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Levels of CD4+ CD25+ T regulatory cells in bronchial mucosa and peripheral blood of chronic obstructive pulmonary disease indicate involvement of autoimmunity mechanisms

Abstract

Introduction: Many theories have been proposed to explain pathogenesis of COPD; however, remains unclear why the majority of smokers (~80%) do not develop COPD, or only develop a mild disease. To explore if COPD has an autoimmune component, the role of T regulatory lymphocytes (Tregs) in the lung tissue of COPD patients is of crucial importance.

Material and methods: Bronchial tissue biopsy samples were prospectively collected from 64 patients (39 COPD and 25 controls — 15 smokers and 10 non-smokers). The patients with COPD were subdivided into mild/moderate (GOLD stage I−II) and severe/very severe (GOLD stage III−IV) groups. Digital image analysis was performed to estimate densities of CD4+ CD25+ cell infiltrates in double immunohistochemistry slides of the biopsy samples. Blood samples were collected from 42 patients (23 COPD and 19 controls) and tested for CD3+ CD4+ CD25+ ^{bright} lymphocytes by flow cytometry.

Results: The number of intraepithelial CD4+ CD25+ lymphocytes mm^{-2} epithelium was significantly lower in the severe/very severe COPD (GOLD III-IV) group as well as in the control non-smokers (NS) group ($p < 0,0001$). Likewise, the absolute number of Treg (CD3+ CD4+ CD25+ ^{bright}) cells in the peripheral blood samples was significantly different between the four groups ($p = 0.032$). The lowest quantity of Treg cells was detected in the severe/very severe COPD and healthy non-smokers groups.

Conclusion: Our findings suggest that severe COPD is associated with lower levels of Tregs in the blood and bronchial mucosa, while higher Tregs levels in the smokers without COPD indicate potential protective effect of Tregs against developing COPD.

Key words: COPD, autoimmunity, T- regulatory cell, bronchial biopsy, flow cytometry

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by poorly reversible airflow limitation and progressive airway inflammation [1]. The most common cause of COPD is cigarette smoking. Many theories have been proposed to explain the mechanisms and development of the disease, however, relatively little attention is being paid to why and how the majority of smokers (~80%) do not develop COPD, or only develop a mild disease [2].

What are the mechanisms that allow the majority of smokers to evade COPD? Autoimmunity has been suspected to play a role in the pathogenesis of COPD, but the antigenic stimulation responsible for lymphocyte activation is not established. Possibilities include viruses or bacteria residing in the airways, or smoking altering the presentation of self-antigens including

elastin [3]. A key clinical observation supporting this hypothesis is that airway inflammation is progressive in many patients, despite smoking cessation [4, 5]. The development of an adaptive immune response to a self-antigen, along with the eventual development of autoimmunity, would depend on the level of tolerance to the antigen: when tolerance is high, no adaptive inflammation would result; when there is no tolerance, a full adaptive immune reaction and autoimmunity would develop [2].

The autoimmunity component of COPD can be evaluated by investigating the function of regulatory T lymphocytes (Tregs) in the lung tissue [6]. Treg lymphocytes are a subset of CD4 positive lymphocytes co-expressing CD25. They suppress autoreactive lymphocytes by a variety of mechanisms, including cell-to-cell contact and secretion of immunomodulatory cytokines [7]. It has been reported that smokers have increased numbers of Treg cells in bronchoalveolar lavage fluid [8, 9] compared to non-smokers. One of these studies also showed decreased Treg numbers in COPD patients compared to smokers with normal lung function, suggesting decreased T regulatory function in COPD. The presence of Tregs in the lungs of COPD patients has been studied by a number of investigators [6, 8, 10−12], but data remain controversial. The contrasting findings regarding Treg cell numbers in different studies may be partly explained by methodology aspects such as the site of sampling and the laboratory methods used to identify Tregs. Another important factor is the heterogeneity of COPD [6].

To our knowledge, quantities of Treg cells in large airways in conjunction with peripheral blood in various stages of COPD patients have not been previously studied. The aim of our study was to evaluate CD4+ CD25+ Treg cells numbers in endobronchial biopsy samples and peripheral blood in all stages COPD patients and compare results with the control group of non-smokers and smokers without COPD.

Materials

Patients and control subjects

The study cohort consisted of 64 persons. All subjects were Caucasians. Thirty-nine COPD patients at the age of 35−77 (mean age 64) were recruited (Table 1). All patients had a post-bronchodilator FEV1/FVC < 70% and < LLN, as well as smoking history of more than 10 pack-years. According to the severity of the disease, the patients with COPD were divided into two following groups — mild/moderate (GOLD stage I−II) group and severe/very severe (GOLD stage III−IV) group, respectively. None had clinical or radiological features of any other lung disease than COPD. Smokers with normal lung function (n = 15) and non-smokers (n = 10) served as controls. All subjects in the control group were volunteers and did not have any lung disease, including lung cancer. The patients with bronchial biopsy examination were the same from which peripheral blood was analysed.

Bronchial tissue biopsy samples were prospectively collected from 64 patients and investigated at the National Center of Pathology. Blood samples were collected from 42 participants: 23 COPD (12 of mild/moderate, 11 of severe/very severe group and 19 controls [11 smokers and 8 non-smokers]). Informed consent was obtained and documented in writing before the study entry. The research was approved by the Regional Bioethics Committee.

Pulmonary function test

All patients and controls underwent spirometry. Pre- and post-bronchodilator spirometry was performed according to the American Thoracic Society (ATS)/European Respiratory Society (ERS) guidelines. PFT parameters, including FEV_1, FEV_1//FVC ratio and FVC were measured using *Vmax Encore* (*Viasys ®Healthcare*, US) equipment. The subsequent blood specimen was obtained and bronchoscopy was performed.

Bronchoscopy and bronchial biopsies

Bronchoscopy procedures were carried out using fiber bronchoscopes manufactured by *Olympus*® and *Pentax*® (Japan). The procedure was performed when the patient was sitting. The subjects received 1.0 ml of atropine sulphate 0.1% subcutaneously for premedication. Anaesthesia of the nose and throat was performed using Lidocaine 10% spray solution administered into both nostrils and the throat. The trachea and bronchi were anaesthetized using 20 ml of Lidocaine 2% solution. Biopsy specimens were taken by use of pulmonary biopsy forceps. From each subject six endobronchial mucosal biopsies were taken from the subsegmental carina of the inferior lobe of the right lung.

Processing and staining of bronchial biopsies

The tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Paraffin sections were cut 2 μm thick. Double IHC was

Table 1. Demographic and lung function characteristics of individuals with and without COPD

	COPD group		Control group	
	Mild/moderate (GOLD I−II) n = 19	Severe/very severe (GOLD III−IV) n = 20	Smokers n = 15	Non-smokers n = 10
Gender, male/female	17/2	20/0	10/5	5/5
Age, years	60 ± 16	67 ± 10	47 ± 14	50 ± 9
Smoking history, pack/years[*]	29 ± 18	40 ± 10	25 ± 12	0
FEV_1/FVC	52.47 ± 9.03[#]	39.63 ± 7.13[#]	80.18 ± 5.91	77.50 ± 7.61
FEV_1% pred	67.58 ± 11.60[#]	40.53 ± 5.94[#]	105.45 ± 9.58[##]	105.83 ± 23.37[##]
FVC% pred	103.11 ± 18.79[#]	81.68 ± 15.86[#]	109.91 ± 10.25	112.17 ± 23.46

Data are presented as mean ± standard deviation (SD). FVC — forced vital capacity; pred, predicted; FEV_1 — forced expiratory volume in one second. [*]One-way Anova not indicated significant difference of lung function parameters in both COPD groups and control group of smokers. [#]Statistically *s*ignificant differences were indicated between GOLD I−II, GOLD III−IV, smokers and non-smokers groups (p < 0.0001) using Kruskal Wallis Test. [##]Statistically *s*ignificant differences in FEV_1 were not indicated between smokers and non-smokers groups using ANOVA post hoc test

performed applying rabbit monoclonal antibodies against CD4 (clone: SP35, Cell Marque) and human interleukin-2 receptor (CD25, clone: 4C9, Leica) with brown (diaminobenzidine) and red (Nuclear fast red) chromogens, respectively (Figure 1). Epitope retrieval was performed using Target buffer solution pH 9.0 at 97°C for 20 min and Cell Conditioning solution (pH 8.5) at 95°C for 64 min. The sections were then incubated with CD4 (1:25) antibody at 37°C for 32 min and CD25 (1:100) antibody at room temperature for 30 min, using EnVision FLEX+ Mouse, High pH visualization kit, based on peroxidase (HRP) with DAB+ as chromogen and ultraView Universal alkaline phosphatase (AP) using Permanent Red as chromogen. DAKO Autostainer Link machine (*DAKO, Agilent technologies*, Santa Clara, CA) and Benchmark Ultra automated staining system (*Ventana Medical Systems*, Tucson, Arizona, USA) was used for the procedure. Finally, the sections were counterstained with Mayer's hematoxylin and mounted. Tissue sections of CD4 and CD25 positive bronchus tissue were used as positive tissue controls, while negative reagent controls were performed by omitting application of the primary antibodies.

Quantification of staining in mucosal biopsy specimens

Digital images were captured using the *Aperio ScanScope XT* Slide Scanner (*Aperio Technologies, Vista*, CA, USA) under 20× objective magnification. Digital image analysis (DIA) was performed with *StrataQuest v.205* (*TissueGnostics* GmbH). The tissue sections were divided into three zones: the epithelium, subepithelial stroma and remaining stroma/other tissue. The epithelial layer was manually annotated; the subepithelial stroma was automatically separated from the remaining stroma as a ribbon of 1 mm in thickness underneath the epithelium by appropriate StrataQuest algorithm setup (Figure 2). Densities of CD4+ CD25+ lymphocytes infiltrate (numbers of cells per square mm) were calculated in the tissue compartments.

Peripheral blood. Immunostaining and flow cytometry

For lymphocyte subpopulation analysis, peripheral blood samples were incubated with monoclonal antibodies for 15 minutes at the
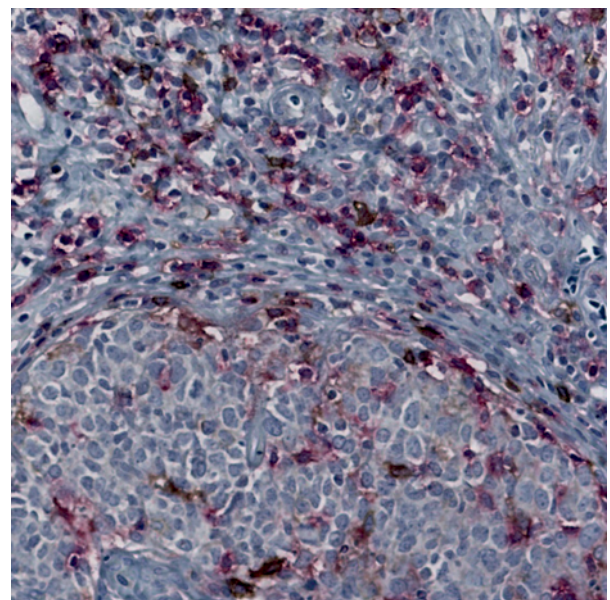

Figure 1. Representative photomicrograph showing bronchial epithelium of patients in the COPD group: double immunohistochemistry for CD4 (brown) and CD25 (red)
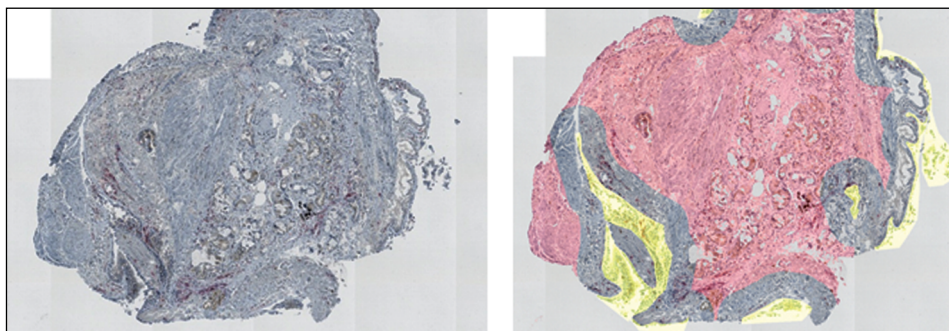
Figure 2. Segmentation of the epithelium (yellow), subepithelial stroma (grey) and the remaining stroma/other tissue (red) in the StrataQuest image analysis platform
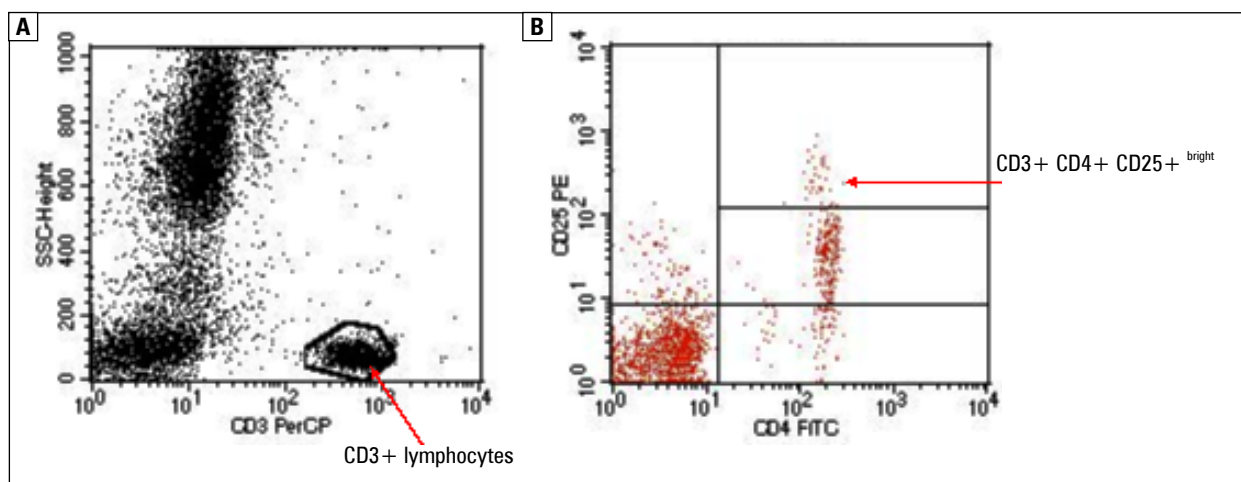

Figure 3. Flow cytometry analysis of CD25 expression on peripheral blood CD4$^+$ T cells. **A.** Lymphocytes were gated on a forward scatter *versus* CD3+ Per CP; **B.** Dot plot of blood CD3+ CD4+ CD25+ lymphocytes: CD25 expression was categorized into CD25 (dim) and CD25 (bright) subpopulations (CD3+ CD4+ CD25+ ^{bright} analyzed as Tregs)

room temperature in the dark. Following the incubation, 2 mL of red cell lysing buffer (BD FACS lysis solution, BD Biosciences San Jose, CA, USA) was added, cells were incubated for 10 minutes at room temperature in the dark. After centrifugation at 300 g for 7 minutes at room temperature, the supernatant was discarded and 2 ml of phosphate buffered saline (PBS) was added to the pellet. Re-suspended cells were washed by centrifugation at 300 g for 7 minutes at room temperature. After fixation in 300 μl of 0.5% paraformaldehyde, prepared samples were analyzed on FACSCalibur flow cytometer (BD Biosciences) using CellQuestPro analysis software. Antibodies used for lymphocytes characterization were: Peridinin Chlorophyll Protein Complex (Per CP) conjugated anti-CD3 monoclonal antibodies, Phycoerythrin (PE) conjugated anti-CD25 monoclonal antibodies and Fluorescein isothiocyanate (FITC) conjugated anti-CD4 monoclonal antibodies (all monoclonal

antibodies BD Biosciences, San Jose, CA, USA). Lymphocytes were gated on a forward scatter *versus* CD3+ Per CP (Figure 3). CD25 expression on CD4 + T cells was categorized into CD25 (dim) and CD25 (bright) subpopulations; the absolute number CD3+ CD4+ CD25+ ^{bright} was analyzed as Tregs.

Statistical analysis

Statistical analysis was performed by using SPSS version 22.0 (*SPSS Inc., Chicago Ill*). Continuous data are given as mean ± SD. Kolmogorov−Smirnov test was applied for assessing the normality of data. For normally distributed data, comparisons between groups were made using analysis of variance one-way ANOVA. If data was not normally distributed, Kruskal–Wallis test was used. Pearson *r* correlation coefficient was applied to evaluate the correlation. Statistical significance was defined at p < 0.05.
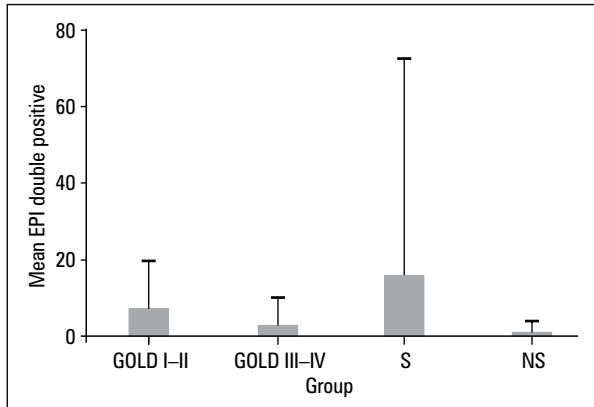
Figure 4. The mean of intraepithelial Tregs mm^{-2} epithelium in bronchial biopsies in the study groups. The number of intraepithelial CD4+ CD25+ lymphocytes mm^{-2} epithelium was significantly lower in the severe/very severe COPD (GOLD III–IV) group as well as in the control non-smokers (NS) group (p < 0.0001). Data are presented as mean ± standard deviation (SD)
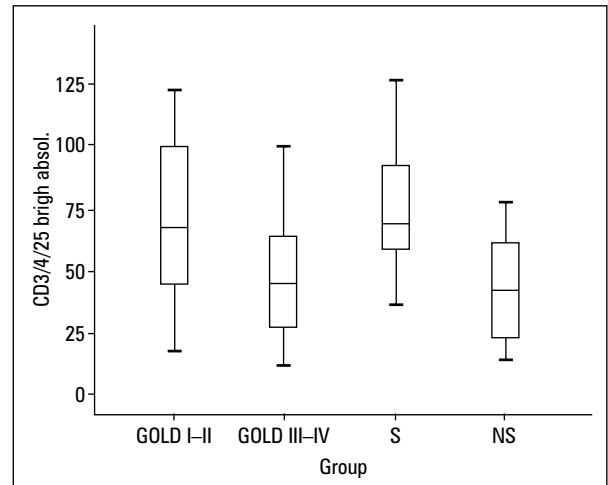

Figure 5. Comparison of the means of Tregs (CD3+ CD4+ CD25+ [bright] number in peripheral blood in groups. Tregs number is significantly lower in severe/very severe COPD (GOLD III–IV) and in the control non-smokers (NS) group. Data are shown as median and interquartile range

Results

Biopsy findings

There was no significant difference in analyzed epithelial length, expressed in mm, between the groups. The number of intraepithelial CD4+ CD25+ lymphocytes mm^{-2} epithelium was significantly different between the groups (p < 0.0001; Kruskall–Wallis), Figure 4. However, there was no difference of subepithelial and stromal CD4+ CD25+ lymphocytes between the groups (p = 0.062 and p = 0.117, respectively).

Peripheral blood findings

Similar to the biopsy results, the absolute number of Treg (CD3+ CD4+ CD25+ [bright]) cells in the peripheral blood samples was significantly different between the four groups (p=0.032), Figure 5.

However, there is no statistically significant difference between the groups in the absolute number and percent of all CD3+ CD4+ CD25+ lymphocytes (p = 0.076 and p = 0.056, respectively).

The lowest quantity of Treg cells was detected in severe/very severe COPD and healthy non-smokers groups. Weak, but not statistically significant correlation between blood and epithelial Tregs numbers was noted (r = 0.241, p = 0.124).

Discussion

Our study was driven by the question why COPD affects only a small proportion of smokers and if immune mechanisms play a role in the

development of this disease. In the study, we demonstrate differences in intraepithelial and peripheral blood Treg cells in mild/moderate and severe/very severe stable COPD compared to smokers with normal lung function and non-smokers. Our data indicate decreased Treg cells in bronchial epithelium in severe/very severe COPD as well as in the control non-smoker group, compared to mild/moderate COPD and healthy smokers. This suggests that severe COPD is diagnosed in patients with lower levels of Tregs in the blood and respiratory tract, while higher Tregs levels in smokers without COPD indicate a potential Treg protective effect against developing COPD.

A role of autoimmune-mediated inflammation in COPD was first suspected by Saetta *et al.* [13] when histological studies of human lung tissue revealed a preponderance of CD8+ T cells in the small and large airway biopsies in ever-smokers with COPD. The intriguing clinical and pathological observations made over the past few years have led to a new concept: in susceptible individuals, cigarette-smoke exposure may trigger long-lasting inflammatory memory T-cell responses that can persist beyond the immediate period of exposure to cigarette smoke. Most importantly, the continuous recruitment of activated lung antigen presenting cells (APCs) could further ensure propagation of auto-inflammatory T-cell responses and the development of chronic progressive lung destruction [14].

It has been shown that Treg cells are important in the control of autoimmunity. Domagala--Kulawik *et al.* [15] observed significantly lower

proportion of peripheral blood CD4+ CD25+ lymphocytes in mild/moderate COPD patients than in healthy controls. This finding drew attention to the fact that, comparing with healthy people, patients with COPD have less of these immune cells in the blood, consequently, a certain level of immune response has been altered in COPD patients. Unlike these authors, we have investigated not only moderate, but also severe COPD patients and the control group was divided into two parts: smokers and non-smokers. The breakdown of subjects into four groups allowed us to assess this chain of immune response in COPD in a more detailed way. We found that the number of Treg cells was significantly different between milder and more severe COPD patients as well as in control smokers and non-smokers groups. These findings give us a possibility to consider the involvement of immune mechanisms for the development of COPD.

An interesting observation was published by Xiang-Nan Li and coworkers [16], who reported imbalances of Th17 and Treg cells and their respective cytokines in COPD patients. The proportion of proinflammatory Th17 cells in peripheral blood and in sputum was significantly higher in acute exacerbation of COPD than in stable COPD patients and the control group of smokers with normal lung function. Additionally, the proportion of Treg cells was lower than in stable COPD but higher than in controls. Thus, COPD patients have shifts in the balance of Th17/Treg cells. In contrast to Th17 cells, Treg cells can be immunosuppressive, secreting the anti-inflammatory cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-ß1 to strengthen immune tolerance and inhibit inflammatory responses.

On the basis of the presence of B-cell lymphoid follicles in patients with advanced COPD and the detection of diverse autoantibodies in a subgroup of patients with COPD, COPD has been regarded as an autoimmune disease [18]. Plumb and coworkers [3] were first to study Tregs in pulmonary lymphoid follicles. It was revealed that there was an increased proportion of Treg cells within pulmonary lymphoid follicles of moderate severity COPD patients compared to smokers and non-smokers. However, in clusters and the subepithelia, there was no difference in the proportion of Treg cells between the groups. Although the number of Treg cells in COPD was elevated, in the opinion of the authors, T-regulatory activity may be altered within COPD lymphoid follicles. However, the study included only moderate COPD patients. This is important because the

prevalence of lymphoid follicles increases sharply from 25% to 30% in severe and very severe COPD for unclear reasons [17]. The progression from moderate to severe COPD, which is associated with a sharp increase in the number of follicles, may arise from a failure of regulatory mechanisms to suppress autoimmunity.

Another study that showed the possible protective function of Treg in the lungs demonstrated that smokers with COPD and emphysema had significantly fewer Treg cells in the lungs and lower interleukin 10 concentrations from the whole lung than controls [10].

CD4+ CD25+ $^{\text{bright}}$ cells were significantly increased in bronchoalveolar lavage fluid in smokers with normal lung function compared with people who had never smoked and patients with moderate COPD in one study [9], whereas bronchoalveolar lavage CD4+ CD25+ $^{\text{bright}}$ cells were increased in smokers and patients with COPD compared with healthy people who had never smoked in another study [8]. The data suggest that cigarette smoking exerts influence on the pulmonary immune system and can affect the development of COPD. The lowest Treg frequency was observed not in severe COPD but in healthy smokers. This suggests that perhaps the best interpretation is that smoking induces CD25 (either induction of Treg or activation of T cells). According to our results, we speculate that the amount of Treg in healthy smokers, compared to non-smokers, increases to protect against harmful effects of smoking on their lungs. Whereas the immune response of these individuals is appropriate, they do not develop COPD. On the other hand, for the group of COPD patients, we can assume that mild/ /moderate COPD is diagnosed in the subjects who have more lung-protecting Tregs. Accordingly, severe COPD affects those with less Tregs.

D'Alessio and coworkers showed that Tregs can play a protective role against lung injuries: Tregs that are CD4+, CD25+, and FOXP3+ resolve experimental lung injury in mice after intratracheal lipopolysaccharide administration and are present in the bronchoalveolar lavage fluid of patients with acute lung injury, suggesting that Treg cells modify innate immune responses during resolution of lung injury [19].

A possible role of CD4+ CD25+ $^{\text{bright}}$ T- cells in COPD pathogenesis was suggested in recent study of Chiappori *et al.* [20], where CD4 + CD25 $^{\text{bright}}$ CD127$^-$ regulatory T-cells percentage was significantly reduced in COPD patients, both current and former smokers, with respect to volunteers. However, in this study, COPD patients were not categorized

according to the severity of the disease, except according to current or previous smoking history.

Our data are in agreement with another study [21], which revealed that in peripheral blood, increased proportions of resting Tregs, activated Tregs and cytokine-secreting Treg cells with proinflammatory capacity were found in smokers compared with never-smokers, whereas patients with COPD showed decreased resting Tregs, activated Tregs and significantly increased pro-inflammatory Treg cells compared with smokers.

There is a debate over Treg numbers in COPD tissue. According to other studies, the number of CD4+ CD25+ FOXP3 Tregs in the bronchial biopsies [22] or lungs [23, 24] of patients with stable COPD is not significantly different compared with healthy controls but is decreased in the small airways of COPD patients, and this negatively correlates with the degree of airflow obstruction [11, 25]. However, in our study we found significantly lower numbers of intraepithelial CD4+ CD25+ lymphocytes in the severe/very severe COPD (GOLD III–IV) group as well as in the control non-smokers group, compared with mild/moderate COPD and control smokers groups. The mentioned authors [11, 23, 25] conducted research on lung tissue from patients undergoing surgical resection for carcinoma and results can be influenced by neoplastic process.

Our data was not affected by cancer, because neither our COPD patients nor the controls had an oncological disease.

In summary, higher Treg frequencies are consistent with a protective role of these cells. But equally, it could be that more severe disease leads to loss of Treg. Normal balance of Treg cells in bronchial mucosa and in peripheral blood is disturbed in patients with COPD. Patients with mild/moderate COPD and healthy smokers exhibit higher quantity of Tregs, while patients with severe/very severe COPD and healthy non-smokers exhibit significantly lower quantity of Tregs. This suggests that severe COPD develops in patients with lower levels of Tregs in the blood and respiratory tract, while a higher Tregs concentration in smokers without COPD indicates a potential Treg protective effect against developing COPD.

Acknowledgement

Conflict of interest and funding

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