Fas+ lymphocytes and CD4+/CD25+ cells in peripheral blood of never smoking patients with chronic obstructive pulmonary disease

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Abstract

The main causative agent for developing chronic obstructive pulmonary disease (COPD) is tobacco smoke. The important pathologic pathways in COPD which are related to smoking were well described. This disease affects mainly the respiratory tract. However, many data confirmed relevant systemic disturbances in course of COPD. Up to 30% of COPD cases are not attributable to smoking, however, little is known about the character of systemic inflammation in never smoking patients with COPD.

The objective of this study was the evaluation of main lymphocyte subtypes currently known to play a possible role in the pathogenesis of COPD in never smokers, and their comparison with smokers with COPD, asymptomatic smokers and healthy nonsmokers. Flow cytometry method with monoclonal antibodies was used for evaluation of lymphocyte subsets: T cells, B cells, T helper and T cytotoxic, T cells with regulatory properties and the expression of Fas (CD95) on T lymphocytes.

There were no differences in the proportion of T cells, B cells and CD4+ cells between the investigated groups. The proportion of CD8+ cells was significantly higher in patients with COPD than in healthy, both in non smokers and smokers group. The proportion of T lymphocytes with expression of Fas was significantly higher in never smoking patients with COPD and smokers with COPD when compared with asymptomatic smokers and nonsmokers (85% vs. 85.1% vs. 72.6% vs. 68.6% for CD4+/Fas+ and 83.2% vs. 89.1% vs. 74.3% vs. 58.8% for CD8+/Fas+ lymphocytes, respectively). The proportion of CD4+/CD25+ was lower in never smoking COPD patients than in never smoking healthy persons (14.2% vs. 19.2%).

Our observation indicates important disturbances in some lymphocyte subtypes not related to smoking and their possible role in systemic inflammation in COPD.

Key words: *never smokers, COPD, Fas, CD4+/CD25+ cells, lymphocytes.*

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Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive disorder, characterized by poorly reversible airway obstruction and persistent inflammation in the lung tissue [1, 2]. The main causative agent for developing COPD is tobacco smoke and the important pathologic pathways in development of COPD which are related to smoking were well described [3]. Many COPD cases are not attributable to smoking and the problem of COPD in never smokers is increaseasing [4]. The proportion of patients with COPD who had never smoked is as high as 25-47% [4, 5]. The risk factors are, among others: environmental tobacco smoke, smoke from biomass fuel, air pollution or manual work [4].

Little is known about the character of inflammation in COPD which is not attributable to smoking. The aim of this study was the evaluation of some aspects of systemic inflammation in never smoking patients with COPD. We

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have previously demonstrated an elevated proportion of CD4+ and CD8+ lymphocyte subtypes with the expression of "death receptor" – Fas derived from peripheral blood of tobacco smoking patients with COPD [6]. On the other hand, we described a significant depletion of CD4+/CD25+ cells in the peripheral blood of COPD patients [7]. Both above observations are strictly connected with the recent hypothesis of the role of autoimmunity in COPD [8-10]. Thus, in this study we focused on the changes in these lymphocyte subpopulations in never smokers with COPD.

Material and methods

In this prospective study 12 never smoking patients with stable COPD were investigated. The diagnosis of COPD was established according to the guidelines [2]. Pulmonary function tests were performed according to the standards [11] (the ABC PNEUMO, abcMED, Poland, Vmax 229, Sensormedix was used). Asthma was excluded on the basis of anamnesis, clinical course of the disease and the negative reversibility of pulmonary function tests. We defined never smokers according to the World Health Organization (WHO) definition: there were subjects who smoked less than 100 cigarettes during their life [12]. We did not included any ex-smokers into this group. The group of smokers with COPD (n = 18), asymptomatic smokers (n = 12) and healthy never smokers (n = 12) served as control groups (demographic data of patients and healthy subjects were collected in Table 1). None of the subjects had symptoms of infection or exacerbation of the disease during one month and at the time of this study. Patients receiving systemic glucocorticoids as well as patients with any chronic disease other than COPD and with malignant disease were excluded from this investigation. The study was approved by the Ethics Committee of the Medical University of Warsaw.

For analysis of lymphocytes subtypes the flow cytometry method was used. All the analysis were performed right after 1 mL of venous blood collection. First, anti-CD45-FITC and anti-CD14-PE was used for the lymphocyte gate setting at FSC/SSC graph. As a negative isotype controls the Ig2a-FITC and Ig2b-PE were applied. We analysed the proportion of following lymphocyte subtypes: T cells, B cells, T helper and T cytotoxic cells and the expression of CD95, CD25 and CTLA4 on CD4+ cells and CD8+ cells with following mixtures of antibodies: CD3-FITC/CD19-PE (Becton-Dickinson Immunocytometry Systems, San Jose, California), CD4-FITC/CD95-PE, CD8-FITC/CD95-PE, CD4-FITC/CD8-PE, CD4-FITC/CD25-PE/CTLA4-Cy5, CD8-FITC/CD25-PE (Dako Cytomation, Denmark). The analyses were performed using three-colour flow cytometry method (FACSCalibur flow cytometer, Becton-Dickinson, San Jose, California). The cells were collected by CELLQuest software. The analysis was performed in the same manner, with the same set of antibody and in the same conditions in patients and controls.

Statistical analysis

For data comparison the Kruskal-Wallis (for data non-normally distributed) test was applied. Statistical significance was set at $p < 0.05$.

Results

The median proportion of circulating lymphocyte subpopulations of never smokers and smokers with COPD and healthy controls are presented in Table 2. There no differences in the proportion of T cells (CD3+), B cells (CD19+) and CD4+ cells between investigated groups. The proportion of CD8+ cells was higher in patients with COPD than in healthy subjects and the difference was significant, both

Table 1. Demographic data and results of pulmonary function tests of patients with COPD and control groups (data expressed as mean ± SD)

		Never smokers		Smokers	
Subjects		COPD	healthy	COPD	healthy
n		12	12	18	12
Age		71.6 ±6	53 ±14	68 ±9	54 ±16
Female/male		7/5	4/8	9/9	8/4
Pack years		0	0	54.5 ±38.1	33 ±23
FEV1	**L**	1.1 ±0.4	3.05 ±0.5	1.3 ±0.5	2.8 ±1.2
	% predicted	47.7 ±16	96.0 ±9.0	50 ±13	95.8 ±11.8
FVC	**L**	1.8 ±0.6	3.4 ±0.8	2.2 ±0.5	3.4 ±1.2
	% predicted	57.5 ±13	93 ±10.5	74.7 ±18.8	103 ±8.4
FEV1%FVC		60.0 ±8	87.8 ±7.0	54.4 ±10.5	86.4 ±5.0

Table 2. Proportion of CD4+, CD8+, CD4+ positive for CD95+ and CD8+ positive for CD95+ lymphocytes as percentage of all lymphocytes and CD4+: CD8+ ratio in the peripheral blood of patients with COPD and both control groups: asymptomatic smokers and healthy non smokers. Data expressed as median (p25-p75)

	Never smokers		**Smokers**	
	COPD (*n* = 12)	**healthy (*n* = 12)**	**COPD (*n* = 18)**	**healthy (*n* = 12)**
CD4+ [%]	38.6 (30.6–47)	47.9 (42.1–51.1)	42.7 (40–52)	44.8 (33.6–49.3)
CD8+ [%]	37[a] (26.9–43)	24[a] (18–25.4	28.8[b] (25–33.7)	25.6[b] (23.4–27.6)
CD4+ : CD8+	1.53 (0.65–2.1)	2.05 (1.4–2.75)	0.96 (0.62–1.7)	1.7 (1.6–2.2)
CD4+/CD95+ [%]	40.8 (30.8–41.6)	30.0 (28.0–34.1)	31.5 (26.6–37.8)	30.4 (26.7–38.7)
CD8+/CD95+ [%]	29.6 (17.9–34.3)	13.5 (12.2–27.9)	32.3[b] (25.6–38.3)	20.0[b] (17.4–37.6)
CD4+/CD25+ [%]	14.2[p = 0.06] (11.1–17.0)	19.2[p = 0.06] (14.9–25.0)	15.9 (10.8–20.3)	16.2 (13.8–27.1)
CD8+/CD25+	0.4[a]	1.4[a]	1.68	4.03
[%]	(0.38–0,46)	(0.84–2.9)	(0.76–4.3)	(1.75–5.5)

[a,b]significant difference between patients with COPD and healthy subjects, $p < 0.05$

in the groups of non smokers and smokers. The CD4+: CD8+ ratio was slightly lower in patients than in healthy (difference not significant).

The proportion of Fas positive lymphocytes was higher in patients when compared with healthy controls what was observed in non smokers and smokers, as well (Table 2). We presented our results in two manners: as the proportion of Fas positive cells of all lymphocytes (Table 2) or as the proportion of all CD4+ or CD8+ cells. On Figure 1 we showed the median proportion of CD4+/Fas+ lymphocytes as a percentage of the whole pool of CD4+ cells and the median proportion of CD8+/Fas+ lymphocytes as a percentage of the whole pool of CD8+ cells. The differences of the proportion of these cells between patients with COPD and healthy persons both, in non smokers and smokers groups, were significant ($p < 0.05$).

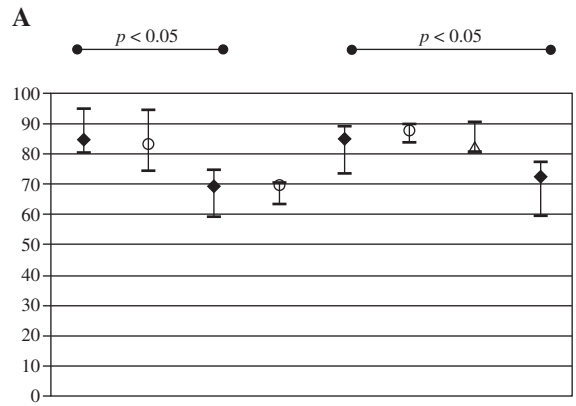
The proportion of CD4+/CD25+ cells was lower in never smoking COPD patients when compared with never smoking healthy persons (difference not significant, $p = 0.06$) (Table 2). On Figure 2 we showed the median proportion of CD4+/CD25+ lymphocytes as a percentage of the whole pool of CD4+ cells and the differences between patients and healthy: in the group of non smokers this proportion was significantly lower in patients than in healthy non smokers (Fig. 2). No significant differences in the proportion of CTLA4+/CD25+ cells between investigated groups were found, however, there was a tendency to increase in the proportion of CTLA+ cells in the pool of CD25+ cells in never smoking COPD patients (Fig. 3). The proportion of CD8+/CD25+ was significantly lower in never smoking COPD patients when compared with healthy.

To eliminate the influence of age on the proportion of lymphocytes we performed another analysis after elimination of subjects which were younger than 45 and older than 75 years. In the groups of patients and healthy controls in the age ranged from 45 to 75 years we observed that the differences remained significant (marked as open circle on Figs. 1 and 2).

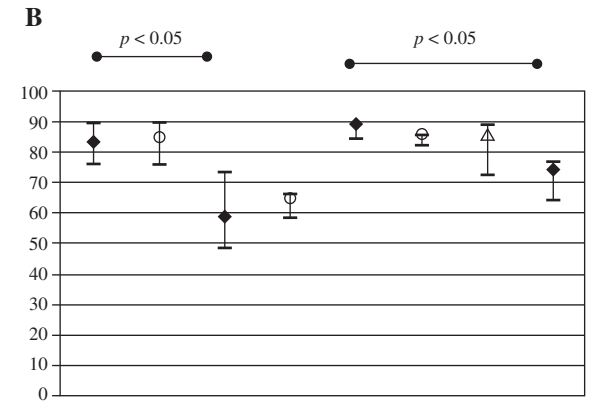
Next we excluded from COPD group the patients with low smoking history (number of pack years smoked lower than 20) and we observed, that the proportion of Fas+ lymphocytes and CD25+ lymphocytes remained comparable between never smokers and smokers with COPD (marked on Figs. 1 and 2).

Discussion

In this study we report for the first time the significant alterations noticed in the population of lymphocytes derived from peripheral blood of never smoking patients with COPD. We found an elevated proportion of T cytotoxic cells (CD8+), of both lymphocyte subtypes: CD4+ and CD8+ with expression of "death receptor" – Fas and a depletion of the proportion of CD4+/CD25+ lymphocytes. We have previously described and widely discussed these alterations in smokers with COPD [6, 7]. The role of these lymphocyte subpopulations in the pathogenesis of COPD was well documented. As far as augmentation of activated

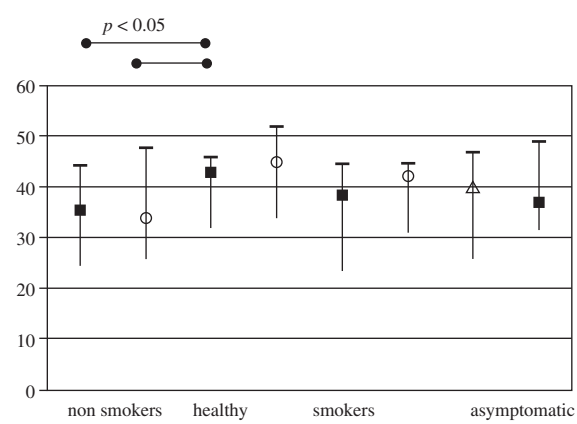


-p25	80.8	75	58.8	63	74.1	84.4	80.9	60.2
◆ median	85	83.6	68.6	68.6	85.1	88	82.5	72.6
-p75	95.1	95	74.2	71	90.2	90	91	77.9



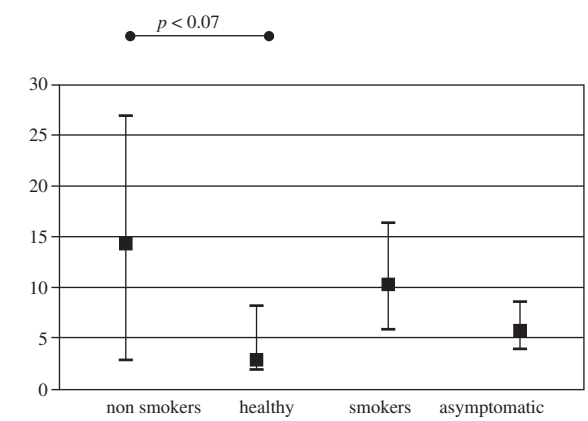
-p25	76	76	49	58.8	84.8	82.7	73	64.6
◆ median	83.2	84.9	58.8	65	89.1	86	86.5	74.3
-p75	89.9	90	73.7	67.2	91.7	86	89	77.3

Fig. 1. A. Proportion of CD4+ cells with expression of Fas in the blood of patients with COPD and healthy controls: non smokers and smokers (median values, p25-p75); **B.** Proportion of CD8+ cells with expression of Fas in the blood of patients with COPD and healthy controls: non smokers and smokers (median values, p25-p75). Cells with expression of CD95 were shown as a percentage of all CD4+ and CD8+ cells open circle – median value after exclusion of subjects younger than 45 and older than 75 years; open triangle – median value in heavy smokers (more than 20 pack years)



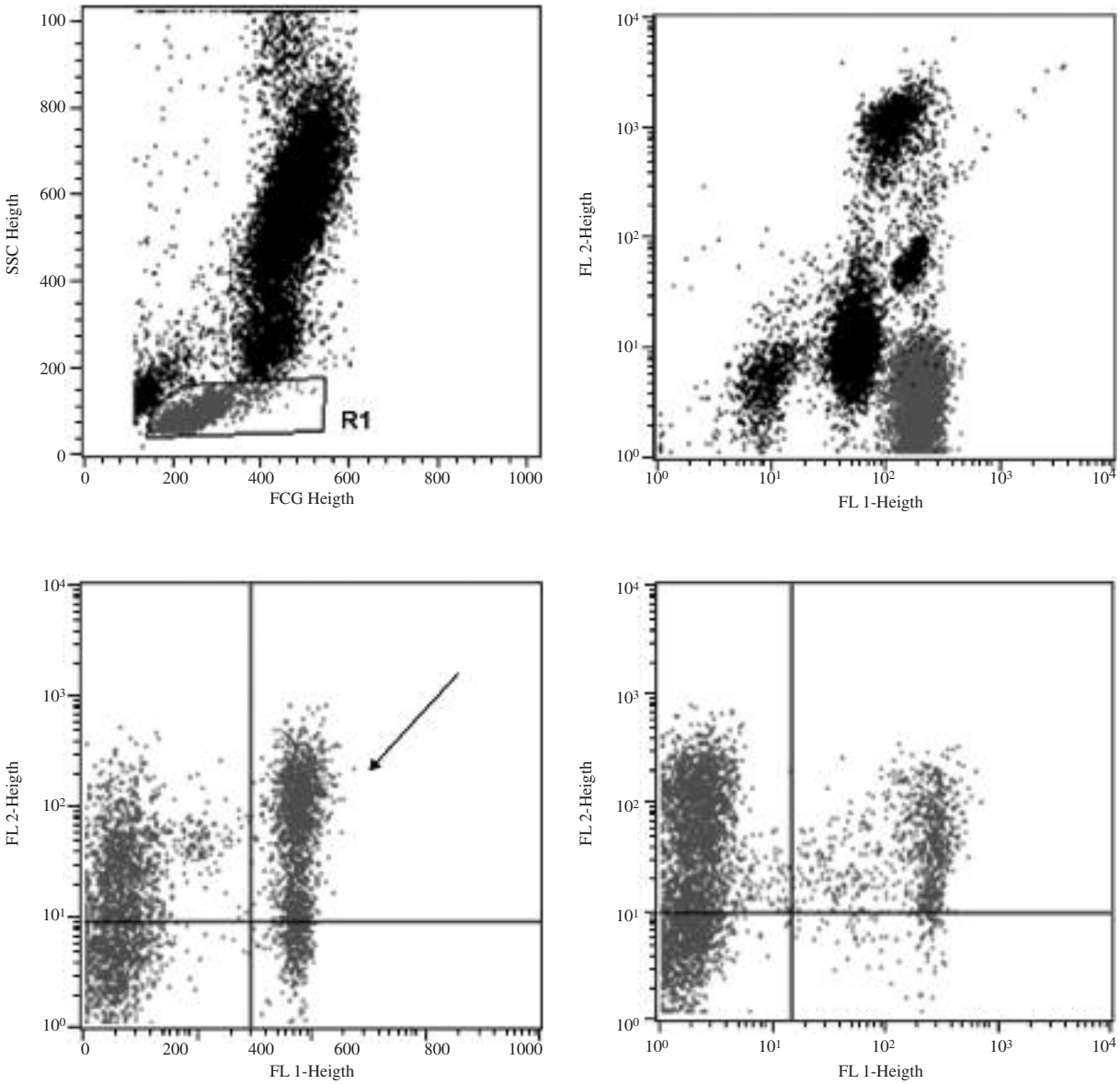
-p25	24.6	26	32.2	34	23.5	31	26	31.5
◆ median	35.4	34	42.8	44.9	38.3	42	40	36.9
-p75	44.5	48	46	51.8	45	45	47	49

Fig. 2. Proportion of CD4+/CD25+ cells in all CD4+ cells in the blood of patients with COPD and healthy controls: non smokers and smokers (median values, p25-p75) open circle – median value after exclusion of subjects younger than 45 and older than 75 years; open triangle – median value in heavy smokers (more than 20 pack years)



-p25	3	1.7	6	4.1
◆ median	14.4	3	104	6
-p75	27	8.3	16.5	8.7

Fig. 3. CTLA4+ cells expressed as proportion of population of all CD4+/CD25+ cells in the blood of patients with COPD and healthy controls: non smokers and smokers (median values, p25-p75)



File 120903d 015
Gated Events: 4623

Quad	Events [%]	Gated [%]
UL	1366	29.55
UR	1988	43.00
LL	927	20.05
LR	342	7.40

File 120903d 016
Gated Events: 4621

Quad	Events [%]	Gated [%]
UL	2605	56.37
UR	840	18.18
LL	1090	23.59
LR	86	1.86

Fig. 4. The diagram from flow cytometry. CD95+ cells marked with an arrow. On the left – COPD patient, on the right – healthy person

lymphocytes is connected with persistent cytotoxic effect and impaired resolution of inflammation [13] the more CD4+/CD25+ cells are involved in the controlling of immune reaction [8, 9].

Our study group consisted only of never smokers, according to the WHO definition [12]. We have collected our group of patients very carefully and during a long time. We did not included any former smokers to this group. In our opinion ex- smokers should be regarded in the groups of smokers since ever smoking is the main risk factor for COPD and it was well documented that quitting smoking does not inhibit persistent immune reaction in this disorder [14, 15]. Nevertheless in many studies never smokers and ex-smokers are included together to the same group of non-smokers [16].

We described changes in lymphocyte subtypes derived from peripheral blood and thus our results supported the presence of systemic inflammation in COPD [17]. In our study we described the elements of systemic immune response which were not related to smoking. This is novelty of this work. The analysis of the pathogenesis of COPD in smokers seems to be much simpler than in non smokers: tobacco smoke is a single and well defined agent while the aetiology of the disease in non smokers is often unknown. Likewise in our patients any potential harmful factor was not recognised. Our work fits in the views on the crucial role of host factors and genetics for COPD development [18, 19].

The analysis of circulating lymphocytes subpopulations revealed important differences between patients with COPD and healthy controls, previously presented by us [6, 7]. In the recent study we focused on the changes in never smokers. Interestingly we did not observed any significant differences between non smokers and smokers in COPD group. More over our experiment with exclusion patients with short smoking history did not change results: there were no differences between never smokers and heavy smokers in the COPD group (more than 20 pack years, Figs. 1 and 2). Our group of patients was not uniform and older than control subjects, but we would like to point, that we excluded the influence of age on the alterations in circulating lymphocytes.

The role of cytotoxic T lymphocytes, CD8+, in the pathogenesis of COPD is well known, these cells play a crucial role in inflammation at the level of airways an lung parenchyma [1, 2]. The role of T cytotoxic cells in systemic inflammation in COPD is not so clear. We found elevated proportion of CD8+ cells in the blood of non smokers with COPD which was higher than in smokers. This observation remains with agreement with the results of de Jong *et al.* [16]. We also observed the tendency to negative correlation of CD8+ cells proportion with degree of obturation in pulmonary function tests (data not shown). This observation adds some arguments to the role of CD8+ in systemic inflammation not related to tobacco smoke but as an integral part of the disease.

Fas protein belongs to the TNF family and presents the susceptibility of cell to apoptosis. The Fas/FasL system plays a role in elimination of activated lymphocytes by apoptosis. Many studies confirmed a role of apoptosis in human lung in course of COPD [20]. We found the elevated proportion of Fas positive cells among CD4+ as well as CD8+ lymphocytes what was also described by Hodge *et al*. [13, 15]. Taken into consideration our previous results and the data from literature we are able to conclude about the importance of activated, Fas positive lymphocytes in the pathogenesis of COPD. This observation in non smokers may indicate underlying genetic (?) alterations in lymphocyte population [21, 22].

We confirmed the depletion of CD25+/CD4+ cells in never smokers with COPD. This population of lymphocytes belongs to regulatory cells family although we did not investigated any functional T regulatory cells characterized by expression of Foxp3 [7, 10]. Lee *et al*. reported the depletion of CD4+/CD25+ cells in the lungs of smokers with emphysema [9]. Depletion of T regulatory cells population was observed in autoimmune diseases and the changes in CD4+/CD25+ population are recently considered as the elements of autoimmunity in COPD [10]. We observed a tendency to increase in the proportion of CTLA4+ CD25+ cells. CTLA4 (CD152) is constitutively expressed on regulatory cells and plays a significant role in regulation of T cell tolerance. There is a down regulation of CTLA4 after activation of T regulatory cells. This may explain our results, however, the role of CTLA4 in COPD needs further investigations.

In conclusion, we observed the important disturbances in some lymphocyte subtypes not related to smoking in COPD. These alterations may have a possible role in systemic inflammation in COPD.

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