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Decreased FOXP3 expression in small airways of smokers with COPD

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ABSTRACT: CD4+CD25+ FOXP3-positive T-regulatory cells have an important role in controlling immune and inflammatory reactions. The present authors hypothesise that these cells may be involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). The aim of the present study was to characterise the expression of FOXP3 in large and small airways of nonsmokers, smokers with normal lung function and COPD patients.

A total of 19 nonsmokers, 20 smokers with normal lung function and 20 smokers with moderate COPD, undergoing lung resection for a solitary peripheral nonsmall cell carcinoma, were enrolled in the study. Immunohistochemical methods were used to evaluate FOXP3 expression in large and small airways.

Smokers with normal lung function and COPD patients had increased numbers of FOXP3-positive cells in large airways compared with nonsmokers. A positive correlation was observed between FOXP3 expression in large airways and smoked pack-yrs. In small airways, COPD patients had decreased numbers of FOXP3-positive cells, compared with asymptomatic smokers and nonsmokers, that negatively correlated with airflow obstruction.

To conclude, chronic obstructive pulmonary disease is characterised by upregulation of FOXP3-positive cells in large airways but a downregulation in small airways that correlated with airflow limitation. The results of the present study contribute to a better understanding of the pathogenesis of chronic obstructive pulmonary disease.

KEYWORDS: Chronic obstructive pulmonary disease, FOXP3 T-regulatory cells, inflammation

Chronic obstructive pulmonary disease (COPD) is characterised by a slowly progressive and irreversible airways obstruction. Cigarette smoking is implicated as a major risk factor for development of COPD and approximately 90% of COPD patients are smokers [1]. However, only a minority of smokers develop COPD. The basic reason for this phenomenon is still poorly understood.

The pathological hallmarks of COPD are inflammation of large and small airways and destruction of lung parenchyma, resulting in the development of pulmonary emphysema [2]. Bronchial biopsies have demonstrated infiltration with mononuclear cells, particularly with CD8+ T-lymphocytes, rather than neutrophils. Similar changes are found in lung parenchyma with a predominance of macrophages and CD8+ T-cells at sites of parenchymal destruction. Inflammation is upregulated by transcription factors, such as nuclear factor (NF)-κB, adhesion molecules such as intercellular adhesion molecule (ICAM)-1 or chemokine receptor, CXCR3 [3–5].

CD4+CD25+ T-regulatory (Treg) cells are important in realising peripheral immunological tolerance, downregulation of persistent inflammation and prevention of autoimmune reactions by inhibition of other T-cell responses [6, 7]. Regulatory CD4+CD25+ T-cells in humans represent between 1 and 3% of total CD4+ T-cells and accumulate at tissue sites of antigen invasion where they exert site-localised immune suppression producing interleukin (IL)-10 and transforming growth factor (TGF)-β1 [6].

There are two different populations of Tregs: natural and adaptive. CD4+CD25+ natural Treg cells are generated in the thymus and reside in the blood and peripheral lymphoid tissues. Adaptive CD4+CD25 Treg cells are generated in the periphery from naïve T-cells after encountering antigens presented by antigen presenting cells. Both the adaptive and natural Tregs depend on a gene called forkhead box P3 (FOXP3) [8, 9].

Intracellular expression of FOXP3 is currently considered as the most specific marker for human Treg cells. Human FOXP3 is localised on the X chromosome encoding scurfin, which binds to the IL-2 promotor and the granulocyte–macrophage colony-stimulating factor enhancer, near the

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nuclear factor of activated T-cell (NFAT) sites. FOXP3 represses these genes, thus reducing IL-2 production by CD4+ T-cells [7, 8]. Although FOXP3 expression was primarily restricted to CD4+CD25+ cells, it was induced following activation of both CD4+ and CD8+ T-cell clones. The large majority of FOXP3-expressing regulatory T-cells is found within the major histocompatibility complex (MHC) class II restricted CD4+ helper T-cell population and express high levels of the interleukin-2 receptor alpha chain (CD25). Recent data indicate that FOXP3 gene expression can be induced also in CD25- T-cells under special conditions. These induced FOXP3-expressing cells also have a suppressive capacity, suggesting that a tight link exists between FOXP3 expression and a regulatory function [8].

Dysfunction of Tregs can lead to autoimmune diseases, allergy and chronic inflammatory diseases [8–11]. In patients with autoimmune diseases, reduced levels of circulating CD4+CD25+ T-cells are described, especially in individuals with juvenile idiopathic arthritis, psoriatic arthritis, hepatitis C, virus-associated mixed cryoglobulinaemia, autoimmune liver disease, systemic lupus erythematosus and Kawasaki disease [6, 11]. Low levels of circulating CD4+CD25+ T-cells also correlate with a higher disease activity or poor prognosis. It has been proposed that downregulation of Treg cells may be caused by impaired proliferation of peripheral CD4+ CD25+ T-cells, as observed *in vitro*. Thereby, the balance between pro-inflammatory and regulatory T-cells could be disturbed, leading to the breakdown of self-tolerance [12–15].

In recent years, several authors have described the presence of an autoimmune component in the pathogenesis of COPD. TARSEVICIENE-STEWART *et al.* [16] showed that rats immunised against endothelial cells developed emphysema. The disease might be passively transferred to naïve rats by blood serum or CD4+ cells. The study suggested that this model provides a proof of principle that an autoimmune attack can cause alveolar destruction. A study by VAN DER STRATE *et al.* [17] examined lymphatic follicules that appear in patients lungs with emphysema and found here B-cells with an oligoclonal, antigen-specific reaction. Similar follicles developed in smoking mice. The development was progressive with time and correlated with the increase in airspace enlargement. The hypothesis was that these B-cells contributed to the inflammatory process and/or the development and perpetuation of emphysema by producing antibodies against either tobacco smoke residues or extracellular matrix components. LEE *et al.* [18] suggested that emphysema could have an autoimmune component characterised by the presence of antielastin antibody and T-helper cell (Th) type 1 responses [18]. Furthermore, anti-epithelial and tobacco anti-idiotypic antibodies have been observed in smoking patients with COPD [19, 20]. All these observations raise the question on the role of Treg cells in the pathogenesis of COPD.

Recently, it has been shown that Treg cells are increased in BAL fluid in healthy smokers and COPD patients [21]. In contrast, BARCELO *et al.* [22] showed that smokers with preserved lung function had a prominent upregulation of Tregs, that was absent in patients with COPD. It was also found that the number of CD4+CD25+ positive Treg cells was decreased in lungs of patients with emphysema that correlated with FOXP3 mRNA expression [18].

However, there are no data in the literature, in which FOXP3 expression is compared in both large and small airways of smoking humans. The nature of the inflammatory process in large airways may be different from that in the small airways. Therefore, the present authors attempted to compare the expression of FOXP3 in large and small airways of nonsmokers, smokers with normal lung function and smokers with COPD.

MATERIAL AND METHODS

Patients

A total of 59 subjects, undergoing lung resection for a solitary peripheral nonsmall cell carcinoma, were enrolled in the study. They were subdivided into three groups: 19 subjects were nonsmokers with normal lung ventilation function, 20 subjects were current smokers with normal lung function and 20 current smoking subjects had moderate COPD. The diagnosis of COPD was established according to the definition of the Global initiative for chronic Obstructive Lung Disease (GOLD) guidelines [1]. COPD patients had a forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) ratio <70% and an FEV_1 between 50 and 80% of predicted. None of the study subjects suffered a recent exacerbation, defined as increased dyspnoea associated with a change in quality and quantity of sputum that would have led them to seek medical attention, during the month preceding the study. All subjects were free of acute upper respiratory tract infections, and none had received glucocorticoids, theophylline or antibiotics within the preceding month. All subjects were nonatopic, had negative skin tests for common allergen extracts and had no past history of asthma or allergic rhinitis.

The study was approved by a local ethical committee (Pauls Stradins Clinical University Hospital ethical committee, Riga, Latvia) and it conforms to the declaration of Helsinki; informed consent was obtained from all subjects. The clinical characteristics of the groups are presented in table 1.

Study design

The lung tissue specimens for small airways evaluation were taken from the subpleural parenchyma but for large airways bronchial rings were taken from the segmental bronchus of the lobe obtained at surgery as far away as possible from the tumour site. Samples were fixed without inflation in 10% neutral buffered formalin, processed and embedded routinely.

Pulmonary function tests

Pulmonary function testing was performed on Jaeger MasterScreen spirograph (Jaeger Gmbh, Hoechberg, Germany) according to British Thoracic Society Guidelines within the week before surgery [23].

Immunohistochemistry

For immunohistochemistry, formalin-fixed paraffin-embedded tissue was cut in 4-μm thick sections. Antigen retrieval was achieved by treatment in domestic microwave for 30 mins in citrate buffer, pH 6.0. Sections were incubated in 0.5% H_2O_2/PBS to quench endogenous peroxidase activity and then blocked with protein block (Dako, Glostrup Denmark). The slides were then incubated for 1 h with primary monoclonal mouse anti-human antibody against FOXP3 (monoclonal mouse antibody, dilution 1:100, clone 221D/D3; AbD Serotec,

TABLE 1 Subject characteristics

Subjects group	Subjects n	Age yrs	Smoking history pack-yrs	FEV_1 % pred	FEV_1/FVC %	Male/female n	Height cm	Weight kg
Control nonsmokers	19	64±10		93±21	79±7	11/8	170±9	81±14
Asymptomatic smokers	20	62±8	27±14	90±17	74±5	18/2	173±6	74±12
Moderate COPD patients	20	64±6	30±15	54±8*	60±7*	16/4	176±5	78±15

Data are presented as mean±SD, unless otherwise stated. FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity. *: $p<0.05$ compared with control smokers and nonsmokers with normal lung function.

Oxford, UK). An LSAB2 kit (Dako) was used for visualisation of bounding with the primary antibody. Briefly, slides were incubated in a humidity chamber for 30 mins each with biotinylated secondary antibody and streptavidin with preceding, interventing and subsequent rinses in isotonic buffer (pH 7.6), three times for 5 mins. 3′3-diaminobenzidine-tetrahydrochloride (DAB) was applied as chromogen (for 7 min). Sections were counterstained in haematoxylin (2 mins). For a positive control, human palatine tonsil tissue was used. Negative controls were performed by omitting the primary antibody.

Analysis of airways was performed using a light microscope connected to a video recorder linked to a computerised image system (Motic Image Advanced 3.2, Xiamen, China). FOXP3 expression was identified as nuclear immunolocalisation. The cases were coded and the measurements made in a blinded fashion by two pathologists (S. Isajevs and A. Kratovska) without knowledge of clinical data for a given patient specimen.

Cell counts in large airways

Bronchial rings were taken from the segmental bronchus of the lobe obtained at surgery, as far away as possible from the tumour site. At least two intact airways with diameter >2 mm were identified for each patient. Large airways were defined as cartilaginous bronchi with submucosal glands. The number of FOXP3-positive cells in large airways was counted randomly by a blinded investigator, in the epithelium and subepithelium of each section, excluding areas of smooth muscle and mucous secreting glands when these were identified. The relevant area was outlined on a captured image and the contained area measured using image analysis software. All cell counts were expressed as cells·mm^{-2}.

Cell counts in small airways

Small airways were considered as membranous bronchioles, without cartilage or glands and with an internal diameter <2 mm, as previously described [2]. At least four intact airways with diameter <2 mm were identified for each patient. In each airway, the number of FOXP3-positive cells in the epithelium, submucosa and in the adventitia were quantified. The submucosa was defined as the area that extends from the distal edge of the basement membrane to the internal edge of the smooth muscle, whereas the adventitia was defined as the area that extends from the outer edge of smooth muscle to the alveolar attachments. All cell counts were expressed as cells·mm^{-2}.

Statistical analysis

Group data are expressed as mean±SD for functional data or median (range) for morphological data. Differences between groups were analysed using ANOVA for functional data and Kruskal–Wallis test for morphological data. When the differences were significant, the ANOVA test was followed by an unpaired t-test and the Kruskal–Wallis test was followed by the Mann–Whitney U-test for comparison between groups. Correlation coefficients were calculated using Spearman's rank method. A p-value of <0.05 was considered statistically significant. At least 3 replicate measurements of FOXP3-positive cells were performed by the same observer in 10 randomly selected slides and the intraobserver reproducibility was assessed with the coefficient of variation and with the interclass correlation coefficient. The intraobserver coefficient of variation was 6% and the intraobserver correlation coefficient was 0.88.

RESULTS

Clinical findings

The clinical characteristics and lung function data of the patients are presented in table 1. As expected from the selection criteria, the values of FEV_1 % pred and the FEV_1/FVC ratio % were significantly different in COPD patients, compared with both nonsmokers and asymptomatic smokers. All patients, except the nonsmokers group, were current smokers. No significant differences in age, weight and height were found among subjects. Furthermore, no substantial differences in pack-yrs of smoking history were found between asymptomatic smokers and COPD patients (27±14 *versus* 30±15; $p>0.05$). The nonsmokers group contained approximately an equal number of males and females but asymptomatic smokers and COPD patients were predominantly males.

FOXP3 expression in large airways

Figure 1a shows FOXP3 expression in large airways. FOXP3 was predominantly expressed in airways subepithelium (fig. 2). The median (range) of FOXP3-positive cells in large airways in COPD patients (26 (14–48) cells·mm^{-2}) and smokers with normal lung function (36 (10–85) cells·mm^{-2}) was significantly higher than that determined in nonsmokers (13 (6–26) cells·mm^{-2}; $p=0.001$ and $p<0.0001$, respectively). In addition, there was a positive correlation between the number of FOXP3-positive cells and the pack-yrs smoked when all patients were analysed together ($\rho=0.697$, $p<0.0001$; fig. 4a). There was a weak negative correlation between the number of

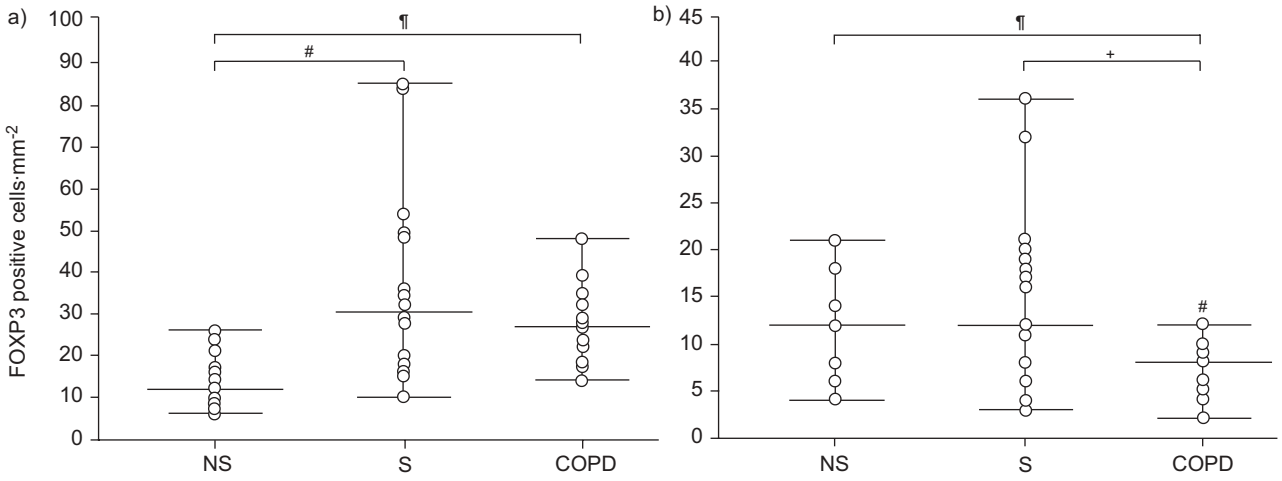

FIGURE 1. FOXP3-positive T-regulatory cells in a) large and b) small airways of nonsmokers (NS; n=19), smokers with normal lung function (S; n=20) and chronic obstructive pulmonary disease (COPD; n=20) patients. Horizontal bars represent medians. #: p<0.0001; ¶: p=0.001; +: p=0.005.

FOXP3-positive cells in large airways and FEV1 % pred (ρ= -0.32, p=0.01; fig. 3a).

FOXP3 expression in small airways

Figure 1b shows FOXP3 expression in small airways. The number of FOXP3-positive cells in small airways in COPD patients (7 (2–12) cells·mm^{-2}) was significantly fewer compared with smokers with normal lung function (14 (3–36) cells·mm^{-2}; p=0.005) and nonsmokers (13 (4–22) cells·mm^{-2}; p=0.001).

There was a weak positive correlation between the number of FOXP3-positive cells in small airways and FEV1% pred (ρ= 0.35, p=0.02; fig. 3b). There was no significant correlation between the number of FOXP3-positive cells and smoked pack-yrs (fig. 4b).

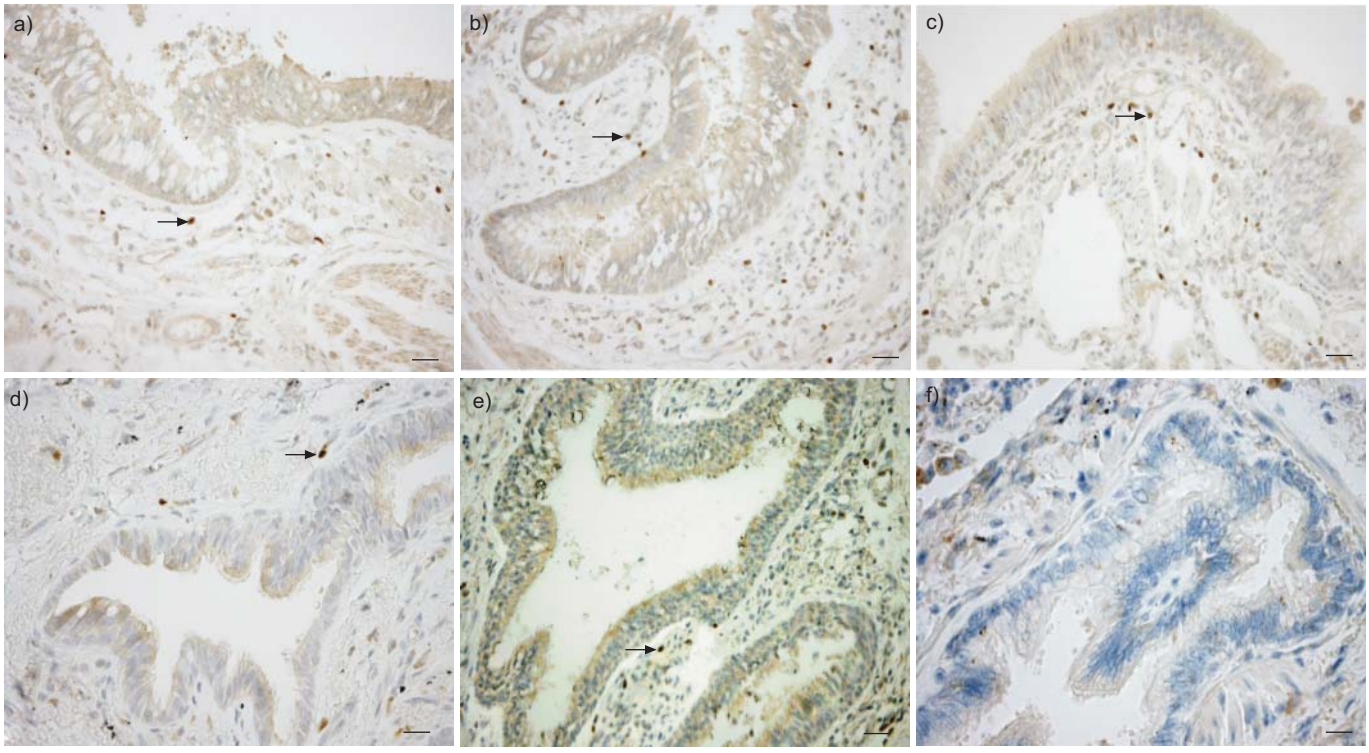

FIGURE 2. Photomicrograph showing FOXP3 expression in large airways of a) nonsmokers, b) smokers with normal lung function and c) patients with moderate chronic obstructive pulmonary disease (COPD); and FOXP3 expression in small airways of d) nonsmokers, e) asymptomatic smokers and f) COPD patients. Arrows indicate representative positively stained cells. Results are representative of those from 19 nonsmokers, 20 smokers with normal lung function and 20 subjects with COPD. a–e) Scale bars=50 μm; f) scale bars=100 μm.

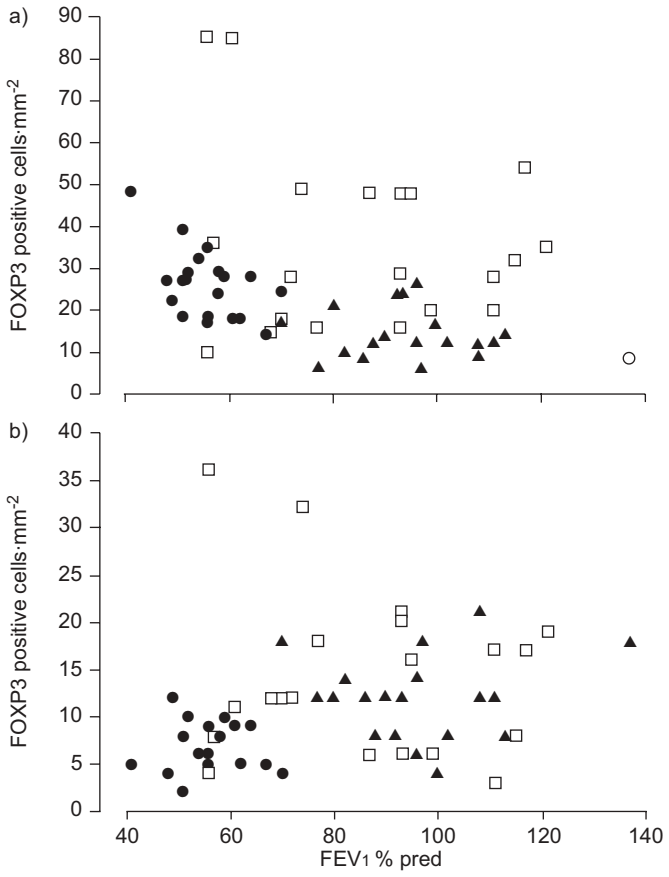

FIGURE 3. Relationship between values of forced expiratory volume in one second (FEV_1) % predicted and number of FOXP3-positive cells in a) large airways and b) small airways. ▲: nonsmokers; □: smokers with normal lung function; ●: chronic obstructive pulmonary disease patients. a) ρ= -0.32, p=0.01; b) ρ= 0.35, p=0.02 from Spearman's rank correlation coefficient.

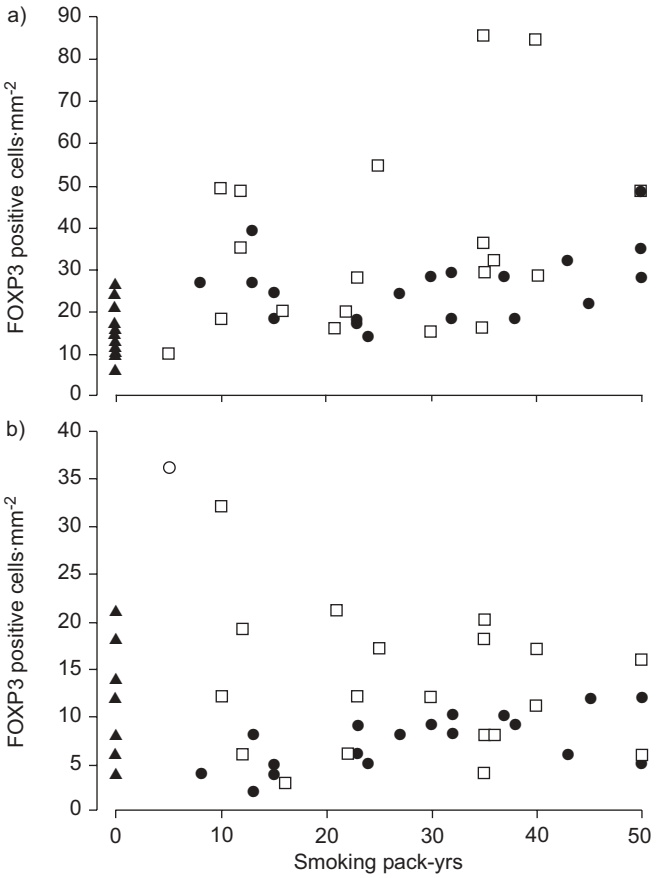

FIGURE 4. Relationship between pack-yrs smoking history and the total number of FOXP3-positive cells in a) large airways and b) small airways. ▲: nonsmokers; □: smokers with normal lung function, ●: chronic obstructive pulmonary disease patients. a) ρ = 0.66, p<0.0001; b) ρ = -0.19, p=0.15 from Spearman's rank correlation coefficient.

The number of FOXP3-positive cells in COPD patients and smokers with normal lung function was significantly fewer in small compared to large airways when analysed within groups (p=0.0002 and p<0.0001, respectively).

DISCUSSION

The present study demonstrates the increased FOXP3 expression in large airways of smokers with and without COPD, but decreased number of FOXP3-positive cells in small airways of COPD patients, which correlated with airflow limitation.

Increased number of FOXP3-positive cells in large airways of all the smokers, that positively correlated with smoked pack-yrs was initially surprising. Given the relevance of this T-lymphocyte subpopulation in tissue homeostasis, this observation is compatible with a physiological response aimed at protecting or repairing the lungs from the injury caused by current tobacco smoking [24, 25]. The specific molecular mechanisms that could lead to this upregulation of Treg lymphocytes in smokers are poorly understood. The authors speculate that a direct stimulatory effect of some component(s) of smoke and/or the products of subsequent inflammatory responses may contribute to this.

SMYTH *et al.* [21] recently showed that in BAL fluid, Treg cells are upregulated in healthy smokers and COPD patients. Furthermore, a significant positive correlation was observed between FOXP3 T-cell count and smoked pack-yrs. By contrast, BARCELO *et al.* [22] demonstrated, in BAL fluid, a prominent upregulation of Tregs in asymptomatic smokers that was absent in COPD patients. The present findings confirm and extend these observations by showing that the increased number of FOXP3-positive cells in large airways of smokers with and without COPD does not correlate with cell numbers in small airways, where fewer Treg cells were found in COPD patients. At the same time, in the present study, there was no difference in smoking pack-yrs history between asymptomatic smokers and COPD patients. Therefore, an effect of smoking should be excluded.

The present study found that small airways of smokers, who manage to preserve their lung function despite their habit, showed an unchanged number of FOXP3-positive cells, whereas smokers with COPD had decreased FOXP3 expression, compared with nonsmokers, which positively correlated with airflow limitation.

The present results are in agreement with study of LEE *et al.* [18], which demonstrated decreased CD4+CD25+ Treg cell number in lung tissue from emphysema patients, which in turn correlated with FOXP3 mRNA expression. This pattern of FOXP3-positive Treg cells response in small airways of COPD patients is in fact very similar to that of γδ T-lymphocytes obtained from bronchoalveolar lavage fluid, which is another subpopulation of T-lymphocytes involved in tissue repair [26]. Furthermore, as illustrated in an animal model, Treg lymphocytes knockout mice (characteristics of this T-cell subset are strikingly similar between mouse and man) exhibit markedly increased inflammatory responses [27].

The present results support evidence that FOXP3 Treg cells restrain inflammatory responses in those smokers who maintain normal lung function, despite considerable smoking consumption. By contrast, in smokers who develop COPD and who are incapable of having an effective Treg response, the inflammatory reaction progresses uncontrolled.

It is very intriguing why FOXP3 T-cells are upregulated in large airways but downregulated in the small airways of smokers with COPD. The present authors hypothesise that antigens or their epitopes can differ between large and small airways. In small airways, for instance, these might be the products of elastolysis or endothelium breakdown products as hypothesised by TARSEVICIENE-STEWART *et al.* [16]. It has been suggested that exposure to cigarette smoke induces secretion of proteolytic enzymes from cells of the innate immune system that liberate lung elastin fragments, which in susceptible individuals could initiate T- and B-cell-mediated immunity against elastin. It was shown that emphysema is characterized by the presence of antielastin antibody and Th1 responses, which correlated with emphysema severity [18]. In addition, it was demonstrated that the volume of elastic fibres was reduced to a similar extent in small airways and alveoli in COPD patients, which correlated with airflow limitation [28].

Furthermore, it is established that tobacco smoking modulates the proliferation and death pathways of lymphocytes and can generate new epitopes by either directly oxidising existing proteins or indirectly by interfering with the clearance of apoptotic cells, thus exposing sequestered intracellular antigens to the immune system. In addition, viral infections and oxidative stress, created by environmental particles, could also contribute to the development of new/altered epitopes [29, 30].

A confusing element in any study could be the unique features of the lung tissue that is taken during surgery from patients with lung cancer. One could suggest that the presence of cancer itself may influence the results. However, surgical specimens are the only material that is available for the examination of both large and small airways in such a study. Lung tissue as far from the tumour site as possible was examined and the control group also consisted of lung cancer patients; therefore, the present authors feel confident that the findings are valid.

To conclude, chronic obstructive pulmonary disease is characterised by upregulation of FOXP3-positive T-regulatory cells in large airways but by downregulation in small airways, thus resulting in persistent and modified inflammatory responses in lung tissue.

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