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CD4⁺CD25^{high}CD127⁻ regulatory T-cells in COPD: smoke and drugs effect

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Background: Chronic obstructive pulmonary disease (COPD) is a progressive lung disorder characterized by poorly reversible airway obstruction and its pathogenesis remains largely misunderstood. Local changes of regulatory T-cell populations in the lungs of COPD patients have been demonstrated although data concerning their pathologic role are contrasting. The aim of our study was to evaluate the relative percentage of regulatory T-cells in the peripheral blood of current and former smoker subjects, affected or not by COPD. Furthermore, the effect of different concentrations of budesonide and formoterol, on regulatory T-cells has been investigated.

Methods: T regulatory lymphocytes were isolated and assessed as CD4⁺CD25^{high}CD127⁻ cells by flow cytometry and cultured for 48 hours in the absence or in the presence of budesonide and/or formoterol at different doses.

Results: CD4⁺CD25^{high}CD127⁻ regulatory T-cells percentage was significantly reduced in COPD patients, both current and former smokers, with respect to volunteers. Furthermore, CD4⁺CD25^{high}CD127⁻ cells of COPD patients showed a not statistically significant response to drugs compared to healthy subjects.

Discussion: Our results evidenced a different behaviour of CD4⁺CD25^{high}CD127⁻ Treg cells in COPD patients after in vitro treatments.

Conclusions: Based on our data, we suggested a possible role of CD4⁺CD25^{high}CD127⁻ T-cells in COPD pathogenesis.

Keywords: Chronic obstructive pulmonary disease (COPD), Inflammation, Regulatory T-cells, Corticosteroids, Budesonide, LABA, Formoterol

Background

Chronic obstructive pulmonary disease (COPD) is a progressive lung disorder characterized by poorly reversible airway obstruction. Tobacco smoking is the main etiological factor inducing oxidative stress and an abnormal inflammatory response leading to mucociliary dysfunction, airway wall thickening and pulmonary parenchymal changes [1]. COPD pathogenesis remains largely unknown and it appears to be the result of smoke exposure and host/defense interaction. In balancing the efficient recognition of pathogens and the control of immune tolerance, regulatory T-cells (Tregs) play a key role. Different

subtypes of Tregs exist. While the forkhead box P3 transcription factor (FOXP3) is the hallmark of regulatory function, interleukin (IL)-2 receptor α -chain (also known as CD25) is a cell surface marker commonly used to distinguish among regulatory (CD25^{high}), activated (CD25^{int}), and naive (CD25^{low}) T-cells in humans [2]. Liu et al. have demonstrated that the downregulation of the α -chain of the IL-7 receptor (CD127) on the majority of the CD4⁺FOXP3⁺ T-cells distinguishes Tregs from activated T-cells. Low CD127 expression, combined with high expression of CD25, therefore enables better isolation and purification of Treg populations among CD4⁺CD25⁺ T-cells. In functional assays, CD4⁺CD25^{high}CD127^{low} T-cells are highly suppressive [3].

Contrasting evidences have been reported concerning different subtypes of CD4⁺FOXP3⁺ T-cells in COPD. Plumb et al. assessed the presence of CD4⁺FOXP3⁺

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Tregs in surgically resected lung tissues from COPD patients, smokers with normal lung function and healthy non smokers showing an increased number of CD4⁺FOXP3⁺ cells in lymphocyte follicles in lung parenchyma of moderate COPD patients [4]. Roos-Engstrand et al., analyzing the bronchoalveolar lavage fluid (BALF), showed no significant differences in CD4⁺CD25⁺ cells between COPD patients and the other healthy smokers and non smokers subjects. Among CD4⁺ T-cells expressing CD25, smokers with normal lung function had significantly decreased percentage of FOXP3 expression compared with those who never smoked. Moreover, the authors found that ex-smokers COPD patients expressed a decreased percentage of CD127⁺ cells in BALF compared to smoking COPD patients and the expression of CD127 on CD4⁺CD25⁺ T-cells was increased in smokers with normal lung function, with respect to non-smokers [5]. Compared with never smokers, smokers with normal respiratory function presented a greater number of regulatory T-cells, absent in COPD subjects [6]. Further, an increased proportion of Tregs in the BALF was found in smokers with COPD compared to the control group [7]. Recently, Lane et al. have found that smokers without COPD have increased numbers of CD4⁺CD25⁺FOXP3⁺ Tregs in the large airways [8]. Besides, another study demonstrated impaired Treg-mediated suppression of CD4⁺ T-cell activation in a group of COPD patients with high body mass index and similar proportions of CD4⁺FOXP3⁺ T-cells in COPD patients compared to controls [9].

Tregs have also been explored in peripheral blood. Xiong et al. showed that CD4⁺CD25⁺, CD4⁺ Treg, CD8⁺CD25⁺ and CD8⁺ Tregs were expressed in the peripheral blood of patients with acute exacerbations of COPD with a significant correlation with age, disease's course, smoking index, quantity of white cells, and blood pH, while no correlations were found between these cells and IL-10 [10]. Barcelò et al. showed no significant differences in peripheral blood samples among healthy smokers, no-smokers and COPD patients, concerning CD4⁺CD25⁺ T-cells [6].

Overall, these data underline a not well understood role of Treg population in the pathogenesis of COPD and further investigations are needed to evaluate the potential effect of drugs on Tregs. Bronchodilators, such as long-acting β_2 -agonists, and inhaled corticosteroids, used in combination, are the recommended treatment for moderate and severe COPD patients with frequent exacerbations. It has been demonstrated that glucocorticoids are able to restore the balance between inflammatory and regulatory cells, increasing the proportion of FOXP3⁺ Treg cells [11, 12] but, to date, not many studies assessed the effects of β_2 -agonists in combination with corticosteroids on these lymphocytes [13].

The aim of our study was to evaluate the relative percentage of CD4⁺CD25^{high}CD127⁻ Tregs in the peripheral blood of current and former smoker COPD patients and healthy volunteers. Furthermore, the in vitro effect of different concentrations of an inhaled corticosteroid (budesonide) and a long-acting β_2 -agonist (formoterol), alone and combined, in modulating CD4⁺CD25^{high}CD127⁻ Tregs cell population has been investigated.

Methods

Study subjects

According with the protocol approved by ethical committees of IRCCS-A.O.U. San Martino-IST of Genoa, healthy volunteers current smokers (CSHV) and never-smokers (NSHV), and COPD patients, former smokers (FSC) and current smokers (CSC), were enrolled from November 2012 to December 2013 among the outpatients attending at Allergy and Respiratory Diseases Clinic of Genoa University for a scheduled visit. COPD diagnosis and functional severity were performed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) document 2011 revision [14]. Inclusion criteria were age ≥ 40 years, clinical diagnosis of COPD and symptoms for more than 2 years, forced expiratory volume in the 1st second (FEV₁)/forced vital capacity (FVC) post-bronchodilator lower than 70 %, FEV₁ between 50-80 % of normal predicted, smoking history of at least 10 pack years, on treatment with long acting bronchodilators. Patients having history of asthma and/or allergic rhinitis before the age of 40 years, or suffering from cancer, infections, autoimmune diseases and other immune-related diseases were excluded. No patients treated with chemotherapeutics, immunosuppressors, oral steroids and antibiotics in the 4 weeks before the enrollment, were recruited. CSHV and NSHV were ≥ 40 years of age with normal spirometry according to American Thoracic Society (ATS)/European Respiratory Society (ERS) criteria. Written informed consent was obtained from all participants before study.

Isolation of peripheral blood mononuclear cells and immunophenotyping

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of COPD patients, CSHV and NSHV by means of a density gradient centrifugation (Lympholyte; Cedarlane, Burlington, USA). PBMCs were suspended in RPMI 1640 cell culture medium (Euroclone S.p.A.; Pero, Milan, Italy) and viable cell counts obtained. Regulatory lymphocytes were stained and assessed as CD4⁺CD25^{high}CD127⁻ cells. Their percentage as a proportion of the total CD4⁺ cells was tested by flow cytometry before treatments (time t₀). The following mAbs were used: CD4-FITC, CD25-PE and CD127-PC5 (Immunotech; Beckman Coulter,

Marseille, France). Tregs $CD4^+CD25^{high}CD127^-$ were gated from $CD4^+$ T-cells (Fig. 1). 100.000 events for each sample were acquired using the Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, USA) and the analysis was performed with Attune Cytometric Software 2.1. The results were expressed as percentage of gated $CD4^+$ cells.

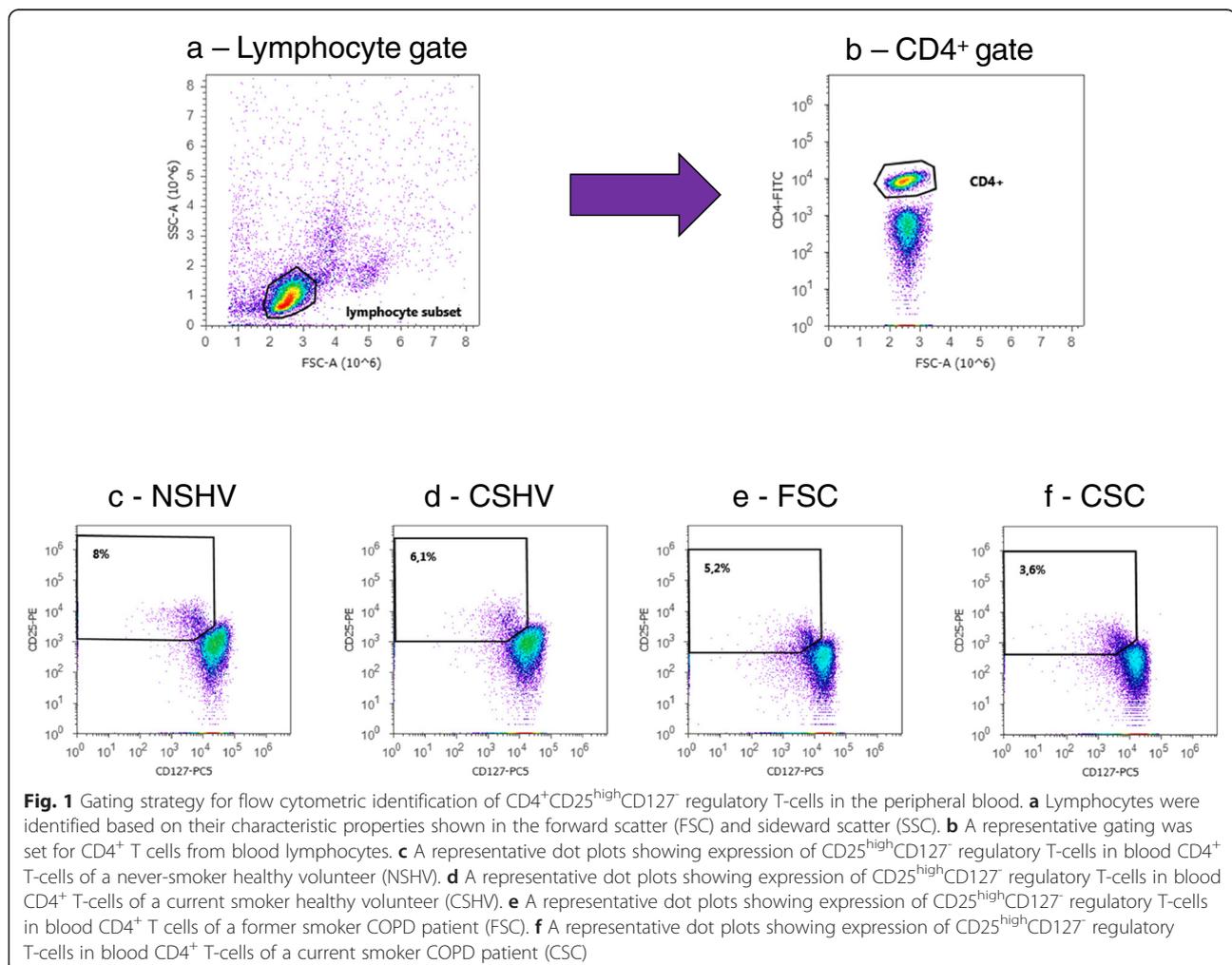
Cell culture and drug treatment

We analyzed $CD4^+CD25^{high}CD127^-$ cells population by flow cytometry using an incubation time of 48 hours according with previously published experiments [15]. Fresh PBMCs were cultured in 24-well round-bottomed microtiter plate at 2×10^6 cells/well in RPMI 1640 (Euroclone S.p.A.; Pero, Milan, Italy) supplemented with 10 % heat-inactivated Human AB Serum (Euroclone S.p.A.; Pero, Milan, Italy), 1 % penicillin/streptomycin (Euroclone S.p.A.; Pero, Milan, Italy) and 2.5 mg/l amphotericin B (Sigma-Aldrich; St. Louis; USA). Cells were treated with IL-2 (100 I.U./ml) (Miltenyi Biotec; Auburn, USA) + transforming growth factor beta-1 (TGF- β 1) (5 ng/ml

(PeproTech EC Ltd.; London, UK) (NT) and in the absence or in the presence of different dosages of budesonide (Bud) and formoterol (For) either alone or in combination (Bud 1 and 0.01 μ M, For 30 and 0.3 nM and Bud 1 μ M + For 30 nM and Bud 0.01 μ M + For 0.3 nM) (Astrazeneca; Basiglio, Italy) [16, 17]. Cell viability was evaluated by trypan blue exclusion dye assay to rule out drugs toxicity. Drugs concentrations have been chosen considering dose-response experiments from our previous study on NK cells population in COPD patients [15]. Following drugs stimulation, cells were harvested, resuspended in PBS, stained with antibodies and analyzed by flow cytometry (time t1) as above described. In order to exclude a drug solvent effect on cells, the effect of the maximum EtOH dose used to dissolve the drugs was also evaluated. We analyzed $CD4^+CD25^{high}CD127^-$ cells population by flow cytometry.

Population sample and data analysis

Population sample was estimated according with available literature and study power calculation. The Kolmogorov-



Smirnov test was applied for assessing the normality of the data distribution. Spearman's rank correlation coefficient was applied to test the correlation between CD4⁺CD25^{high}CD127⁻ Treg cells ratios and FEV₁ values. For multiple comparisons, one-way analysis of variance (ANOVA) was performed, followed by post hoc Duncan's test. Statistical significance was defined as a p value below 0.05. Statistical analysis was performed using STATISTICA version 6.0 (StatSoft) and GraphPad Prism version 5.0 (GraphPad Software Inc.).

Results

Demographic characteristics of study population

PBMCs were obtained from 28 moderate (14 current smokers and 14 former smokers) COPD patients and 20 healthy volunteers (10 current smokers and 10 never-smoker). Clinical and demographic data of study population are reported in Table 1. The mean ages in the patients groups were statistically significant different than in the control groups. The unequal sex ratio is in line with higher prevalence of COPD in men than in women observed in real life.

Circulating Tregs in COPD patients and healthy volunteers

The expression of Treg cells (CD4⁺CD25^{high}CD127⁻) in peripheral blood was different among groups (Fig. 2). In particular CD4⁺CD25^{high}CD127⁻ percentage was significantly reduced in current smokers COPD patients (CSC) and former smokers COPD patients (FSC) with respect to healthy volunteers never-smokers and current smokers (Fig. 2). Correlating CD4⁺CD25^{high}CD127⁻ percentages to FEV₁ values, we observed a statistically significant correlation ($r = 0.6075$; $p < 0.0001$), showing that the lower Treg cells are circulating in peripheral blood, the greater will be the FEV₁ decline (Fig. 3).

Effect of budesonide and formoterol in cultured PBMCs

Statistical analysis of data shows that CD4⁺CD25^{high}CD127⁻ cells in COPD patients have not a statistically significant response to budesonide, alone and in combination with

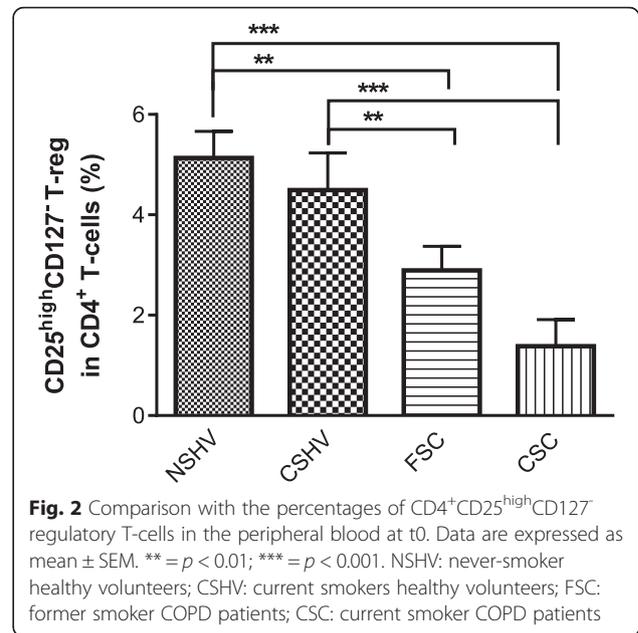


Fig. 2 Comparison with the percentages of CD4⁺CD25^{high}CD127⁻ regulatory T-cells in the peripheral blood at t0. Data are expressed as mean \pm SEM. ** = $p < 0.01$; *** = $p < 0.001$. NSHV: never-smoker healthy volunteers; CSHV: current smokers healthy volunteers; FSC: former smoker COPD patients; CSC: current smoker COPD patients

formoterol, compared with healthy volunteers. In fact, no treatment significantly modulated the proportion of these cells (Fig. 4). Cell culture supernatants were used to investigate the production of IL-10 by ELISA test (data not shown). There were no differences in IL-10 production between groups ($p = 0.1051$).

No difference with untreated cells was evidenced with the maximum EtOH dose used.

Discussion

Several mechanisms have been proposed to be involved in the development of COPD: oxidative stress due to tobacco smoking [18], activation of neutrophils and macrophages, apoptosis of endothelial and epithelial cells [19], defective efferocytosis of residual apoptotic debris [20], viral infections [21] and genetic susceptibility [22].

Many of these hypothesis ascribe to environmental factors a central role in the inflammatory response observed in COPD, nonetheless this inflammatory state is a self-perpetuating process able to persist for years after

Table 1 Demographic characteristics

	Never-smoker healthy volunteers	Current smoker healthy volunteers	Former smokers COPD patients	Current smoker COPD patients
n	10	10	14	14
Age (years)	61.4	57.8	72.5 §§,***	69.9 §,**
Sex (F/M)	6/4	1/9	1/13	3/11
FEV ₁ (%predicted)	102.6 \pm 8.45	94.6 \pm 11.9	61.71 \pm 8.2 §§, **	57.21 \pm 7.3 §§,**

COPD chronic obstructive pulmonary disease; FEV₁ forced expiratory volume in the 1st second

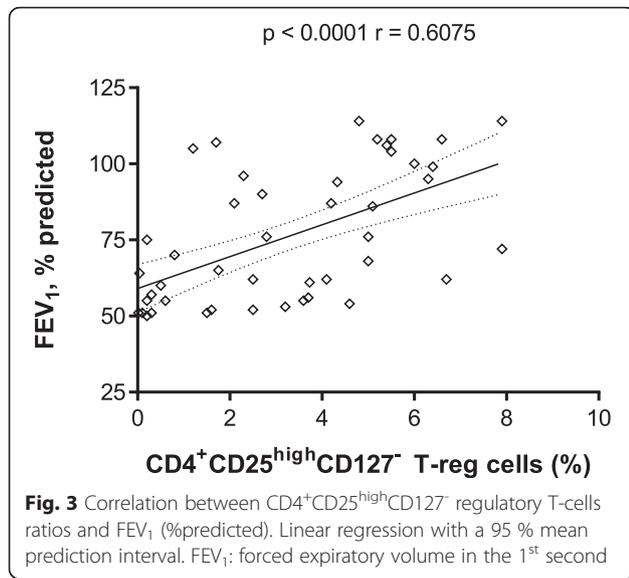
Data are presented as mean \pm SD

§ = $p < 0.05$ vs never-smoker healthy volunteers

§§ = $p < 0.01$ vs never-smoker healthy volunteers

** = $p < 0.01$ vs current smoker healthy volunteers

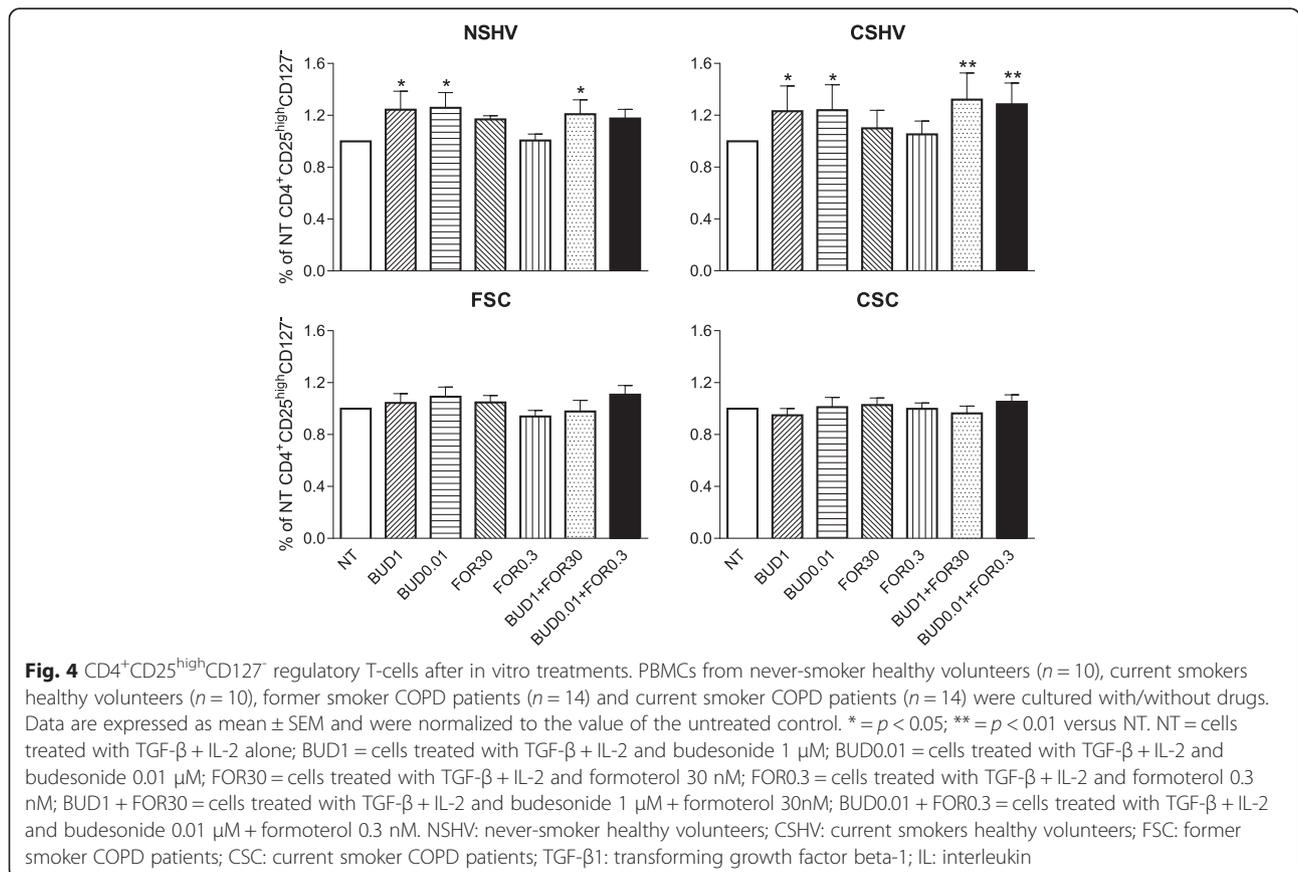
*** = $p < 0.001$ vs current smoker healthy volunteers



cessation of smoking. For this reason it has been proposed that COPD may derive from a shift from the non-specific innate response present in every smoker toward an adaptive immune response and may present an autoimmune component. Exposure to infections or smoking-induced lung injury could release sequestered autoantigens and

DNA from apoptotic cells and alter proteins [23]. T lymphocytes can recognize these products as foreign antigens and maintain a prolonged inflammatory state in the airways in response to self-antigens [24]. Activation of T-cells is highly controlled by negative regulatory mechanisms. Disturbed homeostasis of regulatory T-cell population was demonstrated in several pathologies with autoimmune etiology such as lupus erythematosus, diabetes mellitus and rheumatoid arthritis [25, 26]. A deficiency of regulatory T-cells can weaken the immune tolerance to self-antigens and thereby support a persistent inflammation mediated by CD8⁺ cells in COPD. Lee et al. hypothesized that, in patients with emphysema, the inflammatory process would be sustained by the presence of anti-elastin autoantibodies and showed that the Treg population, detected as CD25^{high}CD62L⁺ cells, was lower in the lungs and in the blood of patients compared to healthy subjects [27].

Data concerning regulatory T-cells in COPD patients are not so numerous and sometimes discordant. In our study we investigated CD4⁺CD25^{high}CD127⁻ proportion in peripheral blood, in current and former smokers with moderate airway obstruction COPD patients and current smokers and never-smokers healthy volunteers. We found a significant depletion in CD4⁺CD25^{high}CD127⁻ proportion



in COPD patients compared to healthy smokers and never-smoker subjects. This might reflect a kind of progression of inflammation status and exhaustion of anti-inflammatory responses from healthy smokers to COPD patients. Evaluating Treg population as CD4⁺CD25^{high}CD127⁻, we confirmed data obtained in other studies [28, 29]. However, our results are in contrast with the findings of Barcelò et al., showing that no differences in CD4⁺CD25⁺ Tregs from peripheral blood were detected among COPD, healthy smokers and controls and with the results of Vargas-Rojas et al. describing increased levels of Treg cells present in COPD and smokers subjects compared to healthy ones [6, 30]. These differences may be caused by patients selection or technical approach to evidence regulatory T-cells. Moreover, we found statistically significant differences in mean age between groups. Based on literature, peripheral CD4⁺CD25^{high}CD127⁻ cells have to increase with patient's age, but our data support the opposite. Thus, we suppose that these results can be mainly related to COPD pathology [31–34]. Other studies are necessary to increase the number of samples and to definitively clarify the role of regulatory T-cells in COPD.

Hopefully, pharmacological treatment might restore the balance between effector T-cells and regulatory T-cells [35]. Profita et al. evaluated the expression of FOXP3 in PBMCs from COPD patients after 48 h of in vitro stimulation with tiotropium and olodaterol. They reported increased levels of CD4⁺CD25⁺FOXP3⁺ in treated PBMCs with respect to untreated ones with both drugs alone or in combination [36].

In our study, stimulated in vitro COPD patients CD4⁺CD25^{high}CD127⁻ cells were not modulated by budesonide or formoterol, both alone and in combination. Interestingly, these cells were significantly modulated by budesonide treatments in never-smoker and current smokers healthy volunteers except by Bud 0.01 µM + For 0.3 nM in NSHV. Yang et al. showed that in patients with moderate or severe COPD receiving treatment with 50/500 µg of salmeterol/fluticasone propionate twice a day for 12 weeks, the proportion of FOXP3⁺ Tregs in the total CD4⁺ T-cell population in the peripheral blood was drastically higher than that before treatment [29]. The different effect of salmeterol/fluticasone and budesonide/formoterol on peripheral Treg cells should be evaluated considering the different methodological approach adopted in the studies.

Nevertheless, if future studies will confirm these results, they should be analyzed bearing in mind the different rate of drug-related adverse events, such as pneumonia, described in clinical research. In fact, among the potential side effects of inhaled corticosteroid (ICS) treatments in COPD patients, the use of fluticasone or fluticasone/salmeterol combination has been associated

with a higher prevalence of pneumonia in the major long-term studies [37–39]. All ICSs can suppress natural and adaptive immunity with a potentially greater inhibition of type-1 innate immunity [40]. On the other hand, no similar increased risk of pneumonia has been reported in patients with COPD treated with the budesonide/formoterol combination [41–43].

Conclusions

Our data pointed out a different behavior of CD4⁺CD25^{high}CD127⁻ T-cells in the four groups evaluated, depending on the presence of COPD inflammatory process. In COPD patients, Treg cells appeared unsusceptible to the action of drugs, whose effect is, on the contrary, clear on cellular components of healthy subjects.

In conclusion, we support the possible role of CD4⁺CD25^{high}CD127⁻ in COPD pathogenesis. Budesonide and formoterol tested in vitro did not have any effects on CD4⁺CD25^{high}CD127⁻ population in our experimental conditions. These results need to further be explored in a direct comparison with other bronchodilators and ICSs in order to better clarify their immunomodulatory properties.

Abbreviations

COPD: Chronic obstructive pulmonary disease; Bud: Budesonide; For: Formoterol; Tregs: Regulatory T-cells; FOXP3: Forkhead box P3 transcription factor; IL: Interleukin; CD127: α-chain of IL-7 receptor; BALF: Bronchoalveolar lavage fluid; CSHV: Current smoker healthy volunteers; NSHV: Never-smoker healthy volunteers; FSC: Former smoker COPD patients; CSC: Current smoker COPD patients; GOLD: Global initiative for chronic obstructive lung disease; FEV₁: Forced expiratory volume in the 1st second; FVC: Forced vital capacity; ATS: American Thoracic Society; ERS: European Respiratory Society; PBMC: Peripheral blood mononuclear cell; TGF-β1: Transforming growth factor beta-1; ICS: Inhaled corticosteroid.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AC and CF contributed to design of the study, acquisition of data, performing the statistical analysis and writing the manuscript. FB recruited the patients and collected clinical data. EC contributed to acquisition of data and analysis of data. AMR and LDF participated in drafting the manuscript and revising it critically. GM helped to perform the statistical analysis and revise the manuscript. FB participated in study design and coordination, contributed to the interpretation of data and writing the manuscript. GWC contributed as lead investigator and was responsible for designing the study and writing the manuscript. All authors read and approved the final manuscript.

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