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Significant increase of CD57$^+$ cells in pulmonary lymphoid follicles of COPD patients

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

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

The density of CD57$^+$ cells within LFs of COPD patients was significantly increased compared to that of nonsmokers 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 LFs.

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

KEYWORDS: Cigarette smoking, follicles, immunohistochemistry, lung inflammation

Chronic obstructive pulmonary disease (COPD) is characterised by 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 in both 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 (LFs) 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 LFs 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 Global Initiative for Chronic Obstructive Lung Disease (GOLD) stage 0 to stage 4 is associated with the number of bronchioles containing LFs [3]. Furthermore, although the nature of the stimuli that trigger the formation of LFs 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].

Conversely, despite increased numbers of macrophages, dendritic cells, natural killer cells and lymphocytes having been reported in lung tissue of COPD patients [10, 11], few data are available on the follicular densities of these cells and the molecules involved in their recruitment to LFs in COPD, especially at the most severe stages of the disease. Indeed, only one study reported a higher percentage of T-regulatory cells within LFs of moderate COPD patients compared with smokers and nonsmokers [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

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are no studies examining which addressins are involved in the migration of inflammatory cells from the blood into the LFs in COPD.

The purpose of the present study is to examine the prevalence, localisation, vascularisation, addressin expression and inflammatory cell densities of LFs in lung tissue of moderate and very severe COPD patients compared to nonsmokers and smokers without COPD.

METHODS

Subjects

The study population comprised 59 subjects who underwent lung resection for non-obstructive peripheral lung tumours or were subjected to double lung transplantation for very severe COPD. The Ethics Committee of Vall d'Hebron Hospital (Barcelona, Spain) approved the study and written informed consent was obtained from all of the patients. Subjects were classified into four clinical groups according to their smoking habits (smokers and nonsmokers) and COPD severity [1]: nine nonsmoking patients (never-smokers) with normal lung function; 18 asymptomatic smokers with normal lung function; 16 smokers with moderate COPD (GOLD stage 2); and 16 smokers with very severe COPD (GOLD stage 4) 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, total lung capacity and carbon monoxide diffusing capacity of the lung ($D_{L,CO}$). All subjects had been free of acute lung infections and none had received chemotherapy before surgery.

Sample processing

The resected lungs or lobes obtained during surgery were immediately inflated with 4% formaldehyde prior to immersion in fixative for 24 h. After fixation, surgical specimens were serially sliced into 1-cm axial sections and the severity of emphysema was graded using the panel grid described by THURLBECK *et al.* [13]. Subsequently, $2 \times 2 \times 1$-cm randomly selected tissue blocks were excised, embedded in paraffin, cut into serial 4-μm sections and mounted on positively charged slides (Starfrost Plus; Menzel-Gläser, Braunschweig, Germany). One section per block was stained with haematoxylin and eosin.

Immunohistochemistry

The following antibodies were used at the indicated dilutions: polyclonal anti-CD3 (DakoCytomation, Glostrup, Denmark; 1:400), monoclonal anti-CD4 (Novocastra, Newcastle, UK; 1:50), monoclonal anti-CD8 (DakoCytomation; 1:100), monoclonal anti-CD20cy (DakoCytomation; 1:800), monoclonal anti-CD57 (DakoCytomation; 1:80), monoclonal anti-follicular dendritic cell (DakoCytomation; 1:20), monoclonal anti-CD83 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:30), monoclonal anti-CD68 (DakoCytomation; 1:50), monoclonal anti-platelet–endothelial cell adhesion molecule (PECAM)-1 (DakoCytomation; 1:200), monoclonal anti-podoplanin (Abcam, Cambridge, UK; 1:150), monoclonal anti-mucosal addressin cell adhesion molecule (MAdCAM)-1 (AbD Serotec, Kidlington, UK; 1:30), monoclonal anti- peripheral node addressin (PNAd) (BD Biosciences, Franklin Lakes, NJ, USA; 1:50), monoclonal anti-Ki-67 (DakoCytomation; 1:150), monoclonal anti-Bcl-6 (DakoCytomation; 1:60), and polyclonal anti-cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA; 1:200).

Immunostaining was performed using the avidin–biotin complex (ABC) immunoperoxidase method (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) with a 3,3′-diaminobenzidine tetrahydrochloride (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 permitted obtention of a differentiating black colour for T-cells. Following this amplification, the sections were incubated with monoclonal anti-CD57, with DAB development giving a brown colour. Hence, this design permitted differentiation between $CD3^+CD57^+$ cells (double-stained with black and brown) and $CD3^-CD57^+$ cells (brown-stained).

Assessment of LFs

Following the criteria previously described by ELLIOT *et al.* [14], LFs were defined as focal collections of >50 lymphomononuclear cells with a cell density (cells per millimetre squared) more than 10 times that of the surrounding tissue. Haematoxylin and eosin staining was used to assess the presence and location of LFs in samples from all patients. Furthermore, two sections per subject were analysed for each antibody.

For the assessment of cell densities, a point-counting method described previously was used [15]. Briefly, at least six fields were randomly and systematically sampled in order to assess cell densities in follicular tissue by using a grid with a known area attached to the eyepiece of the microscope. The number of points hitting each LF profile were counted and converted into square millimetres using a conversion factor calculated for the specific magnification used. 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 LFs. Cell densities were expressed as the number of cells per square millimetre of tissue examined.

In addition, the percentage of patients with positive staining for vascular structures and addressins within LFs was determined by considering two different locations of these markers: either at the LF periphery or inside it. The percentage of patients with positive staining for the markers of proliferation and apoptosis in LFs were also averaged. The cases were coded and the measurements made without knowledge of clinical data.

Statistical analysis

The descriptive statistical analysis included mean±SEM for each parameter. Clinical data were compared between groups using ANOVA. The prevalence, vascularisation, proliferation and apoptosis of LFs were compared between case groups using the Chi-squared test. Differences in LF cell densities were analysed 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. The significance level was set at $p<0.05$. All analyses were

performed using Statgraphics Centurion XV (StatPoint, Inc., Warrenton, VA, USA).

RESULTS

Demographic and clinical data

Table 1 shows the characteristics of the patient groups. The four clinical groups were similar with regard to age, and no significant difference was found in cumulative cigarette consumption among smokers with and without COPD. By contrast, the values of FEV_1, FEV_1/FVC and $D_{L,CO}$ were significantly different in COPD patients, the very severe COPD subjects showing the lowest values. Additionally, as expected, there was a significantly increased macroscopic emphysema degree (% predicted) in COPD patients, especially those at a very severe GOLD stage (table 1).

LF assessment

Structure and localisation

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

LFs were essentially distributed within three tissue compartments: bronchiolar and arteriolar adventitias, and parenchymal interstitium. Occasionally, some samples showed LFs in bronchial adventitias and lobular septa, which were not included in further analyses because of their low frequency.

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

Vascularisation

The presence and distribution of blood vessels in LFs was determined by the immunolocalisation of PECAM-1 (fig. 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 centre of the LF and also the adjacent connective tissue, both the flat and the high endothelial venule (HEV) phenotypes of endothelial cells being observed (fig. 1b).

The immunolocalisation of podoplanin permitted assessment of the presence of lymphatic vessels in the LFs (fig. 1c). The results showed that lymphatic endothelium was detected in around half (56%) of the LFs, without significant differences among groups. When the distribution of lymphatic vessels was assessed in these LFs, it was observed that only 12% showed lymphatic irrigation inside the LFs (fig. 1c), whereas 91% of the LFs 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 LFs, immunolocalisation of PNAd and MAdCAM-1 was carried out in the pulmonary samples. It was found that 35% of LFs 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 LFs (fig. 1D), as well as in some adventitial vessels of bronchioles and arterioles without tertiary lymphoid tissue.

Regarding the PNAd distribution in LFs, it was found that 82% showed peripheral PNAd-positive vessels, whereas 54%

TABLE 1 Clinical and demographic data

	Nonsmokers	Smokers		
		No COPD	Moderate COPD	Very severe COPD
Subjects n	9	18	16	16
Males/females n	1/8	14/4	16/0	13/3
Age yrs	61.8±4.6	60.9±2.6	63.3±2.0	55.9±1.3
Smoking history pack-yrs	0.0±0.0	49.9±5.9	61.4±5.4	47.9±5.7
Current smokers/ex-smokers n		6/12	9/7	1/15
Inhaled corticosteroids n	1	1	7	14
Lymphoid follicle prevalence %	88.9	83.3	100.0	93.8
FEV_1 % pred	99.3±8.2	84.9±2.6	69.3±4.5$^{\#,\P}$	20.8±1.2$^{\#,\P,+}$
FEV_1/FVC % pred	80.1±2.8	78.9±1.9	61.8±4.6$^{\#,\P}$	35.2±2.0$^{\#,\P,+}$
RV % pred	107.5±10.2	98.1±7.7	146.1±7.8	282.7±23.0$^{\#,\P,+}$
TLC % pred	101.0±2.8	90.5±3.8	113.8±3.7	135.2±9.0$^{\#,\P,+}$
$D_{L,CO}$ % pred	82.1±7.9	71.6±4.2	57.6±3.0$^{\#,\P}$	34.8±4.0$^{\#,\P,+}$
MED % pred	1.7±1.1	13.9±3.8	59.1±1.9$^{\#,\P}$	67.5±4.0$^{\#,\P,+}$

Data are presented as mean±SEM unless otherwise indicated. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in 1 s; % pred: % predicted; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; $D_{L,CO}$: carbon monoxide diffusing capacity of the lung; MED: macroscopic emphysema degree. $^{\#}$: $p<0.001$ *versus* nonsmokers; ¶: $p<0.001$ *versus* smokers without COPD; $^{+}$: $p<0.001$ *versus* smokers with moderate COPD.

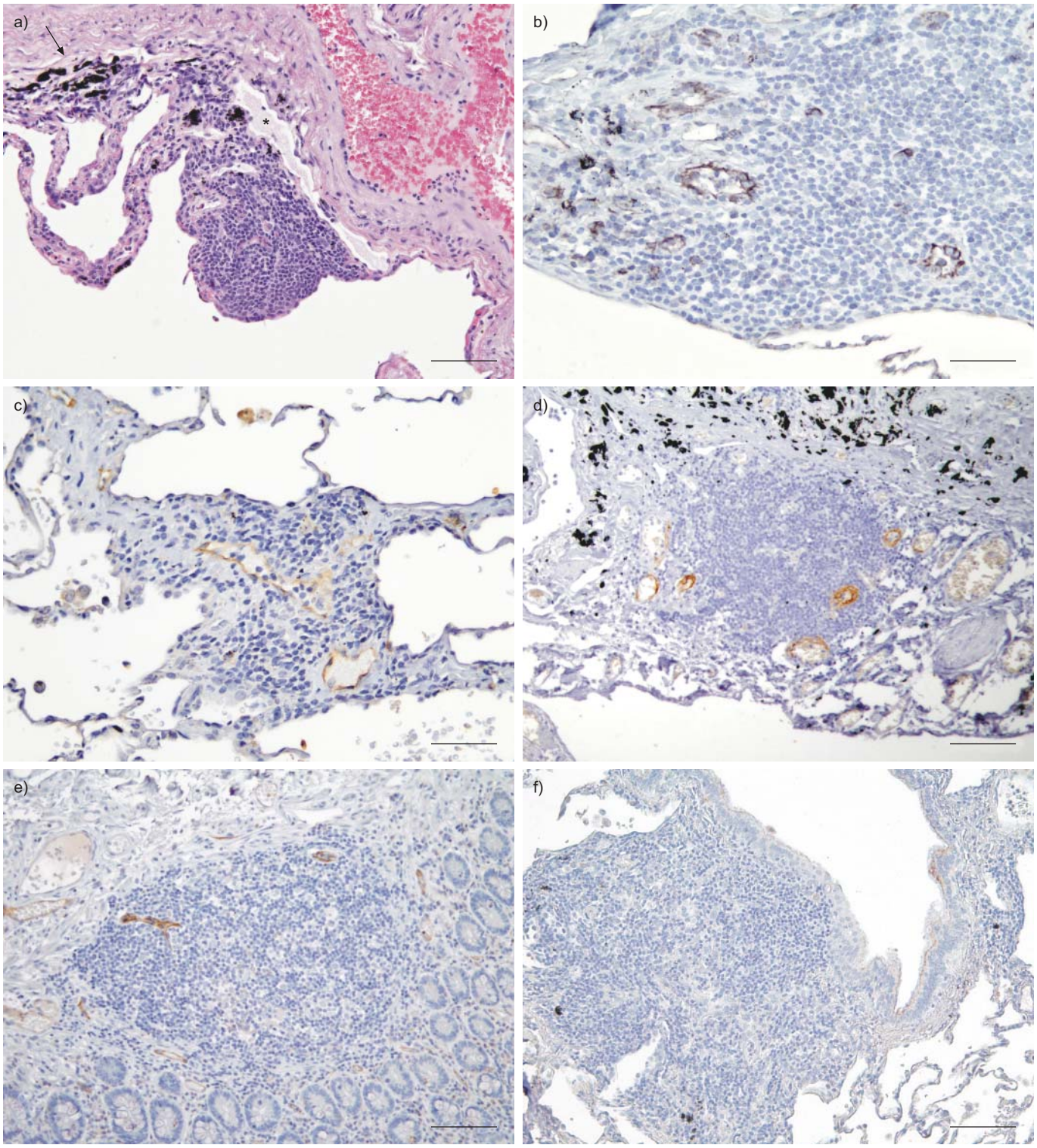

FIGURE 1. Vascularisation and addressins of lung lymphoid follicles (LFs). a) Haematoxylin–eosin staining of a LF in an arteriolar adventitia. Anthracotic granules were frequently found in the periphery of the lymphoid masses (arrow). A lymphatic vessel surrounds the LF (asterisk). b) Immunolocalisation of platelet–endothelial cell adhesion molecule (PECAM)-1 in lung LFs. Blood vessels were found inside and in the periphery of lymphoid masses. PECAM-1 was expressed in both the flat constitutive and high endothelial venule (HEV) phenotypes of endothelial cells. c) Immunolocalisation of podoplanin in lung LFs. Detail of a peripheral lymphatic vessel that penetrates into the lymphoid mass. d) Immunolocalisation of peripheral node addressin (PNAd) in lung LFs. PNAd was expressed in both HEVs and conventional vessels. e) Immunolocalisation of mucosal addressin cell adhesion molecule (MAdCAM)-1 in a Peyer's patch (positive control). f) Immunolocalisation of MAdCAM-1 in lung LFs. None of the LF profiles was MAdCAM-1-positive. Immunostained sections were counterstained with Friedlander's haematoxylin. Scale bars: a, d and e) 100 μm; b, c and f) 50 μm.

showed a central localisation, without significant differences among clinical groups. Nevertheless, severe COPD patients showed a higher number of LFs with central PNAd staining than the other groups (fig. 2).

In order to assess the reactivity of lung LFs for MAdCAM-1, a section of a small intestine with a Peyer's patch was used as positive control (fig. 1e). It was found that none of the pulmonary LF profiles were MAdCAM-1-positive, whatever the location or clinical group considered (fig. 1f).

Cell densities

$CD3^+$, $CD4^+$ and $CD8^+$ cells and B-lymphocytes, as well as macrophages and $CD57^+$ 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 LFs.

All LFs were characterised by a central core of B-lymphocytes, the most abundant follicular cells (fig. 3a), and a peripheral zone mainly infiltrated by T-lymphocytes (fig. 3b). Among these, $CD4^+$ and $CD8^+$ T-cells (fig. 3c and d) were distributed diffusely, in either the follicular core or surrounding germinal centres. 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 LF profiles (fig. 3e–h). By contrast, isolated follicular dendritic cells were only found in samples belonging to very severe COPD patients.

Importantly, when the cell densities of LFs were compared, it was found that COPD patients showed a significantly higher density of $CD57^+$ cells than did nonsmokers and smokers without COPD ($p<0.05$) (table 2, and figs 3g and h, and 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 immunostaining of CD3 and CD57 antigens was performed in the present series (fig. 5). Subsequently, the follicular density of both the $CD57^+CD3^-$ and $CD57^+CD3^+$ subpopulations was assessed. The results showed that the mean ratio of $CD57^+CD3^-$ cells to $CD57^+CD3^+$ plus $CD57^+CD3^-$ cells in LFs was 0.23. No significant differences among the clinical groups were found (0.21 in nonsmokers, 0.33 in smokers without COPD, 0.16 in moderate COPD patients and 0.29 in very severe COPD patients).

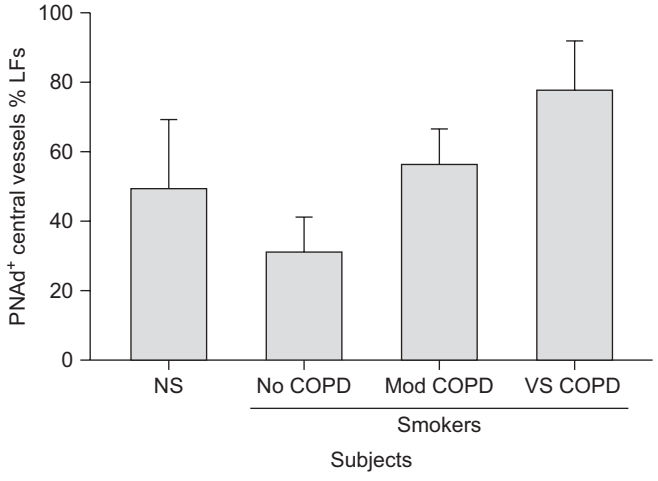

FIGURE 2. Percentage of lung lymphoid follicles (LFs) with peripheral node addressin $(PNAd)^+$ central vessels. Data are presented as mean ± SEM. Very severe chronic obstructive pulmonary disease patients showed a higher percentage of LFs with central PNAd staining, although no significant differences among groups were found ($p>0.05$). NS: nonsmokers; Mod: moderate; VS: very severe.

Proliferation and apoptosis

In order to examine whether or not LFs 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 LF was determined.

Only two LF profiles of a nonsmoker and a very severe COPD patient were positive for Bcl-6 staining (fig. 6a). By contrast, the Ki-67 staining (fig. 6b) demonstrated proliferation in the majority of LFs (ranging 56–91%), without significant differences among groups.

Regarding apoptosis, COPD patients showed a significantly higher percentage of samples with positive staining for activated caspase 3 than the other groups ($p<0.05$) (Figs. 4 and 6c). Indeed, 25% of moderate COPD patients and 30% of very severe COPD patients showed activated caspase 3 staining, whereas no LFs with positive staining were found in nonsmokers and smokers without COPD (fig. 4).

DISCUSSION

For a long time, the presence of LFs has been associated with COPD progression [3], but only recently has some attention 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, the present work represents a complete study of LFs, including their prevalence, vascularisation, cell composition and proliferation/apoptosis in patients with moderate and very severe COPD. The present results show, for the first time, that COPD patients exhibit a significant increase in the follicular density of $CD57^+$ cells compared to nonsmokers and smokers without COPD. Moreover, the percentage of LF profiles with cell apoptosis is also significantly increased in COPD patients.

A principal objective of the present study was to quantify the main cellular types inside LFs in order to compare their densities among clinical groups. Importantly, the results showed a significant and specific increase in the follicular density of $CD57^+$ cells in patients with COPD. This finding adds new knowledge about the role that LFs 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 recently been demonstrated that CD57 antigen is also a marker of terminally differentiated cells with a high cytolytic potential [18]. Indeed, CHATTOPADHYAY *et al.* [18] reported that CD57 expression was strongly correlated with simultaneous expression of pro-apoptotic molecules, such as granzyme A, granzyme B and perforin. Thus an increased density of the follicular $CD57^+$ cells in COPD patients may explain the high percentage of LF profiles with cell apoptosis

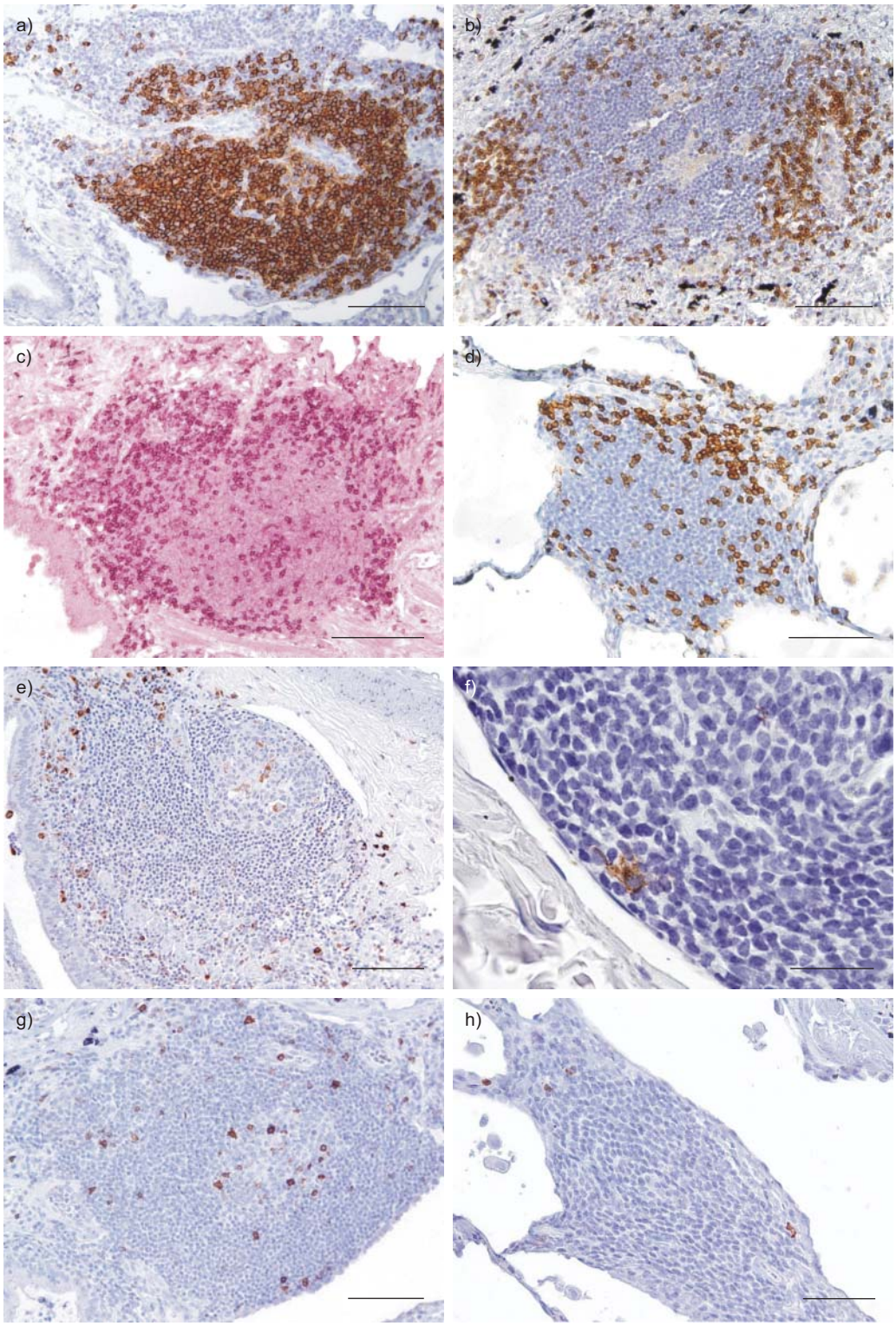

FIGURE 3. Immunolocalisation of the main cell types inside lung lymphoid follicles (LFs). a) B-lymphocytes were the most abundant cells within LFs, and followed a central distribution. b) T-lymphocytes were mainly found in the follicular periphery, with $CD4^+$ cells the most abundant subset (c). Both $CD4^+$ and $CD8^+$ (d) cells were also found in the follicular core. e) Macrophages were diffusely distributed through the lymphoid aggregates, whereas the presence of mature dendritic cells was sparse in most LF profiles (f). g) $CD57^+$ cells were found in both the periphery and the centre of LFs, and their density was significantly higher in LFs of COPD patients than in those of patients without COPD (h). Sections were counterstained with Friedlander's haematoxylin, except one (c), which was counterstained with eosin. Scale bars; a, d, e and g) 100 μm; b and c) 125 μm; f) 250 μm; and h) 250 μm.

TABLE 2 Lymphoid follicle cell densities

	Nonsmokers	Smokers		
		No COPD	Moderate COPD	Very severe COPD
Subjects n	9	18	16	16
$CD20^+$ B-lymphocytes 10^3 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 10^3 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 10^3 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 10^3 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 cell 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)$^{*,\#}$	110.1 (0.0–470.7)$^{*,\#}$

Data are presented as median (range). COPD: chronic obstructive pulmonary disease. *: $p<0.05$ *versus* nonsmokers; #: $p<0.05$ *versus* smokers without COPD.

that was observed in the moderate and very severe COPD groups.

Apart from their cytotoxicity, KIM *et al.* [19] showed that a subset of $CD57^+$ cells present in germinal centres of human lymphoid tissues induces B-cell differentiation and immunoglobulin production. In addition, LAFFONT *et al.* [20] demonstrated that natural killer cells act as regulators of alloreactive T-cell priming in allotransplantation by killing allogeneic dendritic cells in draining lymph nodes.

Recently, there has been increased interest in a better understanding of the pathophysiological role of LFs in COPD. There are several observations pointing to the fact that these structures could arise in response to chronic bacterial and viral colonisation or even against neo- or self-antigens, which have been hypothesised 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

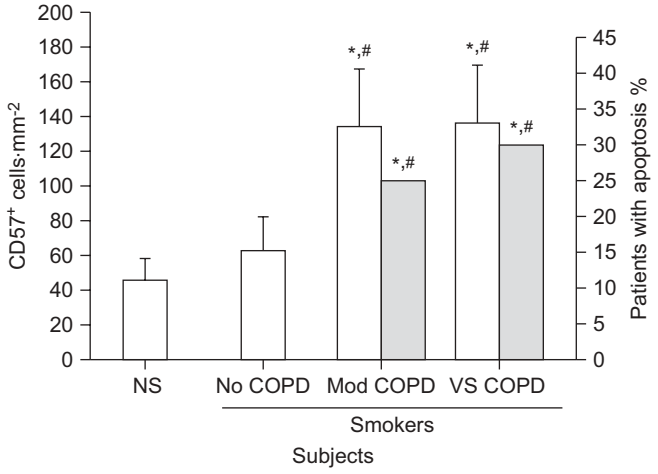

FIGURE 4. Follicular $CD57^+$ cell density (□) and apoptosis (caspase 3^+ lymphoid follicles (LFs); ■). Data are presented as mean±SEM. Moderate (Mod) and very severe (VS) chronic obstructive pulmonary disease (COPD) patients showed a significantly higher density of follicular $CD57^+$ cells and a significantly higher percentage of LF profiles with apoptosis than nonsmokers (NS) and smokers without COPD. *: $p<0.05$ *versus* NS; #: $p<0.05$ *versus* smokers without COPD.

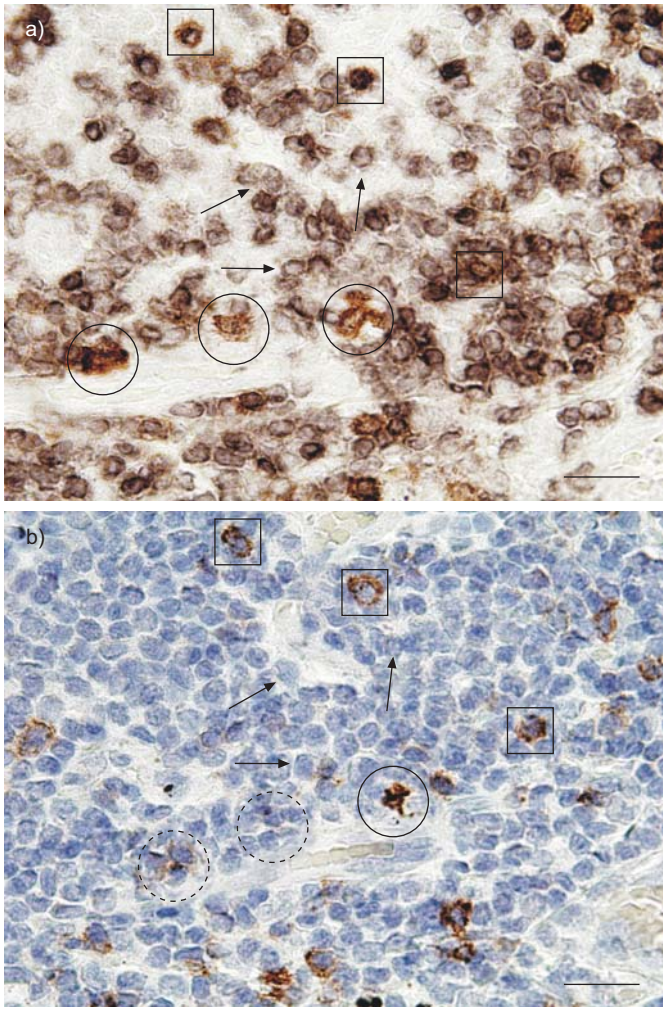

FIGURE 5. Double immunostaining of CD3 and CD57 antigens on lymphoid follicles (LFs). a) Representative micrograph of double immunostaining of CD3 (black) and CD57 (brown) antigens on a LF of a COPD patient. b) Immunostaining of CD57 (brown) on a control consecutive section (3 μm apart) of the same LF. Arrows indicate $CD3^+CD57^-$ cells, circles $CD3^-CD57^+$ cells and squares $CD3^+CD57^+$ cells. Scale bars=20 μm.

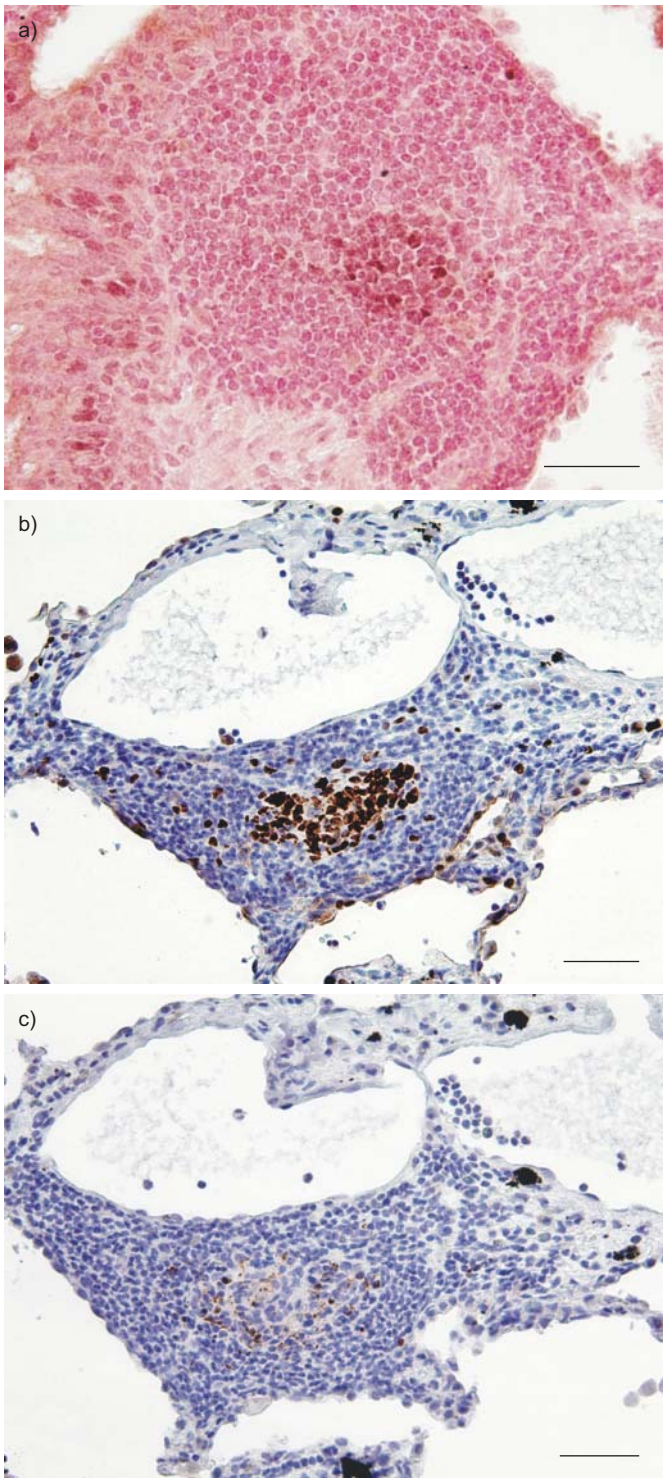

FIGURE 6. Proliferation and apoptosis in lung lymphoid follicles (LFs). a) Immunolocalisation of Bcl-6 in a LF of a nonsmoker. Immunolocalisation of Ki-67 (b) and activated caspase 3 (c) in consecutive sections of a LF from a very severe chronic obstructive pulmonary disease patient. Sections were counterstained with eosin (a) and Friedlander's haematoxylin (b and c). Scale bars=50 μm.

antigen is expressed by many cells during chronic immune activation [21] or under clinical conditions, such as infections or immune dysfunction [17].

By contrast, although previous data of PLUMB *et al.* [5] indicated an increase in $CD4^+$ T-cells in parenchymal LFs of moderate COPD patients, the present 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 the present study was to examine the structure and localisation of LFs in order to find existing differences between subjects with and without COPD. We have stated that LFs were located in both the adventitia of airways and the parenchyma of pulmonary samples, as previously described [2–6, 14]. However, the presence of LFs was also observed in the connective tissue of lobular septa and in the arteriolar adventitia, the later compartment being the most frequently occupied by LFs 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 *et al.* [3] reported an association between the number of small airways containing LFs and COPD severity. However, when the number and distribution of LF profiles for each compartment (small airways, arterioles and parenchyma) were compared, 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 *et al.* [22], found a negative association between steroid therapy and the percentage of airways containing LFs.

Although no data are available regarding the effect of inhaled steroids on infiltrated $CD57^+$ cells, it has been shown that steroid therapy drastically reduces 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 reduces these lymphocyte counts [24]. Hence, if inhaled steroids could have influenced the present results, their hypothetical effect would make the differences found in the follicular density of $CD57^+$ cells more remarkable.

Immunolocalisation of PECAM revealed that LFs of subjects with and without COPD exhibit a defined pattern of vascularisation, 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 [26] and subjects with asthma [14]. Conversely, the immunolocalisation of podoplanin showed that most LF profiles did not present lymphatic vessels inside, suggesting that these LFs lack their own lymphatic drainage. This could be related to the absence of the 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, permitting 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 regarding the expression of PNAd and MAdCAM-1 in LFs of COPD patients. The results showed that 35% of the LF profiles were $PNAd^+$, the very severe COPD

patients showing the highest number of LF profiles with central PNAd staining. Conversely, none of the LF profiles showed positive staining for MAdCAM-1. These results add new knowledge to previous studies reporting the expression of PNAd in LFs of patients with idiopathic pulmonary fibrosis [26] and to others reporting an absence of MAdCAM-1 expression in bronchus-associated lymphoid tissue of mice [29]. This specific expression of PNAd correlates with that which is characteristic of the ectopic lymphoid tissue found in chronic inflammatory diseases [30, 31].

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

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STATEMENT OF INTEREST

None declared.

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