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

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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 \cite{1,2}. By contributing to the innate immune system it provides an efficient host defence system at the mucosal surface \cite{3}.

IL-8 is of particular importance in a number of inflammatory lung diseases\cite{4}, and considered a meaningful endpoint to measure in airway epithelial cell research \cite{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 \cite{7,8}. IL-6 is induced in lung tissue after cigarette smoke exposure in murine studies \cite{9}. Systemic IL-6 is heightened in COPD subjects with an
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
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).

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 β-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).

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. 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 1 cigarette 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,
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 \[^{56-59}\].

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 et al, by comparing epithelial cells from the nasal and bronchial mucosas 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 PNECs 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 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 4h then with increasing concentrations of *Pseudomonas aeruginosa* LPS [0 – 25 µg/ml] for 24h (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).
are relevant to the research hypothesis. Published data on this issue are inconsistent [37, 38].

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
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. 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.

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, 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, 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 and in PNEC cultures, but not by all investigators. 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. 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.

Opinions as to the mode of cell death after CSE exposure are divergent. 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
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,
apoptosis of alveolar walls in COPD leading to parenchymal destruction has also been reported from histological studies \cite{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 \cite{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 \cite{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 \cite{86}. Interestingly, diesel exhaust particle (DEP)-induced cell death by necrotic mechanisms in cultured human bronchial epithelial cells \cite{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 \cite{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,\cite{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 \cite{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
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

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*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.
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...
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 previously 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.

References
1. Diamond G, Legarda D, Ryan LK. The innate immune response of the respiratory epithelium. Immunol Rev 2000; 173:27-38.
2. Schulz C, Farkas L, Wolf K, Kratzel K, Eissner G, Pfeifer M. Differences in LPS-induced activation of bronchial epithelial cells (BEAS-2B) and type II-like pneumocytes (A-549). Scand J Immunol 2002; 56:294-302.
3. Bals R, Beisswenger C, Blouquit S, Chinet T. Isolation and air-liquid interface culture of human large airway and bronchiolar epithelial cells. J Cyst Fibros 2004; 3 Suppl 2:49-51.
4. Pease JE, Sabroe I. The role of interleukin-8 and its receptors in inflammatory lung disease: implications for therapy. Am J Respir Med 2002; 1:19-25.
5. Schulz C, Wolf K, Harth M, Kratzel K, Kunz-Schughart L, Pfeifer M. Expression and release of interleukin-8 by human bronchial epithelial cells from patients with chronic obstructive pulmonary disease, smokers, and never-smokers. Respiration 2003; 70:254-261.
6. Mortaz E, Henricks PA, Kraneveld AD, Givi ME, Garssen J, Folkerts G. Cigarette smoke induces the release of CXCL-8 from human bronchial epithelial cells via TLRs and induction of the inflammasome. Biochim Biophys Acta 2011; 1812:1104-1110.
7. Baggionini M, Dewald B, Moser B, Interleukin-8 and related chemotactic cytokines—CXCl and CC chemokines. Adv Immunol 1994; 55:97-179.
8. Jorrens PG, Richman-Eisenstat JB, Housset BP, Graf PD, Ueki IF, Olesch J et al. Interleukin-8 induces neutrophil accumulation but not protease secretion in the canine trachea. Am J Physiol 1992; 263:L708-713.
9. Vlahos R, Bozinoiski S, Jones JE, Powell J, Gras J, Lilja A et al. Differential protease, innate immunity, and NF-kappaB induction profiles during lung inflammation induced by subchronic cigarette smoke exposure in mice. Am J Physiol Lung Cell Mol Physiol 2006; 290:L931-945.
10. Broekhuizen R, Wouters EF, Creutzberg EC, Schols AM. Raised CRP levels mark metabolic and functional impairment in advanced COPD. Thorax 2006; 61:17-22.
11. Hansen MJ, Gualano RC, Bozinowski S, Vlahos R, Anderson GP. Therapeutic prospects to treat skeletal muscle wasting in COPD (chronic obstructive lung disease). Pharmacol Ther 2006; 109:162-172.
12. Scheidt-Nave C, Bismar H, Leidig-Bruckner G, Woitge H, Seibel MJ, Ziegler R et al. Serum interleukin 6 is a major predictor of bone loss in women specific to the first decade past menopause. J Clin Endocrinol Metab 2001; 86:2032-2042.
13. Yende S, Tuomanen EI, Wunderink R, Kanaya A, Newman AB, Harris T et al. Preinfection systemic inflammatory markers and risk of hospitalization due to pneumonia. Am J Respir Crit Care Med 2005; 172;1440-1446.
14. Kode A, Yang SR, Rahman I. Differential effects of cigarette smoke on oxidative stress and proinflammatory cytokine release in primary human airway epithelial cells and in a variety of transformed alveolar epithelial cells. Respir Res 2006; 7:132.
15. Phillips J, Kluss B, Richter A, Massey E. Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses. Altern Lab Anim 2005; 33:239-248.

16. Betsuyaku T, Hamamura I, Hata J, Takahashi H, Mitsuhashi H, Adair-Kirk TL et al. Bronchiolar chemokine expression is different after single versus repeated cigarette smoke exposure. Respir Res 2008; 9:7.

17. Glader P, Moller S, Lilja J, Wieslander E, Lofdahl CG, von Wachenfeldt K. Cigarette smoke extract modulates respiratory defence mechanisms through effects on T-cells and airway epithelial cells. Respir Med 2006; 100:818-827.

18. Beisswenger C, Platz J, Seifert C, Vogelmeier C, Bals R. Exposure of differentiated airway epithelial cells to volatile smoke in vitro. Respiration 2004; 71:402-409.

19. Hellermann GR, Nagy SB, Kong X, Lockey RF, Mohapatra SS. Mechanism of cigarette smoke condensate-induced acute inflammatory response in human bronchial epithelial cells. Respir Res 2002; 3:22.

20. Richter A, O'Donnell RA, Powell RM, Sanders MW, Holgate ST, Djkunovic R et al. Autocrine ligands for the epidermal growth factor receptor mediate interleukin-8 release from bronchial epithelial cells in response to cigarette smoke. Am J Respir Cell Mol Biol 2002; 27:85-90.

21. Mio T, Romberger DJ, Thompson AB, Robbins RA, Heires A, Rennard SI. Cigarette smoke induces interleukin-8 release from human bronchial epithelial cells. Am J Respir Crit Care Med 1997; 155:1770-1776.

22. Luan M, Bozinovski S, Anderson GP. Cigarette smoke inhibits lipopolysaccharide-induced production of inflammatory cytokines by suppressing the activation of activator protein-1 in bronchial epithelial cells. J Immunol 2004; 173:4164-4170.

23. Li W, Xu YJ, Shen HH. Effect of cigarette smoke extract on lipopolysaccharide-activated mitogen-activated protein kinase signal transduction pathway in cultured cells. Chin Med J (Engl) 2007; 120:1075-1081.

24. Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD. Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. Am J Respir Cell Mol Biol 2004; 30:500-509.

25. Moretto N, Facchinetti F, Southworth T, Civelli M, Singh D, Patacchini R. alpha, beta-unsaturated aldehydes contained in cigarette smoke elicit IL-8 release in pulmonary cells through mitogen-activated protein kinases. Am J Physiol Lung Cell Mol Physiol 2009; 296:L839-848.

26. Pera T, Gosens R, Lesterhuis AH, Sami R, Toorn M, Zaatgsma J et al. Cigarette smoke and lipopolysaccharide induce a proliferative airway smooth muscle phenotype. Respir Res 2010; 11:48.

27. Heijink IH, Brandenburg SM, Postma DS, van Oosterhout AJ. Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery. Eur Respir J 2012; 39:419-428.

28. Wang JH, Kim H, Jang YJ. Cigarette smoke extract enhances rhinovirus-induced toll-like receptor 3 expression and interleukin-8 secretion in A549 cells. Am J Rhinol Allergy 2009; 23:5-9.

29. Lan MY, Ho CY, Lee TC, Yang AH. Cigarette smoke extract induces cytotoxicity on human nasal epithelial cells. Am J Rhinol 2007; 21:218-223.

30. Sarir H, Mortaz E, Karimi K, Kraneveld AD, Rahman I, Caldenhoven E et al. Cigarette smoke regulates the expression of TLR4 and IL-8 production by human macrophages. J Inflam (Lond) 2009; 6:12.

31. Karimi K, Sarir H, Mortaz E, Smit JJ, Hosseini H, De Kimpe SJ et al. Toll-like receptor-4 mediates cigarette smoke-induced cytokine production by human macrophages. Respir Res 2006; 7:66.

32. Sarkar P, Hayes BE. Induction of COX-2 by acrolein in rat lung epithelial cells. Mol Cell Biochem 2007; 301:191-199.

33. Zhang H, Forman HJ. Acrolein induces heme oxygenase-1 through PKC-delta and PI3K in human bronchial epithelial cells. Am J Respir Cell Mol Biol 2008; 38:483-490.

34. McMaster SK, Paul-Clark MJ, Walters M, Fleet M, Anandarajah J, Sriskandan S et al. Cigarette smoke inhibits macrophage sensing of Gram-negative bacteria and lipopolysaccharide: relative roles of nicotine and oxidant stress. Br J Pharmacol 2008; 153:536-543.

35. Comer DM, Elborn JS, Ennis M. Inflammatory and cytotoxic effects of acrolein, nicotine, acetylateddehyde and cigarette smoke extract on human nasal epithelial cells. BMC Pulm Med 2014; 14:32.

36. Comer DM, Kidney JC, Ennis M, Elborn JS. Airway epithelial cell apoptosis and inflammation in COPD, smokers and nonsmokers. Eur Respir J 2013; 41:1058-1067.

37. Comer DM, Elborn JS, Ennis M. Comparison of Nasal and Bronchial Epithelial Cells Obtained from Patients with COPD. PLoS One 2012; 7:e32924.

38. Facchinetti F, Amadei F, Gepetti P, Tarantini F, Di Serio C, Dragotto A et al. Alpha, beta-unsaturated aldehydes in cigarette smoke release inflammatory mediators from human macrophages. Am J Respir Cell Mol Biol 2007; 37:617-623.

39. Kang MJ, Homer RJ, Gallo A, Lee CG, Crothers KA, Cho SJ et al. IL-18 is induced and IL-18 receptor alpha plays a critical role in the pathogenesis of cigarette smoke-induced pulmonary emphysema and inflammation. J Immunol 2007; 178:1948-1959.

40. Demirjian L, Abboud RT, Li H, Duronio V. Acute effect of cigarette smoke on TNF-alpha release by macrophages mediated through the erk1/2 pathway. Biochim Biophys Acta 2006; 1762:592-597.

41. Kubo S, Kobayashi M, Masunaga Y, Ishii H, Hirano Y, Takahashi K et al. Cytokine and chemokine expression in cigarette smoke-induced lung injury in guinea pigs. Eur Respir J 2005; 26:993-1001.

42. Winkler AR, Nocka KH, Sulahian TH, Kobzik L, Williams CM. In vitro modeling of human alveolar macrophage smoke exposure: enhanced inflammation and impaired function. Exp Lung Res 2008; 34:599-629.

43. Valavanidis A, Haralambous E. A comparative study by electron paramagnetic resonance of free radical species in the mainstream and sidestream smoke of cigarettes with conventional acetate filters and 'bio-filters'. Redox Rep 2001; 6:161-171.
44. Birrell MA, Wong S, Catley MC, Belvisi MG. Impact of tobacco smoke on key signaling pathways in the innate immune system. J Cell Physiol 2008; 214:27-37.

45. Shapiro SD. Smoke gets in your cells. Am J Respir Cell Mol Biol 2004; 31:481-482.

46. Rahman I, MacNee W. Oxidant/antioxidant imbalance in smokers and chronic obstructive pulmonary disease. Thorax 1996; 51:348-350.

47. Igishi T, Hiduta Y, Kato K, Sako T, Burioka N, Yasuda K et al. Elevated urinary 8-hydroxydeoxyguanosine, a biomarker of oxidative stress, and lack of association with antioxidant vitamins in chronic obstructive pulmonary disease. Respirology 2003; 8:455-460.

48. Pryor WA, Prier DG, Church DF. Electron-spin resonance study of mainstream and sidestream cigarette smoke: nature of the free radicals in gas-phase smoke and in cigarette tar. Environ Health Perspect 1983; 47:345-355.

49. Vassallo R, Kroening PR, Parambili J, Kita H. Nicotine and oxidative cigarette smoke constituents induce immune-modulatory and pro-inflammatory dendritic cell responses. Mol Immunol 2008; 45:3321-3329.

50. Koarai A, Yanagisawa S, Sugiuira H, Ichikawa T, Akamatsu K, Hirano T et al. Cigarette smoke augments the expression and responses of toll-like receptor 3 in human macrophages. Respirology 2012.

51. Boat TF, Kleinerman JJ, Fanarooff AA, Matthews LW. Toxic effects of oxygen on cultured human neonatal respiratory epithelium. Pediatr Res 1973; 7:607-615.

52. Boat TF, Kleinerman JJ, Carlson DM, Maloney WH, Matthews LW. Human respiratory tract secretions. 1. Mucous glycoproteins secreted by cultured nasal poly epithelium from subjects with allergic rhinitis and with cystic fibrosis. Am Rev Respir Dis 1974; 110:428-441.

53. Wiesel JM, Gamiel H, Vladosky I, Gay I, Ben-Bassat H. Cell attachment, growth characteristics and surface morphology of human upper-respiratory tract epithelium cultured on extracellular matrix. Eur J Clin Invest 1983; 13:57-63.

54. Wu R, Yankaskas J, Cheng E, Knowles MR, Boucher R. Growth and differentiation of human nasal epithelial cells in culture. Serum-free, hormone-supplemented medium and proteoglycan synthesis. Am Rev Respir Dis 1985; 132:311-320.

55. Yankaskas JR, Cotton CU, Knowles MR, Gatzky JT, Boucher RC. Culture of human nasal epithelial cells on collagen matrix supports. A comparison of bioelectric properties of normal and cystic fibrosis epithelia. Am Rev Respir Dis 1985; 132:1281-1287.

56. Bridges MA, Walker DC, Davidson AG. Cystic fibrosis and control nasal epithelial cells harvested by a brushing procedure. In Vitro Cell Dev Biol 1991; 27A:684-686.

57. Hull J, Harris A. Limitations of cell culture of airway epithelium collected by a nasal brushing technique. In Vitro Cell Dev Biol Anim 1994; 30A:488-489.

58. McDougall CM, Blaylock MG, Douglas JG, Brooker RJ, Helms PJ, Walsh GM. Nasal epithelial cells as surrogates for bronchial epithelial cells in airway inflammation studies. Am J Respir Cell Mol Biol 2008; 39:560-568.
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extract induced apoptosis in human bronchial epithelial cells. Exp Cell Res 2010; 316:3501-3511.

74. Sugiuira H, Liu X, Togo S, Kobayashi T, Shen L, Kawasaki S et al. Prostaglandin E(2) protects human lung fibroblasts from cigarette smoke extract-induced apoptosis via EP(2) receptor activation. J Cell Physiol 2007; 210:99-110.

75. Manzel LJ, Shi L, O'Shaughnessy PT, Thorne PS, Look DC. Cigarette Smoke Inhibition of the NF-(kappa)B-Dependent Response to Bacteria in the Airway. Am J Respir Cell Mol Biol 2010.

76. Comer D, Kidney J, Ennis M, Elborn J. Airway epithelial cell apoptosis and inflammation in COPD, smokers and non-smokers. Eur Respir J 2012.

77. Hoshino Y, Mio T, Nagai S, Miki H, Ito I, Izumi T. Cytotoxic effects of cigarette smoke extract on an alveolar type II cell-derived cell line. Am J Physiol Lung Cell Mol Physiol 2001; 281:L509-516.

78. Kim H, Liu X, Kobayashi T, Conner H, Kohyama T, Wen FQ et al. Reversible cigarette smoke extract-induced DNA damage in human lung fibroblasts. Am J Respir Cell Mol Biol 2004; 31:483-490.

79. Demedts IK, Demoor T, Bracke KR, Joos GF, Brusselle GG. Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. Respir Res 2006; 7:53.

80. Yokohori N, Aoshiba K, Nagai A. Increased levels of cell death and proliferation in alveolar wall cells in patients with pulmonary emphysema. Chest 2004; 125:626-632.

81. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. Eur Respir J 2005; 25:447-454.

82. de Souza PM, Lindsay MA. Apoptosis as a therapeutic target for the treatment of lung disease. Curr Opin Pharmacol 2005; 5:232-237.

83. Bardales RH, Xie SS, Schaefer RF, Hsu SM. Apoptosis is a major pathway responsible for the resolution of type II pneumocytes in acute lung injury. Am J Pathol 1996; 149:845-852.

84. Majo J, Ghezzo H, Cosio MG. Lymphocyte population and apoptosis in the lungs of smokers and their relation to emphysema. Eur Respir J 2001; 17:946-953.

85. Pletz MW, Ioanas M, de Roux A, Burkhardt O, Lode H. Reduced spontaneous apoptosis in peripheral blood neutrophils during exacerbation of COPD. Eur Respir J 2004; 23:532-537.

86. Kaushik G, Kaushik T, Khanduja S, Pathak CM, Khanduja KL. Cigarette smoke condensate promotes cell proliferation through disturbance in cellular redox homeostasis of transformed lung epithelial type-II cells. Eur J Clin Invest 2008; 38:120-131.

87. Matsuo M, Shimada T, Uenishi R, Sasaki N, Sagai M. Diesel exhaust particle-induced cell death of cultured normal human bronchial epithelial cells. Biol Pharm Bull 2003; 26:438-447.

88. Greene CM, Carroll TP, Smith SG, Taggart CC, Devaney J, Griffin S et al. TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. J Immunol 2005; 174:1638-1646.

89. Armstrong L, Medford AR, Uppington KM, Robertson J, Witherden IR, Tetley TD et al. Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. Am J Respir Cell Mol Biol 2004; 31:241-245.

90. Guillot L, Medjane S, Le-Barillec K, Balloy V, Danel C, Chignard M et al. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. J Biol Chem 2004; 279:2712-2718.

91. MacRedmond RE, Greene CM, Dorscheid DR, McElvaney NG, O'Neill SJ. Epithelial expression of TLR4 is modulated in COPD and by steroids, salmeterol and cigarette smoke. Respir Res 2007; 8:84.

92. Kirkham P, Rahman I. Oxidative stress in asthma and COPD: antioxidants as a therapeutic strategy. Pharmacol Ther 2006; 111:476-494.

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