=3).

Figure 5. IL-8 release from bronchial epithelial cells from COPD subjects after stimulation with *Pseudomonas aeruginosa* LPS ± CSE in well differentiated well differentiated cultures and in submerged models. Control release of IL-8 from ALI cultures was 33,449 pg/ml (Apical supernatants) and 893 pg/ml for the submerged model. Both cell cultures were stimulated with 25 *µ*g/ml *Pseudomonas aeruginosa* LPS. After stimulating ALI well differentiated PBEC cultures with 25 *µ*g/ml *Pseudomonas aeruginosa* LPS for 24 h, there was a 1.29 fold increase in IL-8 release. This was reduced to 0.53 fold change relative to control after pre-treatment with CSE for 24 h. The corresponding values for the submerged model were 2.72 and 1.84 respectively. Data are displayed as median ± IQR; * (*p* <0.05, Mann-Whitney test; n=3).

are relevant to the research hypothesis. Published data on this issue are inconsistent [37, 58].

We have demonstrated that PNECs, at least in subjects with COPD, cannot satisfactorily be used as surrogates for PBECs for inflammation research. By comparing IL-8 release, under both resting and stimulated conditions, although there were differences in absolute mediator release, a correlation was present. However, significant differences were present for IL-6, and for the responses of the cell cultures to CSE and to acrolein. Although acrolein had the capacity to stimulate the release of IL-8 in PNEC cultures, it was without effect in the PBEC cultures [37].

The responses of PNEC cultures to CSE, in themselves, remain important to study. For many smokers the entire airway is exposed to volatile cigarette smoke. Furthermore, sinonasal symptoms are prominent in smokers and COPD subjects. Therefore, although we do not support the use of PNEC cells as an alternative for PBEC cells, these experiments provide useful information on the responses of PNEC cultures to CSE. This allows us to further understand the mechanisms as to why this group of subjects develop sinonasal symptoms.

Merits of the air liquid interface and the submerged cell culture models

The most widely utilised system enabling the cells to undergo mucociliary differentiation involves growing them on porous supports at an air liquid interface (ALI), first shown by Whitcut et al [61]. Tracheobronchial cells from a number of hosts, including guinea pigs, rats, cows, and humans [62-64] have been cultured in this configuration and successfully retain many morphological and functional characteristics of an in vivo airway epithelium. Recent years have seen a significant
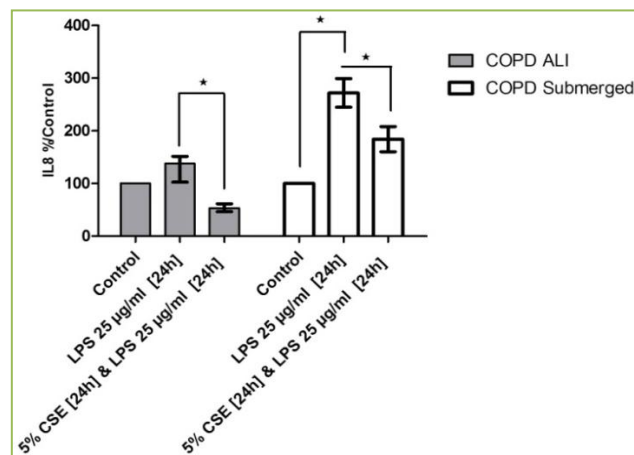
increase in such cultures whereby they demonstrate vectoral mucus transport [65], high resistance to gene therapy vectors [66], and cell-type-specific infection by viruses, functions that cannot be studied using undifferentiated cells on plastic. The complex process of airway epithelial differentiation involves cell-matrix and cell-cell interactions, the differentiation of mucous and goblet cells, and the acquisition of characteristic epithelial ion transport properties. Retinoic acid, which can be added to the cell culture medium, can suppress squamous metaplasia in culture [67].

As primary nasal and bronchial epithelial cells are cultured in ALI cultures, the degree of differentiation, and hence the cell phenotype, changes with time.[68] It is likely that any experimental results are dependent on the timing of the study, and on the degree of differentiation. It has already been reported that in human nasal epithelial cells, the percentages of ciliated cells increases steadily up to 28 days [68]. Although it is difficult to know with absolute certainty what the ideal amount of ciliated cells should be, Chapelin et al reported the percentage of ciliated cell in vivo to be of the order of 59% [69]. In contrast, the amount of mucin increased abruptly on the 14th day, with minimal changes at later time points.[68] It therefore seems that for in vitro studies using nasal epithelial cells, the time point is critical as, depending on the endpoints, will have an influence on the outcome of
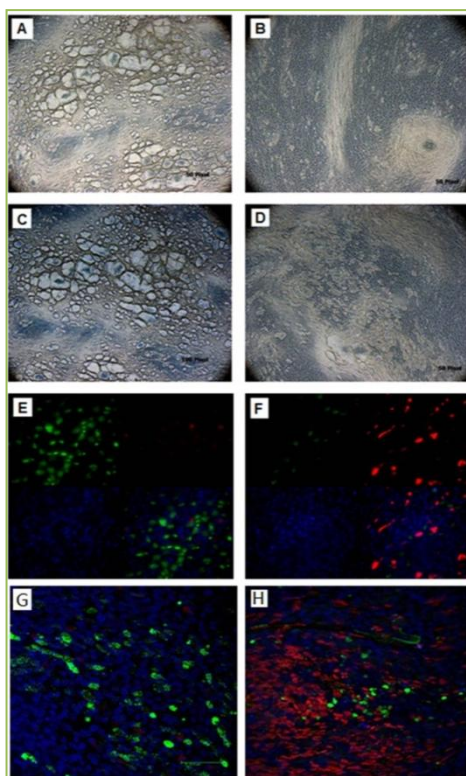
Figure 6. Confirmation of the presence of goblet cells using light microscopy and immunofluorescence. Well differentiated PBEC cultures from 4 healthy subjects had significant differences in the amount of goblet cells and cilia as demonstrated by (A-D) direct light microscopy (x32) and (E-H) immunofluoresence. Cultures were differentiated for 28 days, fixed and stained against mouse anti-MUC5AC Ab (dilution 1:200) and rabbit anti-acetylated β-tubulin proteins (dilution 1:150). Primary antibodies were detected using the polyclonal secondary antibodies goat anti-mouse conjugated with FITC (green, diluted 1:400), and goat anti-rabbit conjugated with alexofluor 568 (red, diluted 1:600). Cultures were counterstained using DAPI (blue). Staining was analyzed on a laser confocal microscope (Leica TCS) at 40 objective lens magnification.

the study. In our research institute, we chose 28 days as this is the time point adopted by other groups and allowed comparison of results [59]. It would be of interest for future work to repeat experiments at earlier time points, particularly for those experiments studying mRNA expression. Interestingly, MUC5AC and MUC5B mRNA are known to be expressed during the second week after confluence of the cell culture is achieved [68].

Using this model, COPD PBEC cultures tend to be immunosuppressed by CSE at concentrations of CSE greater than 5% as determined by release of IL-8. This is also evident in submerged cultures (Figure 5). ALI cultures demonstrated a much higher release of IL-8 rendering it more challenging to measure absolute

differences in its release using commercial soluble mediator ELISA assays.

These different responses in submerged and ALI models are not very surprising. As the cells in the ALI model form many layers, it seems intuitive that not all cells would be equally stimulated by any given agent. In the submerged monolayer model on the other hand, it is more likely that a greater portion of the cells are exposed to the stimuli contained within the supernatant. The ALI model has been reported by others to be particularly useful when investigating morphological differences in different cell types [70], although this was not our experience. We have demonstrated striking differences between separate healthy control cultures themselves from different individuals (Figure 6), and we question if the ALI model is a robust and adequate model when considering this particular end point. Submerged models, more widely used in the context of CSE research [22-24, 71-72], may in fact be a more meaningful model when functional endpoints are to be considered. It therefore seems that the model chosen should depend on the hypothesis and the aims of the study.

Cigarette smoke extract is cytotoxic to epithelial cells & disrupts cellular tight junctions

CSE has been reported to induce apoptosis in PBEC [73-74] and in PNEC cultures,[29] but not by all investigators [75]. The methods used to establish the presence or absence of apoptosis varies among investigators, which can make comparisons between publications challenging. For example, the particular study which used PNECs detected cell viability and apoptosis by a number of assays, which included a FACS technique, in parallel with the activation of downstream pathways, including levels of caspase activity. Manzel et al., however, measured the presence or absence of apoptosis in PBECs by mitochondrial activity in conjunction with additional apoptosis assays, the details of which were not provided.[75] In our hands, CSE is cytotoxic to epithelial cells, particularly when used for extended periods of time (Figure 7 & Figure 8). We have demonstrated that the addition of NAC to the CSE inhibits these toxic effects (Figure 9). Furthermore, we have published differences in cytotoxic responses in cultures derived from healthy subjects and individuals with COPD [76].

Opinions as to the mode of cell death after CSE exposure are divergent [77-78]. Possible explanations for this include the varied methods used to study the mode of cell death, the concentration of the CSE, the exposure time, the particular cells used and the design of the experiment. Furthermore, and possibly of greater
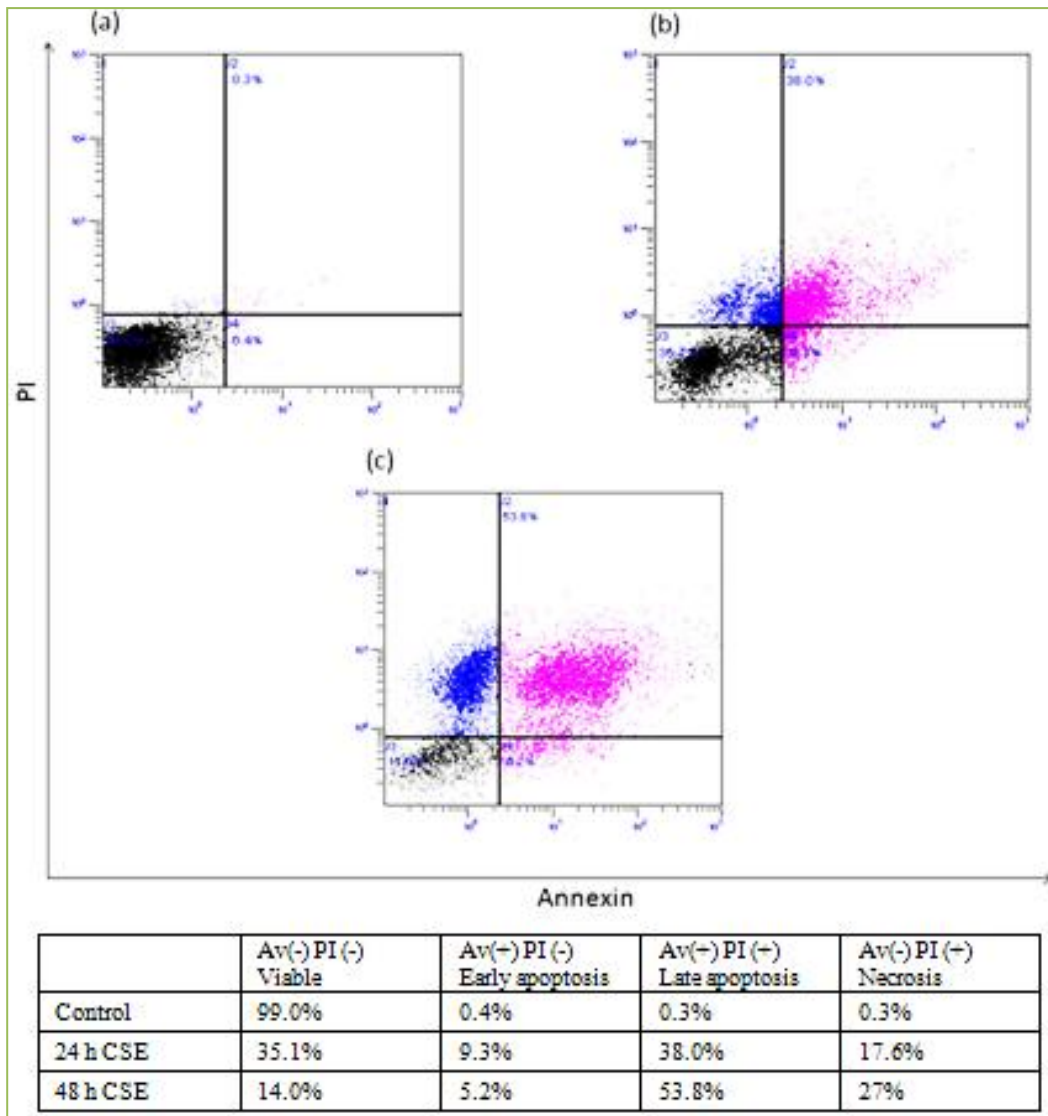
Figure 7. Effect of increasing CSE exposure time on cell death in COPD PBEC well differentiated
cultures. Representative dot-plot demonstrating predominantly necrosis in PBEC ALI cultures after 5%
CSE treatment for (a) 24 h and (b) 48 h demonstrated using the Annexin V/Propidium iodide assay.
Representative dot plots are shown of n=3.

	Av(-) PI (-) Viable	Av(+) PI (-) Early apoptosis	Av(+) PI (+) Late apoptosis	Av(-) PI (+) Necrosis
Control	99.0%	0.4%	0.3%	0.3%
24 h CSE	35.1%	9.3%	38.0%	17.6%
48 h CSE	14.0%	5.2%	53.8%	27%

importance, is that features of secondary necrosis may be incorrectly interpreted as the index event after either treatment with high concentration of CSE or after prolonged exposure. We have demonstrated that CSE induces apoptosis initially, and after a more prolonged exposure, the cells become necrotic. In this case, if the more prolonged exposure to CSE were used initially, the results could misleadingly been interpreted as necrosis of the cells as occurring in the first instance.

Studies which have been performed in subjects with COPD have described an increase in apoptosis in airway epithelial cells [79-81]. Perhaps a shortcoming of these studies is that levels of cell proliferation have not been measured in parallel. In health, the amount of apoptosis is in balance with the amount of proliferation and differentiation. To meaningfully interpret the amount of apoptosis, it would be ideal to measure both aspects. This provides a measurement of the net amount of apoptosis (which itself is counterbalanced by proliferation of the cells).

There has only been a single report of both parameters having been measured, which demonstrated increased levels of apoptosis in alveolar epithelial cells in end-stage emphysema, although the amount of proliferation of the alveolar walls remained constant between patients with and without emphysema.[79] It could be speculated that the resultant oxidant stress induced by cigarette smoke leads to apoptosis in COPD epithelial cells [82]. In fact,
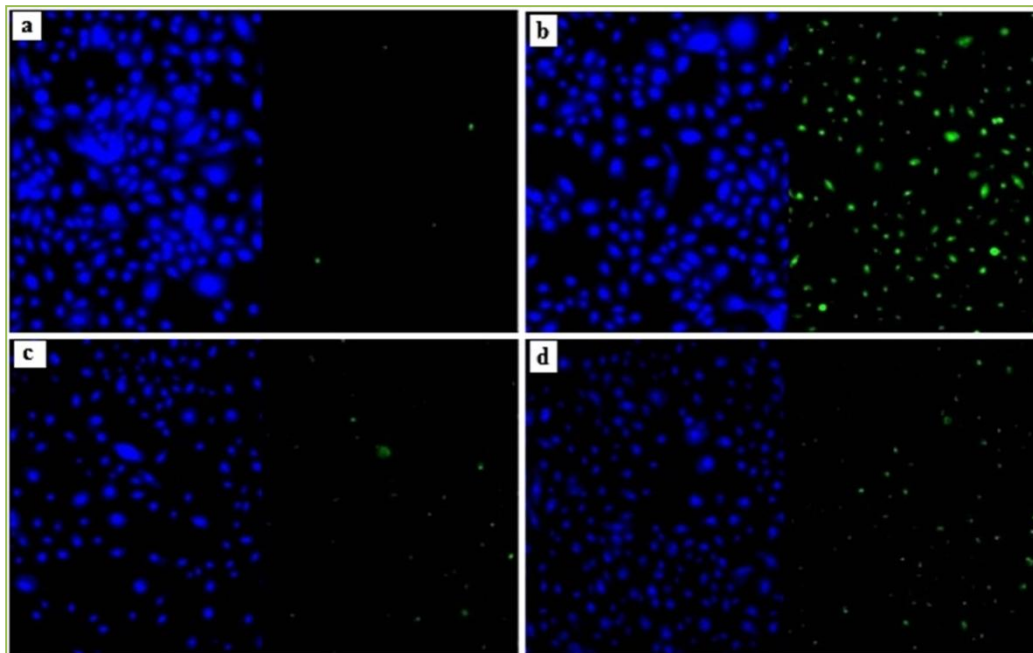
Figure 8. Effect of CSE on apoptosis in COPD PBEC well differentiated cultures using TUNEL assay. Representative images of bronchial epithelial cells grown on coverslips and treated with PBS (a) or DNase I solution for the negative and positive control respectively. Separate samples were treated with (c) 5% CSE (d) and 70% CSE for 4 hrs and the number of apoptotic cells counted using the Click-iT reaction according to manufacturer's instructions. The number of apoptotic cells determined by this technique was 14% and 68% respectively. Representative images are shown of n=3.

apoptosis of alveolar walls in COPD leading to parenchymal destruction has also been reported from histological studies [80, 83-84]. Furthermore, there has also been reports of a reduction in the amount of apoptosis in T lymphocytes obtained from COPD patients, as well as macrophages and neutrophils [85].

It is not yet clear which particular constituents contained within cigarette smoke are responsible for these injuries, but the use of oxidant scavengers in cell culture models with a CSE preparation have demonstrated that oxidants and aldehydes are important, at least in cell line experiments [77]. Cigarette smoke condensate, which also contains the lipid soluble fractions of volatile smoke, induces apoptosis in A549 cells. At a concentration of 50 μg/ml of the condensate, approximately 20% of the cells had undergone apoptosis, which was nominal at 0.1 μg/ml of CSC [86]. Interestingly, diesel exhaust particle (DEP)-induced cell death by necrotic mechanisms in cultured human bronchial epithelial cells [87]. This was attributed, at least in part, to a reduction in cellular glutathione. These observed effects were reduced if the relevant cultures were pre-treated with antioxidants.

CSE also disrupts tight junctions in COPD ALI cultures after CSE exposure on the basis of immunofluorescence findings and measuring trans-epithelial resistance (Figure 10). This was present, to the same degree, in the COPD cultures and those derived from healthy control subjects. Recent work using an electric cell-substrate impedance sensing model to determine trans-epithelial resistance in cell lines after treatment with CSE reported similar findings with reduced transepithelial resistance in 24 well plates after CSE exposure [27]. This is of importance in COPD, as damage to the airway epithelial surface would facilitate penetration of antigens to immune cells in the submucosa.

Cigarette smoke extract and TLR-4 expression

It is controversial as to if epithelial Toll-like receptor (TLR)-4 is expressed on the surface or intracellularly. Although there is evidence that this receptor is present on the surface of cells and is functional at this location,[88-89] other studies indicate that it has a predominantly intracellular localization, and that in order to become activated, internalisation of *Pseudomonas aeruginosa* LPS is necessary [90]. It is quite conceivable that these observed differences can be accounted for by the cell type used, whereby the data which suggests that TLR-4 was expressed on the cell surface used cystic fibrosis airway cell lines and primary alveolar type 2 cells, whereas, on the other hand, the data indicating TLR-4
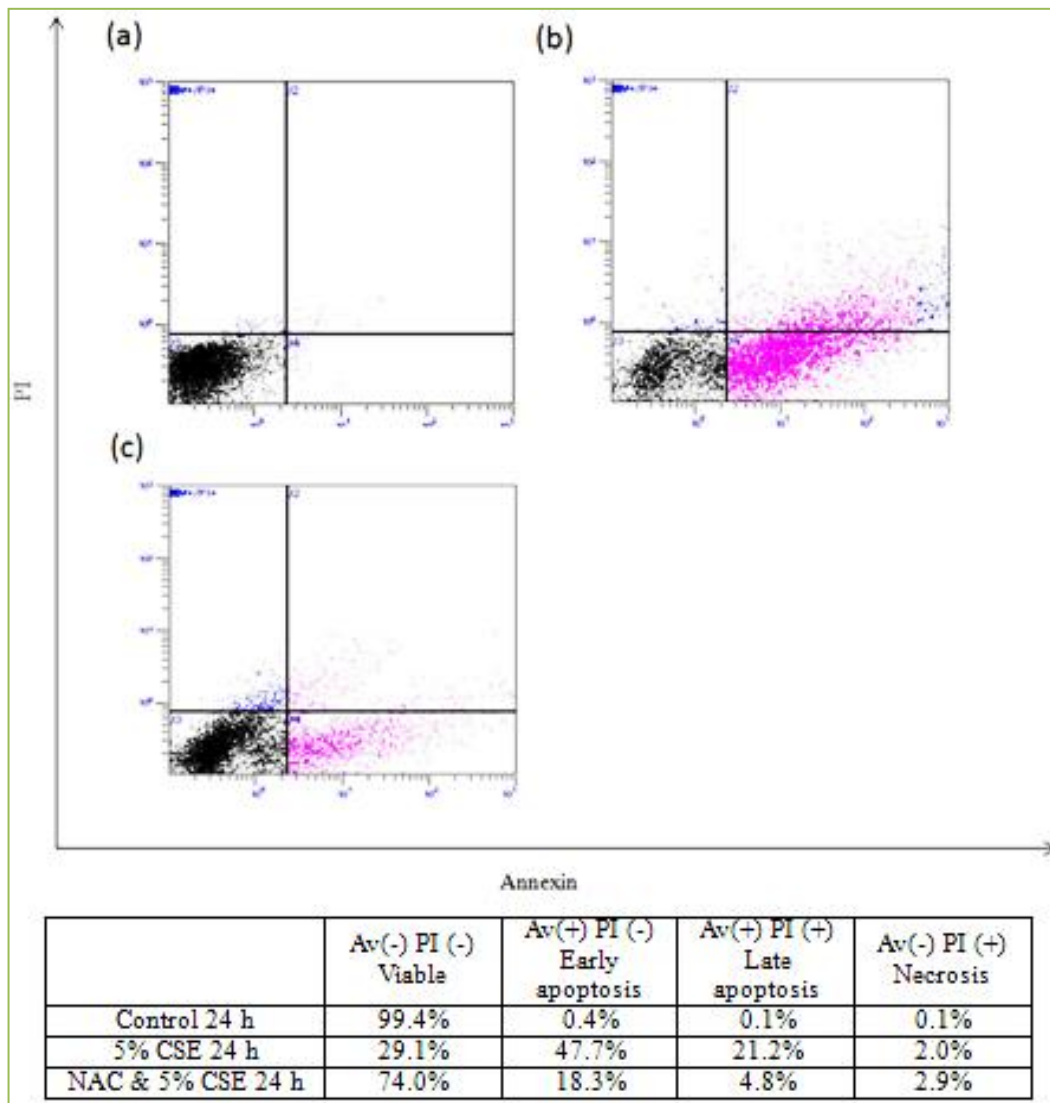
	Av(-) PI (-) Viable	Av(+) PI (-) Early apoptosis	Av(+) PI (+) Late apoptosis	Av(-) PI (+) Necrosis
Control 24 h	99.4%	0.4%	0.1%	0.1%
5% CSE 24 h	29.1%	47.7%	21.2%	2.0%
NAC & 5% CSE 24 h	74.0%	18.3%	4.8%	2.9%

Figure 9. Annexin-V/Propidium Iodide analysis of CSE ± NAC treatment in PNEC Cultures. In each plot, horizontal axis represents intensity of staining for Annexin V and vertical axis intensity of staining for PI (determined in the FL1 and FL3 plot respectively, both logarithmic scale). Dot plots represent (a) untreated cells stained with isotope control, (b) cells treated with 5% CSE for 24 h showing early apoptosis, c) cells treated with 5% CSE & 20 mM NAC for 24 h. Percentages of events positive for apoptosis and necrosis are shown in the table (n=3). Representative dot plots are shown of n=3.

had an intracellular location used human bronchial epithelial cell lines and alveolar carcinoma epithelial cell lines in the relevant experiments.

Previous investigators using flow cytometry demonstrated that it is unlikely that TLR-4 is recruited to the cell surface of epithelial cells after their activation such as with *Pseudomonas aeruginosa* LPS [90]. However, it is possible that other inflammatory mediators may also contribute to TLR-4 relocalisation. We have demonstrated that in COPD nasal epithelial cells (and bronchial cultures, data not shown), that TLR-4 is present both on the surface and intracellularly (Figure 11). It could be speculated that the intracellular localization of

TLR-4 may serve to prevent an inappropriate amount of activation of bronchial epithelial cells after exposure to ambient air which may contain sufficient quantities of *Pseudomonas aeruginosa* LPS and potentially contribute to a chronic inflammatory state. TLR-4 also has a reduced expression at a protein level after CSE pre-treatment as determined by flow cytometry. It therefore is possible that the cytotoxic effects of CSE are not the isolated explanation for the reduced release of IL-8 after CSE exposure in COPD cultures.

There were no differences in TLR-4 at a message level in PBEC and PNEC cultures after CSE exposure using RT-PCR [37]. This suggests that the differences in TLR-4
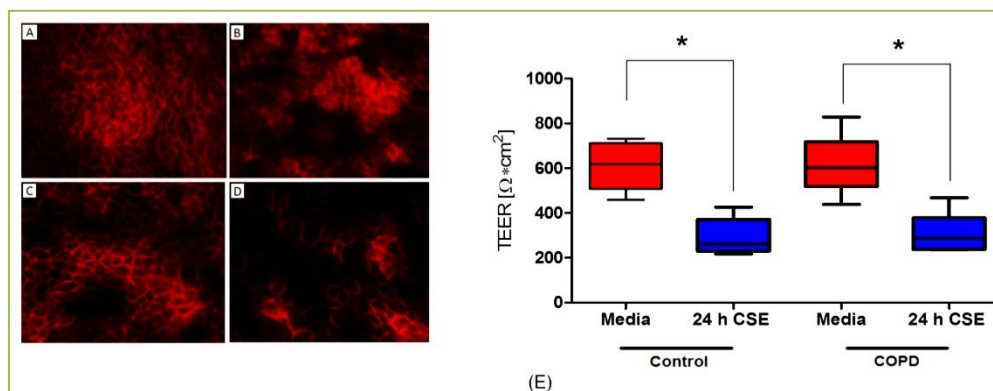
Figure 10. 4 h CSE exposure damages the integrity of tight junctions in well differentiated COPD PBEC cultures. Well differentiated PBEC cultures were stained again E-Cadherin (red) demonstrating the presence of tight junctions from (A) a healthy subject were treated with media, (B-D) a healthy subject, healthy smoker and a COPD suject were treated with 5% CSE for 24 h. (E) Transepithelial resistance (TEERs) was also measured before and after CSE treatment with a statistically significant fall in resistance indicative of a loss of tight junction integrity. Data are displayed as mean ± SEM and * indicates a significant difference ($p < 0.05$; Paired t-test; n=5).

were a consequence of post-translational events. Alternatively, the time point adopted to lyse the cell cultures to collect the RNA may not have been ideal, and the changes in message level may have passed. This would be a valuable area for future study. It would be most interesting to evaluate if CSE induced conformational changes to TLR-4, or damaged the integrity of the protein itself, which may render the protein itself more difficult to be measured. Interestingly, TLR-4 appears to be more avidly expressed in PNECs than PBECs (Figure 12). This may partly account for the higher IL-8 release in the former cultures which we have reported before [37]. It is of interest that with increased

severity of COPD, there are reductions in TLR-4 expression in both nasal and the lower airways.[91] This would serve to reduce inflammation at times of stability and so reduce, at least to a degree, undesirable inflammation.

It is not particularly surprising that the amount of IL-8 release from PNEC cultures is higher than PBEC cultures with the knowledge that nasal epithelial cells are for many individuals first exposed in vivo to inflammatory respirable particles in the ambient air. In this context, the nasal mucosa serves to protect the lower airways from inhaled noxious agents from the environment.
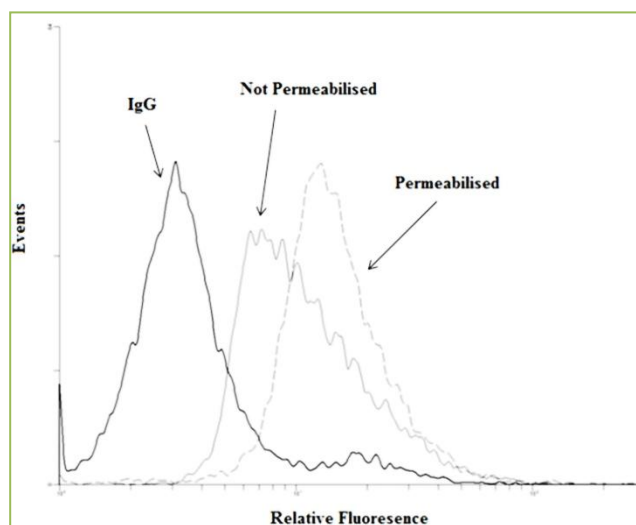
Conclusion

CSE is a particular complicated substance which, despite its shortcomings, contains many compounds which are inhaled by smoking subjects. However, CSE remains different from gaseous smoke, and this renders it challenging to determine what experimental conditions should be adopted in order to optimally reproduce the impact of cigarette smoke on the airway epithelium. The use of whole cigarette smoke (WCS) rather than aqueous cigarette smoke in cell culture experiments has obvious advantages. CSE prepared by our method used may not reflect the true in vivo interaction between cigarette smoking and epithelial cells in smokers [35, 37, 76]. WCS, on the other hand, encapsulates the 4,700 chemical compounds and the high concentration of oxidants (10^{14} molecules/puff) per puff of cigarette.[92] Therefore, the use of WCS, by more closely modeling the in vivo exposure of the airway to volatile smoke, could be regarded as of greater relevance.

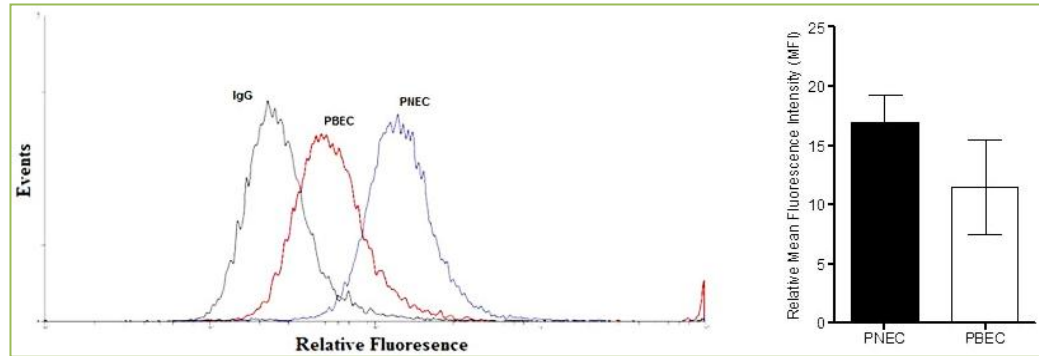Almost certainly as a consequence of the varying

Figure 11. Localization of TLR-4 protein in COPD PNECs using flow cytometry. PNEC cells were permeabilized (or not) and subsequently stained with PE conjugated anti-TLR-4 (or isotype control) antibodies.

Figure 12. Comparison of the expression of TLR-4 in PBEC and PNEC cultures. PBEC and PNEC cells were permeabilised and stained with anti-TLR-4 or isotype control antibodies conjugated with PE. The relative mean fluorescence intensity of for TLR-4 was greater in PNEC cells than PBEC cells, but this did not reach statistical significance.

protocols used for preparing CSE there remains no consensus on the fundamental effects CSE may have on airway epithelial cells. However, it does appear that cigarette smoke is likely to have an impact on mucosal epithelial defence. We have previous reported that if CSE is prepared by bubbling volatile smoke from a single cigarette through 25 ml of media over a 5 minute period, filtering the resulting solution and diluting to a working concentration of 5%, results in a consistent CSE solution in terms of optical density, and this protocol ensures that exposure time is the only determinant as to if CSE treated epithelial cells are either stimulated or immunosuppressed. By using physiologically relevant and attainable concentrations of acrolein and nicotine, we have also demonstrated that these chemicals are responsible, at least in part, for the stimulatory and immunosuppressive effects respectively.

Conflicting Interests

The authors declare that they have no Conflicting Interests.

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