SIGNIFICANT INCREASE OF CD57^{+} CELLS IN PULMONARY LYMPHOID FOLLICLES OF COPD PATIENTS

Jordi Olloquequi[1], Juan F. Montes[1], Ana Prats[1], Esther Rodríguez[2], M. Angeles Montero[3], José García-Valero[1], Jaume Ferrer[2].

[1]Departament de Biologia Cel·lular, Facultat de Biologia, Universitat de Barcelona, Barcelona, Spain; [2]Servei de Pneumologia, Hospital General Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain; CIBER Enfermedades Respiratorias, Instituto de Salud Carlos III, Spain; [3]Departament d'Anatomia Patològica, Hospital General Universitari Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain.

Corresponding author: Dr. José García-Valero

Tel: +34 93 403 4487

Fax: +34 93 403 4607

E-mail: jgarcia@ub.edu

Correspondence and requests for reprints should be addressed to José García-Valero, PhD, Departament de Biologia Cel·lular, Facultat de Biologia, Avda. Diagonal, 645, 08028 Barcelona, Spain; e-mail: jgarcia@ub.edu.

Mr. Olloquequi and Dr. Montes contributed equally to the design of the study and writing the article.

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ABSTRACT

Although the presence of pulmonary lymphoid follicles (LF) has been associated with the progression of chronic obstructive pulmonary disease (COPD), there is no information about the pattern of vascularization, expression of addressins or inflammatory cell densities within these structures in COPD.

Histological and immunohistochemical techniques were used to assess the prevalence, structure, localization, vascularization and cell proliferation/apoptosis of LF, as well as the follicular density of B and T lymphocytes, macrophages, dendritic cells and CD57$^+$ cells in lung tissue of 9 non-smokers, 18 smokers without COPD, 16 smokers with moderate COPD and 16 patients with very severe COPD.

The density of CD57$^+$ cells within LF of COPD patients was significantly increased compared to non-smokers and smokers without COPD ($p < 0.05$). Moreover, the percentage of LF profiles with cell apoptosis was also significantly higher in COPD patients ($p = 0.03$). By contrast, no significant differences among groups were observed in the follicular densities of other inflammatory cells, nor in the distribution of blood and lymphatic vessels within LF.

Since CD57$^+$ cells are important effectors of cytotoxicity and immune regulation, an increase of their follicular density supports the hypothesis of a local immune dysfunction in COPD.

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INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by a progressive and not fully reversible airflow limitation, associated with an abnormal inflammatory response of the lung to noxious particles and gases, mainly cigarette smoke (1). The main pathological features of COPD are found both in peripheral airways and lung parenchyma, as well as in pulmonary vasculature.

It is now established that COPD is a chronic inflammatory condition resulting from complex interactions between cells belonging to the innate and adaptive immune systems. Recently, this inflammatory process has been associated with the development of ectopic lymphoid follicles (LF) in lungs of COPD patients (2-7), which are similar to the tertiary lymphoid tissue found in other inflammatory or autoimmune diseases (8, 9).

Since LF were described in the airways of COPD patients (2), increasing attention has been focused on the study of these structures and their possible role in the pathogenesis of COPD. Progression of COPD from GOLD stage 0 to GOLD stage 4 was associated with the number of bronchioles containing LF (3). Furthermore, although the nature of the stimuli that trigger the formation of LF remains unknown, an oligoclonal process in follicular B lymphocytes of COPD patients and mice with emphysema has been reported, which suggests an antigen-specific proliferation in these structures (4).

On the other hand, despite increased numbers of macrophages, dendritic cells, NK cells and lymphocytes having been reported in lung tissue of COPD patients (10, 11), little data is available on the follicular densities of these cells and the molecules involved in their recruitment to LF in COPD, especially at the most severe stages of the disease. In fact, only one study reported a higher percentage of T-regulatory cells within

LF of moderate COPD patients compared with smokers and non-smokers (5), but no information is available regarding vascular supply, lymphatic drainage or the follicular densities of other key inflammatory cells in subjects with and without COPD. Moreover, there are no studies examining which addressins are involved in the migration of the inflammatory cells from the blood into the LF in COPD.

The purpose of the present study is to examine the prevalence, localization, vascularization, addressin expression and inflammatory cell densities of LF in lung tissue of moderate and very severe COPD patients compared to non-smokers and smokers without COPD.

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 Ethics Committee of the Vall d'Hebron Hospital (Barcelona, Spain) approved the study and a written informed consent was obtained from all patients. Subjects were classified into 4 clinical groups according to their smoking habits (smokers and non-smokers) and COPD severity (1): 9 nonsmoking patients (never smokers) 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) undergoing lung transplantation.

Standard procedures (12) and equipment (Masterlab; Jaeger, Würzburg, Germany) were used to assess pulmonary function in all patients, including measurements of forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), residual volume (RV), total lung capacity (TLC) and carbon monoxide diffusing capacity (DL_{CO}). All subjects had been free of acute lung infections and none had received chemotherapy before surgery.

Sample Processing

The resected lungs or lobes obtained in surgery were immediately inflated with 4% formaldehyde prior to immersion in fixative for 24 h. After fixation, surgical specimens were sliced serially into 1-cm axial sections and the severity of emphysema was graded using the panel grid described by Thurlbeck and coworkers (13). After that, 2 x 2 x 1

cm randomly selected tissue blocks were excised, embedded in paraffin, cut into serial 4-µm sections and mounted on positive charged slides Starfrost Plus (Menzel-Gläser, Braunschweig, Germany). One section per block was stained with hematoxilyn and eosin.

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 (1:100; DakoCytomation), monoclonal anti-CD20cy (1:800; DakoCytomation), monoclonal anti-CD57 (1:80; DakoCytomation), monoclonal anti-Follicular Dendritic Cell (1:20; DakoCytomation), monoclonal anti-CD83 (1:30; Santa Cruz Biotechnology Inc, Santa Cruz, USA), monoclonal anti-CD68 (1:50; DakoCytomation), monoclonal anti-PECAM-1 (1:200; DakoCytomation), monoclonal anti-Podoplanine (1:150; Abcam, Cambridge, UK), monoclonal anti-MAdCAM-1 (1:30; AbD Serotec, Kidlington, UK), monoclonal anti-PNAd (1:50; BD Biosciences, New Jersey, USA), monoclonal anti-Ki-67 (1:150; DakoCytomation), monoclonal anti-BCL6 (1:60; DakoCytomation), polyclonal anti-Cleaved Caspase 3 (1:200; Cell Signaling Technology, Danvers, USA).

Immunostaining was performed using the ABC immunoperoxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) with a DAB reaction. For double immunostaining of CD3 and CD57 antigens, the sections were first incubated with polyclonal anti-CD3. After DAB development, this marker was amplified with a nickel ammonium sulphate solution, which allowed us to obtain a differentiating black color for T cells. After this amplification, the sections were newly

incubated with monoclonal anti-CD57 with a DAB development giving a brown color. Hence, this design allowed us to differentiate between the $CD3^+CD57^+$ cells (double stained with black and brown) and $CD3^-CD57^+$ cells (brown stained).

Assessment of lymphoid follicles

Following the criteria previously described by Elliot and colleagues (14), LF were defined as focal collections of more than 50 lymphomononuclear cells with a cell density (cells/mm^2) of more than 10 times that of the surrounding tissue. Hematoxilin and eosin staining was used to assess the presence and location of LF in samples of all patients. Furthermore, for each antibody, two sections per subject were analyzed.

For the assessment of cell densities, a point-counting method previously described was used (15). Briefly, at least six fields were randomly and systematically sampled to assess cell densities in follicular tissue by using a grid with a known area attached to the eye piece of the microscope. The number of points hitting each LF profile 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. Mean patient values were obtained by averaging the results of all lymphoid follicles. Cell densities were expressed as number of cells per square millimetres of tissue examined.

In addition, the percent of patients with positive staining for vascular structures and addressins within LF was determined by considering two different locations of these markers: either at the follicle periphery or inside it. We also averaged the percent

of patients with positive staining for the markers of proliferation and apoptosis in LF. The cases were coded and the measurements made without knowledge of clinical data.

Statistical Analysis

Descriptive statistical analysis included means and standard errors for each parameter. Clinical data was compared between groups using the analysis of variance (ANOVA). The prevalence, vascularization, proliferation and apoptosis of LF were compared between case groups using the Chi-square test. Differences in LF cell densities were analyzed using the Kruskal-Wallis test. When differences were significant, the Kruskal-Wallis test was followed by the Mann-Whitney U test for comparison between groups. Significance level was set at $p < 0.05$. All analyses were performed using Statgraphics Centurion XV (StatPoint Inc., Virginia, USA).

RESULTS

Demographic and Clinical Data

Table 1 shows the characteristics of patient groups. The four clinical groups were similar with regard to age and no significant difference was found in pack-years among smokers with and without COPD. By contrast, the values of FEV_1, FEV_1/FVC and DL_{CO} were significantly different in COPD patients, the very severe COPD subjects having the lowest values. Additionally, as expected, there was a significantly increased macroscopic emphysema degree (MED, %) in COPD patients, especially in those in a very severe GOLD stage (Table 1).

Assessment of lymphoid follicles

Structure and localization

The presence of at least one lymphoid follicle was observed in 89% of samples studied, their prevalence ranging from 83 to 100% in the four clinical groups, without significant differences among them (Table 1). LF were characterized by a high density of mononuclear infiltrates without a fibrous covering layer. Furthermore, anthracotic granules were frequently located at the periphery of LF, especially in patients with very severe COPD (Figure 1A).

Lymphoid follicles were essentially distributed in three tissue compartments: bronchiolar and arteriolar adventitias and parenchymal interstitium. Occasionally, some

samples showed LF in bronchial adventitia and lobular septa, which were not included in further analysis because of their low frequency.

No significant differences among groups were observed on the frequency of follicles present in each compartment. However, it is interesting to note that nonsmokers showed a higher prevalence of bronchiolar follicles, whereas in COPD patients, the arteriolar compartment was more frequently infiltrated by LF (Figure 1A).

Vascularization

The presence and distribution of blood vessels in LF was determined by the immunolocalization of the platelet-endothelial cell adhesion molecule (PECAM, Figure 1B). It was found that 95% of LF sections were positive for this marker and there were no significant differences in the number of positive profiles among clinical groups. Blood vessels were found in both the center of the follicle and also in the adjacent connective tissue, both the flat and the high endothelial venules (HEVs) phenotypes of endothelial cells being observed (Figure 1B).

The immunolocalization of podoplanine allowed us to assess the presence of lymphatic vessels in the follicles (Figure 1C). Results showed that lymphatic endothelium was detected in around half (56%) of the LF, without significant differences among groups. When the distribution of lymphatic vessels was assessed in these follicles, it was observed that only 12% showed a lymphatic irrigation inside the LF (Figure 1C), while 91% of the follicles had surrounding lymphatic vessels lying on the connective tissue of their periphery. No significant differences among groups were observed in the distribution of lymphatic vessels.

Vascular Addressins

In order to determine which addressin was expressed by the vessels of lung lymphoid follicles, immunolocalizations of both peripheral node addressin (PNAd) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) were carried out in the pulmonary samples. We found that 35% of lymphoid follicles in lung samples were PNAd positive, although no significant difference was found among clinical groups. It is noteworthy that PNAd expression was demonstrated, not only in HEVs, but also in conventional endothelium of lymphoid follicles (Figure 1D), as well as in some adventitia vessels of bronchioles and arterioles without tertiary lymphoid tissue.

Regarding the PNAd distribution in LF, it was found that 82% had peripheral PNAd positive vessels, while 54% showed a central localization, without significant differences among clinical groups. Nevertheless, severe COPD patients showed a higher number of follicles with central PNAd staining than the other groups (Figure 2).

To assess the reactivity of lung lymphoid follicles for MAdCAM-1, a section of a small intestine with a Peyer patch was used as positive control (Figure 1E). It was found that none of the pulmonary lymphoid follicle profiles was MAdCAM-1 positive, whatever the location or the clinical group considered (Figure 1F).

Cell densities

CD3$^+$, CD4$^+$, CD8$^+$ and B lymphocytes, as well as macrophages, CD57$^+$ and dendritic cells were immunostained in order to assess whether, among clinical groups, there were qualitative or quantitative differences in the main inflammatory cells constituting the lymphoid follicles.

All LF were characterized by a central core of B lymphocytes, the most abundant follicular cells (Figure 3A), and a peripheral zone mainly infiltrated by T lymphocytes (Figure 3B). Among these, $CD4^+$ and $CD8^+$ T cells (Figures 3C and 3D) were distributed diffusely either in the follicular core or surrounding germinal centers. In every case, both B and T lymphocytes were found in close contact with blood and lymphatic vessels.

Macrophages, mature dendritic cells and $CD57^+$ cells were diffusely distributed in most of the follicle profiles (Figures 3E-3H). By contrast, isolated follicular dendritic cells were only found in samples belonging to very severe COPD patients.

Importantly, when cell densities of lymphoid follicles were compared, it was found that COPD patients had a significantly higher density of $CD57^+$ cells than non-smokers and smokers without COPD ($p < 0.05$, Table 2, Figures 3G, 3H and Figure 4). By contrast, no significant differences in the densities of the other cell types were found (Table 2).

Additionally, in order to differentiate the subpopulations of $CD57^+$ cells, double immunostainings of CD3 and CD57 antigens were performed in our series (Figure 5). After that, the follicular density of both $CD57^+CD3^-$ and $CD57^+CD3^+$ subpopulations was assessed. The results showed that the mean ratio $CD57^+CD3^-/CD57^+CD3^+ + CD57^+CD3^-$ in lymphoid follicles was 0.23. No significant differences among the clinical groups were found (0.21 in non-smokers, 0.33 in smokers without COPD, 0.16 in moderate COPD patients and 0.29 in very severe COPD patients).

Proliferation and apoptosis

In order to examine whether lymphoid follicles were active in terms of proliferation and/or apoptosis, the percentage of samples with positive cell staining for BCL-6, Ki-67 and activated caspase 3 in at least one lymphoid follicle was determined.

Only two follicle profiles of a non-smoker and a very severe COPD patient were positive for BCL-6 staining (Figure 6A). By contrast, the Ki-67 staining (Figure 6B) demonstrated proliferation in the majority of LF (ranging from 56% to 91%), without significant differences among groups.

Regarding apoptosis, COPD patients showed a significantly higher percentage of samples with positive staining for activated caspase 3 compared to the other groups ($p < 0.05$, Figures 4 and 6C). Indeed, 25% of moderate COPD patients and 30% of very severe COPD patients showed activated caspase 3 staining, while no lymphoid follicle with positive staining was found in non-smokers and smokers without COPD (Figure 4).

DISCUSSION

For a long time, the presence of LF has been associated with COPD progression (3), but only recently some attention has been paid to the study of the cellular and molecular patterns of this tertiary lymphoid tissue in COPD patients (5, 6). Following this research line, our present work raises an integral study of LF, including its prevalence, vascularization, cell composition and proliferation/apoptosis in patients with moderate and very severe COPD. Our results show for the first time that COPD patients have a significant increase in the follicular density of $CD57^+$ cells compared to non-smokers and smokers without COPD. Moreover, the percentage of LF profiles with cell apoptosis is also significantly increased in COPD patients.

A principal objective of this study was to quantify the main cellular types inside LF in order to compare their densities among clinical groups. Importantly, our results showed a significant and specific increase of the follicular density of $CD57^+$ cells in patients with COPD. This finding adds new knowledge about the role that LF could play in the disease, since there is evidence pointing toward CD57 as a marker of lung inflammation (16), or even as a marker of general immune dysfunction, independent of the underlying disease (17). Moreover, it has been demonstrated recently that CD57 antigen is also a marker of terminally differentiated cells with a high cytolitic potential (18). Indeed, Chattopadhyay and colleagues (18) reported that CD57 expression was strongly correlated with a simultaneous expression of pro-apoptotic molecules, such as granzime A, granzime B and perforin. Thus, an increased density of the follicular $CD57^+$ cells in COPD patients may explain the high percentage of follicle profiles with cell apoptosis that we observed in moderate and very severe COPD groups.

Apart from their cytotoxicity, Kim and colleagues (19) showed that a subset of CD57$^+$ cells present in germinal centers of human lymphoid tissues induces B cell differentiation and immunoglobulin production. Also, Laffont and colleagues (20) demonstrated that NK cells act as regulators of alloreactive T-cell priming in allotransplantation by killing allogenic dendritic cells in draining lymph nodes.

Recently, there has been increased interest in understanding better the pathophysiological role of lymphoid follicles in COPD. There are several observations pointing to the fact that these structures could arise in response to a chronic bacterial and viral colonization or even against neo or self antigens which have been hypothesized to be present in lungs of COPD patients (7). In this respect, the higher density of follicular CD57$^+$ cells found in COPD groups is noteworthy, since CD57 antigen is expressed by many cells during chronic immune activation (21) or under clinical conditions, such as infections or immune dysfunctions (17).

By contrast, though previous data of Plumb and colleagues (5) indicated an increase in CD4$^+$ T cells in parenchymal LF of moderate COPD patients, our results do not show any significant difference in the follicular densities of B cells, T cells, macrophages or mature dendritic cells among clinical groups.

Another objective of this study was to examine the structure and localization of LF in order to find existing differences between subjects with and without COPD. We stated that LF were located in both the adventitia of airways and the parenchyma of the pulmonary samples, as previously described (2-6, 14). However, we also observed the presence of LF in the connective tissue of lobular septa and in the arteriolar adventitia, the later compartment being the most frequently occupied by LF in COPD patients. This finding reveals that LF development is not restricted to the connective tissue of airways

and parenchymal interstitium, other lung compartments also being susceptible to infiltration by these cellular aggregates.

Hogg and colleagues reported an association between the number of small airways containing lymphoid follicles and COPD severity (3). However, when we compared the number and distribution of follicle profiles for each compartment (small airways, arterioles, and parenchyma), no significant differences were found among clinical groups. After data analysis, we considered that one factor interfering with these results could be the inhaled corticosteroid treatment given to most COPD patients (see Table 1). Correspondingly, Hogg and colleagues (22), found a negative association between steroid therapy and the percentage of airways containing lymphoid follicles.

Although no data is available about the effect of inhaled steroids on infiltrated $CD57^+$ cells, it has been shown that steroid therapy drastically lowers lymphocyte numbers in the airways (23). Moreover, CD57 levels in patients with relapsing-remitting Multiple Sclerosis, Systemic Lupus Erythematosus and Rheumatoid Arthritis appear to be influenced by corticosteroid therapy, which lowers these lymphocytes counts (24). Hence, if inhaled steroids could have influenced our results, its hypothetical effect would make more remarkable the differences found in the follicular density of $CD57^+$ cells.

Immunolocalization of PECAM revealed that LF of subjects with and without COPD have a defined pattern of vascularization, adding further information to previous studies that reported a high number of vascular structures in lung lymphoid masses of smokers (25), subjects with idiopathic pulmonary fibrosis (IPF) (26) and subjects with asthma (14). On the contrary, the immunolocalization of podoplanine showed that most follicle profiles did not present lymphatic vessels inside, suggesting that these LF lack

their own lymphatic drainage. This could be related to the absence of fibrous covering that is characteristic of tertiary lymphoid tissue (27).

The addressins PNAd and MAdCAM-1 are expressed in HEVs of lymph nodes and Peyer's patches, respectively, and play a key role in lymphocyte homing, allowing the passage of lymphocytes from the blood into secondary lymphoid tissues (28). To the best of our knowledge, this is the first study to provide data about the expression of PNAd and MAdCAM-1 in LF of COPD patients. Our results showed that a 35% of the follicle profiles were PNAd$^+$, the very severe COPD patients showing the highest number of follicle profiles with central PNAd staining. On the contrary, none of the follicle profiles showed positive staining for MAdCAM-1. These results add new knowledge to previous studies reporting the expression of PNAd in LF of patients with IPF (26) and to others reporting an absence of MAdCAM-1 expression in BALT of mice (29). This specific expression of PNAd correlates with that which is characteristic of ectopic lymphoid tissue found in chronic inflammatory diseases (30, 31).

Finally, after exhaustive analysis of LF from COPD patients and control groups, no significant differences were found in the prevalence, vacularization and cell composition of this ectopic lymphoid tissue, except for CD57$^+$ cells. Therefore, the specific increase of these immune cells within lymphoid follicles of diseased subjects allows us to conclude that CD57$^+$ cells could play a key role in COPD pathogenesis.

ACKNOWLEDGMENTS

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TABLE 1. CLINICAL AND DEMOGRAPHICAL DATA

	Non-smokers (n = 9)	Smokers without COPD (n = 18)	Moderate COPD (n = 16)	Very severe COPD (n = 16)
Sex, Male / Female	1 / 8	14 /4	16 / 0	13 / 3
Age, years	61.8 ± 4.6	60.9 ± 2.6	63.3 ± 2.0	55.9 ± 1.3
Smokig history, pack-years	0.0 ± 0.0	49.9 ± 5.9	61.4 ± 5.4	47.9 ± 5.7
Smoking status, current / ex-smoker	-	6 / 12	9 / 7	1 / 15
Inhaled corticosteroids use, yes / no	1 / 8	1 / 17	7 / 9	14 / 2
Prevalence of lymphoid follicles, %	88.9	83.3	100.0	93.8
FEV_1, % predicted	99.3 ± 8.2	84.9 ± 2.6	$69.3 \pm 4.5^{*\dagger}$	$20.8 \pm 1.2^{*\dagger\ddagger}$
FEV_1/FVC, % predicted	80.1 ± 2.8	78.9 ± 1.9	$61.8 \pm 4.6^{*\dagger}$	$35.2 \pm 2.0^{*\dagger\ddagger}$
RV, % predicted	107.5 ± 10.2	98.1 ± 7.7	146.1 ± 7.8	$282.7 \pm 23.0^{*\dagger\ddagger}$
TLC, % predicted	101.0 ± 2.8	90.5 ± 3.8	113.8 ± 3.7	$135.2 \pm 9.0^{*\dagger\ddagger}$
DL_{CO}, % predicted	82.1 ± 7.9	71.6 ± 4.2	$57.6 \pm 3.0^{*\dagger}$	$34.8 \pm 4.0^{*\dagger\ddagger}$
MED, % predicted	1.7 ± 1.1	13.9 ± 3.8	$59.1 \pm 1.9^{*\dagger}$	$67.5 \pm 4.0^{*\dagger\ddagger}$

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.

* Different from control non-smokers ($p < 0.001$).

† Different from smokers without COPD ($p < 0.001$).

‡ Different from moderate COPD patients ($p < 0.001$).

TABLE 2. CELL DENSITIES OF LYMPHOID FOLLICLES

	Non-smokers (n = 9)	Smokers without COPD (n = 18)	Moderate COPD (n = 16)	Very severe COPD (n = 16)
$CD20^+$ (B lymphocytes), $x10^3$ cells/mm^2	11.7 (11.1-13.9)	11.4 (12.7-14.2)	13.2 (1.6-24.0)	12.7 (7.2-16.1)
$CD3^+$ (Total T lymphocytes), $x10^3$ cells/mm^2	9.0 (4.0-14.0)	6.2 (2.2-14.2)	7.8 (3.8-13.2)	6.4 (3.0-12.8)
$CD4^+$ (CD4 T lymphocytes), $x10^3$ cells/mm^2	7.4 (0.8-8.7)	5.6 (0.8-9.5)	5.7 (0.8-9.1)	4.2 (0.8-12.4)
$CD8^+$ (CD8 T lymphocytes), $x10^3$ cells/mm^2	1.9 (0.4-3.7)	1.7 (0.5-4.5)	2.8 (1.6-4.8)	1.8 (0.6-3.0)
$CD68^+$ (Macrophages), cells/mm^2	110.53 (0.0-197.5)	115.34 (0.0-448.5)	224.27 (84.1-384.4)	116.14 (12.7-271.9)
$CD83^+$ (Mature dendritic cells), cells/mm^2	12.14 (0.0-20.0)	0.00 (0.0-76.8)	2.57 (0.0-122.3)	0.0 (0.0-47.9)
$CD57^+$, cells/mm^2	63.27 (0.0-64.4)	48.05 (0.0-160.2)	105.7 (0.0-316.3)$^{*\,\dagger}$	110.1 (0.0-470.7)$^{*\,\dagger}$

Values expressed as median (range).

* Different from control non-smokers ($p < 0.05$).

† Different from smokers without COPD ($p < 0.05$).

‡ Different from moderate COPD patients ($p < 0.05$).

FIGURE LEGENDS

Figure 1. Vascularization and addressins of lung lymphoid follicles. **A)** Haematoxylin-eosin staining of a LF in arteriolar adventitia. Anthracotic granules were frequently found in the periphery of the lymphoid masses (→). A lymphatic vessel surrounds the LF (*). **B)** Immunolocalization of PECAM in lung lymphoid follicles. Blood vessels were found inside and in the periphery of lymphoid masses. PECAM was expressed in both the flat constitutively and the HEV phenotypes of endothelial cells. **C)** Immunolocalization of PDP in lung lymphoid follicles. Detail of a peripheral lymphatic vessel that penetrates into the lymphoid mass. **D)** Immunolocalization of PNAd in lung lymphoid follicles. PNAd was expressed both in HEVs and in conventional vessels. **E)** Immunolocalization of MAdCAM-1 in a Peyer Patch (positive control). **F)** Immunolocalization of MAdCAM-1 in lung lymphoid follicles. None of the LF profiles was MAdCAM-1 positive. Immunostained sections were counterstained with Friedlander's haematoxylin. **A, D, E)** Scale bars = 100 μm; **B, C**, **F)** Scale bars = 50 μm.
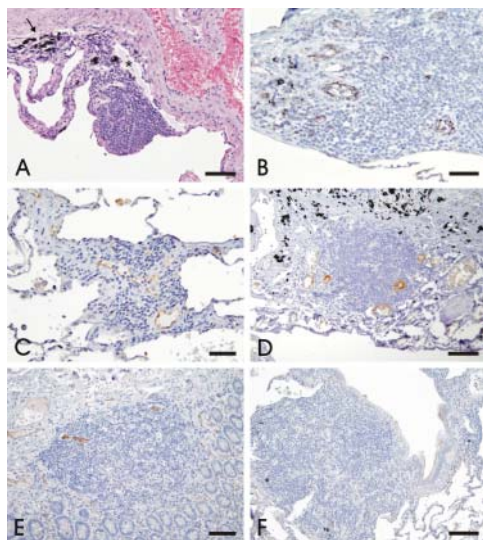
Figure 2. Percentage of lung lymphoid follicles with PNAd$^+$ central vessels. Very severe COPD patients showed a higher percentage of LF with central PNAd staining, although no significant differences among groups were found (p > 0.05).
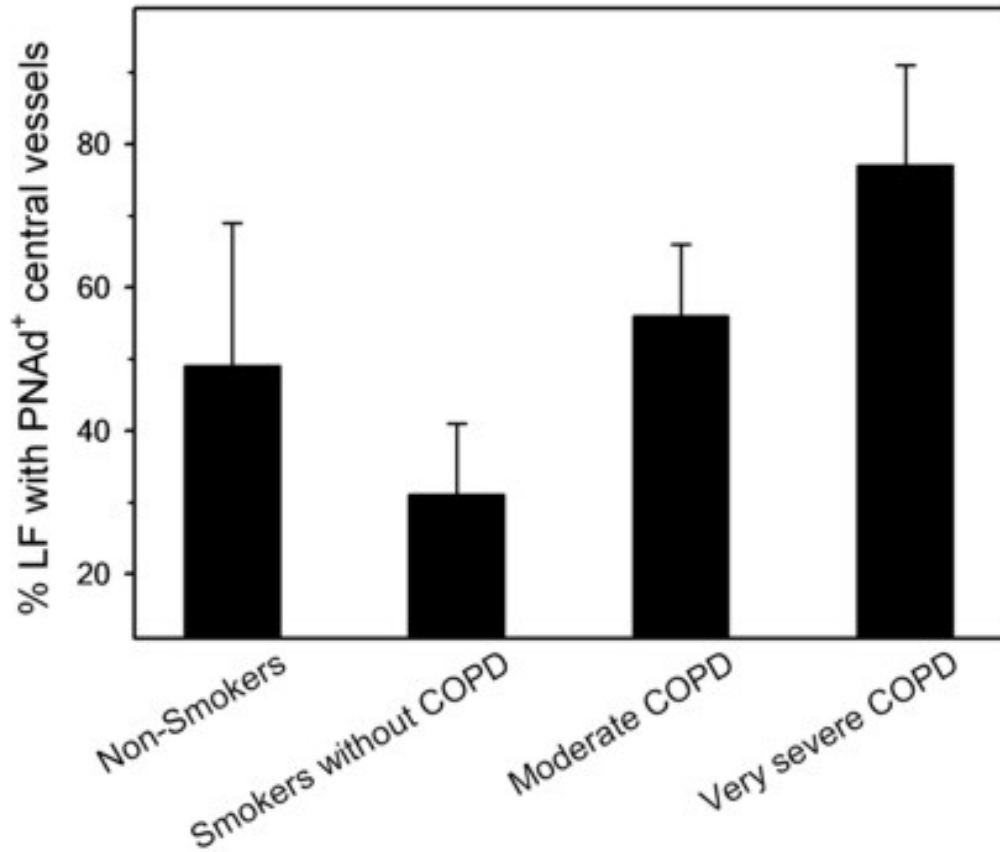
Figure 3. Immunolocalization of the main cell types inside lung lymphoid follicles. **A)** B lymphocytes were the most abundant cells within LF and followed a central distribution. **B)** T lymphocytes were mainly found in the follicular periphery, being the **C)** CD4$^+$ cells the most abundant subset. Both CD4$^+$ and **D)** CD8$^+$ cells were also found in the follicular core. **E)** Macrophages were diffusely distributed through the lymphoid aggregates, whereas the presence of **F)** mature dendritic cells was sparse in most follicle profiles. **G)** CD57$^+$ cells were found both in the periphery and the center of follicles, and its density was significantly higher in LF of COPD patients than **H)** in those of

patients without COPD. Sections were counterstained with Friedlander's haematoxylin, except **C)** that was counterstained with eosin. **A, D, E, G)** Scale bars = 100 μm; **B, C)** scale bars = 125 μm; **F)** scale bar = 250 μm and **H)** scale bar = 250 μm.
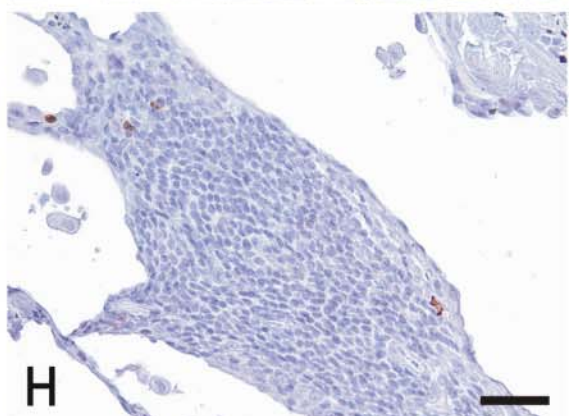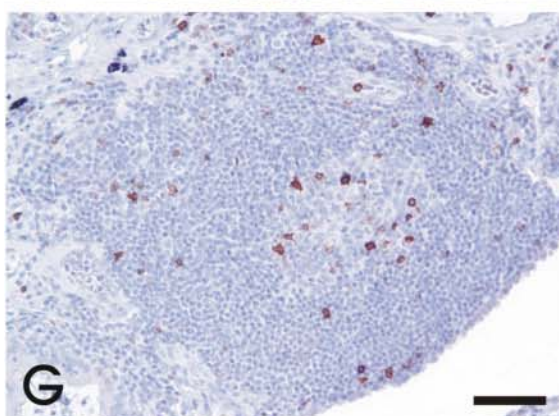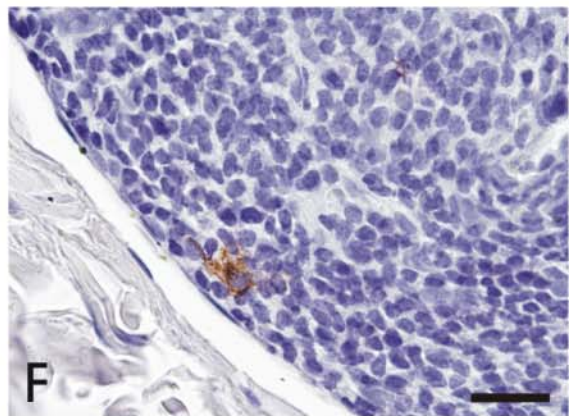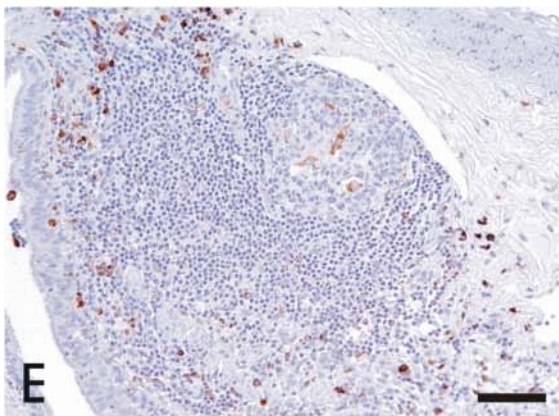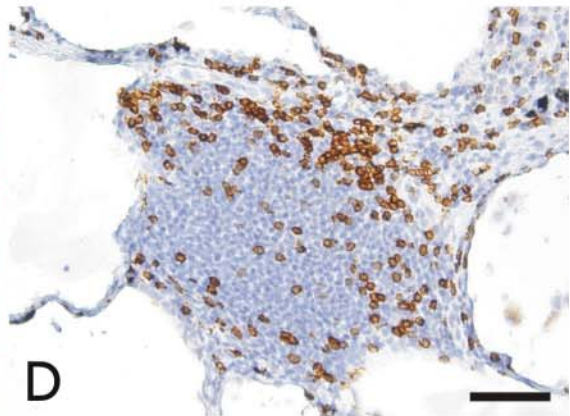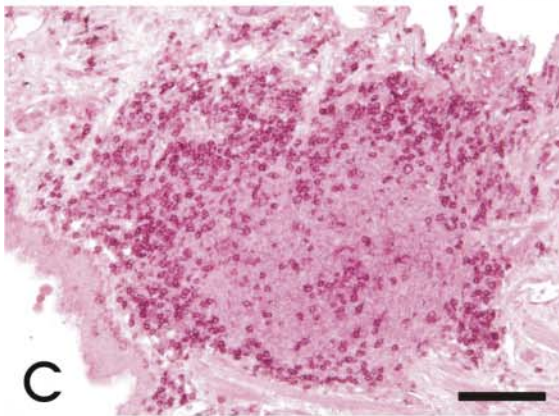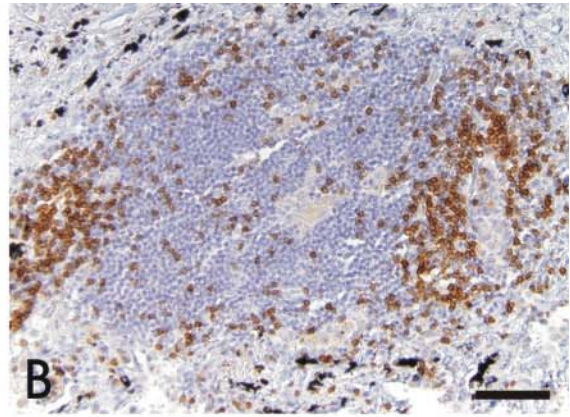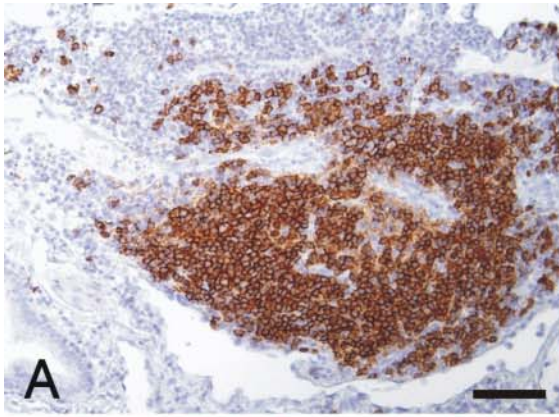
Figure 4. Follicular CD57$^+$ cells density and cell apoptosis. Moderate and very severe COPD patients showed a significantly higher density of follicular CD57$^+$ cells and a significantly higher percentage of follicle profiles with apoptosis than non-smokers and smokers without COPD. Cell density expressed as mean ± SEM. * Different from control non-smokers ($p < 0.05$). † Different from smokers without COPD ($p < 0.05$).
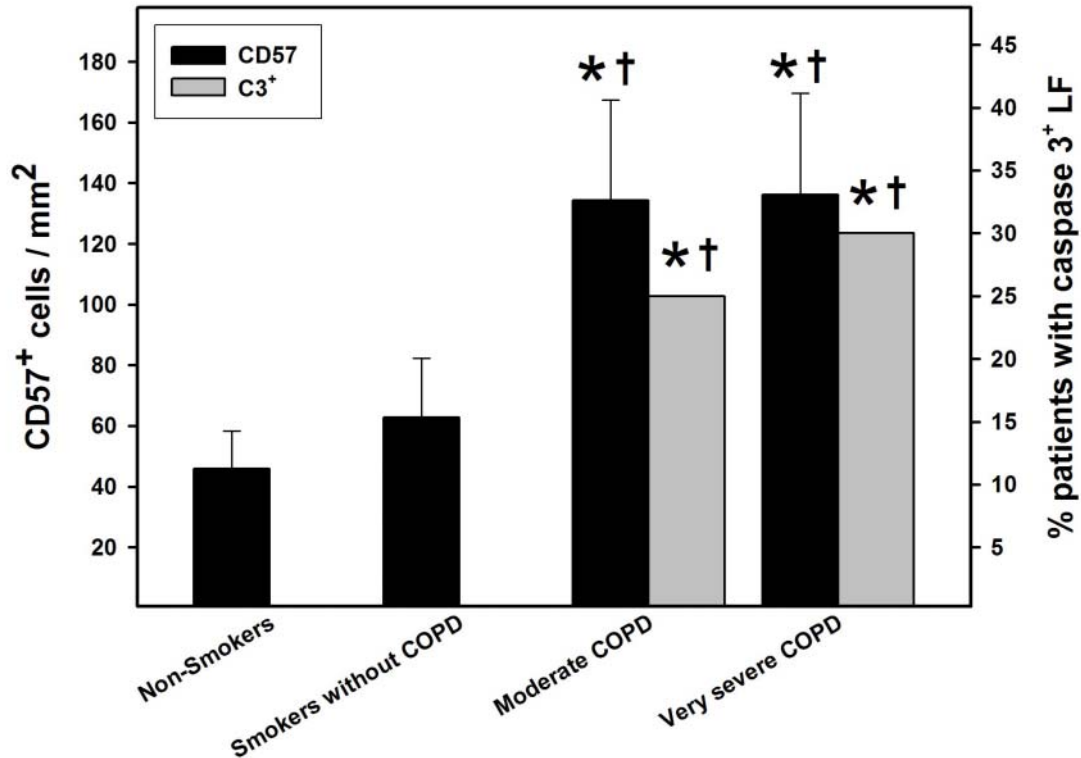

Figure 5. Double immunostaining of CD3 and CD57 antigens on LF. **A)** Representative micrograph of a double immunostaining of CD3 (black stained) and CD57 (brown stained) antigens on a LF of a COPD patient. **B)** Immunostaining of CD57 (brown stained) on a control consecutive section (3 μm apart) of the same follicle. **A, B)** Arrows indicate a CD3$^+$CD57$^-$ cell, circles indicate a CD3$^-$CD57$^+$ cell, and squares indicate a CD3$^+$CD57$^+$ cell. Scale bars = 20 μm.
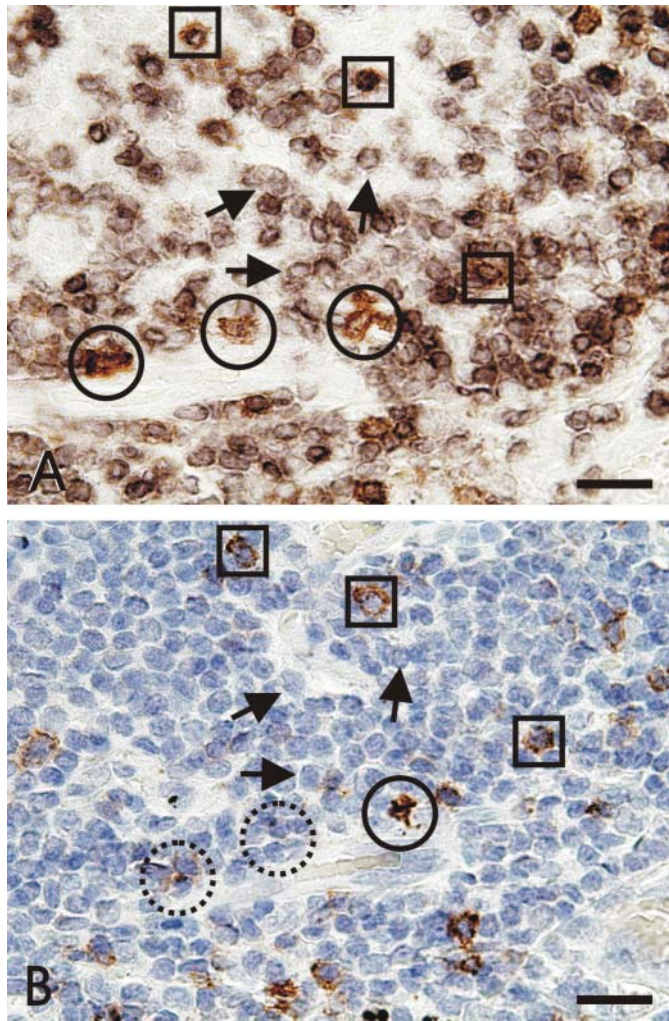
Figure 6. Proliferation and apoptosis in lung lymphoid follicles. **A)** Immunolocalization of BCL-6 in a LF of a non-smoker. **B** and **C)** Immunolocalizations of Ki-67 (**B**) and activated caspase 3 (**C**) in consecutive sections of a LF from a very severe COPD patient. Sections counterstained with eosin (**A**) and Friedlander's haematoxylin (**B** and **C**). Scale bars = 50 μm.