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Characterisation of a Mouse Model of Cigarette Smoke Extract-Induced Lung Inflammation

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Abstract

Aim: The aim of this study was to develop a lung-targeted, mouse model of cigarette smoke-induced inflammation that can be used to study the pathophysiological changes that occur in the lungs of human smokers and patients with chronic obstructive pulmonary disease (COPD).

Materials & methods: Cigarette smoke extract (CSE) was prepared freshly daily. Intranasal administration into female mice was performed once daily for up to 3 weeks.

Results: CSE significantly increased airway macrophages after 3 and 4 days of dosing, and then declined over the subsequent 2 weeks. However, airway neutrophils were elevated after a single dose of CSE, and at all subsequent time points. Muc5AC was significantly increased in the Bronchoalveolar lavage (BAL) of CSE-treated animals compared to control mice ($P<0.05$), but TNF-α concentrations decreased in a dose-dependent manner. In animals challenged with CSE for 4 consecutive days, a PDE4 inhibitor (Roflumilast; 10 mg/kg BID) significantly inhibited both macrophages ($P<0.01$) and neutrophils ($P<0.001$), a steroid (prednisolone; 10 mg/kg BID) had no effect on either macrophages or neutrophils and an oral p38 inhibitor (PHA-818637; 10 mg/kg BID) inhibited macrophages ($P<0.05$), but not neutrophils. CSE inhibited lipopolysaccharide-induced airway neutrophilia.

Conclusion: This model reflects many aspects of human COPD including pulmonary leucocytes, mucin, TNF-α and response to clinical therapeutic agents and may be useful in assessing the efficacy of potential therapies.

Keywords: Cigarette smoke extract; Mouse; Neutrophils; Mucin

Introduction

In the United States, chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death [1] and is predominantly associated with chronic cigarette smoking [2]. Early characteristics of the disease include excessive mucus production, chronic inflammation, and progressive decline in lung function. Bronchoalveolar lavage (BAL) fluids from COPD patients have increased levels of neutrophils, mucin, interleukin (IL)-8, and tumor necrosis factor-α (TNF-α) [3]. Exacerbations are an important cause of the morbidity and mortality found in patients with COPD [4], are associated with increasing disease severity [5], and are frequently caused by viral infection and bacterial colonization [6].

Currently, the research required to investigate the underlying cellular mechanisms and design future pharmacological interventions is hampered by the lack of standardized translational animal models. A number of reports have described acute and chronic lung inflammation in various species following exposure to whole cigarette smoke [7-12]. Exposure is either by a 'nose only' or by a 'whole body' methodology, but neither the type of cigarettes, the number of cigarettes/day nor the duration of the exposure has been standardized. The obvious advantage of these models is that the whole cigarette smoke stimulus bears a direct resemblance to smoke exposure in humans. However, whole body exposure lacks the specific organ targeting associated with cigarette smoking and requires the use of complicated and expensive smoke exposure apparatus. One alternative is to generate an aqueous extract of cigarette smoke that enables dosing specifically to the lung area. An early study showed that high doses of oro-trachea (o.t.) administered CSE was associated with greater chronic respiratory pathology than lower doses of CSE or controls [13]. Exposure of rats to CSE resulted in an increase in lung neutrophils and epithelial permeability [14] and a decrease in lung glutathione [15]. Intraperitoneal administration of CSE in ovalbumin-challenged mice reduced plasma levels of OVA-specific antibodies by 80% following immunisation [16]. In a recent study, administration of CSE to BALB/c mice intranasally for 40 days increased BAL content of neutrophils, lymphocytes, KC, TNF-α and mucin [17]. This was associated with changes in pulmonary responses to methacholine and histological changes in the lung consistent with human COPD.

The aim of this study was to develop a mouse model of cigarette smoke-induced inflammation that can be used to study the pathophysiological changes that occur in the lungs of human smokers and patients with COPD. Such a model may be useful in assessing the efficacy of potential therapies.

Materials & Methods

Chemicals

Research Cigarettes (2R4F) were purchased from the Tobacco Research Institute (University of Kentucky, Kentucky, USA). Monteleukast and Roflumilast were purchased from Sequoia Research Products (Pangbourne, Berkshire, UK). All other regents were purchased from Sigma unless otherwise stated.

Cigarette smoke extract preparation

Cigarette smoke extract (CSE) was prepared daily in a custom designed smoking apparatus. A lighted cigarette was placed within a glass "smoking chamber". Mainstream smoke was withdrawn from the

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cigarette under negative pressure from a peristaltic pump and passed through Dulbecco's phosphate buffered saline without calcium or magnesium (PBS) within a glass "collection chamber". Cigarettes were smoked to within 3 mm of the cigarette filter, with an average burn time of 7 minutes. Smoke from 3 cigarettes was drawn through 3 ml of PBS and the resulting extract designated 100% CSE. Subsequent dilution was performed in PBS immediately prior to use. In one experiment, 300% CSE was prepared from the smoke of 9 cigarettes in 3ml of PBS.

Cigarette smoke extract (100%) was extracted into an equal volume of dichloromethane and analysed by gas chromatography (GC) coupled with flame ionisation detection (FID) and mass spectroscopy (MS). Five peaks were consistently identified within CSE, the second most abundant being identified as nicotine.

Animals

Female BALB/c and C57BL/6 mice, aged 8 weeks and weighing 20g were purchased from Charles River (Manston, Kent, UK). Animals were housed and handled according to Home Office legislation and local ethical regulations and allowed food and water *ad libitum*.

CSE administration

Mice were transiently anaesthetized with 2.5% isoflurane in 100% O_2. CSE was administered by intranasal instillation (50 µl) directly into the nares as the animal was held in a vertical position. Oro-tracheal instillation was achieved by hooking the front incisors over a wire frame so that the mouse was held in a vertical position. A cold light source was used to illuminate the outside of the throat. The tongue was gently displaced and the trachea visualized through a binocular dissection microscope. A Hamilton syringe with a 26 GA blunt needle was inserted between the vocal cords of the larynx into the trachea and 20 µl CSE dispensed. Control animals received PBS.

In experiments where LPS was combined with the CSE challenge, the LPS was added to the CSE immediately (<30 seconds) before dosing. In experiments where ozone was combined with CSE challenge, mice were exposed to 0.5 ppm ozone, generated by an ozone generator (model 0L80F/RT; Ozone Services, Burton, B.C., Canada) for 3 hours.

Compound administration

Dexamethasone was dissolved in sterile DPBS to a concentration of 200 µg/ml and 0.1ml administered i.p. 4 hours prior to CSE. Prednisolone was dissolved in water to a concentration of 1mg/ml. Roflumilast® and PHA-8186370 were suspended in 0.5% methocellulose/0.5% Tween-80 to a concentration of 1mg/ml and 0.5 mg/ml respectively. Prednisolone, Roflumilast® and PHA-818637 were administered in a 0.2 ml volume p.o. 4 hours prior to and after CSE.

Bronchoalveolar lavage (BAL) and cell count

Mice were euthanised with 0.1 ml i.p. PentoJect® 24 hours following the final CSE challenge (unless otherwise stated). The thoracic cavity was opened and blood withdrawn by cardiac puncture. The trachea was cannulated with a 20 GA Insyte I.V. catheter (Becton Dickinson, Oxford, UK) and the lungs lavaged with 4 x 0.5 ml DPBS containing 10mM EDTA. The 4 lavages from single animals were pooled and yielded a consistent return of approximately 1.9 ml that contained 90% of lavagable cells (data not shown). An aliquot of BAL was diluted 1:1 in trypan blue and cell density counted in a haemocytometer. Cytospins were prepared on microscope slides (Thermo Shandon, Runcorn, UK), air dried and stained with DiffQuik (Dade Behring, Milton Keynes, UK). Differential cell counts were performed manually using a light microscope counting at least 300 cells from each slide. The remaining BAL sample was centrifuged and the cell-free fraction frozen at -20°C for cytokine analysis.

TNF-α and Muc5AC ELISA

Murine TNF-α was measured in serum and lavage samples using Cytoset ELISA kits (Biosource, Nivelles, Belgium) according to instructions. The mucin ELISA was performed as described previously [18].

Histone deacetylase activity

Lungs perfused via the heart with 60 ml PBS containing 50 IU/ml heparin (prewarmed to 37°C) at a rate of 16 ml/minute (equivalent to the cardiac output in mouse) until run-off was clear. The lungs were excised, weighed, homogenised and the nuclear and cytosolic fractions fractions isolated using a Nuclear Extraction kit (Active Motif, Carlsbad, CA, USA) according to manufacturer's instructions. Protein concentration was determined using the Bradford reagent. Histone deacetylase activity in the nuclear fraction was measured using a Colorimetric HDAC Activity Assay Kit (Biovision Inc, Mountain View, CA, USA) according to manufacturer's instructions.

Low density array Taqman gene expression analysis

Lungs were excised, coarsely chopped and stored in RNA at 4°C for 24 hours, at -20°C for a further 24 hours and finally -80°C. Samples were shipped to Aros Applied Biosystems (Aarhus, Denmark) for mRNA extraction and expression analysis on 96-well mouse inflammation taqman low density array microfluidic cards that profile 96 genes associated with inflammation according to their in-house protocols.

Data analysis and presentation

Data was considered not normally distributed and underwent log transformation. ANOVA analysis was performed using LabStats, a Microsoft Excel add-in created by the Biostatistics and Reporting Group at Sandwich Laboratories in collaboration with Tessella Support Services plc (Abingdon, Oxon, UK). All analyses were performed on advice from non-clinical statisticians.

Results

Characterisation of CSE

The nicotine content of CSE was 57.5 ± 5.6 ug nicotine/ml as determined by GC-MS/GC-FID. Spectrophotometric absorption of CSE at 405 nm was routinely performed to ensure consistency between preparations. The mean absorbance (path length 1cm) was 0.287 (range 0.236 - 0.351; CV = 9.78%).

Effect of CSE administration route and mouse strain on BAL cell content

Administration of CSE via the intranasal route for 4 consecutive days resulted in a statistically significant increase in BAL macrophages compared to control animals (Table 1; P<0.001). Neutrophils constituted approximately 2% of total BAL cells. Nevertheless,

	Intranasal		Oro-tracheal	
	Macrophages	Neutrophils	Macrophages	Neutrophils
PBS	56 ± 34	1.4 ± 1.1	179 ± 58	0.8 ± 0.5
CSE	248 ± 70**	4.1 ± 3.2*	217 ± 95	0.7 ± 0.9

Macrophage and neutrophils in BAL ($x10^3$) from BALB/c mice treated for 4 days with 50l intranasal or 20 µl oro-tracheal PBS or 100% CSE. Values are mean ± S.D. of 5 mice. **P*<0.05; ***P*<0.001 (vs PBS)

Table 1: Effect of administration route on CSE-induced BAL cell number.

	BALB/c		C57BL/6	
	Macrophages	Neutrophils	Macrophages	Neutrophils
PBS	147 ± 9	1.7 ± 0.6	111 ± 42	0.5 ± 0.5
CSE	254 ± 53**	9.9 ± 4.5***	257 ± 80***	4.5 ± 3.5*

Macrophage and neutrophils in BAL (x10^3) from BALB/c and C57BL/6 mice treated for 4 days with 50 µl intranasal PBS or 100% CSE. Values are mean ± S.D. of 5 mice. **P*<0.05; ***P*<0.005, ****P*<0.001 (vs PBS)

Table 2: Effect of mouse strain on CSE-induced BAL cell number.

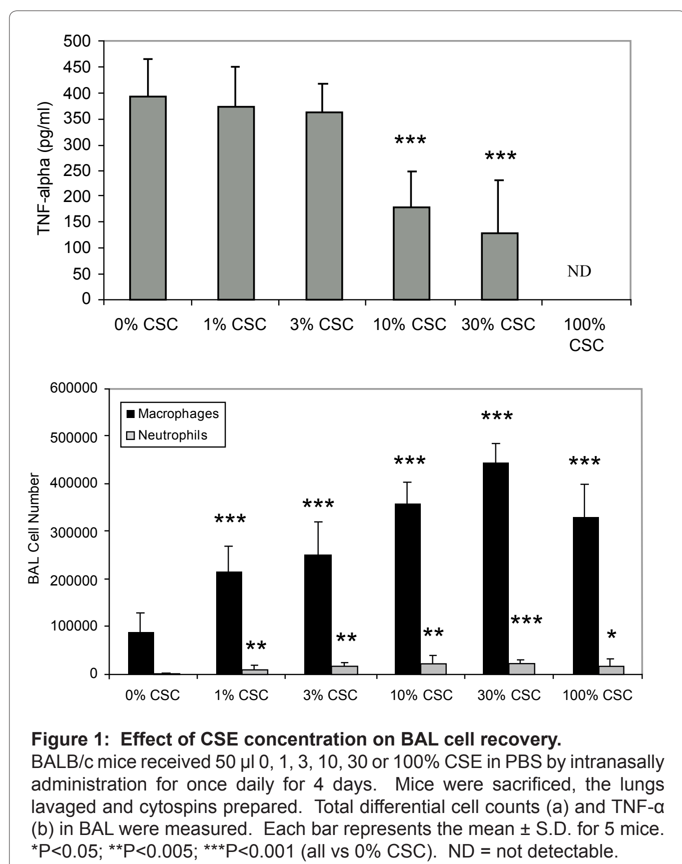

Figure 1: Effect of CSE concentration on BAL cell recovery.
BALB/c mice received 50 µl 0, 1, 3, 10, 30 or 100% CSE in PBS by intranasally administration for once daily for 4 days. Mice were sacrificed, the lungs lavaged and cytospins prepared. Total differential cell counts (a) and TNF-α (b) in BAL were measured. Each bar represents the mean ± S.D. for 5 mice. *P<0.05; **P<0.005; ***P<0.001 (all vs 0% CSC). ND = not detectable.

intranasal administered CSE significantly increased BAL neutrophils (P<0.05). Neither eosinophils nor lymphocytes were detected in BAL of PBS- or CSE-treated animals. Oro-tracheal administration of PBS alone increased BAL macrophages approximately 3-fold compared to intranasal administration, and no significant difference in BAL macrophages was observed between CSE- and PBS-treated animals dosed by this route. BAL neutrophils were slightly lower in animals dosed with PBS via the oro-tracheal route, and this was not increased with CSE treatment.

Intranasal CSE administration to BALB/c and C57BL/6 mice increased both BAL macrophages (P<0.005 and P<0.001, respectively) and BAL neutrophils (P<0.001 and P<0.05, respectively) to a similar degree in both mouse strains (Table 2).

Effect of CSE dose on BAL cell and TNF-α content

Iranasal CSE dose-dependently increased both BAL macrophages and neutrophils up to a concentration of 30% (Figure 1a). The higher dose of 100% CSE subsequently reduced both BAL macrophages and neutrophils in a biphasic manner. No further changes were observed with higher doses up to 300% CSE (data not shown). Removal of the filter from the cigarettes before smoking increased the tar content of the CSE, as evidenced by increased absorbance at 405 nm, but did not alter the number of cells recovered in BAL (data not shown). In contrast, increasing concentrations of CSE dose-dependently decreased the concentration of TNF-α in BAL up to 100% CSE where TNF-α was no longer detectable (Figure 1b).

Changes in BAL cell content over time following CSE administration

BAL macrophages then increased on days 3 and 4 of CSE dosing (Figure 2a), and then declined over the following 2 weeks. In contrast, BAL neutrophils were elevated after a single day of CSE dosing (Figure 2b), were observed to further increase following 3 and 4 days of dosing and then returned to the level recorded on day 1 for the subsequent 2 weeks.

Effect of CSE on lung mucin and HDAC activity

Muc5AC was significantly increased in the BAL of CSE-treated animals compared to control mice (P<0.05; Table 3), however, whilst there was a trend to increased mucin in the lung homogenate, no significant difference was observed. Histone deacetylase (HDAC) activity in the nuclear extract of lungs from CSE- and PBS-treated animals was very similar (Table 3).

Effect of CSE administration on lung gene expression

Mice challenged with a single dose of CSE exhibited changes in gene expression in only a small number of genes from the 96 analysed, showing only low-fold changes. Arbitrary copy numbers for selected genes (normalised by 18S rRNA) are shown in Table 4. Significant reductions were observed in transcripts for TNF-α (30%; P<0.001), inhibitor of IκB-β (21%; P<0.0001), inducible nitric oxide synthase

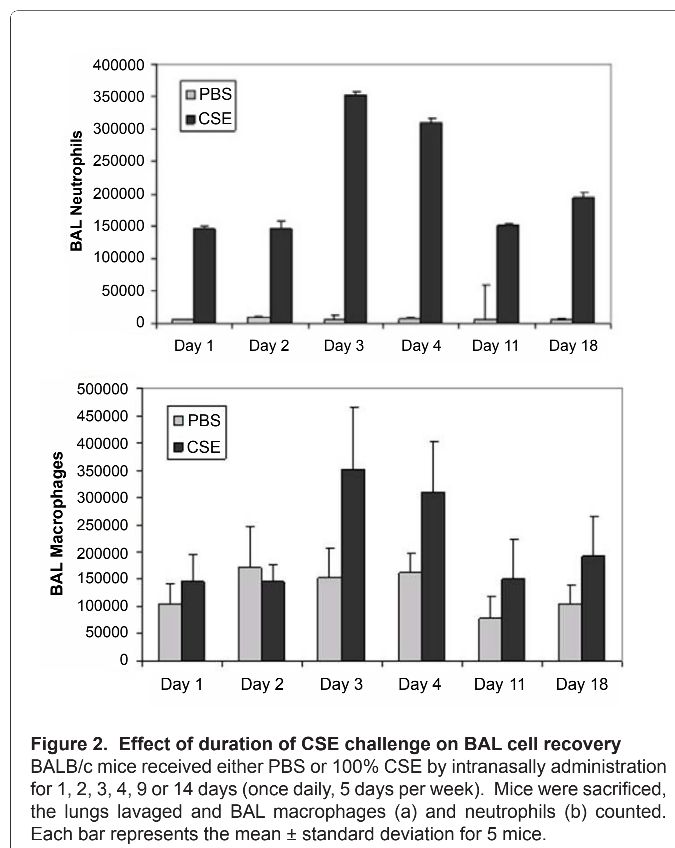

Figure 2. Effect of duration of CSE challenge on BAL cell recovery
BALB/c mice received either PBS or 100% CSE by intranasally administration for 1, 2, 3, 4, 9 or 14 days (once daily, 5 days per week). Mice were sacrificed, the lungs lavaged and BAL macrophages (a) and neutrophils (b) counted. Each bar represents the mean ± standard deviation for 5 mice.

	Muc5AC		HDAC Activity
	BAL	Lung	
PBS	328 ± 209	12.0 ± 1.2	1001 ± 120
CSE	701 ± 508*	19.4 ± 5.7	1013 ± 223

Table 3: Muc5AC and HDAC activity in the lung of CSE-treated mice.
Muc5AC in BAL and muc5AC and HDAC activity in homogenised lung tissue from BALB/c mice treated for 4 days with 50 µl intranasal PBS or 100% CSE. Values are mean ± S.D. of 5 mice. *P<0.05 (vs PBS).

	Copy Number		Percentage change	P Value
	PBS	CSE		
BCL2	22278	17784	-20%	0.002
TNF-α	1549	1098	-29%	0.0008
I-κB kinase-β inhibitor	5830	4624	-21%	0.00006
iNOS	742	488	-34%	0.02
Transferrin Receptor	11675	9115	-22%	0.01
TGF-β1	19487	15426	-21%	0.002
VEGF-A	151739	117842	-22%	0.003

Arbitrary copy numbers for selected genes (normalised by 18S rRNA) are shown 2 hours post-challenge. Values are mean ± S.D. of 4 mice. Percentage change in expression is calculated.

Table 4: Changes in expression of selected genes 2 hours following a single challenge of 100% CSE.

(34%; P<0.05) and transforming growth factor-β (20%; P<0.005) 2 hours post-challenge, but not at 4 hours or 24 hours post-challenge.

Effect of Prednisolone, Monteleukast, Roflumilast and p38i (PHA-818637) on CSE -induced BAL cell content

CSE-treated animals were treated orally twice daily with 10mg/kg Prednisolone, 10 mg/kg Roflumilast and 10 mg/kg of the p38 inhibitor PHA-818637. These doses were previously shown to be effective in a mouse LPS model (data not shown). Prednisolone did not inhibit either CSE-elicited BAL macrophages or neutrophils (Figure 3). In contrast, Roflumilast significantly inhibited both CSE-elicited BAL macrophages (P<0.01) and neutrophils (P<0.001) compared to vehicle-treated animals. However, the oral p38 inhibitor, PHA-818637, inhibited CSE-elicited BAL macrophages (P<0.05), but not airway neutrophils.

Effect of co-challenge with LPS or ozone on CSE-induced airway neutrophil accumulation

LPS induced a dose-dependent increase in BAL neutrophils (Figure 4a) which was significantly inhibited by CSE at the 0.1 µg LPS dose, but not at either the 0.01 or 1.0 µg doses. Ozone exposure synergistically increased BAL neutrophils in the CSE treated animals (Figure 4b).

Discussion

The aim of these studies was to develop a model of pulmonary inflammation induced by CSE that shares aspects of cigarette smoke models and clinical COPD. Cigarette smoke contains in excess of 1200 chemical entities [19], yet CSE contained far fewer constituents with 3 major components (one of which was identified as nicotine) and several hundred smaller peaks. Nevertheless, our data presented here show that CSE administration results in a dose-dependent steroid-insensitive lung inflammation. The increase observed in CSE-elicited BAL inflammatory cells is predominantly due to increases in airway macrophages (96% of BAL cells), and to a lesser extent to increases in neutrophils (3% of BAL cells). One clinical study reports that induced sputum from healthy volunteers and smokers contains 44% and 51% neutrophils respectively [20]. However, studies that sample BAL, rather than sputum, from patients with COPD report lower neutrophilic contents of 5% [20], 7.6% [21] and 13-26% [22]. The magnitude of neutrophilia reported in this study is therefore consistent with that reported in clinical BAL samples. Furthermore, the observed neutrophilic inflammation is similar in magnitude to that reported in other acute mouse models that employ cigarette smoke as a challenge [23-25]. There are a number of reports that mouse strain influences the inflammatory or airway enlargement response in cigarette smoke models [17,26]. However, we found no significant difference in inflammatory response between the 2 mouse strains tested, indicating that this model is suitable for use in the BALB/c strain and also permit evaluation of gene knockout, in which mice are often cross-bred onto the C57BL/6 strain. We compared administration routes, primarily because intranasal administration can be associated with significant gastric, and therefore loss of pulmonary exposure. The vehicle itself was associated with a higher number of macrophages and no CSE-elicited neutrophilia was observed in the oro-tracheal compared with the intranasal delivery route. The reason for this observation is unclear; one possibility is that the higher macrophage counts may be a consequence of tissue damage as a result of multiple oro-tracheal doses through the sensitive larynx, but this would be expected to be accompanied by additional neutrophilia (which was not observed). No reduction in inflammatory response in the intranasal versus the oro-tracheal treated animals was observed.

The dose-dependent inflammatory cell influx observed in response to CSE was accompanied by a concomitant decrease in BAL TNF-α protein and lung tissue TNF-α transcript. This was a surprising finding, given that acute cigarette smoke challenge in mice upregulates TNF-α

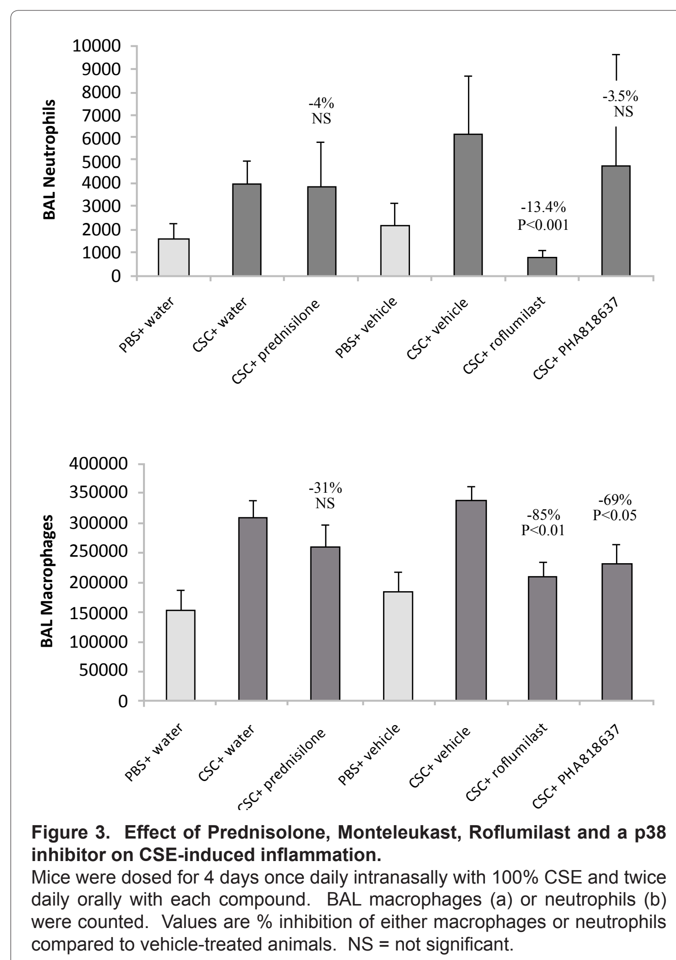

Figure 3. Effect of Prednisolone, Monteleukast, Roflumilast and a p38 inhibitor on CSE-induced inflammation.
Mice were dosed for 4 days once daily intranasally with 100% CSE and twice daily orally with each compound. BAL macrophages (a) or neutrophils (b) were counted. Values are % inhibition of either macrophages or neutrophils compared to vehicle-treated animals. NS = not significant.

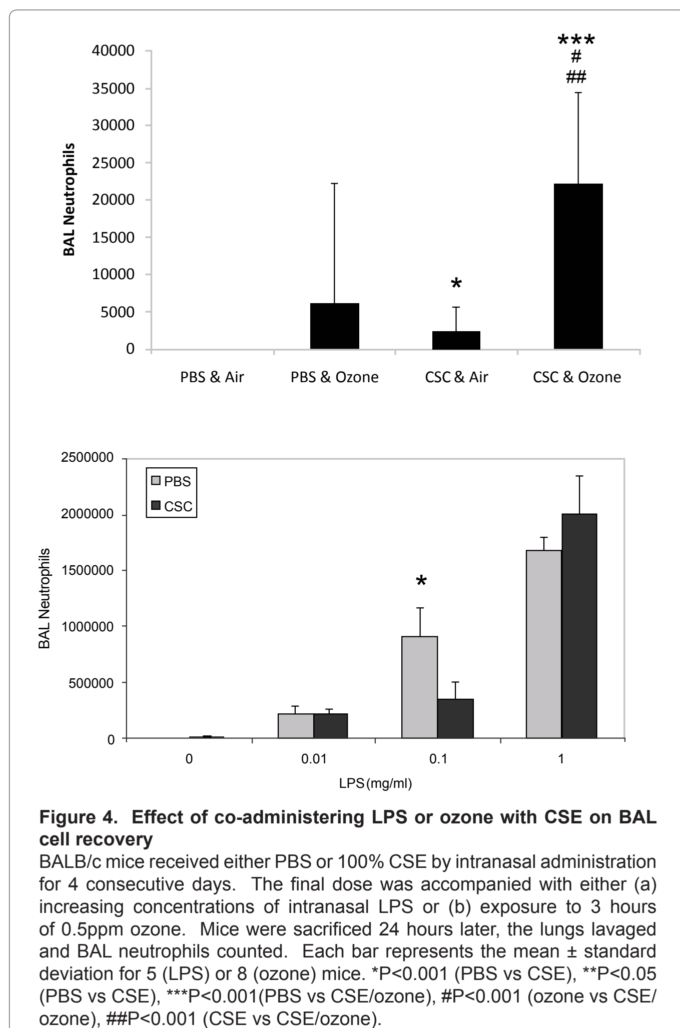

Figure 4. Effect of co-administering LPS or ozone with CSE on BAL cell recovery
BALB/c mice received either PBS or 100% CSE by intranasal administration for 4 consecutive days. The final dose was accompanied with either (a) increasing concentrations of intranasal LPS or (b) exposure to 3 hours of 0.5ppm ozone. Mice were sacrificed 24 hours later, the lungs lavaged and BAL neutrophils counted. Each bar represents the mean ± standard deviation for 5 (LPS) or 8 (ozone) mice. *P<0.001 (PBS vs CSE), **P<0.05 (PBS vs CSE), ***P<0.001(PBS vs CSE/ozone), #P<0.001 (ozone vs CSE/ozone), ##P<0.001 (CSE vs CSE/ozone).

transcript in lung tissue [27] and that cigarette smoke induced matrix metalloproteinases are ablated in TNF-α receptor deficient mice [28]. Indeed, we show here that CSE challenge results in a reduction in lung tissue I-κB kinase-β inhibitor transcript, which may activate the NF-κB pathway resulting in the up regulation of several genes, including TNF-α. However, in clinical sputum samples, TNF-α has not been found to be elevated in patients with COPD [29] and studies looking for genetic evidence linking alleles for TNF-α and TNF-α receptor with the presence of COPD have reported equivocal or conflicting findings [30-32]. Furthermore, macrophages isolated from the sputum of patients with COPD produce less basal TNF-α compared with macrophages from control subjects [33]. The lower TNF-α signal in animals challenged with CSE is thus consistent with reported clinical observations. We also observed decreases in the transcripts for BCL2, transferrin receptor, TGF-β1, VEGF-A and iNOS. It has been reported that exhaled nitric oxide is higher in patients with COPD and correlates negatively with lung function which is not consistent with our findings in this mouse model [34]. However, technical issues with collecting exhaled samples together with the natural variability and anatomical compartmentalisation of inflammation make these data difficult to interpret [35].

The increase in BAL neutrophils and macrophages elicited by CSE challenge was not attenuated by prednisolone at doses that we have shown previously to ablate LPS-elicited neutrophia (data not shown). In contrast, the PDE4 inhibitor, Roflumilast, significantly inhibited both CSE-elicited neutrophils and macrophages, and the p38 MAPK inhibitor significantly reduced macrophages. Steroid insensitivity in clinical COPD has been well-documented [36] and recent reports [37] suggest that Roflumilast bestows a significant improvement in FEV_1 on top of standard of care bronchodilators in patients with chronic bronchitis. Furthermore, it has very recently been reported that SB-681323, a p38 inhibitor, reduces inflammatory biomarkers in blood from patients with COPD [38]. The pharmacological pathways tested with the agents used in this study appear to respond in a similar manner in the CSE-challenge model compared to what has been reported in the clinical literature. Interestingly, whilst prophylactically-administered Roflumilast has been reported to ablate neutrophilia in an acute mouse cigarette smoke challenge model and prevent emphysematous-like changes in a chronic exposure model [23], it has recently been shown to be ineffective against established cigarette smoke-elicited inflammation in a 14-week model [39]. The relative timings of challenge and pharmacological intervention may therefore be important in determining the outcome when a particular pathway is tested in a preclinical challenge model, and care should be taken in interpreting such data when considering potential clinical translation. It has been hypothesised that corticosteroid insensitivity is associated with cigarette-smoke induced inactivation of histone deacetylase (HDAC) [34] and inactivation of HDAC activity has been reported in both rat [7] and mouse [40] cigarette smoke models. Despite demonstrating steroid insensitivity in CSE-elicited inflammation, we were unable to demonstrate any difference in lung HDAC activity. In this study, we used total lung nuclear extract, so it is possible that any reduction in HDAC activity may have been restricted to a sub-set of pulmonary cells such as the epithelium, and therefore not detectable against the background of total lung activity.

Finally, we assessed the interaction of CSE with other pro-inflammatory triggers. Firstly, we combined CSE with LPS as a bacterial (and potential exacerbation) mimic. We demonstrated that CSE attenuated LPS-elicited neutrophilia in the mouse lung. This is consistent with our previous internal observations that CSE inhibits LPS-elicited cytokine release from human macrophages (data not shown) and also with published reports that sputum macrophages from patients with COPD are not responsive to LPS stimulation [33]. We also examined the effect of further oxidative stress in the model by exposing the mice to ozone 16 hours prior to euthanasia (one potential consequence of making a cigarette smoke extract is the potential loss of short-lived oxidative radicals). Interestingly, ozone synergistically increased CSE-elicited neutrophilia in a similar manner to that reported in a whole cigarette smoke mouse model [41]. It has been reported that daily levels of environmental ozone are positively correlated with pooled asthma/COPD hospital admission rates in an elderly Finnish population [42] and the rate of decline in lung function in patients with α1-anti-trypsin deficiency [43], although other publications do not concur [44,45].

In summary, we have developed a cigarette smoke extract mouse model of pulmonary inflammation that negates the requirement for expensive smoking machines. CSE administration resulted in a dose-dependent steroid-insensitive neutrophilic lung inflammation that was ablated by the PDE4 inhibitor, Roflumilast. The inflammatory changes were associated with increases in airway mucin, and decreases in TNF-α, iNOS, TGF-β and inhibitor of I-κB kinase-β inhibitor. The inflammation could also be exacerbated by exposure to ozone, but not to LPS.

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