Neutrophil chemotaxis in Wegener’s granulomatosis and idiopathic pulmonary fibrosis

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Abstract

**Background:** The presence of anti-neutrophil cytoplasmic antibodies (ANCA) in Wegener’s granulomatosis (WG), implicate the neutrophil as a key effector cell. Previous studies have reported elevated neutrophil counts in the lung although the determinants of neutrophil chemotaxis in the WG lung are unknown.

**Methods:** BALF cell counts, myeloperoxidase (MPO) and chemokines were measured in 27 patients with WG, 20 disease controls with idiopathic pulmonary fibrosis (IPF) and 6 healthy controls. CXCL-8, IL-1β, ENA-78, G-CSF and GM-CSF were measured by ELISA. The neutrophil chemotactic potential of BALF was investigated using the under agarose method and specific antibodies examined the role of CXCL-8 and IL-1β.

**Results:** WG BALF had an increased neutrophil percentage and elevated MPO, CXCL-8 and G-CSF concentrations compared with healthy controls. Chemotaxis of control neutrophils towards BALF from patients with active (p=0.006) and remission WG (p=0.077) and IPF patients (p=0.001) was increased compared with normal controls. BALF induced chemotaxis correlated with BALF IL-1β (r=0.761, p=0.001) and CXCL-8 (r=0.640, p=0.012) in WG and was inhibited by anti-CXCL-8 (85%, p<0.001) and anti-IL-1β (69%, p<0.001).

**Conclusions:** Our study confirms a neutrophilia and pro-inflammatory alveolar milieu that persists in clinical remission. CXCL-8 and IL-1β appear to play important roles in the neutrophil chemotactic response to BALF.
Keywords: Broncho-alveolar lavage, Chemokines, Interstitial lung disease, neutrophil
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INTRODUCTION:

Wegener’s granulomatosis (WG) is a small vessel systemic vasculitis which is characterised immunologically by anti-neutrophil cytoplasmic antibodies (ANCA) predominantly directed against the neutrophil serine proteinase-3 (PR3), but also myeloperoxidase(1). The presence of ANCA implicates the neutrophil as a key effector cell in the pathogenesis of WG. In vitro, ANCA activate primed neutrophils resulting in an enhanced respiratory burst and cytokine release(2). An animal model of ANCA-associated vasculitis suggested that neutrophils play a direct role in the induction of disease(3) and an in vivo study revealed that activated neutrophils, within glomeruli, correlated with the severity of renal injury(4).

WG frequently involves the lungs (5;6), with one series suggesting up to 90% of patients suffer from lower respiratory tract involvement at some stage during the disease course(7). Increased neutrophil levels have been previously reported in the bronchoalveolar lavage fluid from WG patients during active disease(8;9). Unchecked neutrophilic inflammation contributes to the tissue damage seen in other chronic lung disorders such as bronchiectasis(11). Improved survival rates following the implementation of cyclophosphamide based treatment regimens(12) has made the long term effects of neutrophilic inflammation on the WG lung more pertinent. The
mechanisms and mediators associated with neutrophil recruitment to the lung in WG are undetermined.

Cytokines play an important regulatory role in neutrophil migration to sites of inflammation(13). Up regulated cytokine concentrations have been implicated in the neutrophilia associated with acute lung injury and pulmonary fibrosis by promoting chemotaxis within the alveolar compartment (14;15). Previous small studies have suggested increased levels of neutrophilic chemokines (16;17) in the serum of WG patients, and elevated CXCL8 in the glomeruli of patients with active WG(18). Neutrophilic chemokine levels have not been established within the lung, although a case report has reported an increase in CXCL8, interleukin 1 beta (IL-1β) and granulocyte colony stimulating factor (G-CSF)(19).

The aims of this study were to determine the dynamics of neutrophilic inflammation during acute and relapsing WG, its relationship to neutrophilic chemokines with BALF and what are the functionally important chemokines in WG BALF. Comparison was made with idiopathic pulmonary fibrosis (IPF) and normal controls. IPF patients were recruited as disease controls because of the well described pulmonary neutrophilia associated with the disorder. The chemotactic potential of BALF was examined using the under agarose method and specific antibodies against CXCL8 and IL-1β

MATERIALS AND METHODS 496 (500)
**Recruitment:** Twenty six patients with WG were recruited from the University Hospital Birmingham between 2003 and 2006. Active patients were sequentially recruited on admission if bronchoscopy was deemed safe according to local guidelines. Remission patients were sequentially enrolled from the vasculitis clinic at routine clinic appointments. All patients complied with the American College of Rheumatology criteria and the Chapel Hill consensus statement definition of WG (20). Patients were assessed for disease activity at the time of inclusion using the Birmingham Vasculitis Activity Score (BVAS), a validated index that comprises a weighted item list, which assesses activity attributable to active vasculitis (21). Remission was defined as a BVAS of $\leq 1$ and active patients had a score $>4$. Where there was a suggestion of disease relapse with negative ANCA, tissue biopsy confirmed the presence of active vasculitis in all cases. The Vasculitis Damage Index (VDI) assessed severity of irreversible injury in specific organ systems.

IPF patients were sequentially recruited from a research interstitial lung disease clinic and diagnosed according to American thoracic society (ATS)/European respiratory society (ERS) guidelines. Healthy controls, free from respiratory disease were recruited from hospital volunteers. The local research ethics committee gave approval for the study, and informed, written consent was obtained (LREC ref 2003/166).

**Radiology:** All WG and IPF patients underwent high resolution computer tomography (HRCT) of the chest. Scans were read blinded to conditions by a pulmonologist (DRT) and a radiologist (PG) with an interest in interstitial lung disease, and were assessed for
the presence or absence of bronchiectasis, fibrosis, cavities, nodules and emphysema; features previous noted in a review of pulmonary findings in WG(1).

**Pulmonary function testing:** Forced vital capacity (FVC) was measured in WG and IPF patients using the Jaeger Compact system (Viasys Healthcare). Total lung diffusing capacity for carbon monoxide (TLCO) was measured by single-breath technique. Results are expressed as the percent of predicted values.

**Bronchoalveolar lavage:** All patients underwent bronchoalveolar lavage (BAL), according to national guidelines(2). Bronchoscopy was performed according a standardized method. 180mls (3X 60ml syringes) of sterile 0.9% saline was instilled into the right middle lobe bronchus and manually aspirated. BALF was filtered through coarse surgical gauze to remove mucous debris and centrifuged at 500g for 6 minutes at 4°C. The supernatant was removed and frozen to -80°C for subsequent analysis. The cell pellet was resuspended in Roswell Park Memorial Institute 1640 (RPMI) media (Sigma). A cell count was performed using a hematocytometer and cell differential analyzed following staining (Diffquick) of a cytospin slide.

**Measurements**

BALF IL-1β, CXCL8, ENA-78, G-CSF and GM-CSF levels were measured by ELISA (R&D systems) according to manufacturers instructions. These assays had an average percentage coefficient of variance of 8% (range 6.6-9.6%). MPO activity was measured using a colorimetric substrate assay(22).
Under agarose chemotaxis

Normal neutrophils were purified from peripheral blood by discontinuous percoll gradients (Sigma-Aldrich). A greater than 98% cell purity and viability was confirmed using a cytospin. The chemotactic activity of BALF on control neutrophils was measured using the under-agarose method as previously described (15;24). The same donor control was used for all neutrophil experiments. In short, agarose plates with imprinted wells were concentrically filled with the chemotactic ligand, the neutrophil suspension (5 x 10^7 cells per ml) and appropriate control. The plates were incubated for 2 hours to allow migration, fixed with glutaraldehyde and then stained with Azure B stain. The furthest point of neutrophil migration, towards the chemoattractant and control, was measured in mm by two individuals, aided by a graded graticule, blinded to the diagnosis or chemoattractant. Chemotaxis was measured as the limit of migration of neutrophils towards the outer chemoattractant ring and chemokinesis the activation of neutrophils and subsequent random migration towards the inner control ring. Net chemotaxis describes the difference between the two. Apart from the initial BALF experiments, where the measurements are individually displayed, the data is presented as net chemotaxis (mm).

For all chemotaxis experiments a positive (10^{-7}M formyl-Met-Leu-Phe [fMLP]) and negative (0.9% saline) chemoattractant control were added and experiments with greater than 10% inter-assay variability were repeated. Experiments were performed in triplicate for each tested condition.
For the cytokine blocking experiments BALF was pre-incubated, for 20 minutes, with anti-IL8 (1 x 10^{-7}M), anti-IL1β (2x10^{-7}M) antibody (R&D systems) and a CXCR2 antagonist (SB225002, 1x 10^{-8} M). Optimum inhibitor concentrations were established in preliminary experiments (data not shown). The effect of the antibodies on chemotaxis was examined to show that their actions were not due to non-specific binding (data not shown).

**Analysis**

The chemotaxis data are presented as mean [standard error (SE)] and between disease cohort comparisons made using an ANOVA test with Tukey’s post hoc analysis. All other data were non-parametric and presented as median [inter-quartile (IQR)]. IQR describes the difference between the lower and upper quartiles. Differences between the disease groups in cell counts and cytokines were explored using a Kruskal-Wallis test with Dunn’s test for between group comparison. Correlations between chemotaxis and cytokines were made using a Spearman rank test. All statistics were performed using SPSS 15. A p value \( \leq 0.05 \) was considered statistically significant.

**RESULTS:**

**Demographics**

Nineteen WG patients with a BVAS > 4 were classified as active. One patient had a BVAS of 3 with biopsy evidence of relapse and was included in the active group. Seven WG patients were recruited and deemed to be clinically in remission. Twenty IPF patients and 6 normal controls were recruited. Smoking history and the presence of
chronic lung damage, evidenced by pulmonary function testing and HRCT chest, did not predict the neutrophil count (data not shown). Serology and HRCT findings expressed as percentage of group (table 1).
Table 1: Demographics

<table>
<thead>
<tr>
<th></th>
<th>WG active</th>
<th>WG remission</th>
<th>IPF</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient numbers</strong></td>
<td>19</td>
<td>7</td>
<td>20</td>
<td>6</td>
</tr>
<tr>
<td><strong>Sex: Male</strong></td>
<td>52%</td>
<td>42%</td>
<td>77%</td>
<td>33%</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>56.6 (28.25)</td>
<td>51.0 (18.8)</td>
<td>70 (18.8)</td>
<td>45 (13.8)</td>
</tr>
<tr>
<td><strong>Smoking: Never/Ex/Current</strong></td>
<td>11/13/1</td>
<td>3/3/0</td>
<td>3/20/3</td>
<td>2/3/1</td>
</tr>
<tr>
<td><strong>Pack years</strong></td>
<td>10.1 (24.3)</td>
<td>6.8 (14.0)</td>
<td>30 (26.2)</td>
<td>21</td>
</tr>
<tr>
<td><strong>FVC</strong></td>
<td>113.0 (23.5)</td>
<td>108.5 (26.0)</td>
<td>69.5 (27.8)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Tlc</strong></td>
<td>85.0 (11.0)</td>
<td>86.0 (31.0)</td>
<td>49.0 (13.0)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Average CRP</strong></td>
<td>23.3 (32.1)</td>
<td>10.0 (40.1)</td>
<td>6 (10.3)</td>
<td>&lt;4</td>
</tr>
<tr>
<td><strong>ANCA positivity</strong></td>
<td>92%</td>
<td>33%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>PR3 positivity</strong></td>
<td>80%</td>
<td>44%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>MPO positivity</strong></td>
<td>12%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>BVAS</strong></td>
<td>4.5 (9.0)</td>
<td>0.0 (2.4)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>VDI</strong></td>
<td>4.2 (0.6)</td>
<td>3.8 (0.8)</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Bronchiectasis</strong></td>
<td>30%</td>
<td>50%</td>
<td>74%*</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Interstitial changes</strong></td>
<td>54%</td>
<td>33%</td>
<td>100%</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Cavities</strong></td>
<td>36%</td>
<td>17%</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Nodules</strong></td>
<td>82%</td>
<td>66%</td>
<td>0%</td>
<td>N/A</td>
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<tr>
<td><strong>Haemoptysis</strong></td>
<td>12%</td>
<td>0%</td>
<td>0%</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Steroids</strong></td>
<td>78%</td>
<td>77%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Cyclophosphamide</strong></td>
<td>43%</td>
<td>11%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Azathioprine</strong></td>
<td>17%</td>
<td>44%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Mycophenolate</strong></td>
<td>9%</td>
<td>33%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Methotrexate</strong></td>
<td>0%</td>
<td>11%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Rituixmab/infliximab</strong></td>
<td>9%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>No medication</strong></td>
<td>13%</td>
<td>22%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>
FVC (Forced vital capacity), TLco (Total lung diffusing capacity for carbon monoxide), CRP (C reactive protein), BVAS (Birmingham vasculitis activity score), VDI (Vasculitis damage index), expressed as mean (standard error). Interstitial changes included scarring, fibrosis and atelectasis. * indicates traction bronchiectasis.

**Neutrophilic inflammation and activation is persistent in WG patients**

BALF percentage neutrophil counts were higher in the active [5.0% (IQR 27.0), p=0.007] and remission [8.0% (IQR 60.9), p=0.072] WG patients compared with normal controls [1.8% (IQR 2.3)]. IPF BALF neutrophil percentage was higher than normal controls [15.3% (IQR 26.8), p<0.001] but not significantly different from active (p=0.210) or remission (p=0.855) WG groups (figure 1a).

The total cell count was statistically increased in active and remission WG groups [108.0 (IQR 159.0) and 74.2 (IQR 170.4) x 10⁴ cells/ml] compared with normal controls [3.8 (IQR 49.9) x 10⁴ cells/ml, p=0.004 and p=0.019 respectively]. The total cell count was higher in the IPF cohort [123.0 x 10⁴ cells/ml (IQR 237.0)] than in normal controls (p=0.001) but there was no difference with either WG activity group.

There was increased MPO activity in the active [0.064 activity units/ml (IQR 0.403), p<0.001] and remission [0.122 (IQR 0.500), p=0.022] WG groups compared with normal controls [0.001 activity units/ml (IQR 0.003)] (figure 1b). IPF BALF MPO activity [0.096 activity units/ml (IQR 0.244)] was elevated compared to normal controls.
(p<0.001) but there was no difference with either WG activity group (figure 1b). BALF neutrophil percentage and MPO units correlated in the active WG group (rho=0.484, p=0.005) and IPF (rho=0.563, p=0.002) but not in the remission WG group (rho=0.069, p=0.832).

There was no relationships between neutrophil percentage, MPO and lung function in WG, but the BALF neutrophil percentage did correlate with serum CRP (rho=0.460, p=0.016).

**BALF and plasma cytokine levels**

There were increased concentrations of BALF CXCL8 and ENA-78 in both the active and remission WG patients and IPF patients both compared to normal controls (table 2a). There was no difference in IL-1β, G-CSF and GM-CSF concentrations between cohorts.

ENA-78 plasma concentrations were elevated and IL-1β, G-CSF and GM-CSF were reduced in the active WG group compared with normal controls. IL-1β concentrations were lower in the remission WG group compared with controls. In contrast to the BALF there was no difference in the plasma concentrations of CXCL8 in active or relapse WG patients compared with controls (Table 2b). There were increased plasma concentrations of CXCL8 and ENA-78 in IPF patients compared with controls but lower concentrations of IL-1β, G-CSF and GM-CSF.
### Table 2a: BALF cytokine levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Active</th>
<th>Remission</th>
<th>IPF</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>422.8 (314.0)*</td>
<td>2725.0 (13527.9)*</td>
<td>329.0 (868.3)*</td>
<td>30.5 (58.0)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>4.0 (41.5)</td>
<td>74.9 (306.3)</td>
<td>18.6 (41.1)</td>
<td>3.8 (1.6)</td>
</tr>
<tr>
<td>ENA-78</td>
<td>259.3 (761.3)*</td>
<td>153.4 (263.5)*</td>
<td>325.7 (475.4)*</td>
<td>7.3 (41.4)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>56.4 (132.3)</td>
<td>74.3 (143.4)</td>
<td>68.6 (78.2)</td>
<td>26.1 (15.7)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>5.9 (6.3)</td>
<td>4.0 (2.8)</td>
<td>8.4 (4.9)</td>
<td>1.0 (6.4)</td>
</tr>
</tbody>
</table>

* Indicates significant difference with normal controls when compared using Kruskal-Wallis and Dunn’s test (p<0.05).  There were no significant differences between disease cohorts.  All cytokine concentrations at pg/ml, measured by ELISA (R&D)

### Table 2b: Plasma cytokine levels

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Active</th>
<th>Remission</th>
<th>IPF</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL8</td>
<td>11.4 (16.1)</td>
<td>19.8 (26.6)</td>
<td>11.9 (11.2)*</td>
<td>8.6 (3.9)</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.0 (0.0)*</td>
<td>0.0 (0.0)*</td>
<td>0.0 (0.1)*</td>
<td>8.6 (3.9)</td>
</tr>
<tr>
<td>ENA-78</td>
<td>1009.7(828.1)*</td>
<td>723.0 (937.8)</td>
<td>984.5 (1209.0)*</td>
<td>151.7 (187.0)</td>
</tr>
<tr>
<td>G-CSF</td>
<td>12.9 (23.7)*</td>
<td>15.5 (22.5)</td>
<td>10.4 (15.6)*</td>
<td>50.8 (49.5)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.6 (2.8)*</td>
<td>2.7 (5.8)*</td>
<td>1.2 (4.4)*</td>
<td>77.5 (12.8)</td>
</tr>
</tbody>
</table>

Data expressed as median (IQR). * Indicates significant difference with normal controls when compared using Kruskal-Wallis and Dunn’s test (p<0.05).  There were no significant differences between disease cohorts.  All cytokine concentrations at pg/ml, measured by ELISA (R&D)
The effect of BALF on neutrophil chemotaxis and chemokinesis

Neutrophil chemotaxis towards BALF from WG [active 6.16mm (IQR 3.67), p=0.04 and remission 8.43mm (IQR 2.34), p=0.003] and IPF patients [8.71mm (IQR 3.71), p=0.002] was increased compared with normal BALF [6.5 mm (IQR 1.21)]. There was no difference in chemotaxis between either WG groups or IPF (p=0.451). WG [active 6.8 mm (IQR 1.53), p=0.041 and remission 7.37mm (IQR 2.23), p=0.007] and IPF BALF [7.62mm (3.71), p=0.04] also exhibited increased neutrophil chemokinesis compared with normal control [5.62mm (IQR 1.33)]. There was a close correlation between chemokinesis and chemotaxis in WG (r=0.941, p<0.001) and IPF (r=0.968, p<0.001).

The net chemotaxis of active WG BALF [1.33mm (IQR 1.17)] and IPF BALF [1.50mm (IQR 1.09)] was elevated compared to normal controls [0.33mm (IQR 0.67)] (figure 2). The net chemotaxis of WG remission BALF [0.67mm (IQR 1.25)] was lower than the active group and higher than normal controls but was not significantly different to either group. The net chemotactic potential of WG BALF correlated with the measured BALF neutrophil % (rho=0.567, p=0.004) and MPO (rho=0.672, p=0.012). There was no such relationship in the IPF patients BALF. BALF chemotactic potential did not relate to the presence of a significant bacterial culture from the BALF in either active or remission groups (data not shown).
**Investigation of a role for IL-1β, CXCL8 in WG BALF induced chemotaxis**

The amount of BALF induced chemotaxis correlated with BALF CXCL8 (Spearman rank correlation, rho=0.640, p=0.001) and IL-1β levels (rho=0.761, p=0.001), but not with GM-CSF (rho=0.171, p=0.413), G-CSF (rho=0.291, p=0.157) or ENA-78 (rho=0.148, p=0.322). As a consequence of these results specific antibodies were used to explore the effects of CXCL8, IL-1β and a CXCR-2 antagonist (SB225002) on WG BALF induced chemotaxis.

The chemotactic response to active WG BALF (n=9) [4.24mm (SE 0.39)] was inhibited by anti-CXCL8 [1.5mm (SE 0.24), p=0.001], anti-IL-1β (1.26mm (SE 0.22), p<0.001) and CXCR-2 [0.98mm (SE 0.25), p=0.001]. The response to BALF was further inhibited by the combination of anti-IL-1β and anti-CXCL8 (0.39mm (SE 0.15), p=0.001) (figure 3).

**Effect of IL-1β and CXCL8 on neutrophil chemotaxis**

IL-1β resulted in a dose dependent increase in normal neutrophil chemotaxis; 20, 200, 2000 pg/ml (2.3, 4.0, 11.0 mm respectively) which could be abrogated to 90% chemotaxis by anti-IL-1β (2.5 x 10^{-7} M) (p<0.001). CXCL8 caused a dose dependent increase in chemotaxis; 10^{-9} M to 10^{-6} M (0.7, 3.7, 5.7, 9.3 mm respectively) which could be inhibited by anti-CXCL8 (2 x 10^{-7} M) (p<0.001). There was a synergistic chemotactic response when IL-1β (200 pg/ml) and CXCL8 (10^{-7} M) were co-incubated; IL-1β (4.0
mm), CXCL8 (5.7 mm), IL-1β + CXCL8 (12.0 mm) (Anova, p=0.013). The antibodies did not influence fMLP stimulated chemotaxis (figure 4).
Discussion:

This large bronchoscopy study has confirmed the findings of previous studies reporting a BALF neutrophilia during active WG disease (8;25) and one small series which demonstrated increased neutrophils during remission (9). Elevated MPO levels indicate that these neutrophils are activated. BALF concentrations of CXCL8 and ENA-78, but not IL-1β, G-CSF and GM-CSF, were elevated in both active and remission WG patients compared with normal controls. BALF from WG patients was found to stimulate neutrophil chemotaxis compared with normal controls and this was inhibited by antibodies against CXCL8 and IL-1β and a CXCR-2 antagonist.

The presence of a neutrophilia has particular relevance to WG due to the strong association of the disease with ANCA (31). The molecular mechanisms contributing to the BALF neutrophilia seen in WG are not well understood. Leukocyte scanning of vasculitis patients has shown that WG patients have increased neutrophil margination in the lung (10). Recruitment of neutrophils into the tissues is a multistep process partially directed by cytokines. The BALF neutrophil percentage correlated with CXCL8 and IL-1β which may suggest a role for cytokines in neutrophil recruitment in WG. This finding was supported by the BALF experiments which found that BALF induced chemotaxis was inhibited by specific antibodies against CXCL8 and IL-1β. The degree of inhibition that resulted from incubation with these antibodies suggested that CXCL8 and IL-1β were the major cytokine determinants of BALF induced chemotaxis.
Plasma CXCL8 levels were elevated in WG and IPF patients compared with normal controls, albeit at a 10 fold lower level than in the BALF suggesting local lung production. CXCL8 is known to have potent chemotactic activity and has an important role in the accumulation of neutrophils in the lungs of patients with IPF(32) and adult respiratory distress syndrome (33) where a persistent neutrophilia is associated with mortality. CXCL8, is able to increase neutrophil adhesiveness and up regulate ICAM expression, potentially promoting margination(17). Aside to neutrophil recruitment, CXCL8 has other mechanisms relevant to WG with ANCA directly stimulating CXCL8 release from both monocytes and neutrophils(18;34). CXCL8 and other cytokines, including TNFα, further stimulate translocation of PR3 to the cell surface increasing the likelihood of ANCA binding (35;36). CXCL8 exerts its actions through the CXCR1 and CXCR2 receptors(37;38) and our experiments suggest a role for the CXCR-2 receptor BALF induced chemotaxis.

Whilst the role of CXCL8 as a neutrophil chemoattractant is well established, the role of IL-β remains controversial although it has established actions on neutrophil adherence(13;39) and induces a number of cells to produce known neutrophil chemoattractants including CXCL8(40). ENA-78 is a potent neutrophil chemotactant that has been found to be up-regulated in inflammatory conditions such as rheumatoid arthritis(41) but although we found increased levels in WG BALF there was no significant relationship with the BALF neutrophilia. Haematopoietic factors regulate neutrophil production and maturation in the bone marrow. This study has shown increased G-CSF levels in the WG BALF a chemokine which has established actions on the proliferation and activation of neutrophils(42). GM-CSF is able to stimulate bone
marrow precursors to proliferate and differentiate neutrophil cell lines (9). GM-CSF, but not G-CSF, is able to up-regulate PR3 expression on neutrophils and thus has specific relevance to WG pathology(43), however, concentrations were not significantly elevated in our WG cohort. Neutrophil chemokines were also elevated in BALF from IPF patients, but only in patients with WG did CXCL8 and IL-1β relate to BALF neutrophil counts.

The consequences of persistent neutrophilic inflammation and unopposed protease action are best understood in chronic obstructive airways disease and bronchiectasis(11) features of which have been reported in both IPF and WG (7) cohorts. Elevated MPO activity has previously been reported in active WG (26), this study confirms these findings and goes on to show that MPO activity remains elevated during clinical remission. Whether this reflects ongoing neutrophil activation secondary to disease activity or chronic lung damage and infection is unknown. We have previously reported that pathogens are commonly grown from both WG and IPF BALF (44), however, bacterial growth was not associated with a higher neutrophil count or BALF chemotactic potential.

There are a number of limitations to this study. Firstly, the under agarose method cannot fully reflect the complexities of the human lung. However, the degree of neutrophil chemotaxis correlated with percentage neutrophil count and MPO levels in the BALF supporting our hypothesis that this model does to some extent reflect in vivo dynamics. Also there was significant variability between under agarose experiments performed at
different time points as seen in the different graphs. For this reason each set of experiments were performed on the same day and each sample and control in triplicate. Second, it cannot necessarily be extrapolated that undiluted epithelial lining fluid (ELF) would give similar results to the bronchoalveolar lavage fluid. Repeating these experiments using a different method to assess chemotaxis with directly sampled epithelial lining fluid and evaluating other cytokines and cytokine receptors would allow this research to be taken forward. Similarly, examining tissue samples, would confirm whether these chemokines are also elevated in the interstitium. Lastly, the median BVAS was lower in the acute patients than had been expected from other studies (7). Our interpretation is that a number of patients had respiratory limited WG and that the BVAS, which is weighted towards renal disease, does not score single organ pulmonary disease highly.

In conclusion, this study provides further evidence that neutrophilic inflammation is a key manifestation of the WG lung during disease activity and also during remission. We demonstrate elevated alveolar cytokine concentrations and evidence that CXCL8 and IL-1β may provide a continuous stimulus for neutrophil chemotaxis. The mechanisms of action of cytokines in the neutrophil chemotactic response warrants further investigation as targeting pathways resulting in the activation and recruitment of neutrophils may offer alternative therapeutic options.
Reference List


Figure 1: BALF neutrophil percentage and MPO activity levels

**Graph 1A:** BALF neutrophil count expressed as a percentage of total cells in BALF

**Graph 1B:** BALF myeloperoxidase activity measured by colorimetric assay

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Figure 2: Chemotactic response of neutrophils to BALF

The under-agarose method was used to measure the distance migrated by normal neutrophils towards either BALF or saline control. Net chemotaxis describes the difference between migration towards the chemoattractant and control. Data were examined using an ANOVA test with Tukey's post hoc analysis. Chemotaxis was measured with all BALF samples.
Figure 3: The effect of anti-IL-1β and anti-CXCL-8 on BALF induced chemotaxis

Chemotaxis to active WG BALF was inhibited by anti-IL-1β (2x10^{-7}M), anti-IL-8 (10^{-6}M) and a CXCR2 antagonist (SB225002, 1x10^{-8}M) (P<0.001 for each experiment). The combination of anti-IL-1β and anti-IL-8 further inhibited the chemotactic response. All experiments were performed on 9 active WG BALF samples and repeated in triplicate.
Figure 4: The effect of IL-1β and CXCL-8 and their specific antibodies upon neutrophil chemotaxis

Chemotactic response demonstrated to IL-1β (200 pg/ml) and IL-8 (10^{-7} M). There was enhanced chemotactic activity when IL-1β and IL-8 were co-incubated. The effects of IL-1β and IL-8 were reduced by specific antibodies which did not affect FMLP stimulated chemotaxis.