Differential lymphocyte infiltration in small airways and lung parenchyma in COPD patients

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Inflammation; Emphysema; Immunohistochemistry; Cigarette smoking

Summary
Background: In COPD, although histological lesions at both the small airways (wall thickening and tissue remodeling) and lung parenchyma (emphysematous destruction) are definitely different, the inflammatory cells involved in both processes are the same. Our study aims to determine if these histopathological phenotypes are related to two different lymphocyte profiles.

Methods: Distribution and cell density of CD3+, CD4+, CD8+ and B lymphocytes were compared in small airways and parenchymal interstitium of 9 non-smokers, 18 smokers without COPD, 16 smokers with moderate COPD and 16 patients with very severe COPD undergoing lung transplantation. Spatial distribution of lymphocytes in periemphysematous parenchyma was also assessed.

Results: CD3+ and B cell densities were significantly higher in small airways than parenchyma interstitium of very severe COPD patients. Furthermore, CD8+ cells were increased in the epithelium of small airways and parenchymal interstitium of 9 non-smokers, 18 smokers without COPD, 16 smokers with moderate COPD and 16 patients with very severe COPD undergoing lung transplantation. Spatial distribution of lymphocytes in periemphysematous parenchyma was also assessed.

Conclusions: In COPD, it is true that the small airways’ wall shows a clear inflammatory pattern, with a high mononuclear infiltration and tissue remodeling. However, parenchymal interstitium shows a milder CD8+ infiltration which, moreover, is not spatially related to emphysematous destroyed areas.

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Differential lymphocyte infiltration in COPD

Introduction

Chronic obstructive pulmonary disease (COPD) is defined as an abnormal inflammatory response of the lung to noxious particles and gases, mainly cigarette smoke.1

The main pathological features of COPD are found in small airways and lung parenchyma, although the histological alterations in these two compartments are different. Thus, COPD is associated with tissue remodeling (fibrosis and smooth muscle hypertrophy) in small airways; while in lung parenchyma tissue destruction predominates, leading to emphysema.2,3 Recent studies show that lymphocytes may play a key role in enhancing and maintaining the inflammatory response in the aforementioned compartments affected in COPD.4

Some information has been published on lymphocyte infiltration in small airways of COPD patients.5–10 Unlike with asthma, CD4+ cells do not seem to be involved in COPD pathogenesis, since the number of both total T cells5,6 and helper T cells6,7 was similar in the small airways of smokers with and without COPD. However, a higher number of CD8+ cells7,8 and B-cells9 has been detected in the wall of small airways of smokers with COPD compared with smokers without COPD. Furthermore, an inverse correlation between the bronchial infiltration of these cells and FEV1 was detected in a cohort of COPD patients in different GOLD stages.10

Few data are available about the extent of lymphocyte infiltration in parenchyma, especially in the most severe forms of COPD. Two studies reported that COPD patients have an increased number of CD8+ cells in alveolar wall compared with control non-smokers.11,12 Another one showed a relationship between the increase of CD4+ and CD8+ cells and the severity of the disease, without differences in B lymphocytes.13

In spite of this evidence, as far as we know, no studies have been carried out analyzing lymphocyte infiltration in the two compartments affected in COPD: i.e., small airways and lung parenchyma. Likewise, although the number of lymphocytes infiltrating lung parenchyma correlates with the degree of microscopic emphysema,14,15 the localization and distribution of lymphocytes in the areas nearest to emphysema remain unexplored.

Given that in COPD small airways and lung parenchyma are infiltrated by the same inflammatory cells (T and B lymphocytes and macrophages), a research question remains to be elucidated: Are there differences in type and/or cell density of infiltrating lymphocytes between the two compartments? Additionally, assuming that CD8+ lymphocytes have been correlated with parenchymal destruction, a second research question emerges: Is there a spatial association of these cells with emphysematous lesions?

In order to shed light on these questions, our study carries out a systematic exploration of the lymphocyte subsets in bronchial compartments (epithelium, smooth muscle and connective tissue) and alveolar interstitium in samples from smokers with moderate and very severe COPD and in subjects without COPD (smokers and non-smokers).

Methods

Subjects

The study population comprised 59 subjects who underwent lung resection for non-obstructive peripheral lung tumors or were subjected to double lung transplantation for very severe COPD. The study was approved by the Ethics Committee of the Vall d’Hebron Hospital (Barcelona, Spain) and a written informed consent was obtained from all patients. Spirometry and diffusing capacity measurements and body plethysmography were performed to assess pulmonary function using standard procedures16 and equipment (Masterlab; Jaeger, Würzburg, Germany). None of the subjects had had a COPD exacerbation nor had received chemotherapy before surgery.

Patients were classified into 4 clinical groups according to their smoking habits (smokers and non-smokers) and COPD severity: 9 nonsmoking patients with normal lung function; 18 asymptomatic smokers with normal lung function; 16 smokers with moderate COPD (GOLD stage II); and 16 smokers with very severe COPD (GOLD stage IV) subjected to double lung transplantation.

Sample processing

Immediately after surgery, the excised lungs or lobes were inflated by intrabronchial injection of 4% formaldehyde at a constant pressure prior to immersion in fixative during 24 h. After fixation, surgical specimens were sliced and the severity of emphysema (macroscopic emphysema degree, MED, %) was graded using a panel grid as previously described.17 This parameter shows a statistically significant correlation with HRCT, reflecting the extent of parenchymal damage.18,19 After that, 2 × 2 × 0.3 cm randomly selected tissue blocks were excised, embedded in paraffin, cut into 4-μm sections and mounted on positive charged slides Starfrost Plus (Menzel-Gläser, Braunschweig, Germany).

Immunohistochemistry

The following antibodies were used at indicated dilutions: polyclonal anti-CD3 (1:400; DakoCytomation, Glostrup, Denmark), monoclonal anti-CD4 (1:50; Novocastra, Newcastle, UK), monoclonal anti-CD8 antigen (1:100; DakoCytomation) and monoclonal anti-CD20cy (1:800; DakoCytomation). Immunostaining was performed using the ABC immunoperoxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) with a DAB reaction.

Cell counts

Cell density for each marker and sample was assessed using a point-counting method previously described.20,21 Briefly, at least twelve fields were randomly and systematically sampled to assess cell densities in alveolar interstitium and bronchial wall by using a grid with a known area attached to the eye piece of the microscope. The number of points hitting parenchymal interstitium and bronchial...
epithelium, connective tissue and smooth muscle were counted and converted into square millimeters using a conversion factor calculated for the specific magnification. In each field, the number of cells was determined by counting the cell profiles that were not in vessels or intersected by the exclusion lines.

To assess the spatial association of lymphocytes with destroyed parenchyma, we measured CD8\(^+\) and B cells in alveolar interstitium of three fields in proximal peri-emphysematous parenchyma (100 to 300 \(\mu\)m away from emphysema), and three more fields in distal peri-emphysematous parenchyma (300 to 600 \(\mu\)m away from emphysema), in patients with COPD.

Cell densities were expressed as number of cells per square millimetres of tissue examined.\(^5\)\(^-\)\(^7\)\(^,\)\(^8\)\(^,\)\(^13\)\(^,\)\(^22\)\(^,\)\(^23\) Mean patient values were obtained by averaging the results of all fields. The cases were coded and the measurements made without knowledge of clinical data.

**Blood analysis**

Blood samples were taken from all patients immediately before surgery. Total cell counts and lymphocyte subsets were determined as previously described.\(^24\)

**Statistical analysis**

Descriptive statistical analysis included means and standard errors for each parameter. Differences among groups were analyzed using the analysis of variance (ANOVA) for clinical data and the Kruskal–Wallis test for cell densities. When differences in cell densities were significant, the Kruskal–Wallis test was followed by the Mann–Whitney U test for comparison between groups. Correlations were calculated by Spearman’s rank correlation test.

Significance level was set at \(p < 0.05\). All analyses were performed using Statgraphics Centurion XV (StatPoint Inc., Virginia, USA).

**Results**

**Demographic and clinical findings**

Patient characteristics are presented in Table 1. The four groups of patients were similar with regard to age, and there was no significant difference in pack-years between smokers with and without COPD. As expected from the selection criteria, non-smokers and smokers without COPD had normal lung function, whereas patients with COPD showed significant alterations in spirometry parameters, such as FEV\(_1\), FEV\(_1\)/FVC and DL\(_{CO}\). In addition, there was a significantly increased MED in COPD patients compared to subjects without COPD (Table 1).

**Immunohistochemistry**

**Small airways**

In order to determine whether the inflammatory response was different in epithelium, connective (subepithelial and adventitial) or smooth muscle tissues of small airways, we assessed the lymphocyte density for each one of these histological compartments for all patients.

Regarding T lymphocytes, only the number of CD8\(^+\) cells revealed some differences when the four groups were compared. Indeed, COPD patients showed more CD8\(^+\) lymphocytes in small airways than subjects without COPD, and a significantly higher density of these cells was found in the epithelial layer of subjects with moderate COPD in comparison to control non-smokers (Figs. 1 and 2A and B).

<table>
<thead>
<tr>
<th>Table 1 Clinical and demographical data.</th>
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<tr>
<td><strong>Sex, Male/Female</strong> ((n = 9))</td>
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<tr>
<td>Age, years</td>
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<tr>
<td>Smokig history, pack-years</td>
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<tr>
<td>Smoking status, current/ex-smoker</td>
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<tr>
<td>Inhaled corticosteroids use, yes/no</td>
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<td>Exacerbations in the previous year</td>
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<tr>
<td>FEV(_1), % predicted</td>
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<td>FEV(_1)/FVC, % predicted</td>
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<tr>
<td>RV, % predicted</td>
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<tr>
<td>TLC, % predicted</td>
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<tr>
<td>DL(_{CO}), % predicted</td>
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<td>MED, % predicted</td>
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*Definition of abbreviations:* FEV\(_1\): forced expiratory volume in 1 second; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; DL\(_{CO}\): carbon monoxide diffusing capacity; MED: macroscopic emphysema degree.

*Values expressed as mean ± SEM.*

\(^{a}\) Different from control non-smokers (\(p < 0.001\)).

\(^{b}\) Different from smokers without COPD (\(p < 0.001\)).

\(^{c}\) Different from moderate COPD patients (\(p < 0.001\)).
shown). Differences were observed among the four groups (data not in infiltration in smooth muscle was minimal and no statistical condition was considered. Thus, we observed that the ratio of CD4+/CD8+ cells was lower than 1.0 in the epithelium of smokers, whereas non-smokers showed a CD4+/CD8+ ratio higher than 1.0 (p = 0.02, Table 2).

Finally, the number of B-lymphocytes in the connective tissue of small airways of subjects with very severe COPD was significantly higher than in the other groups (Figs. 1B and 2C, D). However, no differences were found in the density of these cells in epithelium or in smooth muscle, nor in the small airway’s wall, among the four clinical groups.

**Lung parenchyma**

As was found in small airways, the density of CD8+ cells in interstitium was significantly higher in patients with COPD compared to patients without COPD (Figs. 3A and 4A and B).

Importantly, when CD8+ cell density was compared between the interstitium closer to emphysematous areas (proximal parenchyma) and the interstitium located farther than 300 μm from the border of emphysematous destruction (distal parenchyma) in COPD patients, no significant differences were observed (Table 3 and Fig. 4C).

Regarding B-lymphocyte densities, patients with very severe COPD had the highest number of these cells (25.17 ± 8.67 cell/mm²), although the difference from the other groups was not significant (Fig. 3D). Furthermore, there were no significant differences in B cell numbers between proximal and distal periemphysematous parenchyma in COPD patients (Table 3).

**Small airways versus lung parenchyma**

When the extent of lymphocytes infiltrating small airways and lung parenchyma interstitium was compared, we observed that COPD patients presented a higher CD3+ density in the bronchiolar tissue. Patients with moderate and very severe COPD showed a significantly higher number of CD3+ cells in bronchiolar wall (433.38 ± 70.73 cell/mm² and 359.77 ± 89.41 cell/mm², respectively) than in parenchymal interstitium (235.67 ± 47.87 cell/mm² and 166.67 ± 32.08 cell/mm², respectively) (p < 0.03, Fig. 3B). Moreover, very severe COPD patients had a significantly increased density of B cells in bronchiolar tissue (99.82 ± 22.07 cell/mm²) compared to parenchyma (25.17 ± 22.07 cell/mm², p < 0.01, Fig. 3D). By contrast, no significant differences were found between bronchiolar and parenchymal densities of CD3+ and B cells in non-smokers and smokers without COPD (Fig. 3C and D).

Additionally, we observed a higher infiltration of CD4+ cells in small airways than in parenchyma of all groups (Fig. 3B). Moreover, smokers without COPD and moderate COPD patients also showed a significant increase of CD8+ in bronchiolar wall compared to parenchymal interstitium (Fig. 3A).

**Correlations**

Some correlations were found between lymphocyte densities and clinical data when all smokers were considered together. The number of pack-years smoked was positively correlated with the cell density of CD8+ lymphocytes infiltrating the total small airway (R = 0.28, p = 0.04), bronchiolar connective compartment (R = 0.32, p = 0.02) and epithelium (R = 0.30, p = 0.03). Moreover, a significant correlation was also found between the number of CD8+ cells in alveolar interstitium and pack-years (R = 0.27, p = 0.03), as well as RV (R = 0.35, p = 0.02) values. On the contrary, there were no significant correlations between the macroscopic emphysema degree and the density of CD8+ in small airway or parenchymal interstitium. Regarding B-lymphocytes, there was an inverse correlation between the density of these cells in the total

![Figure 1](image1.png)
Figure 2  Immunolocalization of lymphocyte subpopulations in small airways. (A, B) CD8\(^+\) T lymphocytes infiltrating small airways of a control nonsmoker (A) and a patient with moderate COPD (B). Bars = 75 µm (A) and 100 µm (B). (C, D) Infiltration of B lymphocytes in a small airway of a smoker without COPD (C) and a patient with very severe COPD (D). Bars = 100 µm.

Figure 3  Lymphocyte densities in bronchiole wall and lung parenchyma. (A) CD8\(^+\) lymphocytes, (B) CD4\(^+\) lymphocytes, (C) CD3\(^+\) lymphocytes, (D) B lymphocytes. *Different from control non-smokers (p < 0.05). † Different from smokers without COPD (p < 0.05). ‡ Different from parenchyma (p < 0.05).
small airway, as well as in the connective compartment, and the FEV₁ values ($R = -0.37$, $p = 0.02$ and $R = -0.39$, $p = 0.01$, respectively).

When control smokers were excluded from the analysis, only the correlation between the density of B-cells in the bronchiolar compartment and the FEV₁ values was maintained ($R = -0.54$, $p = 0.008$). No other correlations were found between lymphocyte densities and clinical parameters, including DLCO and MED.

**Blood lymphocytes**

The estimation of the number of each lymphocyte subset per μL of blood showed no significant differences among the four clinical groups (data not shown). Moreover, no correlations have been found between lymphocyte numbers in blood and those infiltrated into the tissue compartments.

**Discussion**

Two histopathological phenotypes have been described in COPD: remodeling in small airways and emphysema in parenchyma. There is emerging evidence that lymphocyte-mediated inflammation in both compartments may contribute to progression of COPD. The present study demonstrates that T and B lymphocyte infiltration is significantly higher in small airways than in parenchymal interstitium of very severe COPD patients. Moreover, no spatial relationship was found between lymphocyte infiltration and emphysematous areas in the examined samples from COPD.

Regarding the CD8⁺ lymphocyte subset, our results suggest that, while cigarette smoke increases CD8⁺ cell density in small airways of all smokers, there is a significant and specific increase of these cells in the parenchymal interstitium of COPD patients. Furthermore, we found that CD8⁺ cell density was always higher in small airways than in lung parenchyma of COPD patients, although significant differences were found only in the moderate COPD group. This is not surprising, as very severe COPD patients had a lower density of CD8⁺ cells in airways than moderate COPD patients, while density in lung parenchyma was similarly increased in both COPD groups. Although some studies report an increase of CD8⁺ cells in small airways with the severity of the disease, previous findings of Di Stefano and colleagues showed that subjects at severe stages of COPD have a lower number of CD8⁺ lymphocytes in bronchial mucosa than mild/moderate COPD patients and control smokers. A possible explanation is that all patients with very severe COPD had stopped smoking some years before, and smoking cessation has been associated with a decrease of CD8⁺ cell numbers in the airways. Another explanation may be the higher number of patients treated with inhaled corticosteroids in the group of very severe COPD (14/16), since these steroids drastically lower CD8⁺ cells in the airways.

The assessment of each bronchiolar compartment allowed us to detect whether lymphocyte infiltration was more severe in epithelium, smooth muscle or connective tissue. Importantly, we have demonstrated that moderate COPD patients have a higher density of CD8⁺ cells in the epithelium of small airways than non-smokers. This finding adds further information to previous observations of Fournier and coworkers, who reported an increase of intraepithelial CD8⁺ lymphocytes in central airways of subjects with chronic bronchitis and airflow limitation. Furthermore, smokers with and without COPD showed a predominance of the CD8⁺ over the CD4⁺ T cell phenotype (CD4⁺/CD8⁺ < 1) in the epithelial layer compared with non-smokers; whereas, in the connective tissue, the ratio CD4⁺/CD8⁺ was reversed only in COPD patients. Additionally, when we considered all smokers as one group, a positive correlation was found between the CD8⁺ cell density in small airways and the pack-years smoked, adding new data to previous findings concerning large airways. Taken together, these results suggest that cigarette smoke induces a modification of the lymphocyte profile in the epithelium of small airways by increasing the number of CD8⁺ cells.

With respect to lung parenchyma, as far as we know, our study is the first to provide data about the number of lymphocytes in patients who underwent double lung transplantation for very severe COPD. We have found an increased density of CD8⁺ cells in the alveolar interstitium, which was specific to COPD patients; however, the CD8⁺ increase in airways was also observed in smokers without COPD. This finding is in line with previous studies reporting higher numbers of CD8⁺ lymphocytes in lung parenchyma of subjects with moderate or severe COPD. Moreover, similar to the finding concerning small airways, the number of CD8⁺ cells in parenchymal interstitium was positively correlated with the number of packs-year smoked, as previously described by Majó and colleagues.

However, when the localization of lymphocytes in the emphysematous lung parenchyma was analyzed, we observed that the number of CD8⁺ and B cells in the interstitium adjacent to the border of emphysematous areas was similar to that found in non-affected
parenchyma. So, in spite of the higher density of CD8\(^+\) lymphocytes in COPD patients, the distribution of these cells was uniform throughout the parenchyma and no accumulations were found in proximal-emphysematous regions. Since the effector functions of these lymphocytes occur after recognition of and adhesion to target cells,\(^{30}\) and lymphocyte proximity to affected area is necessary to induce cell apoptosis, these results do not support the hypothesis linking CD8\(^+\) cells with development of emphysema. Furthermore, no correlation was found between the parenchymal density of CD8\(^+\) or B cells and the macroscopic emphysema degree or the FEV\(_1\) values.

We believe our data rather support the previously formulated speculation that the increase of CD8\(^+\) and, to a lesser extent, B cells in lung parenchyma of COPD patients might be a consequence of emphysema. As previously suggested,\(^{8,10,11,13,31}\) it is likely that repeated viral or bacterial infections, known to be frequent in COPD patients, could account for the increased lymphocyte numbers in the lungs of these subjects. Moreover, Hogan and colleagues\(^{32}\) demonstrated in a murine model that antigen-specific CD8\(^+\) cells can persist in lung tissue and airways for several months after clearance of a respiratory virus. Nonetheless, we are aware that emphysema is a long-term process and further studies are necessary to understand whether CD8\(^+\) lymphocytes have a direct role in its progression.

Finally, the comparison between bronchiolar and parenchymal compartments showed that all lymphocyte densities were higher in bronchiolar wall than in alveolar interstitium, regardless of cell type and the group considered. Statistically, the difference was significant in CD3\(^+\) and B lymphocytes in very severe COPD patients. Histopathological patterns in small airways and lung parenchyma are different in COPD, with both alterations coexisting in most patients. Since lymphocyte types are the same, their contribution to the different histopathological patterns in both compartments is difficult to understand with available data. However, although the structure of alveolar interstitium and small airways is different, there is no evidence that lymphocyte function varies depending on the infiltrated tissue. Consequently, the simplest explanation for these results is probably that lymphocytes take part in different processes leading to chronic airflow obstruction.

We think that a plausible hypothesis is that, while the lymphocytes are involved in a high inflammatory response to smoking in the small airways, with a great number of infiltrating cells producing fibrosis and smooth muscle hypertrophy, in lung parenchyma, lymphocytes are the result, but not the cause, of a mild inflammation with a lower number of infiltrating cells resulting from the emphysematous areas. In summary, our results show that responses in small airways and lung parenchyma to cigarette smoke in COPD patients are different in terms of lymphocyte infiltration. Both CD8\(^+\) and B cells are the main lymphocyte subsets

<table>
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<th>Table 3 Lymphocyte densities in periemphysematous parenchyma.</th>
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<tr>
<td>CD8(^+), cells/mm(^2)</td>
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<td>CD20(^+), cells/mm(^2)</td>
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Values expressed as mean ± SEM. No significant differences were found.

Figure 4 Immunolocalization of lymphocyte subpopulations in lung parenchyma. (A, B) Infiltration of CD8\(^+\) T lymphocytes in a parenchyma of a smoker without COPD (A) and a patient with very severe COPD (B). (C) Periemphysematous lung parenchyma immunostained for CD8\(^+\) T lymphocytes. Alveolar walls of periemphysematous parenchyma did not achieve a higher infiltration of CD8\(^+\) cells than those of non-affected parenchyma. Bars = 125 \(\mu\)m.
involved in the pathogenesis of small airways in COPD, where these cells could play a role in tissue remodeling and airway obstruction. By contrast, although COPD patients showed an increased density of CD8^+ cells in lung parenchyma, the fact that these cells were not preferentially infiltrated into the alveolar walls adjacent to emphysematous destruction, even in patients at the most severe stage of COPD, challenges the hypothesis attributing emphysema to CD8^+ lymphocytes. Further investigation on the role of other cells, such as neutrophils, and processes, such as oxidative stress, could provide important data in order to elucidate the pathogenesis of the disease.

**Conflict of interest**

None of the authors have any conflicts to disclose.

**Acknowledgments**

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