Title: Increased Phosphorylated p38 Mitogen Activated Protein Kinase in COPD Lungs.

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Abstract

The p38 Mitogen Activated Protein Kinase (MAPK) pathway is upregulated in COPD. To date dual-labelling to identify cell-type specific presence of phosphorylated (phospho-) p38 MAPK has not been carried out.

Phospho-p38 MAPK was quantified in a variety of cell types in the lung tissue of 20 COPD patients, 12 smokers and 12 non-smokers using immunohistochemistry. Paired blood and sputum neutrophils (n=7 COPD), and CD8 and epithelial cells (n=3 COPD) were cultured with a p38 MAPK inhibitor. Supernatant TNFα, CXCL8 levels were analysed by ELISA. Sputum and blood neutrophil cytospins were analysed for phospho-p38 MAPK.

Phospho-p38 MAPK was increased in bronchial epithelial cells, macrophages and CD20+ and CD8+ lymphocytes in COPD lungs. Sputum and lung tissue neutrophils were devoid of phospho-p38 in all patient groups. The p38 MAPK inhibitor SB100 attenuated pro-inflammatory mediator release in COPD lung CD8 cells and airway epithelia but there was no effect on COPD sputum neutrophils. Our data indicates cell specific anti-inflammatory effects of p38 MAPK inhibition in the lung.

Key words:

Airway inflammation, neutrophils, p38 MAPK. CD8 lymphocytes, epithelial cells
Introduction

Chronic obstructive pulmonary disease (COPD) is characterised by poorly reversible airflow obstruction. There is also evidence of progressive airway inflammation [1]. Glucocorticoids are the most commonly used anti-inflammatory drug in COPD, but have limited effects on airway inflammation and disease progression [2, 3]. Novel therapeutic approaches targeting inflammation in COPD are needed.

The p38 mitogen activated protein kinase (MAPK) intracellular signalling pathway is activated by a variety of extracellular stimuli, including pro-inflammatory cytokines and toll like receptor (TLR) agonists [4, 5]. p38 MAPK activation causes histone modifications within the promoter regions of a subset of genes; This increases accessibility for transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) to these regions enhancing inflammatory gene expression [6]. Additionally, p38 MAPK acts post-transcriptionally by stabilising mRNAs, and promotes protein translation [7]. p38 MAPK inhibitors reduce cytokine production from alveolar macrophages [8-11] and are in clinical development for the treatment of COPD [12, 13].

There are increased numbers of inflammatory cells in the lungs of COPD patients, including lymphocytes, macrophages and neutrophils [1]. COPD patients also have increased numbers of pulmonary lymphoid follicles [1], which may function as antigen presenting sites that promote auto-immune processes [14]. Renda et al used single label immunohistochemistry to demonstrate increased expression of activated p38 MAPK in the alveolar macrophages of COPD patients compared to controls [15]. The specific expression of activated p38 MAPK on other relevant lung cells such as epithelial cells, lymphocytes and neutrophils has not been described. Furthermore, although the anti-inflammatory effects of p38 MAPK inhibitors on
cytokine production from COPD alveolar macrophages is well documented [8-11], there is little data on the effects of this class of drug on other relevant immune cell types within the lungs of COPD patients.

There are 4 isoforms of p38 MAPK which are encoded by separate genes; p38α, p38β, p38δ and p38γ. The expression of these isoforms varies between tissues and cell types [16]. The p38α and p38β isoforms play predominant roles in immune cell activation, so the majority of p38 MAPK inhibitors developed for the treatment of inflammation have been targeted against these isoforms in order to avoid unwanted physiological effects through p38γ and p38δ inhibition. It has been shown in glomerulonephritis that p38α is the most highly expressed isoform in infiltrating leukocytes in the kidney [17], but there was also evidence of p38β and p38γ isoform expression in structural cell types. The expression levels of p38 MAPK isoforms in the lungs of COPD patients have not been quantitatively studied; this would identify the isoforms relevant to the pathophysiology of COPD.

The aims of the current study were as follows; (1) to characterise the cell specific expression of activated p38 MAPK in COPD lungs compared to controls by using dual labelled immunofluorescence (2) to investigate the anti-inflammatory effects of p38 MAPK inhibition on cytokine production from neutrophils, epithelial cells and lymphocytes isolated from COPD lungs (3) to investigate p38 MAPK isoform expression in lung tissue from COPD patients compared to controls.
Methods

Study subjects

Patients undergoing surgical resection for suspected or confirmed lung cancer were recruited. Patients with a previous diagnosis of COPD according to the GOLD guidelines [18] with at least a 10 cigarette pack year history and airflow obstruction defined as FEV1 <80% and FEV1:FVC < 70% were recruited. Controls consisted of smokers (S) with > 10 pack year smoking history and normal pulmonary function and lifelong non-smokers (NS). Some controls had evidence of FEV1:FVC < 70% due to obstruction by tumour. In a separate study COPD, S and NS for sputum induction, and COPD patients for isolated neutrophil work were recruited. Additionally 3 COPD patients were recruited for bronchoscopy for the isolation of airway epithelial cells. The demographics are shown in table 1. All patients gave written informed consent. The study was approved by the local ethics committee.

Analysis of p38 mRNA levels

Levels of p38α, p38β, p38δ and p38γ were measured by qPCR in COPD, S and NS patients. Levels of expression were normalised to GAPDH for analysis as described in the online supplement.

Immunohistochemistry

Tissue blocks were obtained from an area of the lung as far distal to the tumour as possible, and processed as described previously [19]. Blocks were labelled using anti-phospho-p38 MAPK primary antibody. Dual label immunofluorescence with phospho-p38 was performed with one of the following primary antibodies: neutrophil elastase, CD20, CD8 or CD4. Further details of methods and antibodies are described in the online supplement.
Image analysis
The percentage of phospho-p38+CD20+, CD8+ and CD4+ cells within inflammatory follicles and within the sub-epithelium was quantified. Total numbers of phospho-p38+neutrophils, small airway epithelial cells and macrophages (identified by morphology) were also quantified. For dual-label images, fluorescent images from the same field were captured and digitally merged to determine the phospho-p38 positive cells. Digital micrographs were obtained through the use of a Nikon Eclipse 80i microscope (Nikon UK Ltd, Surrey, UK) equipped with a QImaging digital camera (Media Cybernetics, Marlow, UK) and ImagePro Plus 5.1 software (Media Cybernetics, Marlow, UK). Cell counts, follicle area and epithelial and sub-epithelial length were quantified using the ImagePro Plus 5.1 software. Cell counts were standardised to the number of positive cells.mm-2 of the area of interest.

Cell culture
Neutrophils
Isolated blood and sputum neutrophils (cell isolation details are provided in the online supplement; the sputum cell counts are shown in table 2) were pre-treated with a p38α and p38β MAPK selective inhibitor SB100 (GlaxoSmithKline, Hertfordshire, UK) using final concentrations of 10-1000nM before the addition of LPS (100ng/ml) (Sigma Aldrich, Poole, UK) for 24 hours. LPS 100ng/ml is commonly used for neutrophil stimulation [20], and we confirmed that this is a sub-optimal concentration for cytokine production (supplementary figure S1a). Supernatants were removed and stored at -80°C for cytokine analysis. Remaining cells were removed and centrifuged at 400g for 10 mins at 4°C. Cell viability and counts were determined before preparing cytospins and fixing in 4% paraformaldehyde prior to immunocytochemical analysis (see online supplement for methods). In order to determine cell viability, trypan blue exclusion was assessed before and after SB100 (1000nM) treatment. To
determine the extent of apoptosis before and after SB100 (1000nM) treatment, DNA fragmentation and presence of condensed nuclei were assessed by TUNEL and nuclear morphology respectively (described in online supplement). Apoptosis was determined by examining the disappearance of chromatin bridges between nuclear lobes (early apoptosis) and shrinkage or fragmentation of the nucleus (late apoptosis). The percentage of normal appearing neutrophils as well as early and late apoptotic neutrophils was assessed by counting a total of 300 neutrophils.

**CD8 Cells**

Isolated pulmonary CD8 cells (isolation protocol detailed in online supplement) were seeded at 5x10^4 cells/well before pre-treatment with SB100 (0.1-1000nM). Cells were then stimulated for 24 hours with IL-12 (10ng/ml, Peprotech, London, UK) and IL-18 (10ng/ml, Peprotech) for 24 hours before harvesting supernatants for the measurement of IFNγ by ELISA (eBioscience, Hatfield, UK). These cytokine concentrations were determined by preliminary concentration-response experiments in isolated blood CD8 cells; sub-optimal IFNγ production coupled with p38 phosphorylation was observed (see supplementary figure 1b and 1c).

**Epithelial Cells**

The isolation of epithelial cells is described in the online supplement. Epithelial cells were cultured in 96 well plates until 80% confluent. Cells were pre-treated with SB100 (1µM) for one hour before culturing with Poly I:C (10µg/ml, Invivogen, San Diego, CA, USA) for 24 hours. Supernatants were harvested for the measurement of CCL5, CXCL8 and IL-6 by ELISA (R&D systems, Abingdon, UK).

**Cytokine measurements**

Supernatant levels of TNFα, CXCL8, IFNγ, IL-6 and CCL5 were determined using ELISA according to the manufacturer’s instructions. The lower limit of detection for
TNFα, CXCL8, IL-6 and CCL5 were 15.6pg/ml, 32.5pg/ml, 9.375pg/ml and 15.6pg/ml respectively (R&D systems, Abingdon, UK). The lower limit of detection of IFNγ was 7.8pg/ml (eBioscience, Hatfield, UK).

**Statistical Analysis**

Normality was assessed using Kolmogorov-Smirnov test. Comparisons between COPD patients, S and NS were performed using one-way ANOVA followed by Bonferroni’s post-test for parametrically distributed immunohistochemistry data. PCR data were analysed by non-parametric ANOVA (Kruskal-Wallis test) for between group comparisons and repeated measures ANOVA (Friedman test) for within group analyses, followed by Dunn’s post-test. Paired t-tests were used to compare the effect of SB100 in cell cultures. Analysis was carried out using GraphPad InStat software version 3.06 (GraphPad Software, Inc., San Diego, CA, USA).
Results

p38 isoform expression in lung tissue.

Gene expression levels of p38 α, β, γ and, δ were analysed by qPCR in RNA extracted from COPD (n=11), S (n=7) and NS (n=10) lung tissue (figure 1). The α and δ isoforms were significantly increased in COPD lung tissue compared to NS (p<0.01 and p<0.05 respectively), while p38α expression was also significantly increased in S compared to NS (p<0.01).

P38α expression was increased compared to p38β and p38γ in COPD patients (p<0.01 for both isoforms), and p38α expression was also increased compared to p38β in S (p<0.01). There was no difference between the expression levels of the isoforms in NS.

Phospho-p38 MAPK in lymphocytes

Follicles

Dual label immunofluorescence showed that phospho-p38 MAPK was present in all the follicles analysed.

B Cells

Bonferroni’s multiple comparisons tests showed that the percentage of CD20+phospho-p38+ cells was significantly higher in COPD patients compared to S and NS (p<0.001 for both comparisons, means 86.6%, 44.1% and 30.9% respectively). Numbers of CD20+phospho-p38+ were also significantly greater in S compared to NS (p<0.05) (see figure 2a and figure 3).

CD8 Cells

Bonferroni’s multiple comparisons tests showed that the percentage of CD8+ phospho-p38+ cells was significantly higher in COPD patients compared to NS (p<0.001) (means 56.2%, and 31.5%, respectively). There was also a numerical trend towards increased numbers of CD8+phospho-p38+ cells in COPD compared to S (mean 43.6%) and in S compared to NS (mean 31.5%), although this difference
was not statistically significant \( (p>0.05 \text{ for both comparisons}) \) (see figure 2b and figure 4).

**CD4 Cells**

The numbers of phospho-p38+ CD4+ cells within inflammatory follicles was much lower in all patient groups, typically less than 20% of CD4+ cells were positive for phospho-p38 MAPK. Statistically, there were no differences between any of the groups (figure 2c)

**Sub-epithelium**

The number of sub-epithelial lymphocytes and the number of sub-epithelial lymphocytes positive for phospho-p38 MAPK was similar across all 3 patient groups (see table 3). Phospho-p38 MAPK was absent in sub-epithelial CD4+ lymphocytes. Numbers of CD20+ and CD8+phospho-p38+ cells were significantly lower compared to phospho38+ lymphocytes within follicles (ANOVA \( P<0.0001 \) for comparisons of both cell types).

**Phospho-p38 MAPK in macrophages**

**Alveolar macrophages**

The percentage of phospho-p38+ alveolar macrophages was significantly greater in COPD patients compared to both S and NS (means 70.0%, 56.4% and 28.5% respectively, Bonferroni’s multiple comparisons tests COPD vs S \( p<0.01 \), COPD vs NS \( p<0.001 \), and in S compared to NS (Bonferroni’s multiple comparions test \( p<0.001 \)) (see figure 2d and 5a-c).

**Sputum macrophages**

The percentage of phospho-p38+ sputum macrophages was significantly greater in COPD patients \((n=8)\) compared to both S \((n=6)\) and NS \((n=4)\) (means 92.8%, 59.3%,
31.8%, respectively; Bonferroni’s multiple comparisons tests p<0.001 for all comparisons) (see figure 2e and 5d-f).

**Phospho-p38 MAPK in epithelial cells**

Phospho-p38 MAPK was present in the majority of small airway epithelial cells analysed in all 3 patient groups. Bonferroni’s multiple comparisons tests showed that there was a significant increased percentage of phospho-p38+ epithelial cells between COPD and NS (p<0.05; means 96% and 81%, respectively). There was also a trend towards increased percentages of phospho-p38+ epithelial cells in COPD patients compared to S (mean 91%) although this was not statistically significant (see figure 2f and 5g-i).

**Phospho-p38 MAPK in neutrophils**

Lung tissue neutrophils were devoid of phospho-p38 MAPK immunoreactivity in all patient groups (see figure 5j-l). Sputum neutrophils also lacked phospho-p38 MAPK (see figure 5d-f). We examined phospho-p38 MAPK in neutrophils isolated from blood of COPD patients. Neutrophils examined immediately after isolation from blood did not have phospho-p38 MAPK, but phospho-p38 MAPK was induced following culture with LPS (see figure 6a and b). Although p38 MAPK expression was observed in sputum neutrophils (Supplementary figure 1d), phospho-p38 MAPK was absent in sputum neutrophils cultured with LPS for 24 hours (see figure 6c and d), indicating that the p38 MAPK pathway is not active in lung neutrophils.

**Cell specific effects of p38 MAPK inhibition**

**CD8 Cells**

CD8 cells isolated from lung tissue of 3 COPD patients were pre-treated with SB100 before stimulating with IL-12 and IL-18 in combination. IFNγ production increased, from mean basal levels of 5.3pg/ml to mean 1780pg/ml. SB100 inhibited IFNγ
production in a dose-dependent manner, with maximal inhibition of 94.8% observed (figure 7a).

*Epithelial Cells*

Cells were pre-treated with SB100 (1000nM) for one hour prior to stimulating with Poly I:C for 24 hours. Mean basal levels of IL-6 and CXCL8 were 85.5 pg/ml and 15258 pg/ml respectively. Basal levels of CCL5 were below the limit of detection. Poly I:C stimulation increased IL-6, CXCL8 and CCL5 release to 1580.1 pg/ml, 56335 pg/ml and 2643 pg/ml respectively. SB100 caused 50.7%, 38.7% and 26.7% inhibition of IL-6, CXCL8 and CCL5 respectively (figure 7b).

*Neutrophils*

Isolated blood neutrophils (n=7 COPD) were cultured for 24 hours with and without LPS. The mean levels of unstimulated TNFα and CXCL8 release were 527 pg/ml and 2857 pg/ml, respectively, increasing to 2706 pg/ml and 7161 pg/ml, respectively, after LPS stimulation. SB100 caused a dose dependant inhibition of cytokine production in both stimulated and unstimulated neutrophils (see figures 7c and d). There were no differences between percent inhibition of TNFα and CXCL8 in stimulated or unstimulated neutrophils.

Sputum samples from these 7 COPD patients were also obtained. Previous studies have shown that LPS has no effect on cytokine production from isolated sputum neutrophils [21], which we also observed (data not shown). The mean levels of unstimulated TNFα and CXCL8 release were 681 pg/ml and 4532 pg/ml in sputum neutrophils. In unstimulated neutrophils isolated from sputum, SB100 only inhibited TNFα at the highest concentration (1000 nM) and had no effect on CXCL8 production (see figure 7e and f). SB100 had a significantly lower effect on sputum neutrophils compared to LPS stimulated and unstimulated blood neutrophils at all
concentrations for both TNFα and CXCL8 (p<0.05 for comparisons of all concentrations using one tailed paired t-tests).

Cell viability was assessed before and after SB100 (1000nM) treatment (n=4). There was no significant effect of SB100 on sputum neutrophil or blood neutrophil viability, measured by trypan blue exclusion, morphological analysis for apoptosis and TUNEL assay (see supplementary figure S2 and S3).
Discussion

We have demonstrated increased phospho-p38 expression in specific cell types within the lungs of COPD patients compared to controls; the proportion of follicular B cells and CD8 lymphocytes, small airway bronchial epithelial cells and macrophages expressing immunoreactivity for phospho-p38 was increased in lung samples from COPD patients compared to controls. In these specific cell types, the overall pattern that we observed was for cigarette smoking to increase phospho-p38 MAPK, and the development of COPD to cause a further increase. It has previously been reported that the proportion of alveolar macrophages expressing phospho-p38 MAPK is increased in COPD alveolar macrophages [15]; we have now identified other lung cell types that also show this pattern of increased phospho-p38 MAPK expression in COPD patients. P38 MAPK inhibition in isolated COPD lung CD8 cells and epithelial cells reduced cytokine production, demonstrating that the expression of phospho-p38 in these cells is associated with pro-inflammatory functions.

Lung neutrophils were devoid of phospho-p38 MAPK immunoreactivity, in both COPD patients and controls. Additionally, p38 MAPK inhibition had no effect on cytokine production from COPD lung neutrophils; this suggests that p38 MAPK signalling does not play a role in the pro-inflammatory activity of COPD lung neutrophils. This contrasts with COPD blood neutrophils, where phospho-p38 was detected and was functionally involved in cytokine production. P38 MAPK inhibitors are currently being developed as anti-inflammatory drugs for COPD; it appears that these drugs can exert anti-inflammatory effects on certain lung cell types such as B cells, CD8 cells, macrophages and epithelial cells, but have no effect on lung neutrophils.
We observed that p38α MAPK isoform gene expression levels were increased in lung tissue from COPD patients and smokers compared to non-smokers, with no difference between COPD patients and smokers; this suggests that cigarette smoking upregulates p38α gene expression. P38α was also the most highly expressed isoform; this is the primary isoform target of the majority of p38 MAPK inhibitors. P38δ expression was increased in COPD patients compared to non-smokers, although there was no difference between COPD patients and smokers. P38δ has previously been shown to be expressed in macrophages [9], and our results indicate upregulation of the expression of this isoform in the lungs of COPD patients. We also demonstrated expression of p38γ in COPD lung tissue, at a similar level to control samples; this isoform appears to play a role in glucocorticoid resistant inflammation [22].

Increased numbers of lymphoid follicles in the small airways of COPD patients are associated with more severe disease [1]. We observed that the B cell cores and CD8 cells within these follicles had increased phospho-p38 MAPK in COPD patients compared to controls. Interestingly, there was no increase in numbers of phospho-p38+ B cells and CD8 cells within the sub-epithelial region of COPD patients compared to controls. This suggests that lymphocytes within the follicles have a different physiological function compared to other lung lymphocytes. This is perhaps not surprising as follicular lymphocytes lie within an environment that resembles lymph nodes rather than normal lung tissue, and function as important sites of antigen presentation [23, 24].

The numbers of follicular phospho-p38+ CD4+ cells were similar in all patient groups. The number of phospho-p38+CD4+ cells within these follicles was much lower than that observed for both CD8+ cells and CD20+ cells. Furthermore, phospho-p38 immunoreactivity was absent in CD4 cells in the subepithelium. This striking
difference in phospho-p38 expression between different lymphocyte subtypes suggests that p38 MAPK signalling may not play a central role in the physiology of lung CD4 cells. However, caution should be applied to the interpretation of these immunohistochemistry data which is taken from a snapshot in time, as it is possible that p38 MAPK signalling is activated in COPD CD4 cells at other times e.g. during exacerbations.

Previous studies have shown that pharmacological inhibition of p38 MAPK can reduce pro-inflammatory cytokine production from COPD macrophages [9-11]. We now show that p38 MAPK inhibition also reduces pro-inflammatory cytokine production from lung CD8 cells and epithelial cells from COPD patients. Inhibition of p38 MAPK significantly reduced IFNγ release from isolated CD8 cells from COPD lungs. It is known that p38 MAPK signalling is involved in cytokine production from blood CD8 cells [25], and we now confirm a similar role for this pathway in lung CD8 cells. It has recently been demonstrated that the effect of glucocorticoids on IFNγ release from broncho-alveolar lavage lymphocytes is reduced in COPD patients compared to controls [26]. Furthermore, IFNγ causes glucocorticoid insensitive cytokine production in COPD alveolar macrophages through STAT1 activation [27]. Inhibiting p38 MAPK signalling in lung CD8 cells can therefore target glucocorticoid insensitive mechanisms; IFNγ production from CD8 cells and hence subsequent STAT1 mediated cytokine production from macrophages.

Cigarette smoke induces the release of a variety of pro-inflammatory mediators from bronchial epithelial cells in vitro [28-31], implicating the epithelium in the pathogenesis of COPD. In the present study, the percentage of small airway epithelial cells positive for phospho-p38 MAPK was significantly higher in COPD lungs. Increased phospho-p38 MAPK expression in airway epithelial cells has also
been found in severe asthma [32]. COPD and severe asthma are characterised by persistently increased levels of pro-inflammatory mediators in the airways [33, 34]. The p38 MAPK pathway is activated by a range of inflammatory stimuli, and it appears that bronchial epithelial cells in both severe asthma and COPD respond to these stimuli by p38 MAPK activation.

Lung neutrophils were devoid of phospho-p38 MAPK immunoreactivity in all patient groups, including smoking and non-smoking controls. Stimulation of lung neutrophils with LPS did not induce phospho-p38 MAPK activation indicating that the pro-inflammatory activity of lung neutrophils is not dependent on p38 MAPK signalling. This was confirmed by the lack of an effect of SB100 on pro-inflammatory cytokine production from lung neutrophils. In contrast, phospho-p38 MAPK activation was observed in blood neutrophils, and cytokine production from these cells was inhibited by SB100. Our results suggest that SB100 inhibits both pre-formed and de-novo synthesised cytokines in blood neutrophils as similar inhibition was observed in both unstimulated and LPS stimulated cells. Our results suggest that neutrophils leaving the bloodstream and entering the lung undergo phenotypic changes, altering the activity of intracellular signalling pathways required for important cell functions. Similarly, we have previously observed normal glucocorticoid receptor expression in blood neutrophils, but depleted glucocorticoid receptor expression in airway neutrophils [35]. These data highlight a potential pitfall of using blood neutrophils as a model for lung neutrophils, as there appear to be differences in the signalling mechanisms responsible for cytokine production.

Previous studies have demonstrated that p38 MAPK inhibition attenuates chemotaxis [36-39] and, superoxide generation [40], in addition to pro-inflammatory mediator generation [41-43] in blood neutrophils. The lack of activated p38 MAPK observed in
lung neutrophils makes it unlikely that p38 MAPK inhibition would have any effect on chemotaxis and superoxide generation in lung neutrophils. The altered phenotype of lung neutrophils, with reduced activation of p38 MAPK and expression of glucocorticoid receptor [35] suggests that specific anti-neutrophil therapies are needed to target this cell type in COPD, rather than broad anti-inflammatory drugs.

In alveolar macrophages, we have previously shown that p38 MAPK activation is glucocorticoid resistant [11]. P38 MAPK inhibitors therefore target a glucocorticoid resistant pathway that is activated within the lungs of COPD patients, and we show here that these drugs can suppress cytokine production from COPD lung lymphocytes and epithelial cells. There are synergistic interactions between p38 MAPK and glucocorticoids; both drugs used together cause a greater than additive inhibition of cytokine production from COPD alveolar macrophages [11]. There are molecular mechanisms that can explain such observations, such as glucocorticoid induced upregulation of MAPK phosphatase [44], which dephosphorylates p38 MAPK. Combination treatment with glucocorticoids and p38 MAPK inhibitors may also have synergistic effects on cytokine production from lymphocytes and epithelial cells.

We and others have reported no differences between COPD patients and controls for the effect of p38 inhibition on the release of inflammatory mediators from alveolar macrophages (9, 11). We now report the effect of p38 inhibition in lung CD8 and epithelial cells from COPD patients, and it would be of interest to know if these effects differ from controls.

In conclusion, we show cell-specific activation of the p38 MAPK pathway in the lungs of COPD patients. P38 MAPK inhibitors suppress cytokine production from COPD lung lymphocytes and epithelial cells, but have no effect on lung neutrophils. These novel drugs therefore target some, but not all, of the inflammatory processes involved in COPD.
Table 1. Subject demography

<table>
<thead>
<tr>
<th></th>
<th>Lung Tissue (PCR and IHC)</th>
<th>Sputum cells (ICC)</th>
<th>In vitro cell culture</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>COPD</td>
<td>S</td>
<td>NS</td>
</tr>
<tr>
<td>Number</td>
<td>31</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Male/Female</td>
<td>17/14</td>
<td>8/11</td>
<td>8/13</td>
</tr>
<tr>
<td>Age (years)</td>
<td>66.4 (7.2)</td>
<td>61.5 (13.1)</td>
<td>59 (15.6)</td>
</tr>
<tr>
<td>Smoking History</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Pack years)</td>
<td>50.9 (25.6)</td>
<td>44.3 (29.4)</td>
<td>0</td>
</tr>
<tr>
<td>FEV1 (L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8 (0.5)</td>
<td>2.3 (0.73)</td>
<td>2.2 (0.8)</td>
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<tr>
<td>FEV1% predicted</td>
<td>66.7 (11.6)</td>
<td>91.3 (16.7)</td>
<td>93.6 (19)</td>
</tr>
<tr>
<td>FEV1/FVC ratio</td>
<td>57.2 (8.6)</td>
<td>73.8 (7.2)</td>
<td>75.7 (10.5)</td>
</tr>
<tr>
<td>Number on ICS</td>
<td>31</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Data are presented as mean (SD). * neutrophils were isolated from both sputum and blood. PCR: polymerase chain reaction; IHC: immunohistochemistry; ICC: immunocytochemistry; COPD: chronic obstructive pulmonary disease; S: smokers; NS: non-smokers; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; ICS: inhaled corticosteroids.
Table 2. Sputum differential cell counts from samples used in cell culture experiments

<table>
<thead>
<tr>
<th></th>
<th>Before enrichment</th>
<th>After enrichment</th>
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<tbody>
<tr>
<td>Total cell count/g sputum *</td>
<td>14.2 (13.5)</td>
<td>8.84 (8.9)</td>
</tr>
<tr>
<td>Total neutrophil count/g sputum *</td>
<td>10.3 (9.7)</td>
<td>8.0 (8.2)</td>
</tr>
<tr>
<td>Neutrophil %</td>
<td>73.5 (4.6)</td>
<td>89.5 (3.1)</td>
</tr>
<tr>
<td>Total macrophage count/g sputum *</td>
<td>3.3 (3.5)</td>
<td>0.7 (0.6)</td>
</tr>
<tr>
<td>Macrophage %</td>
<td>22.5 (4.4)</td>
<td>9.0 (3.6)</td>
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<tr>
<td>Eosinophil% #</td>
<td>1.0 (0.5-1.8)</td>
<td>0 (0-1.75)</td>
</tr>
<tr>
<td>Lymphocyte % #</td>
<td>0.3 (0-1.8)</td>
<td>0 (0-0.5)</td>
</tr>
<tr>
<td>Squamous cell% #</td>
<td>1 (0-1.75)</td>
<td>0.25 (0-3.75)</td>
</tr>
</tbody>
</table>

Differential cell counts for sputum samples used for cell culture pre- and post-neutrophil isolation step (n=7). Data are presented as mean (SD). Cell numbers are normalised per gram of sputum, * denotes number of cells x10⁶ and # denotes data presented as median (range).
Table 3. Mean number of CD20+, CD8+ and CD4+ lymphocytes/mm² sub-epithelia and percentage of cell-specific phosphorylated p38 MAPK.

<table>
<thead>
<tr>
<th></th>
<th>Number of lymphocytes per mm² sub epithelia</th>
<th>Percentage phospho-p38 MAPK presence</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>COPD*</td>
<td>S#</td>
</tr>
<tr>
<td>CD20</td>
<td>181.64</td>
<td>113.47</td>
</tr>
<tr>
<td>CD8</td>
<td>438.82</td>
<td>400.31</td>
</tr>
<tr>
<td>CD4</td>
<td>84.44</td>
<td>60.36</td>
</tr>
</tbody>
</table>

Summary of the mean number of CD20+/CD8+/CD4+ lymphocytes identified per mm² of sub-epithelial tissue. The percentage of phospho-p38+ sub-epithelial lymphocytes is also shown. ANOVA p values shown for comparisons between patient groups for each cell types. COPD: chronic obstructive pulmonary disease; S: smokers; NS: non-smokers. *: 20 samples analysed; #: 20 samples analysed; +: 12 samples analysed.
Figure 1. P38 isoform expression in lung lysates from COPD patients, smoking (S) and non-smoking (NS) controls. $\beta$, $\gamma$, $\delta$ and $\alpha$ isoforms were analysed by qPCR and normalised to GAPDH expression in NS ($n=10$), S ($n=7$) and COPD ($n=11$) patients' lung lysates. Data is expressed as median ± range. # indicates difference between groups reached statistical significance $p<0.05$, ###$p<0.01$ (unpaired data). * indicates difference within groups reached statistical significance **$p<0.01$ (paired data).

Figure 2. The mean percentage of phosphorylated (phospho-) p38 MAPK+ (A) follicle CD20+ B cells, (B) follicle CD8+ cells, (C) follicle CD4+ cells, (D) lung tissue macrophages, (E) sputum macrophages and (F) small airway epithelial cells. Differences between patient groups for each cell type: *$p<0.05$, **$p<0.01$, ***$p<0.001$. 
Figure 3. Representative images for the dual label immunofluorescent detection of phosphorylated (phospho-) p38 MAPK in CD20+ B cells in inflammatory follicles within human lung tissue. Representative images from (A-C) 20 COPD patients, (D-F) 12 smokers and (G-H) 12 non-smokers are shown. Cell nuclei were counterstained with 4', 6-diamidino-2-phenylindole (blue). CD20+ cells were identified using an Alexa-488 conjugated goat anti-mouse secondary antibody (Red; A, D, G) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti-rabbit secondary antibody (Green; B, E and H). Composite images are shown (C, F and I). Green/yellow fluorescence is caused by intrinsically fluorescent tissue
components such as elastic fibres and red blood cells. Autofluorescence can be distinguished from positive fluorescence by forming a composite image of the red, green and blue channels. Autofluorescence is visible in all 3 channels and so appears an amalgamation of the three colours. Positive fluorescence is visible in only one channel and thus appears as the pure colour. Magnification x200
Figure 4. Representative images for the dual immunofluorescent detection of phosphorylated (phospho-) p38 MAPK in CD8+ cells within inflammatory follicles in
lungs tissue. Representative images from (A-C) 20 COPD patients, (D-F) 12 smokers and (G-I) 12 non-smokers are shown. Cell nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (Blue). CD8+ cells were identified using an Alexa-488 conjugated goat anti mouse secondary antibody (Red; A, D, G) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti rabbit secondary antibody (Green; B, E, H). Composite images are also shown (C, F, I). Magnification x200
Figure 5. Representative images for the immunohistochemical and dual label immunofluorescent detection of phosphorylated (phospho-) p38 MAPK in human lung tissue and sputum cytospins. Representative images shown for (A-I) immunocytochemical and (J-L) immunofluorescence tissue analysis from (A, G, J; n=20, D; n=8) COPD patients, (B, H, K; n=12, E; n=6) smokers and (C, I, L; n=12 F; n=4) non-smokers are shown. Cell nuclei were counterstained with either Mayer’s haematoxylin (Blue; A-I) or 4′, 6-diamidino-2-phenylindole (Blue; J-L). For immunohistochemical analysis phospho-p38 MAPK expression was detected using 3, 3’- diaminobenzadine (Brown; A-I). For dual label immunofluorescence (J-L) lung tissue neutrophils were identified using an Alexa-488 conjugated goat anti mouse secondary antibody (Red; J) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti rabbit secondary antibody (Green; K). Composite images for dual label immunofluorescence are also shown (L). Phospho-p38 MAPK expression in alveolar macrophages (Brown; A-C), sputum macrophages (Brown; D-F) and small airway epithelial cells (Brown; G-I). Lung tissue neutrophils (Red) expressing phospho-p38 MAPK (Green; J-L). Magnification x200.
Figure 6. Representative images for the immunohistochemical detection of phosphorylated (phospho-) p38 MAPK in isolated COPD blood and sputum neutrophils. Cell nuclei were counterstained with Mayer’s haematoxylin (Blue). Phospho-p38 MAPK expression was detected using 3, 3’-diaminobenzadine following direct immunohistochemistry (Brown). (A) Phospho-p38 MAPK expression is absent in basal blood neutrophils. (B) Phospho-p38 MAPK (Brown) is induced in blood neutrophils following stimulation with 1μg/ml LPS. Phospho-p38 MAPK is absent in (C) basal and (D) LPS stimulated sputum neutrophils.
Figure 7. Effect of p38 MAPK Inhibition in COPD cells. (A) Inhibition of IFNγ release from COPD lung CD8 cells (n=3) by SB100 (0.1nM – 1000nM) following 24 hour stimulation with IL-12 (10ng/ml) and IL-18 (10ng/ml). (B) Inhibition of CCL5, CXCL8 and IL-6 from epithelial cells isolated from COPD lung tissue (n=3) by SB100 (1000nM) following 24 hour stimulation with poly I:C (10ug/ml). Inhibition of (C) TNFα and (D) CXCL8 in stimulated and unstimulated isolated COPD blood neutrophils (n=7) following incubation with SB100 (0.1-1000nM) for 24 hours. Inhibition of (E) TNFα and (F) CXCL8 release from sputum neutrophils (n=7) following incubation with SB100 (0.1-1000nM) for 24 hours. Data is presented as mean ± SEM. A significant reduction in cytokine release following SB100 treatment is denoted by * p<0.05, ** p<0.01 and *** p<0.001.
**Supplementary Figure S1.** (A) Blood neutrophils (n=7 COPD) were cultured with LPS (0-10,000ng/ml) for 24 hours. Levels of IFNγ and CXCL8 were measured by ELISA. Data is presented as mean +/- s.e.m. (B) Blood CD8 cells (n=3) were cultured with IL-12 (0-100ng/ml) and IL-18 (0-100ng/ml) alone and in combination for 24 hours and IFNγ release measured by ELISA. (C) Representative images for the dual staining of phospho-p38 in blood CD8 cells following 1 hour treatment with IL-12 and IL-18 (10ng/ml for both). CD8 cells were identified using an Alexa-488 conjugated goat anti-mouse secondary antibody (red) and phospho-p38 MAPK was detected using an Alexa 468 conjugated goat anti-rabbit secondary antibody (green). Composite images are shown. DAPI was used as a nuclear counter stain. Magnification X200. (D) Expression of p38 MAPK in sputum neutrophils, detected by immunocytochemical staining using 3, 3'- diaminobenzadine (brown). Magnification X200.

**Supplementary Figure S2.** (A, C, E) Sputum neutrophil and (B, D, F) blood neutrophil viability before culture (basal) and 24 h after culture with DMSO (0.05%) or SB100 (1000nM). (A, B) Percentage of live cells analysed by trypan blue exclusion. (C, D) Percentage of cells showing morphologically normal, early or late stage apoptotic nuclei. (E, F) Percentages of cells staining positive for TUNEL as a measure of apoptosis.

**Supplementary Figure S3.** Representative photomicrographs from (A-F) sputum cytospins and (G-L) blood neutrophil cytospins analysed by (A-C and G-I) nuclear morphology and (D-F and J-L) TUNEL (Green). Basal samples were analysed immediately after collection, while 24 hr samples were cultured with SB100 (1000nM) and control vehicle (DMSO 0.05%). Arrows indicate cells showing evidence of apoptosis (loss of chromatin bridges between nuclear lobes or dual TUNEL (Green) and neutrophil elastase (Red) positivity). * indicates normal appearing neutrophils.
(neutrophil elastase positive and TUNEL negative) in close proximity to apoptotic cells. Magnification x 200.


