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Lung CD57+ cell density is increased in very severe COPD

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Summary. Among all inflammatory cells involved in COPD, those with a cytolytic or elastolytic activity are thought to play a key role in the pathogenesis of the disease. However, there is no data about the infiltration of cells expressing the CD57 marker in small airways and parenchyma of COPD patients.

In this study, surgical specimens from 43 subjects undergoing lung resection due to lung cancer (9 non-smokers, 18 smokers without COPD and 16 smokers with moderate COPD) and 16 patients undergoing double lung transplantation for very severe COPD were examined. $CD57^+$ cells, neutrophils, macrophages and mast cells infiltrating bronchioles (epithelium, smooth muscle and connective tissue) and parenchymal interstitium were localized and quantified by immunohistochemical analysis.

Compared to the other groups, the small airways of very severe COPD patients showed a significantly higher density of $CD57^+$ cells, mainly infiltrated in the connective tissue ($p=0.001$), and a significantly higher density of neutrophils located characteristically in the epithelium ($p=0.037$). Also, the density of neutrophils was significantly higher in parenchyma of very severe COPD patients compared with the rest of the groups ($p=0.001$). Finally, there were significant correlations between the bronchiolar density of $CD57^+$ cells and the FEV_1 values ($R=-0.43$, $p=0.022$), as well as between the parenchymal density of neutrophils and macroscopic emphysema degree ($R=0.43$, $p=0.048$) in COPD groups.

These results show that $CD57^+$ cells may be involved in COPD pathogenesis, especially in the most severe stages of the disease.

Key words: Cigarette smoking, Cytolytic cells, Immunohistochemistry, Lung inflammation

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major health problem which is expected to be the third leading cause of death worldwide by 2020 (López et al., 2006). COPD is defined as a progressive and not fully reversible airflow limitation, which is associated with an abnormal inflammatory response of the lung to noxious particles and gases, mainly cigarette smoke (Global Initiative for Chronic Obstructive Pulmonary Disease, GOLD, 2010). Although cigarette smoking is the most commonly encountered risk factor in COPD, others including genetic susceptibility, oxidative stress and respiratory infections are thought to contribute to the pathogenesis of the disease (GOLD, 2010). The characteristic chronic inflammatory pattern (mononuclear infiltration and tissue remodeling) in COPD affects mainly small airways and lung parenchyma, resulting in bronchiolitis and emphysema development.

Several types of inflammatory cells, belonging both to the innate and the adaptive immune systems, are thought to be implicated in the complex inflammatory status of COPD. Among these cells, those with cytolytic or elastolytic capability are relevant in COPD pathogenesis, due to their role in enhancement of the inflammatory response and development of emphysema (Barnes and Cosío, 2006).

Increased numbers of $CD8^+$ cells have been documented in pulmonary tissue of COPD patients,

which correlated with lung function parameters and disease severity (O'Shaughnessy et al., 1997; Saetta et al., 1999; Hogg et al., 2004; Olloquequi et al., 2010). Furthermore, a recent study (Urbanowicz et al., 2010) has shown a higher proportion and an increased lytic activity of NK cells and NKT-like cells in the induced sputum of COPD patients as compared to non-smokers and smokers without COPD. However, very little is known about the possible role that other cells with a cytolytic potential, such as cells expressing CD57, could play in COPD. CD57 is a marker of terminally differentiated cells with a killer activity (Brenchley et al., 2003; Focosi and Petrini, 2007; Chattopadhyay et al., 2009). It has been demonstrated that $CD57^+$ cells infiltrating lung tissue of smokers both with and without COPD express granzymes A and B (Vernooy et al., 2007), and a high density of these cells have been found in both the bronchial mucosa (Di Stefano et al., 1998) and lung lymphoid follicles (Olloquequi et al., 2011) of COPD patients. Although these findings point to an involvement of $CD57^+$ cells in the pathology of COPD, there is no data about the density and distribution of these cells in the two main compartments affected in the disease, i.e., small airways and lung parenchyma.

Regarding the involvement of elastolytic cells in COPD, the hypothesis that neutrophils and macrophages play a key role in the pathogenesis of the disease is one of the most widely accepted. However, the results published to date are not conclusive, since some findings show significantly increased numbers of neutrophils or macrophages in small airways and lung parenchyma of COPD patients (Finkelstein et al., 1995; Grashoff et al., 1997; Pilette et al., 2001; Retamales et al., 2001; Turato et al., 2002), while other findings rule out a relationship between these cells and the disease (Bosken et al., 1992; Lams et al., 1998; Saetta et al., 1998; Saetta et al., 1999; Majó et al., 2001). The lack of conclusive results could be largely due to the scarcity of data from the group of very severe COPD patients.

In order to shed light on these questions, our present study systematically examines the densities and localization of cells with cytolytic/elastolytic capabilities infiltrating bronchiolar compartments and parenchymal interstitium of moderate and very severe COPD patients.

Materials and methods

Patients

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 written informed consent was obtained from all patients. Spirometry and diffusing capacity measurements and body plethysmography were performed to assess pulmonary function using standard procedures (Crapo et al., 1995) and equipment (Masterlab; Jaeger, Würzburg, Germany). None of the subjects had had a COPD exacerbation during a minimum of 3 weeks prior the surgery, 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 (GOLD, 2009): 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.

Sample processing

Immediately after surgery, the excised lungs or lobes were inflated and fixed with 4% formaldehyde. After fixation, surgical specimens were sliced and the severity of emphysema (macroscopic emphysema degree, MED, %) was graded using a panel grid as previously described (Thurlbeck et al., 1970). After that, 2x2x0.3 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).

In samples from patients with non-obstructing peripheral masses, sections were taken from sites macroscopically free from tumor, always farther than 2 cm from the masses. A further microscopic examination was also performed by an expert pathologist in order to exclude a tumor infiltration in the sample.

Immunohistochemistry

The following antibodies were used at indicated dilutions: monoclonal anti-CD57 (Clone TB01, 1:80; DakoCytomation, Glostrup, Denmark), monoclonal anti-CD68 (for macrophages, Clone KP1, 1:50; DakoCytomation), monoclonal anti-Human Neutrophil Elastase (Clone 121-3E10, 1:250; Calbiochem, San Diego, USA) and monoclonal anti-Human Mast Cell Tryptase (Clone AA1,1:800; DakoCytomation). Primary antibody binding was detected using the ABC immunoperoxidase method (Vectastain Elite ABC kit, Burlingame, USA) with a DAB reaction. Negative controls were carried out on parallel sections by omission of the primary antibody and by substitution of the primary antibody with the appropriate isotype control (Negative control Mouse IgM, clone DAK-G08 and Negative control Mouse IgG1, clone DAK-G01, Dako).

Cell counts

For the assessment of cell densities, the numbers of cell profiles including a nucleus were counted and expressed per tissue area, the last determined by point counting, as previously described (Olloquequi et al., 2010). Briefly, twelve fields were randomly and

systematically sampled to assess cell densities in parenchymal interstitium, while in small airways, several non-overlapping fields were assessed until all the available area of each bronchiole was covered. By using a grid with a known area attached to the eye piece of the microscope, the number of points hitting the tissue compartment of interest (parenchymal interstitium and bronchiolar epithelium, smooth muscle and adventitia) were counted and converted into square millimeters. In turn, for 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 fields. Cell densities were expressed as number of cells per square millimetres of tissue examined.

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 clinical groups were similar with regard to age and no significant difference was found in pack-years among 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}. Moreover, there was a significantly increased macroscopic emphysema degree (MED, %) in COPD patients, especially those in very severe GOLD stage (Table 1).

Immunohistochemistry

Small airways

In all patients, the density of $CD57^+$ cells, neutrophils, macrophages and mast cells was assessed in the entire bronchiolar wall, as well as in the bronchiolar epithelium, connective (subepithelial and adventitial) and smooth muscle layers. Results are summarized in Table 2 and Fig. 1.

Patients with very severe COPD showed a significantly higher density of $CD57^+$ cells in small airways than non-smokers and smokers without COPD ($p=0.021$, Table 2, Fig. 1A,B). Furthermore, the density of neutrophils was significantly increased in the bronchiolar wall of very severely diseased patients when compared with the rest of the groups ($p=0.049$, Table 2).

The assessment of each bronchiolar compartment revealed a significantly higher density of neutrophils in the epithelium of very severe COPD patients compared with the rest of the groups ($p=0.037$, Table 2, Fig. 1C,D). Although the difference was not statistically significant, the density of $CD57^+$ cells, macrophages and mast cells was also higher in the epithelium of COPD patients compared to patients without COPD.

Regarding the bronchiolar connective tissue, there was a significantly increased density of $CD57^+$ cells in very severe COPD patients compared to the rest of the groups ($p=0.001$, Table 2). No differences were found among clinical groups for the other inflammatory cells in the connective compartment. Therefore, the small airways of very severe COPD patients are characterized by two kinds of cell infiltration: an epithelial infiltrate of

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	61.78±4.62	60.94±2.58	63.34±2.01	55.93±1.34
Smoking history, pack-years	0.00±0.00	49.89±5.86	61.44±5.35	47.87±5.72
Smoking status, current / ex-smoker	-	6 / 12	9 / 7	1 / 15
Inhaled corticosteroids use, yes / no	1 / 8	1 / 17	7 / 9	14 / 2
FEV1, % predicted	99.34±8.16	84.98±2.57	69.34±4.52*†	20.78±1.22*†‡
FEV1/FVC, % predicted	80.07±2.77	78.99±1.86	61.78±4.64*†	35.18±2.01*†‡
RV, % predicted	107.53±10.17	98.08±7.69	146.06±7.82	282.65±23.02*†‡
TLC, % predicted	101.02±2.82	90.5±3.75	113.76±3.70	135.17±9.00*†‡
DLCO, % predicted	82.13±7.86	71.62±4.21	57.56±3.03*†	34.79±4.01*†‡
MED, % predicted	1.67±1.10	13.89±3.81	59.14±1.99*†	67.50±4.03*†‡

Definition of abbreviations: FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; DLCO: 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 ($p<0.001$).

neutrophils near airway lumen, and another of $CD57^+$ cells located deeper in the connective tissue.

Finally, the infiltration in smooth muscle was minimal and no statistical differences were observed among the four groups (Table 2).

Parenchymal interstitium

When the density of inflammatory cells was assessed in lung parenchymal interstitium, it was found that very severe COPD patients had a higher number of neutrophils per square millimeter of parenchymal interstitium than the other groups (p=0.001, Table 2). Although the density of $CD57^+$ cells was also higher in patients with very severe COPD, no statistical differences were found for these cells, neither for macrophages nor mast cells, among clinical groups (Table 2).

Correlations

When moderate and very severe patients were considered as one group, some significant correlations were found between cell densities and clinical data. The density of $CD57^+$ cells in the bronchiolar wall was inversely correlated with FEV1 (% of predicted) values (R=-0.43, p=0.022). Moreover, the density of neutrophils in parenchymal interstitium was significantly correlated with the FEV_1 (R=-0.46, p=0.041) and with the macroscopic emphysema degree values (R=0.43, p=0.048).

Finally, although the correlations were maintained when all smokers were considered as a group, the values were, mainly, lower than those found when only COPD patients were grouped. There was an inverse correlation between the density of bronchiolar $CD57^+$ cells and the FEV_1 (% of predicted) values (R=-0.36, p=0.003). Moreover, the density of neutrophils in the bronchiolar wall was also correlated with a decrease in FEV_1 values (R=-0.44, p=0.006). With reference to parenchymal interstitium, there was an inverse correlation between the density of neutrophils and the FEV_1 values (R=-0.37, p=0.026) as well as with the macroscopic emphysema degree (R=0.49, p=0.002).

Discussion

Our present study shows for the first time that very severe COPD patients have a significantly higher density of $CD57^+$ cells and neutrophils in small airways than non-smokers and smokers without COPD. Furthermore, the bronchiolar density of these cells is inversely correlated with the FEV_1 (% of predicted) values. Additionally, our results show a significant increase of

Table 2. Bronchiolar and parenchimal densities of innate immune cells.

	Non-smokers (n=9)	Smokers without COPD (n=18)	Moderate COPD (n=16)	Very severe COPD (n=16)
Bronchiolar wall				
$CD57^+$, cells/mm^2	0.00 (0.00-21.66)	6.91 (0.00-60.00)	8.75 (0.00-118.18)	26.67 (0.00-78.57) *†
$CD68^+$ (Macrophages), cells/mm^2	142.79 (68.06-236.07)	126.86 (26.20-339.66)	152.51 (41.66-236.07)	120.10 (48.33-324.97)
$Elastase^+$ (Neutrophils), cells/mm^2	0.00 (0.00-0.00)	6.30 (0.00-65.60)	2.37 (0.00-15.85)	46.82 (0.00-238.14) *†‡
$Tryptase^+$ (Mast cells), cells/mm^2	155.11 (102.65-367.14)	187.03 (70.00-328.17)	209.09 (33.75-506.99)	204.12 (43.21-400.83)
Bronchiolar epithelium				
$CD57^+$, cells/mm^2	0.00 (0.00-16.66)	0.00 (0.00-100.00)	4.51 (0.00-366.66)	8.33 (0.00-105.66)
$CD68^+$ (Macrophages), cells/mm^2	13.72 (0.00-45.47)	45.86 (0.00-311.55)	50.00 (8.33-262.38)	63.00 (0.00-129.00)
$Elastase^+$ (Neutrophils), cells/mm^2	0.00 (0.00-0.00)	12.05 (0.00-100.00)	14.28 (0.00-125.00)	91.53 (0.00-396.87) *†‡
$Tryptase^+$ (Mast cells), cells/mm^2	34.22 (10.11-112.37)	50.00 (7.50-212.05)	101.85 (12.50-152.11)	103.85 (0.00-350.00)
Bronchiolar smooth muscle				
$CD57^+$, cells/mm^2	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.00 (0.00-12.00)	0.00 (0.00-12.50)
$CD68^+$ (Macrophages), cells/mm^2	0.00 (0.00-10.00)	0.00 (0.00-33.00)	1.66 (0.00-90.92)	1.92 (0.00-18.19)
$Elastase^+$ (Neutrophils), cells/mm^2	0.00 (0.00-3.20)	0.00 (0.00-12.16)	0.00 (0.00-17.02)	0.00 (0.00-15.22)
$Tryptase^+$ (Mast cells), cells/mm^2	5.55 (0.00-526.58)	0.00 (0.00-556.67)	0.00 (0.00-60.00)	0.00 (0.00-11.11)
Bronchiolar connective tissue				
$CD57^+$, cells/mm^2	0.00 (0.00-25.00)	2.20 (0.00-72.98)	9.52 (0.00-64.43)	25.00 (0.00-92.50) *†‡
$CD68^+$ (Macrophages), cells/mm^2	219.69 (96.89-531.31)	191.65 (20.35-510.56)	250.46 (41.66-511.12)	96.26 (36.57-462.06)
$Elastase^+$ (Neutrophils), cells/mm^2	0.00 (0.00-0.00)	6.44 (0.00-96.66)	5.18 (0.00-83.35)	13.75 (0.00-151.25)
$Tryptase^+$ (Mast cells), cells/mm^2	310.26 (154.49-494.52)	278.59 (91.51-472.22)	330.91 (41.66-647.97)	235.41 (86.21-504.13)
Parenchymal interstitium				
$CD57^+$, cells/mm^2	14.16 (1.81-75.75)	11.01 (0.00-80.95)	8.33 (0.00-76.42)	23.00 (0.00-56.89)
$CD68^+$ (Macrophages), cells/mm^2	9.31 (0.00-33.68)	11.92 (0.00-51.54)	12.38 (0.00-78.80)	8.09 (0.00-28.97)
$Elastase^+$ (Neutrophils), cells/mm^2	2.38 (0.00-18.89)	2.70 (0.00-15.39)	2.65 (0.00-20.02)	4.25 (0.00-21.42)*†‡
$Tryptase^+$ (Mast cells), cells/mm^2	109.67 (32.91-271.65)	106.46 (33.64-208.33)	101.49 (17.16-220.71)	122.96 (0.00-267.98)

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).

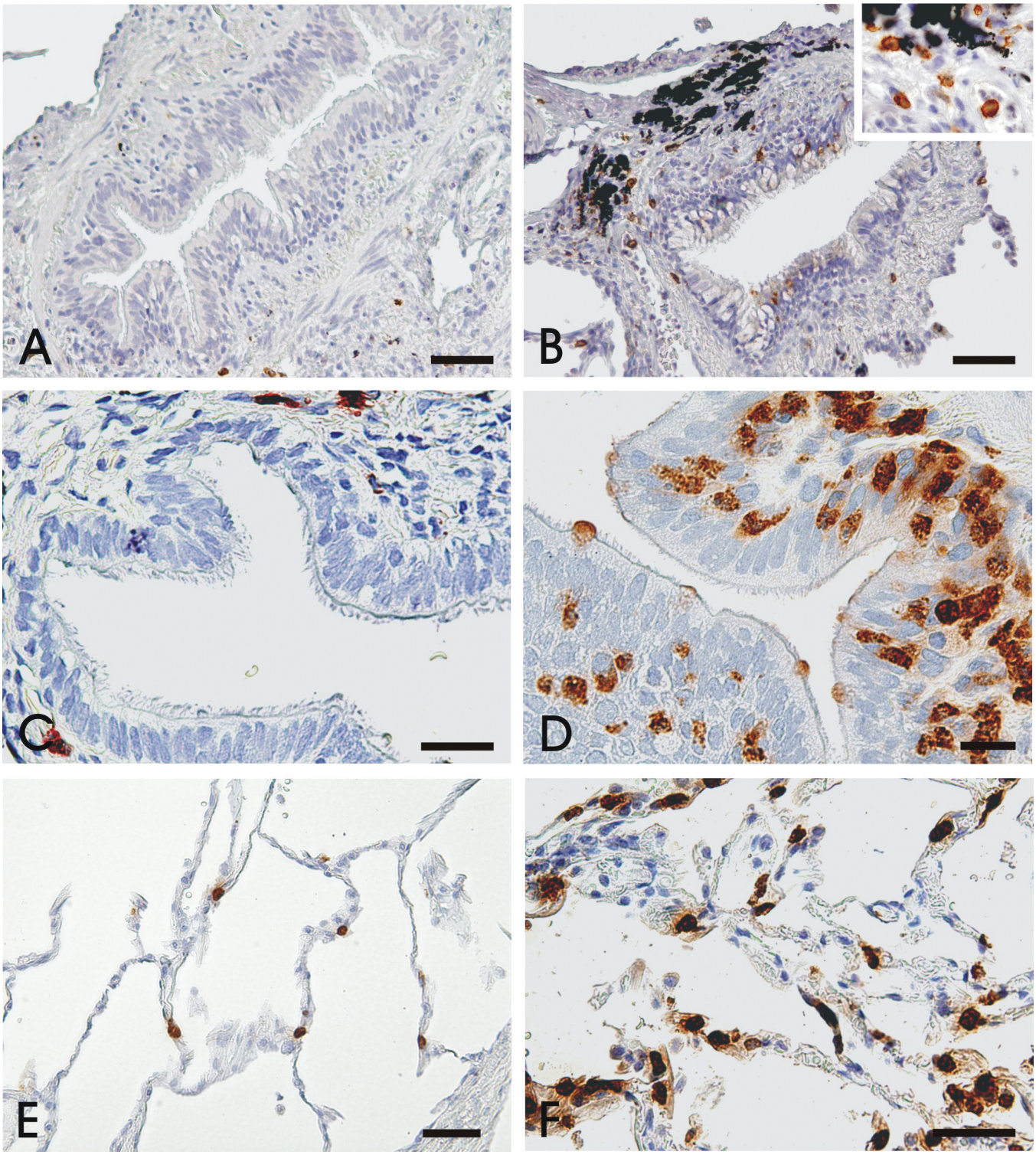

Fig. 1. Immunolocalization of CD57⁺ cells and neutrophils in peripheral lung tissue. **A, B.** CD57⁺ cells infiltrating small airways of a control smoker **(A)** and a patient with very severe COPD **(B)**. Very severe COPD patients showed a significantly higher infiltration of CD57⁺ cells in the bronchiolar connective tissue compared to the rest of the groups (p=0.001). Inset in **(B)** represents a close-up image of bronchiolar CD57⁺ cells. **C, D.** Infiltration of neutrophils in a small airway of a non-smoker **(C)** and a patient with very severe COPD (**D**). The density of neutrophils in the epithelial layer was higher in very severe COPD compared to the rest of the groups (p=0.037). **E, F.** Neutrophil infiltration in lung parenchyma of a non-smoker (**E**) and a patient with very severe COPD (**F**). The density of neutrophils was higher in parenchymal interstitium of very severe COPD patients than in the rest of the groups (p=0.001). Sections were counterstained with Friedlander's haematoxylin. Scale bars: A, B, 75 μm; C, D, 25 μm; E, F, 50 μm.

neutrophils in parenchymal interstitium of very severe COPD patients, which is also correlated with both the FEV1 and with the degree of macroscopic emphysema.

The CD57 epitope was first described to be selectively expressed on human NK and NK T cells (Abo and Balch, 1981). Importantly, in light of recent findings, CD57 appears to be a good marker of differentiated inflammatory cells with cytolytic activity. It has been demonstrated that CD57 expression is strongly correlated with a simultaneous expression of pro-apoptotic molecules, such as granzyme A, granzyme B and perforin in peripheral blood mononuclear cells (Chattopadhyay et al., 2009). Parallel to this, it is noteworthy that Vernooy et al. (2007) showed that $CD57^+$ cells infiltrating lung tissue of smokers with and without COPD express granzymes A and B.

To the best of our knowledge, this is the first study providing data on $CD57^+$ cell density in small airways and lung parenchyma of COPD patients. We have found a significant increase of these cells in small airways of very severe COPD patients compared to smokers without COPD and non-smokers. Moreover, the assessment of each bronchiolar compartment allowed us to observe that $CD57^+$ cells were mainly infiltrated in the bronchiolar connective tissue. These data extend the previous findings of Di Stefano et al. (1998), who found increased $CD57^+$ cells in bronchial subepithelium of severe COPD subjects, as well as extending the findings of our previous study (Olloquequi et al., 2011) reporting an increased density of $CD57^+$ cells in lung lymphoid follicles in COPD. In addition to the aforementioned alterations in cell population, our results show an inverse correlation between the bronchiolar density of $CD57^+$ cells and the FEV_1 values, concurring with the results obtained by Di Stefano et al. (1998) in large airways. However, our results show no statistical differences in parenchymal $CD57^+$ cells among groups, unlike our results obtained in small airways.

Athough our findings do not show a higher density of pulmonary $CD57^+$ cells in smokers without COPD compared to non-smokers, it is known that smoking *per se* increases the number of inflammatory cells in lung tissue (Tanni et al., 2010). This suggests that, although the inflammatory pattern initiated by cigarette smoke could induce the recruitment of cytolitic cells in the lungs, other stimuli are also required to enhance the infiltration of $CD57^+$ cells.

As CD57 is mainly expressed on activated cytolytic cells, one question emerges from our results: which mechanisms are involved in the activation of these cells? It has been recently reported that dendritic cells can enhance NK cell cytotoxicity through Jagged2-Notch interaction in a murine model (Kijima et al., 2008). Furthermore, although some authors reported that cigarette smoke may suppress dendritic cell maturation (Tsomakidou et al., 2009) others have shown an increase of dendritic cells in lung tissue of COPD patients (Van Pottelberge et al., 2010; Vassallo et al., 2010). Thus, it is conceivable that an accumulation of dendritic cells in lung could account for an activation of NK and NK T cells.

Although there is evidence pointing toward CD57 as a marker of lung inflammation (Palmer et al., 2007), it is difficult to discuss the functional consequences of the presence of $CD57^+$ cells in COPD, since studies of these cells in respiratory diseases are scarce. However, both the population increase of $CD57^+$ cells in bronchioles of very severely diseased patients and the inverse correlation with FEV_1 suggest an important role for these cells in COPD pathogenesis. $CD57^+$ cells may act by promoting an inflammatory status in small airways of COPD patients, as was proposed for $CD8^+$ or B-lymphocytes (Barnes and Cosio, 2006; Olloquequi et al., 2010), through the release of some inflammatory mediators. For instance, cells expressing CD57 are capable of immediate functional activity by producing IFN-γ and TNF-α (Freeman et al., 2010). Moreover, $CD57^+$ cells express granzyme A, a serine protease that can directly activate certain cytokines (Irmler et al., 1995), as well as induce secretion of IL-6 and IL-8 (two major mediators of the inflammatory response) in lung fibroblasts, among other cell types (Sower et al., 1996a,b). In addition, this hypothesis for a role of $CD57^+$ cells in enhancing the inflammatory response in COPD is reinforced by the increase of CD57 expression observed during chronic immune activation (Tarazona et al., 2000). Nevertheless, since the percentage of $CD57^+$ cells increases in some infectious diseases (Focosi et al., 2010), it is also possible that the increased bronchiolar density of $CD57^+$ cells in COPD may occur in response to repeated and worsening viral or bacterial infections, which are frequent in COPD patients (Di Stefano et al., 1998; Sethi and Murphy, 2008).

Regarding the distribution of other key cells in COPD pathogenesis, either by their elastolytic activity and/or by their inflammatory potential, the present study provides relevant data on a seldom studied group: patients submitted to double lung transplantation for very severe COPD. Concurring with previous studies (Bosken et al., 1992; Lams et al., 1998; Saetta et al., 1998, 1999; Turato et al., 2002), our results showed no significant differences in bronchiolar or parenchymal densities of macrophages and mast cells among patients with or without COPD. However, there are studies (Finkelstein et al., 1995; Grashoff et al., 1997; Retamales et al., 2001; Turato et al., 2002; Andersson et al., 2010) reporting increased numbers of these cells in small airways or lung parenchyma of COPD patients. These contradictory findings may be attributable to the origin of the samples and the different clinical groups considered. By contrast, we have found a significant increase of neutrophils in both small airways and parenchymal interstitium of very severe COPD patients compared with the other groups. Moreover, both the bronchiolar and the parenchymal densities of neutrophils correlated with a decrease of FEV_1, whereas a positive correlation was found between parenchymal neutrophils and the degree of macroscopic emphysema. Although an

inverse relationship between neutrophils and FEV_1 has been reported in large airways of COPD patients (O'Shaughnessy et al., 1997; Di Stefano et al., 1998; Baraldo et al., 2004), to the best of our knowledge, this is the first study describing a similar correlation in small airways and lung parenchyma in COPD.

Our results from very severely diseased patients (GOLD stage IV) extend the results of previous studies reporting high numbers of neutrophils in bronchioles (Pilette et al., 2001) and parenchyma (Retamales et al., 2001) obtained in severe COPD. In one of these studies, Retamales et al. (2001) described a relationship between the number of neutrophils in parenchymal tissue and the amount of emphysematous destruction assessed by computed tomography.

Despite these results, there are also studies showing no differences in bronchiolar (Bosken et al., 1992; Grashoff et al., 1997; Lams et al., 1998; Saetta et al., 1998; Turato et al., 2002) or parenchymal (Saetta et al., 1999) densities of neutrophils among patients with or without COPD.

These inconclusive results could be explained by the short lifespan of the infiltrating neutrophils (O'Donnell et al., 2006). Furthermore, unlike our present work, none of the aforementioned studies included a group of patients with very severe COPD (GOLD stage IV). Although all the patients enrolled in our study were in a stable condition, it is conceivable that an increase in neutrophil density may be more easily detected in very severe COPD patients, since these subjects are likely to suffer repeated infections which could enhance the recruitment of neutrophils to lung tissue (Sower et al., 1996; Retamales et al., 2001). In any event, neutrophils have been largely related to airway inflammation and parenchymal destruction in COPD (O'Donnell et al., 2006; Di Stefano et al., 2009; Murugan and Peck, 2009). Hence, our results of increased neutrophil densities in both small airways and parenchyma in very severe COPD patients, together with their correlation with FEV_1 and emphysema, contribute new evidence to the arguments for a key role of these cells in COPD.

The possibility that corticosteroids treatment could have influenced the numbers of inflammatory cells should be taken into account, since most patients in our study received inhaled steroids. Indeed, it is known that corticosteroids may decrease the apoptosis of neutrophils *in vitro* (Liles et al., 1996; Cox and Austin, 1997). Moreover, Gizycki et al (2002) showed a significant increase of neutrophils in bronchial biopsies of COPD patients treated with inhaled corticosteroids as compared to those treated with placebo. However, there are also studies showing no significant differences in airway neutrophil counts between inhaled corticosteroids-treated and placebo-treated asthmatics (Hoshino and Nakamura, 1996; Nguyen et al., 2005), and even a reduction of neutrophil counts have been found in sputum of COPD patients treated with inhaled corticosteroids versus those treated with placebo (Yildiz et al., 2000). Although it has been shown that steroid therapy drastically lowers lymphocyte numbers in lung tissue (Reid et al., 2008), no data are available about the effect of inhaled steroids on infiltrated $CD57^+$ cells. Nevertheless, Palmer et al. (2007) reported no differences in CD57 expression on blood and BAL T lymphocytes between corticosteroid-treated subjects and corticosteroid-untreated subjects with beryllium-induced disease. Therefore, the hypothetical effect of inhaled steroids on these inflammatory cells requires further study.

A potential bias in our study is that moderate COPD patients and control groups had peripheral lung cancer whereas very severe COPD patients did not, and there is evidence that lung cancer can influence T cell differentiation (Reepert et al., 2010). However, Domagala-Kulawik et al. (2003) reported no significant differences in $CD57^+$ cell counts on bronchoalveolar lavage fluid among patients with peripheral lung tumors and control smokers and non-smokers. Moreover, it has been reported that metastasis-free regional lymph nodes draining different human epithelial tumors present a reduction in almost all immune cells, except $CD57^+$ cells (Di Girolamo et al., 2008). Taken together, we believe that our present results are valid.

In summary, our study shows that very severe COPD patients have an increased density of $CD57^+$ cells in small airways, as well as an increased density of neutrophils in both small airways and parenchymal interstitium. Moreover, the number of these cells is correlated with the FEV_1 and the macroscopic emphysema degree values, which suggests a possible role for $CD57^+$ cells and neutrophils in the pathogenesis of COPD.

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