Inhaled LPS challenges in smokers: a study of pulmonary and systemic effects

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT
• Neutrophilic airway inflammation is a feature of chronic obstructive pulmonary disease (COPD). Inhaled lipopolysaccharide (LPS) challenge in healthy non-smokers has been used to assess the anti-inflammatory effects of novel drugs on neutrophilic airway inflammation.

WHAT THIS STUDY ADDS
• We performed inhaled LPS challenge in smokers to study an inflammatory response in subjects who more closely resemble COPD patients. Inhaled LPS caused reproducible pulmonary inflammation in smokers, associated with changes in systemic biomarkers. Inhaled LPS appears to be a suitable model for studying exacerbations of COPD.

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

WHAT THIS STUDY ADDS

AIMS
Lipopolysaccharide (LPS) is a TLR4 agonist which activates NFXB dependent cytokine production. We investigated LPS inhalation in healthy smokers as a model of COPD bacterial exacerbations. We studied safety, reproducibility, the translocation of the NFXB subunit p65 in sputum cells and changes in systemic biomarkers of inflammation.

METHODS
Twelve smokers inhaled 5 and 30 μg LPS and safety was monitored over 24 h. IL-6, CRP, CCI-18, SP-D, CC-16 and β-defensin 2 were measured in serum samples collected at baseline, 4, 8 and 24 h. Sputum was induced at baseline, 6 and 24 h for cell counts and p65 expression. Repeated challenges were performed after a 2 week interval in 10 smokers.

RESULTS
LPS inhalation was well tolerated. Significant increases occurred in sputum neutrophil counts with both doses, with a maximum increase of 21.5% at 6 h after 30 μg which was reproducible, r (intraclass correlation coefficient) = 0.88. LPS increased sputum cell nuclear p65 translocation and phospho-p65 expression. All of the serum biomarkers increased following challenge but with different temporal patterns.

DISCUSSION
Inhaled LPS challenge in smokers causes pulmonary and systemic inflammation that involves NFXB activation. This appears to be a suitable model for studying bacterial exacerbations of COPD.

Introduction
The bacterial endotoxin lipopolysaccharide (LPS) is recognized by the pathogen receptor Toll like receptor 4 (TLR4), which is expressed on a range of immune and structural cells. TLR4 signalling activates transcription factors such as NFXB that upregulate the production of inflammatory cytokines involved in the innate immune response [1]. LPS
has been administered by inhalation into the lungs of healthy subjects to model the innate immune response. This is a safe procedure that causes an increase in airway neutrophil numbers [2–4]. This model has been used to evaluate the pharmacological effects of anti-inflammatory drugs on neutrophilic airway inflammation [5, 6].

Cigarette smokers with normal lung function (‘healthy smokers’) have increased numbers of airway neutrophils compared with non-smokers [7–9]. There is a further increase in airway neutrophil numbers in smokers with chronic obstructive pulmonary disease (COPD) [7, 8]. Exacerbations caused by bacterial infections are a common event in COPD patients and are associated with an acute upregulation of airway neutrophil numbers [10]. The inhalation of LPS in smokers may serve as a relevant model of bacterial COPD exacerbations, provoking acute neutrophilic inflammation on a background of chronic airway inflammation. This model may be useful for studying the mechanisms responsible for inflammation in exacerbations and for evaluating the effects of anti-inflammatory drugs on such events.

LPS challenge has been performed in healthy smokers with airway inflammation assessed by bronchoscopy [11], which is an invasive procedure. Airway inflammation in LPS challenge studies can also be assessed using induced sputum [2, 3], which is less invasive and can be easily repeated to assess the time course of airway inflammation. In order for LPS challenges in healthy smokers to be used in clinical trials in future, it would be important to know the time course of the inflammatory response in the airways and systemic circulation. Furthermore, the reproducibility of the challenge in this population should be determined.

There has been much interest recently in protein biomarkers for COPD that are secreted mainly by the lungs and are measurable in the systemic circulation. Such biomarkers include surfactant protein D (SP-D), CCl-18 (PARC), CC-16 (Clara cell protein) and \(\beta\)-defensin 2. SP-D is produced by type 2 pneumocytes and Clara cells [12] and plays a role in surfactant homeostasis and pulmonary immunity [13]. CCI-18/PARC is produced by macrophages and dendritic cells [14] and has been associated with lung fibrosis and acute coronary syndromes [15, 16]. SP-D and CCI-18/PARC levels are increased in the serum of COPD patients compared with controls [17, 18]. CC-16 is produced by Clara cells and functions as a protective immunosuppressant during lung injury. Serum CC-16 concentrations show an acute increase after the inhalation of LPS (and ozone) in healthy subjects [19, 20]. Interestingly, serum and induced sputum CC-16 concentrations are reduced in COPD patients compared with controls [21, 22]. \(\beta\)-defensin 2 is a neutrophil chemo-attractant produced by lung epithelial cells that is involved in host innate immune defence [23]. There are conflicting reports of \(\beta\)-defensin 2 levels in the lungs of COPD patients, as increased levels have been observed in broncho-alveolar lavage from COPD patients compared with controls, but decreased levels in the induced sputum of COPD patients compared with controls [22, 24].

This paper reports the effects of LPS challenges in healthy smokers as a potential model of bacterial exacerbations in COPD patients. The novelty of this study is that we measured the time course of airway and systemic inflammation and the reproducibility of the challenge. Airway inflammation was assessed by sputum cell counts and NFkB activation. Biomarkers of inflammation in the circulation including SP-D, CCI-18/PARC, CC-16 and \(\beta\)-defensin 2 were measured.

### Methods

#### Subjects

Twenty-two current smokers (11 males and 11 females) with no history of respiratory disease and normal pulmonary function participated. Their mean age was 53.4 years (SD 6.4 years) and mean smoking history 34.6 pack years (SD 19.0 years). All the subjects were current smokers with >10 pack year history. We also used historical induced sputum and lung function data from 10 healthy nonsmokers who had previously undergone LPS challenge in our laboratory. Written informed consent was obtained and the study was approved by the local research ethics committee (Greater Manchester South, 09/H1004/8).

#### Study design

Two studies were performed. The safety and effects of inhaled LPS on airway and systemic inflammation were assessed in study 1, while study 2 investigated the reproducibility of inhaled LPS challenges. In study 1, 12 subjects underwent baseline sputum induction, and were administered 5 \(\mu\)g inhaled LPS (Escherichia coli serotype O26:B6, Sigma-Aldrich, UK reconstituted in 0.9% w/v saline and delivered a Mefar dosimeter; Markos Mefar, Bresica, Italy) after at least 48 h. Sputum induction was performed at 6 and 24 h post LPS challenge. Subjects returned after a 2 week period for a further LPS challenge with 30 \(\mu\)g with sputum inductions at 6 and 24 h. Pulse, blood pressure, oxygen saturations, temperature and spirometry (using Vitalograph, Buckinghamshire, UK) were performed pre LPS challenges and every hour for 6 h post LPS challenge. All these measurements, except spirometry, were continued until 8 h post LPS challenge. Blood samples for biomarkers were collected at pre-challenge (baseline), 4, 8 and 24 h post LPS challenge. Subjects were required to refrain from smoking for 2 h before LPS challenges or sputum induction, and for 8 h post LPS challenges.

Ten healthy smokers participated in study 2. Baseline sputum induction was performed, followed by inhalation of LPS 30 \(\mu\)g after an interval of at least 48 h. Sputum induction was performed at 6 h post LPS. The baseline sputum followed by LPS challenge was repeated after 2 weeks. Subjects were required to refrain from smoking for
2 h before LPS challenges or baseline sputum induction and for 6 h post LPS challenges. Historical data from 10 healthy non-smokers with normal pulmonary function who had undergone LPS challenge using 30 μg were used. Induced sputum samples were obtained at 6 and 24 h post LPS challenge.

**Induced sputum**

Sputum was induced and dithiothreitol (DTT) processed using established methods [25]. The supernatant was stored at −80°C for later analysis. Cytospin preparations were made (Cytospin 4, Shandon, Runcorn, UK) and stained with Rapi-diff (Triangle, Skelmersdale, UK). Four hundred non-squamous cells were counted and differential cell counts obtained as percentage of total non-squamous cells. Cell viability was analyzed by trypan blue exclusion.

**P65 immunocytochemistry**

NFκB activation was assessed by calculating the nuclear translocation of p65 and phospho-p65 in sputum cells. Cytospins were incubated in 0.2% w/v Triton X-100 (Sigma, UK) followed by 15 mM glycine for cell permeabilization. Slides were incubated in 0.2% v/v Triton X-100 (Sigma, UK) followed by PGS (1% PBS, 1% w/v gelatine and 0.2% w/v saponin) and incubated with anti-NFκB (p65 1/50 in PGS, Lab Vision, Runcorn, UK) or anti-phospho-NFκB p65 (ser536, Cell Signalling, Hitchen, UK). Following washing, slides were incubated with Alexa 488 conjugated anti-rabbit secondary antibody (Invitrogen, Paisley, UK) followed by 15 mM glycine for cell permeabilization. Cytospins were incubated in 0.2% v/v Triton X-100 (Sigma, UK) followed by PGS (1% PBS, 1% w/v gelatine and 0.2% w/v saponin) and incubated with anti-NFκB (p65 1/50 in PGS, Lab Vision, Runcorn, UK) or anti-phospho-NFκB p65 (ser536, Cell Signalling, Hitchen, UK). Following washing, slides were incubated with Alexa 488 conjugated anti-rabbit secondary antibody (Invitrogen), counterstained with propidium iodide and mounted in citifluor (Citifluor Ltd, London, UK).

P65 expression was examined (×200 magnification) by confocal microscopy (LAS SP5, Leica Microsystems, Milton Keynes, UK). Green nuclear and cytoplasmic fluorescence intensities normalized for numbers of cells were measured using image analysis software (Leica Microsystems), running a customized macro.

**Measurement of serum biomarkers**

Serum was assayed using the following ELISA kits according to manufacturers instructions: IL-6 (Quantikine hs-IL-6; R&D Systems, Abingdon, UK), β-defensin-2 (Phoenix Pharmaceuticals, Burlingame, USA), CC-16 (Biovendor, Modrice, Czech Republic), CCI-18/PARC (Duo set kit; R&D Systems, Abingdon, UK), SP-D (Biovendor, Modrice, Czech Republic). Serum C-reactive protein (CRP) was measured by high sensitivity particle enhanced immunonephelometry (Cardiophase; BN systems, Dade Behring, Newark, USA) with a lower limit of detection of 0.175 mg l⁻¹.

**Data analysis**

Sputum differential neutrophil and macrophage counts were normally distributed and so differences pre and post LPS inhalation were compared using paired t-tests. Other sputum cell counts were non-parametric and analyzed using Wilcoxon tests. Immunocytochemistry data were normally distributed; differences pre and post LPS were compared using unpaired t-tests as evaluable slides for all subjects at each time point were not available. Paired t-tests were used for the subset of patients where all samples were available. Comparisons between healthy smokers and healthy non-smokers were performed using unpaired t-tests. SP-D and CC-16 are presented as means, while hs-CRP, CCI-18/PARC, IL-6 and β-defensin-2 are presented as geometric means. The effects of LPS challenge were assessed using paired t-tests. Safety data were evaluated using one way ANOVA with Bonferroni correction. The reproducibility of LPS challenges was analyzed using Bland Altman plots and intraclass correlation coefficient (r) for the increase in sputum neutrophil percentage. The differences between baseline sputum cell counts were assessed using paired Student’s t-tests. Power calculations were performed using the within subject standard deviation of the increase in sputum neutrophil percentage after repeated challenges.

**Results**

**Study 1: effects of 5 and 30 μg LPS**

**Safety** Only minor symptoms such as headache (n = 1) and rhinorhoea (n = 1) were observed following inhalation of either 5 or 30 μg LPS, which resolved within 24 h. Figure 1 shows an increase in temperature and pulse rate within 8 h which returned to normal by 24 h. Blood pressure did not change. There was a significant decrease in FEV₁. The mean maximal decrease was 8.2% with 5 μg and 14.0% with 30 μg. FEV₁ returned to normal by 24 h. There was a similar mean maximal decrease in healthy non smokers (11.9%, P = 0.6) following 30 μg LPS. Additionally, analysis of the area under the curve (AUC) for the FEV₁ decrease from 0–6 h showed no difference (P = 0.5) between healthy smokers and healthy non-smokers (38.2 FEV₁, % h vs. 29.9 FEV₁, % h respectively).

**Sputum cell counts** Inhaled LPS increased sputum neutrophil counts (see Table 1 and Figure 2 for individual data). The mean percentage of neutrophils increased by 18.8% and 8.9% at 6 and 24 h respectively, after inhalation of 5 μg LPS. The increases at 6 and 24 h after 30 μg LPS were 21.7% and 7.6% respectively. These data show greater increases at 6 h compared with 24 h, which was statistically significant at the 30 μg dose (P < 0.01). The absolute neutrophil count increased 4.5 and 5.2 fold over baseline at 6 and 24 h respectively, after 5 μg LPS, and 9.9 and 3.5 fold at 6 and 24 h respectively, after 30 μg LPS. The greatest fold change was observed after 30 μg LPS at 6 h (P < 0.05 for comparisons with other time points at both LPS doses). There was a decrease in the percentage of macrophages and no significant change in other cells types after LPS challenges. In comparison, in healthy non-smokers the mean baseline neutrophil percentage was 44.5% which increased to
77.8% and 71.0% at 6 and 24 h post LPS, respectively. These mean increases of 33.3% and 26.5% were greater than observed in healthy smokers (P = 0.18 and P = 0.02, respectively).

**p65 immunohistochemistry** Figure 3 shows the data from all available samples. P65 was expressed in the nuclei and cytoplasm of both neutrophils and macrophages at baseline. LPS inhalation increased the nuclear translocation of p65 in both macrophages and neutrophils (Figure 3A and B). The intensity of nuclear phospho-p65 staining was significantly increased in macrophages 24 h post 30 μg LPS inhalation with a trend to significance post 5 μg LPS inhalation (Figure 3C). Neutrophils did not stain with phospho-p65. In the subset of patients who had paired samples (pre and post LPS challenge), similar numerical differences were observed to those presented in Figure 3, but statistical significance (P < 0.05) was only reached for phospho-p65 intensity due to the reduction in sample size which varied from n = 5 to n = 9 depending on the time point and LPS dose.

**Serum biomarkers** The time profiles of the serum biomarkers following LPS inhalation are shown in Figure 4. LPS 30 μg had a greater effect on these biomarkers, in particular CRP and IL-6. IL-6, CC-16 and β-defensin-2 were increased at 4 h. These three proteins subsequently showed different patterns of regulation. β-defensin-2 levels were persistently raised, IL-6 returned to baseline levels by 24 h, whilst CC-16 levels were significantly below baseline at 24 h. Following LPS inhalation, CRP, SP-D and CCl-18/PARC all showed a delayed increase, with CRP being increased at 8 and 24 h, and SP-D and CCl-18/PARC being increased at 24 h only.

**Study 2: reproducibility of LPS challenge**
Repeated challenges were undertaken using 30 μg of LPS, as this produced greater effects than 5 μg in study 1. Mean sputum cell count data are shown in Table 2 and the Bland–Altman plot for the increase in neutrophil percentage are shown in Figure 5. The mean difference was 1.2% (within subject SD 10.0%). The intraclass correlation coefficient (r) was 0.88, indicating very good reproducibility. Power calculations using the within subject SD were performed. Sample sizes of n = 5, 7 and 20 are required to demonstrate 75%, 50% and 25% reductions of the increase in percentage neutrophils with 80% power in a crossover study design. There were no significant differences between differential cell counts at baseline and at 2 weeks post LPS challenge.

**Discussion**
Inhaled LPS in healthy smokers increases airway neutrophil numbers and NFkB activation. This challenge was reproducible and well tolerated. Inhaled LPS increased serum concentrations of IL-6, CRP, CC-16, β-defensin-2, CCI-18/PARC and SP-D, but with different time profiles observed for these proteins as IL-6, CC-16 and β-defensin-2 were upregulated at 4 h, whilst CRP, CCI-18 and SP-D showed later upregulation. These effects of inhaled LPS on both pulmonary and systemic inflammation in smokers...
greater duration of inflammation of the 5 μg LPS dose on sputum absolute cell counts at 24 h compared with the higher LPS dose, although this difference between doses at 24 h was not observed for neutrophil percentage. This might suggest that lower doses of LPS cause a more prolonged time course of inflammation.

The effects of repeated LPS challenges on neutrophil counts were very reproducible, with a mean difference between challenges of only 1.2%. LPS and ozone challenges have been used to investigate the effects of drugs on acute neutrophilic inflammation in healthy subjects [5, 6, 26]. The advantage of the model described in the current study is that healthy smokers have a background of chronic airway inflammation [7, 8] and so the LPS challenge may more closely resemble the acute neutrophilic inflammation that occurs during exacerbations in COPD patients [10]. The power calculations using the within subject SD show that well powered studies can be performed using this reproducible model in limited numbers of subjects, e.g. 20 subjects are required to demonstrate 25% inhibition of the increase in sputum neutrophil percentage with 80% power.

The changes in neutrophil counts in healthy non-smokers were greater than those observed in healthy smokers because of the lower pre LPS baseline neutrophil percentage in healthy non-smokers. Previous LPS challenge studies have used doses ranging from 0.5 to 300 μg [2–4, 27–30], with more side effects observed using higher doses. The doses of 5 and 30 μg of LPS in the current study were well tolerated, and we would advise that higher doses in healthy smokers are not needed.

Environmental LPS exposure concentrations have been reported up to 10 000 endotoxin units (EU)/m³ [31] although ambient levels are usually below 1000 EU/m³ [32–34]. 1 EU is equivalent to approximately 0.1 ng LPS [35] and so 1000 EU is equivalent to 0.1 μg LPS. Our LPS challenge method with 5 and 30 μg was therefore higher than the level of exposure that would usually occur in the environment [31–34], but may represent an appropriate

Table 1
Sputum cell counts following LPS inhalation

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>6 h</th>
<th>5 μg LPS</th>
<th>24 h</th>
<th>6 h</th>
<th>30 μg LPS</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum weight (g)</td>
<td>1.2 (0.4–5.9)</td>
<td>1.5 (0.3–6.1)</td>
<td>2.3 (0.5–7.0)</td>
<td>2.1 (0.2–11.5)</td>
<td>1.7 (0.2–6.1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cells (x10⁶)</td>
<td>2.7 (1.4–24.1)</td>
<td>11.5 (2.0–43.3)</td>
<td>15.4 (2.4–66.8)*</td>
<td>23.4 (2.8–41.1)*</td>
<td>10.0 (2.0–47.0)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute neutrophil count (x10⁶)</td>
<td>1.9 (0.6–18.4)</td>
<td>8.4 (1.7–40.1)*</td>
<td>9.7 (1.5–63.0)*</td>
<td>18.5 (2.2–37.5)*</td>
<td>6.5 (1.0–38.9)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute macrophage count (x10⁶)</td>
<td>1.2 (0.2–4.4)</td>
<td>2.2 (0.2–7.9)</td>
<td>3.0 (0.8–12.4)*</td>
<td>2.7 (0.3–6.4)*</td>
<td>2.3 (0.3–21.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophil (%)†</td>
<td>62.0 (21.9)</td>
<td>80.8 (11.9)*</td>
<td>70.9 (15.8)*</td>
<td>83.7 (7.0)*</td>
<td>69.6 (18.0)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macrophage (%)†</td>
<td>31.8 (20.2)</td>
<td>16.0 (9.7)*</td>
<td>25.2 (14.7)</td>
<td>13.1 (6.1)*</td>
<td>26.3 (16.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.9 (0–16.5)</td>
<td>0.9 (0–3.5)</td>
<td>1.3 (0.25–6.5)</td>
<td>0.8 (0–2.3)</td>
<td>1.3 (0–5.3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>0.5 (0–1.3)</td>
<td>0.4 (0–1.5)</td>
<td>0.5 (0–1.8)</td>
<td>0.5 (0–2.8)</td>
<td>0.3 (0–1.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelial cells (%)</td>
<td>1.1 (0–10)</td>
<td>0.8 (0–5.3)</td>
<td>0.3 (0–4.5)</td>
<td>1.5 (0–5.0)</td>
<td>1.9 (0–6.0)</td>
<td></td>
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</tr>
</tbody>
</table>

Data are presented as median (range). Results at different time points were compared against baseline using Wilcoxon matched pair test. *Indicates P ≤ 0.05. †Data are presented as mean (SD) and results at different time points compared with baseline using paired t-test.

**Figure 2**
Sputum neutrophil counts following challenge with LPS 5 or 30 μg. Individual data and mean values (horizontal lines) are shown. * indicates P ≤ 0.05; ** indicates P ≤ 0.001.

Figures and diagrams are not included in this text. The text continues to discuss the results and conclusions of the study, emphasizing the reproducibility of the model and the differences in neutrophil counts observed between healthy smokers and non-smokers. The study highlights the potential for using LPS challenges to investigate the effects of drugs on acute neutrophilic inflammation in healthy subjects, with a focus on the power calculations to determine the required number of subjects for well-powered studies.
TLR4 signalling activates the NFκB pathway [1]. We observed an increase in p65 nuclear translocation and phospho-p65 expression in sputum cells after LPS challenge. P65 displayed high levels of nuclear staining in pre-LPS sputum samples, giving a smaller ‘window’ to observe a change in the nucleus : cytoplasm ratio after LPS challenge. However, there was an increase in phospho-p65 staining intensity of >20% after LPS challenge, which clearly demonstrated p65 activation. Previous studies have shown that NFκB activation is increased in the airways of COPD patients in the stable state and in sputum macrophages during exacerbations [36, 37]. Consequently, inhibitors of the NFκB pathway, such as IKK2 inhibitors, are being developed for the treatment of COPD [38, 39]. We could not detect phospho-p65 in sputum neutrophils, although we could detect total p65. This suggests that there was a technical issue with the sensitivity of phospho-p65 staining in sputum neutrophils, rather than an absence of p65 in neutrophils. Nevertheless, our findings demonstrate that NFκB activation is involved in the inflammatory response of healthy smokers due to inhaled LPS. The effects of NFκB inhibitors could therefore be evaluated rapidly in early phase studies using this challenge model.

We studied the serum concentrations of four proteins that are thought to be predominantly secreted by the lungs: SP-D, CCl-18/PARC, CC-16 and β-defensin-2. Serum concentrations of SP-D and CCl-18/PARC are increased in COPD patients [17, 18] and we have shown that acute lung inflammation in smokers causes upregulation of both of these proteins at 24 h. In contrast, there was a more rapid increase in β-defensin-2 concentrations. β-defensin-2 is an endogenous TLR ligand [40] and it is possible that a positive feedback loop exists after LPS inhalation to promote the rapid production of this protein. Previous reports of β-defensin-2 concentrations in the lungs of COPD patients are conflicting, and it is possible that the sampling techniques may influence these results, as increased concentrations were observed in broncho-alveolar lavage but decreased concentrations in induced sputum [22, 24].

LPS inhalation in healthy non-smokers increases CC-16 concentrations at 6 h, returning to normal at 24 h [19]. We observed a similar time profile, but CC-16 concentrations were below baseline at 24 h. This suggests differential regulation of CC-16 in smokers compared with non-smokers. Smokers and COPD patients have lower serum and induced sputum CC-16 concentrations compared with non-smoking controls [21, 22], and our results indicate that acute lung inflammation caused by TLR4 signalling in smokers is a mechanism by which serum CC-16 concentrations are downregulated.

Systemic IL-6 and CRP concentrations are increased after LPS challenges in non-smokers [3, 27, 28, 41] and we observed similar findings in smokers. IL-6 regulates CRP

Figure 3
Nuclear translocation of p65 following challenge with LPS 5 or 30 μg. (A) shows the mean ratio of p65 between the nucleus and cytoplasm (p65 N : C) in sputum macrophages. (B) shows the p65 N : C ratio in sputum neutrophils. (C) shows the mean nuclear phospho-p65 staining intensity in macrophages following LPS challenge. Error bars represent SEM. * indicates P ≤ 0.05, NS not significant.
Figure 4

Time profiles of systemic biomarker levels following challenge with LPS 5 or 30 µg. The systemic responses following inhalation of 5 µg or 30 µg LPS are shown IL-6 (pg/ml), CRP (mg/ml), CCL-18/PARC (ng/ml), CC-16 (ng/ml), SP-D (ng/ml) and β-Defensin-2 (pg/ml) levels are shown in (a)–(f) respectively. Grey columns represent the biomarker levels following inhalation of 5 µg LPS and the black columns represent the biomarker levels following 30 µg LPS. Data are means (CC-16, SP-D) or geometric means (IL-6, CRP, CCL-18/PARC, β-Defensin-2) and error bars represent SEM. * indicates $P \leq 0.05$.
production by the liver [42], and our study suggests that IL-6 promotes a later increase in CRP concentrations after LPS challenge. Serum IL-6 and CRP concentrations are known to increase after COPD exacerbations [43, 44] and our findings suggest a temporal course for the relative induction of these proteins during such events. Indeed, it appears that inhaled LPS challenge in healthy smokers causes an early increase in sputum neutrophils and serum IL-6, CC-16, and β-defensin-2, followed by a later increase in NFκB activation in airway cells coupled with increased serum concentrations of CRP, SP-D and CCl-18/PARC.

In conclusion, LPS challenges in healthy smokers upregulated neutrophilic airway inflammation and NFκB signalling. These pulmonary effects were accompanied by changes in systemic IL-6, CRP, CC-16, β-defensin-2, SP-D and CCl-18/PARC concentrations. We suggest that LPS challenge in healthy smokers can be used as a model of COPD exacerbations, both to study the mechanisms involved in pulmonary and systemic inflammation and to evaluate the effects of anti-inflammatory drugs.

### Authorship contributions

Participated in research design: Aul, Armstrong, Lomas, Miller, Singh
Conducted experiments: Aul, Armstrong, Duviox, Hayes, Jagger
Performed data analysis: Aul, Duviox, Hayes, Jagger
Wrote or contributed to the writing of the manuscript: Aul, Jagger, Lomas, Miller, Singh
Guarantor: Singh

### Competing Interests

DS has received sponsorship to attend international meetings, honoraria for lecturing or attending advisory boards and research grants from various pharmaceutical companies including AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline (GSK), Almirall, Forest, Pfizer, UCB, Novartis, and Cipla. DL has received an educational grant and fees for speaking and acts as a consultant for GlaxoSmithKline. BH and BM are employees of GSK. BH owns GSK stock. BM owns GSK stock and stock options. BM’s spouse is also an employee of GSK and owns GSK stock and stock options. All other authors have no competing interests to declare.

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