Chapter 5

**Characterization of Interstitial Macrophages Around Airways of Current and Ex-smoking COPD Patients and Controls**

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Abstract
Macrophages significantly contribute to the development of chronic obstructive pulmonary disease (COPD). Little is known about their relationship with airway remodeling and which polarization types of macrophages are present in airways of COPD patients. Therefore, we studied numbers of airway interstitial macrophages, their polarization states M1, M2, or M2-like and basement membrane thickness in central airways of COPD patients and non-COPD controls.

Large airway sections from ex- and current smoking individuals with COPD (n=27) or without COPD (n=29), that underwent resection surgery for suspected cancer, were stained for macrophages with CD68 in combination with a macrophage polarization marker: IRF5+ for M1 macrophages, CD206+ M2 macrophages and IL-10+ for M2-like macrophages. Effects of disease and smoking status were assessed and macrophage numbers were correlated with lung function (FEV₁ %pred.) and basement membrane thickness.

Numbers of total macrophages and the three subsets as well as basement membrane thickness were similar between COPD patients and controls. Lower FEV₁ was associated with having more IRF5+ M1 macrophages and a thicker basement membrane in COPD patients. Basement membrane thickness correlated negatively with M2 macrophages in ex-smoking COPD patients. Current smoking was associated with lower numbers of total and M1 macrophages compared to ex-smoking.

In conclusion, having COPD was not associated with changes in interstitial airway macrophage phenotypes or greater basement membrane thickness, whereas current smoking did lead to lower numbers of interstitial M1 macrophages. The negative associations of lung function with M1 macrophages and basement membrane thickness in COPD patients stress the importance and the possible contributions of the different macrophage subsets to obstructive airway disease.
Introduction

A number of reports have shown that macrophages in the lung play an important role in maintaining tissue homeostasis but that they can also significantly contribute to the development of chronic obstructive pulmonary disease (COPD) 1-4. COPD is characterized by pulmonary inflammation, often caused by smoking, leading to a varying combination of chronic bronchitis with fibrosis around airways and emphysema with loss of parenchymal lung tissue 1-7.

Macrophages can polarize into various phenotypes depending on tissue-derived or exogenous signals and these phenotypes present themselves as a spectrum rather than distinct phenotypes 8,9. Within this spectrum three main phenotypes are recognized: 1. A proinflammatory type (also known as M1), induced by IFNγ, TNFα, and microbial products, and characterized by expression of interferon regulatory factor 5 (IRF5) 10,11; 2. A tissue repair-associated type (also known as M2), induced by IL4 and/or IL13, and characterized by expression of uptake receptors like CD206 and Mfge8 12,13; 3. An anti-inflammatory type (also known as M2-like or M2c), induced by IL10, corticosteroids and PGE2, and characterized by production of high levels of IL10 and expression of CD163 8,14. For the sake of simplicity, the names M1/M2/M2-like are adopted for these three main subsets throughout this report.

Although a role of macrophages in the development of COPD is generally accepted, little is known about the presence of the different phenotypes in lung tissue of COPD patients and their association with different aspects of the disease. To the best of our knowledge no quantification of macrophage phenotypes in COPD compared to controls have been published for either alveolar or interstitial macrophages. Alveolar macrophages have been studied most in COPD with respect to changes on a genetic, cytokine-producing and cellular level. Shaykhiev et al. demonstrated that bronchoalveolar lavage macrophages of COPD patients show substantial suppression of M1-related genes and partial induction of genes associated with M2 polarization as compared to healthy individuals 15. This partial suppression/induction of the M1/M2 repertoire was also shown by Hodge et al. in studies showing a mixed phenotype in alveolar macrophages of smoking COPD patients with some M1 (MHC II expression) and M2 (efferocytosis) markers going down and some going up (proinflammatory cytokine production and DC-SIGN expression) compared to healthy individuals 2-5,16,17.

None of the studies to date, however, have investigated polarization changes in interstitial macrophages around airways. This is of interest because COPD is characterized by chronic bronchitis with remodeling of the airways. Especially M2 macrophages are associated with fibrotic processes and we therefore hypothesized that the macrophages around the airways in COPD patients would show preferential polarization towards M2. To investigate this hypothesis and to gather information about changes in interstitial macrophage polarization, we quantified the three main polarization states of macrophages in the central airways of subjects undergoing lung resection for suspected cancer with or without mild to moderate COPD (GOLD stage II and III). Macrophage phenotypes were identified based on their expression of IRF5, CD206, or IL10 in combination with the general macrophage marker CD68 and we correlated their presence with various clinical parameters and basement membrane thickness as a marker of airway remodeling.
Materials and Methods

Subjects
The study protocol was consistent with the Research Code of the University Medical Center Groningen (UMCG) and Dutch national ethical and professional guidelines. Lung tissue included in this study was obtained from individuals with COPD (n=27) and non-COPD control subjects (n=29) undergoing lung surgery for suspected cancer. These subjects were part of a larger cohort and the material was previously collected by the UMCG (for more detailed information on this study see Hao 2012). The group of COPD patients included patients with GOLD stage II and III and both ex- and current smokers were included. COPD was defined as FEV1/FVC ratio <70%. The control group consisted of subjects with normal lung function (FEV1/FVC >70%), no history of lung disease and included ex-smokers (min 5 years of smoking cessation) and current smokers. Subjects with other lung diseases such as asthma, cystic fibrosis, or interstitial lung diseases were excluded. Patient characteristics are reported in table 1.

Table 1. Characteristics of patients whose central airway lung tissue was used to determine the number of macrophages in airway walls.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=26)</th>
<th>COPD (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, male/female</td>
<td>13/13</td>
<td>24/3</td>
</tr>
<tr>
<td>Age, years</td>
<td>60 (45-76)</td>
<td>64 (35-82)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ex-smokers</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>Current smokers</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>FEV1, % predicted</td>
<td>98 (84-114)</td>
<td>66 (45-78)</td>
</tr>
<tr>
<td>FEV1/FVC</td>
<td>76 (71-90)</td>
<td>55 (40-69)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Suspected lung cancer</td>
<td>COPD + suspected lung cancer</td>
</tr>
<tr>
<td>GOLD stage</td>
<td>Stage II: 25 Stage III: 2</td>
<td></td>
</tr>
<tr>
<td>Corticosteroid use</td>
<td>1/26</td>
<td>2/27</td>
</tr>
</tbody>
</table>

Patients underwent surgical resection for suspicion of carcinoma. Noncancerous normal lung tissue was obtained as far distant from the tumor as possible and lung tissue of each patient was stained with a standard haematoxylin and eosin staining and checked for abnormalities by a lung pathologist (WT). Collected tissue was fixed with 4% formalin and paraffin imbedded. Lung tissue sections of 2.5 μm thickness were used for all analyses.

Histology
We assessed the total number of macrophages and the three main subsets in the submucosa of a transversally cut large airway (average internal diameter of 5 mm ±SD 2.0 mm). The total number of macrophages in each section was identified by a single CD68 staining.
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(anti-CD68, DAKO, Heverlee, Belgium) with a haematoxylin nuclear counterstain. Numbers of M1 macrophages were determined by a double staining of CD68 and IRF5 (anti-IRF5, ProteinTech Europe, Manchester, UK), M2 macrophages by combining CD68 (anti-CD68, Abnova, Heidelberg, Germany) with CD206 (anti-CD206, Serotec, Puchheim, Germany), and M2-like macrophages by a combination of CD68 and IL10 (anti-IL10, Hycult Biotech, Uden, The Netherlands) using standard immunohistochemical procedures. The double stainings were not counterstained with haematoxylin to facilitate identification of double-positive cells.

To stain for IRF5+ or CD206+ macrophages, sections were deparaffinized and antigen retrieval was performed by overnight incubation in Tris-HCL buffer pH 9.0 at 80°C; thereafter sections were incubated with rabbit anti-IRF5 followed by mouse anti-CD68 or mouse anti-CD206 followed by rabbit anti-CD68. Next, sections were incubated with the two secondary antibodies together: horseradish peroxidase (HRP)-conjugated goat-anti-mouse antibody and alkaline phosphatase (AP)-conjugated goat-anti-rabbit antibody.

To stain for IL10+ macrophages, antigen retrieval was performed by heating the sections in citrate buffer at pH 6.0 for 10 minutes at sub-boiling temperatures. Sections were pretreated with 1% bovine serum albumin (Sigma Aldrich, St Louis, MO) and 5% milk powder in PBS for 30 minutes and incubated with rabbit anti-IL-10 overnight. The next day, sections were incubated with mouse anti-CD68 followed by the two secondary antibodies together: HRP-conjugated goat-anti-rabbit antibody and alkaline AP-conjugated goat-anti-mouse antibody.

First the AP-conjugated antibodies were visualized using 5-bromo-4-chloro-3-indolyl-phosphate/ nitro blue tetrazolium (BCIP/NBT) as chromogen (Vector, Burlingame, CA). Next, the HRP-conjugated antibodies were visualized with ImmPACT NovaRED (Vector, Burlingame, CA) as chromogen. Figure 1 displays representative pictures of each double staining (arrows indicate double positive cells).

Stainings were quantified by morphometric analysis using ImageScope analysis software (Aperio, Vista, CA). Sample slides were blinded for an observer who manually counted CD68-single positive cells or double positive cells in a central airways section per length of intact basement membrane, extending 100 μm into the intact submucosa, excluding vessel and smooth muscle. Data are expressed as the number of positive cells per 0.1 mm². Along the same length, we measured the basement membrane thickness at 4 different, randomly picking points per mm airway in the CD68+hematoxylin stained sections.

**Statistics**

Data were non-normally distributed; therefore, the data were log-transformed to obtain a Gaussian distribution. Comparisons between two groups were then conducted using a two-sided, unpaired Students T-test. The effects of COPD and smoke exposure were determined with a two-way ANOVA. When significant, a Sidak’s multiple comparisons test was used to test whether differences between groups were significant. When log-transformation did not result in a Gaussian distribution (basement membrane thickness) groups were compared using a Kruskal-Wallis and a Dunn’s post-test to correct for multiple testing (P<0.05).
Correlations were calculated for normally distributed data or log-transformed data using a Pearson test. A Pearson Rho was calculated for the interactions between macrophage subtypes and lung function (FEV₁ and FEV₁/FVC), age and pack years. Lung function correlations were solely determined for COPD patients, as lung function is more variable in COPD and is a marker of disease severity. A nonparametric Spearman coefficient was calculated for the correlations between basement membrane thickness (no Gaussian distribution) and FEV₁ or macrophage subtypes. P-values <0.05 were considered significant. Data was analyzed using Graphpad Prism 6 (Graphpad Software, La Jolla, CA).

Results

Macrophages and the three subsets were identified by double staining central airway sections of patients with and without COPD. Figure 1 shows representative pictures of each of the double staining: (A-B) CD68+IR5+, (C-D) CD68+CD206+ and (E-F) CD68+IL-10+ cells (double positive cells are indicated by the arrows). We then related the numbers of double positive macrophages to having COPD or not, smoking status, age, packyears and basement membrane thickness.

No differences in macrophage numbers or polarization states in central airways between COPD patients and controls

First we investigated whether patients with COPD had different numbers of total macrophages compared to controls and found no significant differences between the groups (figure 2A). Furthermore, no differences were found with respect to the three macrophage polarization states (figure 2B-D). Correlations of FEV₁ with macrophage polarization states (only calculated for COPD patients as FEV₁ is variable among these patients) showed a trend towards a negative correlation between FEV₁ and numbers of M1 macrophages (p=0.10, data not shown).

When we subsequently correlated the number of macrophages and FEV₁ in patients with COPD separately for ex- and current smokers, we found a significant correlation between FEV₁ and the number of IRF5+ M1 macrophages in ex-smoking COPD patients (figure 3B, Pearsons coefficient: -0.56, P<0.04), but no correlation was found in current smoking COPD patients. No correlations were found for any of the other populations (figure 3A, C and D). In addition, we found no correlations between the number of macrophages and FEV₁/FVC, packyears and age (data not shown).

Lower numbers of total and IRF5+ macrophages in central airways of current smokers

Smoking is a major risk factor for COPD. We therefore assessed the effect of smoking status on the number of macrophages around the central airways. Figure 4A shows that overall, current smokers had lower numbers of macrophages around central airways as compared to ex-smokers. Among the different macrophage phenotypes, the number of IRF5+ M1 macrophages was significantly lower in current smokers compared with exsmokers (figure 4B). Specifically, current-smoking COPD patients showed a significantly lower number of IRF5+ M1 macrophages than ex-smoking COPD patients (figure 4B). A similar effect was observed
as a trend among the smoking control individuals as compared to ex-smokers (p=0.06). No differences were found between current and ex-smokers regarding CD206+ M2 and IL10+ M2-like macrophages (figure 4C-D).

**Basement membrane thickness was unchanged in COPD**

Thickening of the basement membrane in the central airways is an indication of remodeling of the airways and this is associated with COPD. Thus, we assessed basement membrane thickness and correlated these findings with the number of macrophages in airways of COPD patients. No correlations were found between any of the macrophage subtypes and basement membrane thickness when assessed separately for all current smokers or ex-smokers (COPD and controls combined, data not shown). Among the COPD patients, the number of CD206+ M2 macrophages showed a significant negative correlation with basement membrane thick-

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**Figure 1.** Representative pictures of large airway sections double stained for CD68 and IRF5 (A-B), CD68 and CD206 (C-D), CD68 and IL-10 (E-F). Double-positive cells are indicated by the arrows.
ness, exclusively in ex-smokers (figure 5C), whereas other macrophage phenotypes did not significantly associate with basement membrane thickness (figure 5A, B and D). In addition, basement membrane thickness did not correlate with total number of macrophages or any of the macrophage subsets in the group of control subjects (data not shown).

COPD patients did not show altered thickening of the basement membrane as compared to controls, nor was there a difference with respect to current or ex-smoking (figure 5E). Moreover, we assessed basement membrane thickness and correlated these findings with lung function (FEV1 %pred.) and showed a trend towards a negative correlation with FEV1 for COPD patients (P<0.06, figure 5F).

Figure 2. No differences were detected in the number of macrophages present 100 μm into intact submucosa of large airways of COPD patients compared with controls. Macrophages were characterized as followed: CD68+ was used as a general macrophage marker (A), M1 macrophages were detected with CD68 and IRF5 (B), M2 macrophage by CD68 and CD206 (C), and M2-like macrophages with CD68 and IL10 (D). Data are presented with a mean and statistical differences were tested with a student-T test and P<0.05 was considered significant.
Discussion

In this study we aimed to investigate whether having COPD or not would be associated with changes in macrophage polarization states around the airways, in particular with having more M2-polarized macrophages as these have been shown to be associated with remodeling processes. Surprisingly, no differences were found in the number of total macrophages or the three main polarization states M1, M2, and M2-like between COPD patients and control subjects. We did find that current smokers have significantly less total macrophages around the airways as compared to ex-smokers and this seemed to be caused by a significant selective decrease in the number of IRF5+ M1 macrophages in current smokers with COPD. We found no evidence for more remodeling of the airways in COPD patients as compared to controls, because basement membrane thickness was similar between the two groups. However, a thicker basement membrane correlated negatively with the number of CD206+ M2 macrophages in the group of COPD patients.

Figure 3. Total numbers of macrophages showed no correlation with FEV1 %pred. in COPD patients (A). FEV1 did negatively correlate with the number of CD68+IRF5+ M1 macrophages in ex-smoking COPD patients (Pearson coefficient -0.56, P<0.04) (B). Numbers of CD68+CD206+ M2 macrophages (C) or CD68+IL10+ M2-like macrophages (D) did not correlate with FEV1. For each correlation a Pearson’s correlation coefficient was calculated and P<0.05 was considered significant.
Current literature has not provided a straightforward view on the presence of different macrophage subsets in COPD. Macrophages are known to be involved in the pathogenesis of COPD, but the presence of each subset separately has hardly been studied. Most studies investigating macrophage polarization have focused on alveolar macrophages and the picture that arises from these studies is that COPD may be characterized by increased numbers of macrophages with dysfunctional M1 and M2 characteristics. Information on polarization changes of interstitial macrophages around the airways is even less available. Airway inflammation plays a key role in the development of COPD and macrophages are important inflammatory cells involved in addition to neutrophils and CD8+ T cells. However, our current results did not show a higher number of macrophages or any changes in their polarization state in the central airways of COPD patients as compared to control subjects. For the total number of macrophages, this is in line with other studies using lung tissue from lung resection surgeries for suspected cancer. However, higher num-

![Figure 4](Image)

**Figure 4.** Smoking status affected numbers of macrophages in the airway wall. Current smokers showed lower numbers of CD68+ total macrophages (p<0.04) (A) and CD68+IRF5+ M1 macrophages (P=0.001) (B) compared with ex-smokers. In COPD patients, current smoking resulted in lower numbers of CD68+IRF5+ M1 macrophages (P=0.017) (B). No differences were detected for CD68+CD206+ M2 macrophages (C) or CD68+IL-10+ M2-like macrophages (D). Data are presented with a mean and statistical differences were tested with a two-way ANOVA with a Sidak’s multiple comparison post-test. P<0.05 was considered significant.
bers of CD68+ macrophages have been found in airway wall biopsies from COPD patients as compared to healthy controls. The differences between these studies are firstly that these three latter studies used airway wall biopsies while we and Saetta et al., Grashoff et al., Turato et al. and Battaglia et al. used large airways in specimens of dissected lung tissue. Secondly, these biopsy studies had genuine healthy control groups as opposed to our control group that consists of macroscopically unaffected tissue patients undergoing lung tissue

Figure 5. Among CD68+CD206+ M2 macrophages, the number of macrophages correlated negatively with basement membrane thickness in ex-smoking COPD patients (Spearman coefficient -0.63, P=0.018) (C). No correlations were detected between basement membrane thickness and CD68+ total macrophages (A), CD68+IRF5+ M1 macrophages (B) or CD68+IL10+ M1 macrophages (D). Statistical differences were tested using a Kruskal-Wallis with a Dunn’s post-test to correct for multiple testing. For each correlation a Spearman correlation coefficient was calculated and P<0.05 was considered significant. Basement membrane thickness was not different between controls and COPD patients (E). We observed a trend (P=0.06) towards a negative correlation between FEV1 and basement membrane thickness (F).
resection surgery for suspicion of a tumor. Comparing the number of total macrophages per 0.1 mm² of tissue between the two types of studies, we find comparable numbers of total macrophages for COPD patients, suggesting biopsy results are comparable to results from dissected airways. However, our non-COPD controls have more total macrophages as compared to healthy controls. This suggests changes in total macrophage numbers due to the presence of a tumor or differences in the clinical phenotype of the control subjects. As we do not have access to dissected lung tissue of healthy controls, this hypothesis is difficult to test. Nevertheless, our COPD patients also underwent lung resection surgery for suspected tumors and we found that having COPD did not additionally affect interstitial macrophages around the airways or their phenotype. A cautionary remark to be made about the data is the fact that both M1 and M2 macrophages were characterized based on phenotypical markers that say little about their function. Disturbed functions of both subsets of macrophages have been suggested before in COPD 3,4,20,23,26,38-42. Interestingly, we did see that current smoking was associated with lower numbers of total macrophages and IRF5+ M1 macrophages. There are several possible explanations for this finding. Cigarette smoke components may directly or indirectly kill macrophages in the interstitium, affect antigen exposure and therefore staining quality or influence chemokine release by bronchial epithelial cells, which may have drawn macrophages out from the interstitium into the lumen of the airway. The latter may seem the more logical explanation as many studies have found higher numbers of alveolar macrophages in (healthy) smokers in BAL/sputum 43-45. Moreover, smoking has been found to induce epithelial expression of chemokine receptors for macrophages such as monocyte chemoattractant protein-1 (MCP-1 or CCL2) and IL-8 (CXCL8) 46-49. Why this appears to affect IRF5+ M1 macrophages more than the other two M2 subsets may be explained by a differential expression of both chemokine receptors by the different macrophage subsets. A recent publication by Xuan et al. showed that M1 macrophages have higher expressions of CXCR1 and CXCR2 (the receptors for CXCL8) than M2 macrophages and are indeed specifically attracted by CXCL8 as opposed to M2 macrophages. CXCL8 is well known for the fact that it is produced by bronchial epithelial cells after smoke exposure, particularly with respect to its role in attracting neutrophils 49. It may therefore also promote M1 migration out of the interstitial-tissue compartment in current smokers. This may also explain the trend we observed in the control group that also pointed towards less M1 macrophages in current smokers.

The lower number of IRF5+ M1 macrophages in specifically current smoking COPD patients as compared to ex-smoking COPD patients, however, is more difficult to explain using the IL-8 hypothesis. Many studies have shown either unchanged or even higher IL-8 levels in sputum of ex-smoking as compared to current smoking COPD patients 50-54. Therefore it is likely that other mechanisms or chemokines may also play a role in explaining the lower numbers of M1 macrophages in smoking COPD patients. As we do not see a concomitant increase in either M2 or M2-like macrophages, we think it is unlikely that cigarette smoke induces repolarization into a M2 subset as has been suggested by the studies of Yuan et al. 55.

We did not find any evidence for our main hypothesis that airway remodeling, as defined as a thicker basement membrane, was associated with the presence of more CD206+ M2
macrophages. On the contrary, our data suggest that lower numbers of CD206+ M2 macrophages were associated with a thicker basement membrane in ex-smoking COPD patients. In addition, a thicker basement membrane was associated with a trend towards a lower FEV₁ in all COPD patients. A possible explanation could be the finding that M2 macrophages also appear to be involved in inhibiting fibrotic processes and can be important in clearing away excess extracellular matrix through mannose receptors and Mfge8 56-60. These differences in function within the M2 subset are not detectable by staining for just two phenotypic markers (CD206 and CD68). A more functional marker or read-out could help to get direction in this discussion. Why the association would only be present in ex-smoking COPD patients cannot be explained at present.

We found no differences in basement membrane thickness between patients with COPD and controls. Results regarding basement membrane thickness in COPD patients compared to controls have been inconsistent going from thicker in COPD, to no differences and even thinner in COPD as compared to control 36,61-64. Of note, our patient populations may differ from these earlier studies due to the presence of cancer as we did find a trend in COPD patients towards lower FEV₁ being associated with a thicker basement membrane. Therefore, within COPD patients, basement membrane thickness does appear to influence lung function. Furthermore, the number of IRF5+ M1 macrophages showed a trend towards a negative correlation with FEV₁ in all COPD patients (which was significant in ex-smokers only). This is in line with data we obtained from a cohort of asthma patients in which IRF5+ M1 macrophages also negatively correlated with FEV₁ (Draijer et al. manuscript submitted). The reason for the stronger effect in ex-smokers is not clear to us at present.

In conclusion, having COPD was not associated with changes in numbers of interstitial airway macrophages, their polarization states, or basement membrane thickness as compared to controls. However, lower FEV₁ was associated with a thicker basement membrane and having more IRF5+ M1 macrophages. Furthermore, a thicker basement membrane was associated with having less CD206+ M2 macrophages in ex-smoking COPD patients. These associations stress the importance and the possible different contributions of macrophage subsets to COPD.
References

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