Enhanced monocyte migration to CXCR3 and CCR5 chemokines in COPD

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ABSTRACT Chronic obstructive pulmonary disease (COPD) patients exhibit chronic inflammation, both in the lung parenchyma and the airways, which is characterised by an increased infiltration of macrophages and T-lymphocytes, particularly CD8⁺ cells. Both cell types can express chemokine (C-X-C motif) receptor (CXCR)3 and C-C chemokine receptor 5 and the relevant chemokines for these receptors are elevated in COPD. The aim of this study was to compare chemotactic responses of lymphocytes and monocytes of nonsmokers, smokers and COPD patients towards CXCR3 ligands and chemokine (C-C motif) ligand (CCL)5.

Migration of peripheral blood mononuclear cells, monocytes and lymphocytes from nonsmokers, smokers and COPD patients toward CXCR3 chemokines and CCL5 was analysed using chemotaxis assays. There was increased migration of peripheral blood mononuclear cells from COPD patients towards all chemokines studied when compared with nonsmokers and smokers. Both lymphocytes and monocytes contributed to this enhanced response, which was not explained by increased receptor expression. However, isolated lymphocytes failed to migrate and isolated monocytes from COPD patients lost their enhanced migratory capacity.

Both monocytes and lymphocytes cooperate to enhance migration towards CXCR3 chemokines and CCL5. This may contribute to increased numbers of macrophages and T-cells in the lungs of COPD patients, and inhibition of recruitment using selective antagonists might be a treatment to reduce the inflammatory response in COPD.

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Introduction
Chronic obstructive pulmonary disease (COPD) is characterised by progressive and largely irreversible deterioration of lung function due to small airway fibrosis and alveolar damage as a result of chronic inflammation [1, 2]. Chronic inflammation in the lung parenchyma and the airways is characterised by increased infiltration of macrophages and T-lymphocytes [3, 4]. Although there are increased numbers of CD4+ and CD8+ lymphocytes, it is CD8+ cells that predominate in COPD [5, 6]. Moreover, the CD8+ T-cell numbers within the lung parenchyma correlate with airflow limitation, suggesting their importance in COPD pathogenesis [4, 7]. Macrophage numbers are increased in bronchoalveolar lavage (BAL) fluid [8, 9] and sputum [10] of smokers with COPD when compared with nonsmokers, suggesting that enhanced macrophage numbers in COPD lungs are a consequence of increased recruitment of monocytes from the circulation [11], since there is little proliferation within the lung [12]. Once in the lung, monocytes differentiate into macrophages and may then orchestrate many of the pathophysiological features of COPD [9].

The precise role of lymphocytes in the pathophysiology of COPD is uncertain. Classically, immune responses to specific antigenic stimuli are regulated by the pattern of cytokines released by activated T-cells. Type-1 immune responses are associated with lung inflammation in COPD [13, 14], in contrast to that in asthma, which has a type-2 immune response. The chemokine receptors expressed by type-1 T-cells differ from those expressed by type-2 cells [15]. Interferon (IFN)-γ producing type-1 T-cells most commonly express chemokine (C-X-C motif) receptor (CXCR)3 and C-C chemokine receptor (CCR)5 [16, 17]. CD8+ lymphocytes in lung tissue express CCR5 and CXCR3 and are correlated with disease severity in COPD [18]. However, these receptors are not restricted to T-lymphocytes. CXCR3 are also expressed by B-cells, monocytes, macrophages and airway epithelial cells [19, 20], whereas CCR5 are expressed predominantly on monocytes and macrophages [19, 21, 22]. Indeed, CXCR3 are highly expressed on B-cells in lymphoid follicles associated with more severe COPD [23]. Such diverse patterns of chemokine receptor expression indicate that these receptors could alter both innate and adaptive immune responses important in COPD pathogenesis [24].

Three chemokines bind and signal through CXCR3: C-X-C motif ligand (CXCL)9 (also known as monokine induced by IFN-γ), CXCL10 (IFN-inducible protein-10) and CXCL11 (IFN-inducible T-cell α-chemoattractant) [21]. The ligands for CCR5 include (C-C motif) ligand (CCL)5 (RANTES), CCL3 (macrophage inflammatory protein (MIP)-1α) and CCL4 (MIP-1β). The expression of CXCR3 and CCR5 on monocytes and T-cells suggests that they may drive recruitment of these cells to inflammatory sites. Moreover, increased concentrations of CXCR3 chemokines and CCL5 in induced sputum of COPD patients [25] further supports a role for CXCR3 and CCR5 in leukocyte recruitment in COPD. The aim of this study was to examine the contribution of CXCR3 and CCR5 to chemotactic responses of lymphocytes and monocytes of nonsmokers, smokers and COPD patients.

Methods
Subject selection
The project was approved by the ethics committees of the Royal Brompton and Harefield Hospitals and East Berkshire National Health Service Trust. Diagnosis of COPD was made using Global Initiative for Chronic Obstructive Lung Disease criteria [26]. Inclusion criteria for COPD included smoking history of ≥10 pack-years, no history of allergic disease and no history of upper respiratory tract infection the preceding 6 weeks. The inclusion criteria for smokers were similar to COPD, but they were required to have a recent lung function test with normal values. Nonsmoker subjects fulfilled the same inclusion criteria as smokers, but without smoking history. All subjects gave written informed consent.

Chemokine receptor expression in leukocytes from whole blood
Leukocytes in whole blood were analysed for the expression of CXCR3 and CCR5 using methods described previously [11]. The data are expressed as the percentage of cells expressing the receptor being evaluated and as relative fluorescence, which was calculated using the fluorescence values (mean channel) of the cells stained with phycoerythrin (PE)-conjugated anti-CXCR3 and anti-CCR5 antibodies (R&D Systems Europe, Abingdon, UK) divided by the fluorescence values (mean channel) for the PE-conjugated IgG2a isotype control.

Isolation of peripheral blood mononuclear cells
Peripheral blood mononuclear cells (PBMCs) were separated from 60 mL venous blood as described previously [11].

Isolation of monocyte and non-monocytic cells
Isolation of monocytes was performed using negative selection with a MACS monocyte isolation kit and magnetic depletion column following the manufacturer’s instructions (Miltenyi Biotec, Bisley, UK).
Non-monocytic cells were obtained by washing the column with isolation buffer (PBS supplemented with 0.5% (w/v) bovine serum albumin (BSA) and 0.4% (w/v) EDTA) in the absence of the magnet. Both monocytes and non-monocytic cells were resuspended at $3 \times 10^6$ cells·mL$^{-1}$ in RPMI 1640 medium supplemented with 0.5% (w/v) BSA.

Measurement of chemotaxis
Chemotaxis was measured in a 48-well microchemotaxis chamber (Neuroprobe Inc., Gaithersburg, MD, USA) by a modification of the methods of Wilkinson [27] and Matsushima et al. [28], as reported previously [11]. Chemoattractant was loaded into the lower compartment of the chemotaxis chamber, and a cellulose nitrate filter (8 μm pore size) was used to separate the two compartments. Cell suspensions ($3 \times 10^6$ cells·mL$^{-1}$) were loaded into the upper chamber and incubated at 37°C for 90 min to allow the migration of cells through the filter. The filter was removed, cells in the filter fixed with 70% (v/v) ethanol, cell nuclei stained with haematoxylin and the filter dehydrated using increasing concentrations of ethanol. The filters were left in xylene overnight, and then mounted onto glass slides using DePex mounting medium. The leading front method of counting [27] was used, with six high-power fields counted for each data point. Alternatively, chemotaxis was measured using 8 μm pore size transwell filters (Becton-Dickinson, Oxford, UK). 150 μL of cell suspension ($10^7$ cells·mL$^{-1}$) were placed in the transwell insert, and chemoattractant in the well, and incubated at 37°C for 1 h to allow migration of the cells through the filter into the well. Cells were harvested and cell numbers determined using flow cytometry. Cells that had passed through the filter were washed with PBS and resuspended in PBS containing 0.5% (w/v) BSA and 0.1% (w/v) sodium azide and incubated with 10 μL of fluorescein isothiocyanate (FITC)-labelled anti-CD14 antibody, anti-CD3 antibody, anti-CD56 antibody, anti-CD19 antibody or murine IgG1 isotype control antibody for 20 min at 4°C. Cells were washed and resuspended in 300 μL fluorescence-activated cell sorter (FACS) flow containing 0.5% (v/v) formaldehyde, and subsequently analysed using forward-scattered light and side-scattered light. Monocytes were identified by CD14+ labelling, lymphocytes as the CD3+ population, natural killer cells as the CD56+ population and B-cells as the CD19+ population.

Measurement of chemotaxis in the presence of antagonists
PBMCs were incubated with either of two different CXCR3 antagonists: A (N-(1-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)ethyl)-2-(3-fluoro-4-(trifluoromethyl)phenyl)-N-(pyridine-2-ylmethyl)acetamide) and B (N-(2-(dimethylamino)ethyl)-N-(1-(3-(4-ethoxyphenyl)-4-oxo-3,4-dihydroquinazolin-2-yl)ethyl)-2-(3-fluoro-4-(trifluoromethyl)phenyl)acetamide), as described in patent WO02/083143A1 of the T487 series [29] or the CCR5 antagonist: C (N-((tert-butyl)-4-chloro-2-fluoro-5-(4-(3-fluorophenyl)-4-(2(1R,3r,5S)-3-(2-methyl-1H-benzo[d]imidazole-1-yl)-8-azabicyclo[3.2.1]octan-8-yl)ethyl)piperidine-1-carbonyl) benzenesulfonyamide; GlaxoSmithKline, King of Prussia, PA, USA) for 1 h prior to chemotaxis. Monocytes were migrated towards suspension buffer or chemoattractant.

Statistical analysis
Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Group data were expressed as mean±SE. Differences between groups were determined using the Kruskal–Wallis test followed by Dunn’s comparison. Spearman rank correlation was used to obtain correlation coefficients and differences with $p<0.05$ were considered statistically significant.

Results
Subject characteristics
There was no statistical difference between the age of healthy smokers and COPD patients, but COPD patients had smoked significantly more than healthy smokers (table 1). Patients with COPD had significantly worse lung function than smokers or nonsmokers, as indicated by lower values for FEV$\text{I}_\text{ % pred}$ and FEV$\text{I}_\text{/FVC}$ ratio (table 1).

Flow cytometric analysis of CXCR3 and CCR5 expression on inflammatory cells
CXCR3 were present on monocytes and CD8$^+$ T-lymphocytes, CD4$^+$ T-lymphocytes and B-cells, with little expression on neutrophils (table 2). CCR5 were present on monocytes, CD8$^+$ T-lymphocytes and CD4$^+$ T-lymphocytes, with little or no expression on the other inflammatory cell types examined (table 2). There were no differences in the levels of CXCR3 expression on any cell types examined between the three subject groups (table 2). Similarly, there were no differences in the numbers of cells expressing each of these receptors between the groups (online supplementary table 1).

For all inflammatory cell types examined the population of cells expressing the receptors was monophasic, with >95% of B-cells and ≥40% of monocytes, 50% of CD8$^+$ T-lymphocytes and 40% of CD4$^+$ T-lymphocytes expressing CXCR3 (table 3). Similarly, there was no difference in the expression of CCR5
on any of the cell types examined between the three subject groups (table 2). Again, expression of receptor was monophasic, with \( \sim 30\% \) of CD8\(^+\) T-lymphocytes expressing CCR5 and \( \sim 15\%–20\% \) of monocytes and CD4\(^+\) T-lymphocytes expressing CCR5, but <10\% of neutrophils and B-lymphocytes on cells from all subject groups expressed CCR5 (table 3). Given the lack of differences in receptor expression between the groups there was no correlation between receptor expression and age of subject or smoking pack-years.

### Chemotactic responses of PBMCs towards CXCR3 chemokines

There was no difference in the basal chemotactic response of PBMCs from nonsmokers, smokers and COPD patients (fig. 1). PBMCs from all subject groups migrated towards CXCR3 chemokines, producing bell-shaped response curves to increasing concentrations of CXCL9, CXCL10 and CXCL11 (fig. 1). Cells from all subject groups migrated towards all three CXCR3 chemokines with no difference in the responsiveness of cells from smokers and nonsmokers. In contrast, PBMCs from COPD patients migrated in greater numbers towards CXCL9 than cells from nonsmokers (fig. 1a), and towards CXCL10 and CXCL11 than cells from smokers and nonsmokers (figs 1b and c). There were no differences in the median effective concentration (EC\(_{50}\)) values of the migratory responses of PBMCs from any of the subject groups to CXCL9, CXCL10 or CXCL11 (online supplementary table 2). In addition, the concentration of chemokine that elicited the maximal chemotactic response was not significantly different between any of the groups (online supplementary table 1).

In order to ensure that the enhanced chemotactic responses of PBMCs from COPD patients were being mediated via CXCR3, migration assays were performed in the presence of the CXCR3 antagonists A and B. The migratory response of PBMCs from COPD patients towards CXCL9, CXCL10 and CXCL11 was completely inhibited by increasing concentrations of compound A with EC\(_{50}\) values of 0.34±0.05 nM, 0.74±0.44 nM and 1.56±0.65 nM, respectively, with no statistically significant difference in the potency of the antagonist against the three CXCR3 chemokines (fig. 2a–c). Similarly, a second CXCR3 antagonist, compound B, also completely inhibited the migratory response of PBMCs from COPD patients towards CXCL9, CXCL10 and CXCL11 with EC\(_{50}\) values of 0.35±0.20 nM, 0.58±0.21 nM and 0.44±0.11 nM, respectively, with no differences in the potency of the antagonist against the three CXCR3 chemokines (fig. 2d–e).

### TABLE 2 Relative expression (mean fluorescence intensity) of chemokine (C-X-C motif) receptor (CXCR3) and C-C chemokine receptor (CCR5) on circulating leukocytes of nonsmokers, smokers and patients with chronic obstructive pulmonary disease (COPD)

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>COPD patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CXCR3</td>
<td>CCR5</td>
<td>CXCR3</td>
</tr>
<tr>
<td>CD14(^+) monocytes</td>
<td>5±2</td>
<td>7±1</td>
<td>7±3</td>
</tr>
<tr>
<td>CD16(^+) neutrophils</td>
<td>2±0.2</td>
<td>1±0.8</td>
<td>2±0.2</td>
</tr>
<tr>
<td>CD8(^+) T-cells</td>
<td>9±1</td>
<td>12±2</td>
<td>12±1</td>
</tr>
<tr>
<td>CD4(^+) T-cells</td>
<td>20±5</td>
<td>9±2</td>
<td>22±3</td>
</tr>
<tr>
<td>CD19(^+) B-cells</td>
<td>53±5</td>
<td>1±0.4</td>
<td>47±4</td>
</tr>
</tbody>
</table>

Data are presented as mean±SEM, n=8–11.
Chemotactic responses to CCL5

In order to determine whether the enhanced migratory response of PBMCs from COPD patients was restricted to CXCR3 chemokines, the effects of a CCR5 ligand, CCL5, was examined. PBMCs from all subject groups migrated towards CCL5, producing a bell-shaped response curve (fig. 3a). Again, PBMCs from COPD patients migrated in greater numbers than cells from nonsmokers and smokers (fig. 3a). As with the CXCR3 chemokines, there was no significant difference in the potency of CCL5 on the migratory responses of PBMCs from any of the subject groups (online supplementary table 1). Similarly, the concentration of CCL5 required for a maximal migratory response was similar for all three subject groups (online supplementary table 1).

CCL5 is promiscuous and will bind a number of receptors including CCR5, CCR1 and CCR3. As CCR1 is expressed on monocytes, the specificity of the enhanced migratory response of PBMCs from COPD patients was assessed using a CCR5-selective antagonist. The migratory response of PBMCs from COPD patients towards CCL5 was completely inhibited by increasing concentrations of antagonist C with an EC50 value of 0.4±0.07 nM (fig. 3b), suggesting that this migration is preferentially via CCR5, and not through any other chemokine receptors, including CCR1.

Identification of migrating PBMCs

PBMCs comprise a variety of cell types, including T-lymphocytes, B lymphocytes, natural killer (NK) cells and monocytes. Expression of CXCR3 and CCR5 receptors is not restricted to a single cell type, therefore within the PBMC fraction a number of different cell types may be migrating and this population may be different in COPD patients. In order to determine which cell population was responsible for the enhanced migratory effect observed in PBMCs from COPD patients, an alternative chemotactic assay was applied which enabled identification of the migratory population of cells. Using PBMCs from COPD patients only and a transwell migratory system, T-lymphocytes (CD3+) and NK cells (CD56+) migrated towards both CXCL10 and CCL5, with fewer monocytes (CD14+) and B-cells (CD19+) migrating (fig. 4).
In order to establish which cell type was responsible for the enhanced migratory response of PBMCs towards CXCR3 chemokines and CCL5 the monocytes were specifically selected from the other cells in the PBMC fraction and both the monocyte fraction and the remainder of the cells (lymphocytes) were migrated towards a single, submaximal concentration (7.5 nM) of chemokine. Cells from COPD patients and nonsmokers were used for these experiments as there were no differences in the responses of cells from smokers and nonsmokers. In both cases, isolated monocytes migrated towards the CXCR3 chemokines, but there was little migration observed in the lymphocyte population over the buffer control (fig. 5). In addition, the enhanced migratory response of cells from COPD patients was lost when the cells were isolated, since monocytes from COPD patients migrated in the same numbers as those from nonsmokers (fig. 5). A similar effect was seen in the responsiveness of isolated cells to CCL5 (data not shown).

Discussion
Chemotaxis of inflammatory cells is part of the normal immune response to various stimuli [30]. However, the increased number of macrophages and T-lymphocytes in the parenchyma and airways of patients with COPD suggests increased cell trafficking from the circulation to the lungs [11]. The present study compared the migration of PBMCs from nonsmokers, smokers and COPD patients toward CCL5 and the CXCR3 chemokines in an attempt to examine the mechanisms of this increased inflammatory infiltrate in COPD.

PBMCs from nonsmokers, smokers and COPD patients migrated towards CXCL9, CXCL10, CXCL11 and CCL5 with similar responses of cells from smokers and nonsmokers. In contrast, the migratory responses...
of cells from COPD patients were heightened compared with control groups for all chemokines studied. There was no difference between the potency of the chemokines studied between the groups analysed, but the efficacy of chemokines was greater in patients with COPD, with more cells migrating to a given chemokine. As the age of the patients with COPD patients matched that of the smokers, it is unlikely that this is a confounding factor in this study since there were no correlations with chemokine efficacy and age in the COPD group (data not shown). Receptor antagonists confirmed the involvement of CXCR3 and CCR5 in these responses.

Differences in the chemotactic responses between PBMCs from COPD patients and nonsmokers were observed, even at the lowest concentration of chemokines studied (0.3 nM), suggesting that at low concentrations of chemokines, cells from COPD patients are activated and migrate in greater numbers. This enhanced migratory response is unlikely to be due to increased receptor numbers on the surface of cells derived from COPD patients as we were unable to detect any differences in cell surface expression of either CXCR3 or CCR5 on any inflammatory cell in whole blood using flow cytometric analysis.

FIGURE 3 Migration response of peripheral blood mononuclear cells (PBMCs) towards chemokine (C-C motif) ligand (CCL5). PBMCs were isolated from nonsmokers (n=6), smokers (n=6) and chronic obstructive pulmonary disease (COPD) patients (n=10). a) Chemotaxis experiments towards CCL5; b) the effect of the C-C motif receptor 5 antagonist GSK222474A (antagonist C) on PBMCs from COPD patients. The PBMCs were pre-incubated for 1 h with the antagonist prior to chemotaxis towards 7.5 nM CCL5. The number of cells present in the filter from 20 μm onward was counted. Data are presented as mean±SEM. *: p<0.05 for COPD versus nonsmokers; #: p<0.05 for COPD versus smokers.

FIGURE 4 Identification of migrated chronic obstructive pulmonary disease (COPD) cell types. Peripheral blood mononuclear cells (PBMCs) from COPD patients were allowed to migrate through an 8 μm pore-size transwell towards 100 nM chemokine (C-X-C motif) ligand (CXCL10) or (C-C motif) ligand (CCL5) for 1 h. Cells collected in the well were identified using positive staining for CD3, CD14, CD56 or CD19 using flow cytometric analysis. Data are presented as mean±SEM of the number of cells migrated after subtraction of background migration to buffer alone. n=6.
The heightened chemotactic response of PBMCs from COPD patients toward CXCR3 chemokines and CCL5 is a novel finding that is likely to be important in the pathophysiology of COPD. Moreover, this increased response involves lymphocyte, NK cell and monocyte components. We have previously demonstrated that PBMCs and monocytes from patients with COPD will migrate in increased numbers to some but not all CXCR2 agonists by a mechanism that involves downregulation and increased cycling of cell surface receptors [11]. In contrast, all CXCR3 agonists and CCL5 elicited similar responses. Although both lymphocytes and monocytes isolated from peripheral blood express CXCR3 and CCR5, the relative expression of these receptors was not altered in COPD. GRUMELLI et al. [22] also reported highly variable expression of CXCR3 and CCR5 on lymphocytes isolated from peripheral blood similar to that presented here, but this contrasts with of KOCH et al. [31], who described increased expression of CXCR3 on CD8+ cells in COPD and BROZYNA et al. [32], who also showed increased expression of CXCR3 and CCR5 in T-helper type 1 cells. Increased expression of CXCR3 and CCR5 has also been reported on lung lymphocytes and macrophages from patients with COPD [22]. However, it is unclear if circulating lymphocytes from patients with COPD are activated, although circulating lymphocytes in emphysema patients do not show any difference in activation markers compared with control subjects [33]. However, the present study did not discriminate between patients with chronic bronchitis and emphysema so differences in activation markers were not assessed directly in the present study.

Our study shows that PBMCs and isolated monocytes migrate toward all of the chemokines we investigated, whereas isolated lymphocytes did not respond to these same chemokines. This concurs with a previous report that only activated lymphocytes migrate towards CXCL11 [34]. One possibility for the data in the present study is that lymphocytes in PBMCs from patients with COPD are activated by the presence of other leukocytes in the PBMC fraction, as they could migrate in the transwell assays.

This study indicates that PBMCs and monocytes from all three groups of subjects migrate toward CXCR3 chemokines and CCL5. However, in COPD, PBMCs and monocytes can be recruited in higher numbers to the chemokines studied and contribute to the accumulation of macrophages and lymphocytes in lung.
parenchyma and airways. Neutralisation of CXCR3 and CCR5 receptors from COPD patients with receptor antagonists could therefore offer a therapeutic option for reducing the inflammatory infiltrate observed. While CXCR3 antagonists are in preclinical development, CCR5 antagonists have been developed for HIV/AIDS and one such drug, maraviroc, is currently approved for clinical use [35]. Therefore, it may be possible to test CCR5 antagonists in clinical studies in COPD patients in the near future. A limitation of our study is the small numbers of subjects, particularly in the control groups; however, the observation that cooperativity occurs between different leukocyte populations with respect to cell migration was observed in all subjects.

In summary, PBMCs from COPD patients migrate in greater numbers towards CCL5 and CXCR3 chemokines. Although the lymphocytes contribute to this response, this is lost when the cells are isolated, suggesting cross-talk between circulating lymphocytes and monocytes in COPD patients that promotes increased migration into the lung leading to the chronic inflammation.

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