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Original Article

Expressions of CD4$^+$CD25$^+$ regulatory T cells and *foxp3* in peripheral blood of patients with chronic obstructive pulmonary disease

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Abstract: Objective: To explore the expression of CD4$^+$CD25$^+$ regulatory T cell (Treg) and Foxp3 in peripheral blood of patients with chronic obstructive pulmonary disease (COPD) and its clinical significance by performing a case-control study. Methods: Eighty-two COPD patients treated in our hospital from Jan 2013 to Jun 2014 were enrolled as case group and 76 healthy individuals as control group. All the subjects underwent pulmonary function testing. Expression of Treg in peripheral blood was detected by Flow cytometry (FCM); serum concentration was detected by Enzyme-Linked ImmunoSorbent Assay (ELISA); and reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect the mRNA expression of Foxp3; finally related statistical analysis was carried out by using SPSS 18.0 software package. Results: A significant decrease of PEV1 and PEV1/FVC levels was found in patients with COPD when compared with control group ($P < 0.01$); comparing with control group, expressions of CD4$^+$ Treg and CD4$^+$CD25$^+$ Treg were significantly decreased ($P < 0.01$), also the percentage of CD4$^+$CD25$^+$ Treg in CD4$^+$ Treg was lower ($P < 0.01$); when compared to control group, the concentration of IL-10 and TGF-β was also decreased in COPD patients ($P < 0.05$); comparing with control group, expression of both Foxp3 mRNA and Foxp3 protein in COPD patients were significantly decreased (both $P < 0.01$). Conclusion: The population of CD4$^+$CD25$^+$ Treg cells and serum concentration in patients with COPD were decreased and Foxp3 genetic expression decreased as well.

Keywords: Chronic obstructive pulmonary disease, pulmonary function, CD4$^+$ Treg, CD25$^+$ Treg, serum concentration, Foxp3

Introduction

Chronic obstructive pulmonary disease (COPD), a chronic inflammation disease triggered by immune response, is characterized by developing airflow limitation, persistent inflammation and progressive destruction of lung tissue [1]. At the moment, COPD ranks third as cause of mortality and disability-adjusted life years around the world, which imposes social and economic load. Moreover, it is presumed that COPD will rise to the third cause of mortality and ranks fifth in terms of economic burden till 2020 [2]. However, the pathogenesis of COPD is far from clear [1]. But studies have showed that the key mechanisms of COPD pathogenesis might greatly associate with immune system disorder [3]. Besides, studies have suggested various inflammatory cells were involved in COPD development, including macrophages, neutrophils and T-lymphocytes [4].

T cells, especially CD4$^+$ T cells, have been involved in mediating many aspects of autoimmune inflammation [5]. CD4$^+$CD25$^+$ regulatory T cells (Treg), that is a novel subset of CD4$^+$T cells discovered in recent years, function the regulation of immune response [6]. Besides maintaining homeostasis, CD4$^+$CD25$^+$ Treg is capable of suppressing immune response triggered by tumor or tumor-derived substance [6]. As an important member of FOX protein family, forkhead/winged helix transcription factor p3 (Foxp3) is specifically overexpressed in CD4$^+$ CD25$^+$ Treg, which is an important switch to regulate the development, activation and function of CD4$^+$CD25$^+$ Treg, and can reflect the number and function of CD4$^+$CD25$^+$ Treg to a

Table 1. General information

Index	COPD (n=82)	Control group (n=76)	*P*
Age (years)	48.1±11.0	51.5±11.8	0.063
Gender (male/female)	50/32	45/31	0.910
Smoking history	14	8	0.311
PEV1 (%)	71.9±8.9	97.8±8.2#	< 0.001
FVC (%)	104.7±13.4	101.4±12.4	0.111
PEF (%)	77.7±11.5	80.9±10.0	0.065
PEV1/FVC (%)	69.6±11.9	98.0±15.2#	< 0.001

Notes: #means $P < 0.01$ comparing with COPD patients; PEV1, forced expiratory volume in one second; FVC, forced vital capacity; PEF, peak expiratory flow; COPD: chronic obstructive pulmonary disease.

Table 2. Comparison of Treg subset between COPD patients and healthy controls ($\bar{x}$±s, %)

Cell type	COPD (n = 82)	Control group (n = 76)	*P*
$CD4^+$	28.65±3.53	30.18±4.43#	0.017
$CD25^+$	4.05±1.01	4.33±0.93	0.073
$CD4^+CD25^+$	2.91±0.97	3.42±1.12#	0.003
$CD4^+CD25^+/CD4^+$	10.27±3.63	11.69±4.60*	0.032

Note: *means $P < 0.05$ comparing with COPD group; #means $P < 0.01$ comparing with COPD group; COPD: chronic obstructive pulmonary disease.

certain extent [7]. Recent studies have reported an alteration in the population of Tregs in autoimmune disease and chronic inflammatory disease, which may indicate Treg cells play an important role in regulating the pathogenesis of these diseases [8]. Despite evidence shows the importance of Treg cells in COPD pathogenesis, the mechanism in details is still unclear [1]. In this study, we explore the changes of $CD4^+CD25^+$ Treg and Foxp3 mRNA and protein expression in patients with COPD, so as to elucidate the potential roles of $CD4^+CD25^+$ Treg and Foxp3 in the occurrence and development of COPD.

Materials and methods

Study subjects

This prospective study was performed in 82 COPD patients (50 males/32 females; mean age, 48.1±11.0 years; age range, range 40-75 years) admitted in our hospital from Jan 2013 to Jun 2014; A total of 76 healthy volunteers were selected as control group, including 45 males and 31 females, with the mean age 51.5±11.8 years, age range 40-70 years. Diagnosis criteria: we referred to *Global Initiative for COPD 2010* and *Guidelines on Diagnosis and Treatment of Chronic Obstructive Pulmonary Disease 2007* made by COPD group belonged to Chinese Thoracic Society [9]: 1. Poorly reversed airflow limitation; after using bronchodilators, a ratio of forced expiratory volume in 1 second to forced vital capacity (FEV1/FVC) < 70%, FEV1 < 0.8 of predicted value (must have); 2. Symptoms such as cough, expectoration and dyspnea (could have); 3. Contact history of risk factors for COPD (could have). Inclusion criteria: 1. In accordance with diagnosis criteria of COPD; 2. Male or female patients aged 30-38 years; 3. Patients volunteering to accept clinical research and signing a letter of informed consent. Exclusion criteria: 1. Patients with chronic cough and asthma caused by tuberculosis, bronchiectasia, pulmonary cystic fibrosis, fungal infection, tumor, irritant gas and allergic factors; 2. Patients complicated with pneumothorax, pulmonary bulla and bleeding tendency; 3. Patients with neuromuscular disease affecting respiratory movement function; 4. Other diseases like severe complications of angiocarpy, urinary, digestive system and metabolic system, hepatic and renal insufficiency, unconsciousness, dementia and various mental diseases; 5. Pregnant or lactating woman. The study has been examined and approved by our Academic Ethics Committee and obtained informed consent from all study subjects. Moreover, every subject signed the written informed consent in prior to enrollment.

Experimental methods

Pulmonary function test: The test was arranged in the morning. Using 200 μg Salbutamol for 15 min, we began to determine parameters of lung function: forced expiratory volume in one second (PEV1), forced vital capacity (FVC), PEV1/FVC and peak expiratory flow (PEF). All indexes were expressed by percentage of actual measurement in predicted value.

Detection of $CD4^+CD25^+$ Treg cells in peripheral blood: The peripheral venous blood of all the patients and control group was collected before

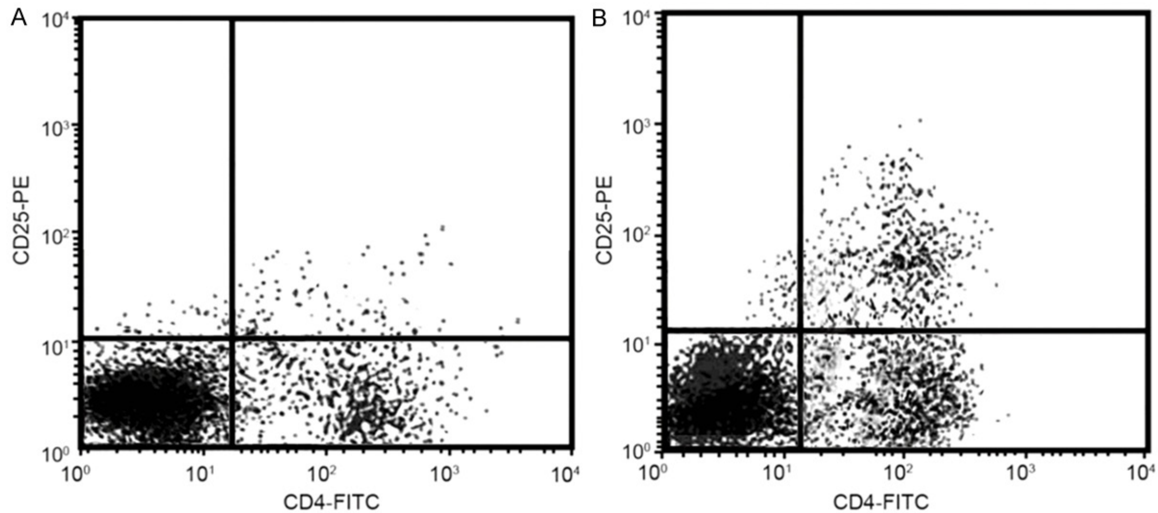

Figure 1. Detection results of Flow cytometry. A. Chronic obstructive pulmonary disease (COPD patients); B. healthy controls.

Table 3. Comparison of serum cytokine level between COPD patients and healthy controls

Serum type	COPD (n = 82)	Control group (n = 76)	*P*
IL-4	68.07±6.97	70.28±7.15	0.051
IL-10	30.53±9.45	33.77±10.25*	0.040
IFN-α	70.76±22.73	65.71±19.31	0.136
IFN-γ	95.65±11.09	92.38±10.07	0.055
TGF-β	165.26±35.52	178.31±43.19*	0.039

Note: *shows $P < 0.05$ when compared with COPD patients; IL-4, interleukin 4; IL-10, interleukin 10; IFN-α, interferon-α; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; COPD: chronic obstructive pulmonary disease.

breakfast, early morning, of which 5 ml blood was isolated by centrifugal separation for serum and was maintained at -70°C for cytokine analysis. For the rest 5 ml blood, we added heparin for anticoagulation. Peripheral blood mononuclear cells (PBMC) were isolated using standard lymphocytes separation medium (AXIS-SHIELD), followed by adding RPMI-1640 with 10% fetal bovine serum (FBS) to adjust the concentration of cells to 1×10^6/mL. Cells (200 μL) were added with 20 μL anti-CD4-FITC and anti-CD25-PE monoclonal antibody (isotype controls were IgG_1-FITC and IgG_1-FE), mixed, incubated in darkness for 25-30 min and washed twice by PBS. Cells were suspended in 0.5 mL PBS buffer and shocked evenly. Data were detected and analyzed by EPICS-XL flow cytometry (Becton Dickinson) using Cell quest software and included the quantity and proportion of each positive cell.

Detection of cytokines: Quantitative Enzyme-Linked Immuno Sorbent Assay (ELISA) method was used to detect the reserved blood abovementioned according to instructions of ELISA kit (Shanghai Enzyme-linked Biotechnology Co., Ltd.). Setting up blank control, absorption value was measured by ELISA (Bio-tek instruments inc.) in double-barrel at 450 nm and determined from the mean value by deleting control A value.

Detection by reverse transcriptase-polymerase chain reaction (RT-PCR): Standard TRIZOL method was used to isolate total RNA [10]. A total of 2 μg isolated RNA was taken for PCR reaction according to instructions of First Strand cDNA Synthesis Kit (MBI) and the overall reaction volume was 20 μL. For *Foxp3*, its positive-sense strand was 5'-TTTCATGCACCAGCTCTCAA-3', and antisense strand was 5'-ATGGCACTCAGCTTCTCCTTC-3' and the product length was 580 bp; for GADP, its positive-sense strand was 5'-AATCCCATCACCATCTTCCA-3' and antisense strand was 5'-CCTGCTTCACCACCTTCTTG-3' and the product length was 475 bp. PCR reaction conditions: both *Foxp3* and GADPH underwent pre-denaturation at 95°C, 3 min; for Foxp3, 35 PCR cycles (including denaturation at 95°C, 30 s, annealing at 60°C, 30 s, extension at 68°C, 90 s), extension at 68°C, 10 min again; for GADPH, 25 PCR cycles at 72°C

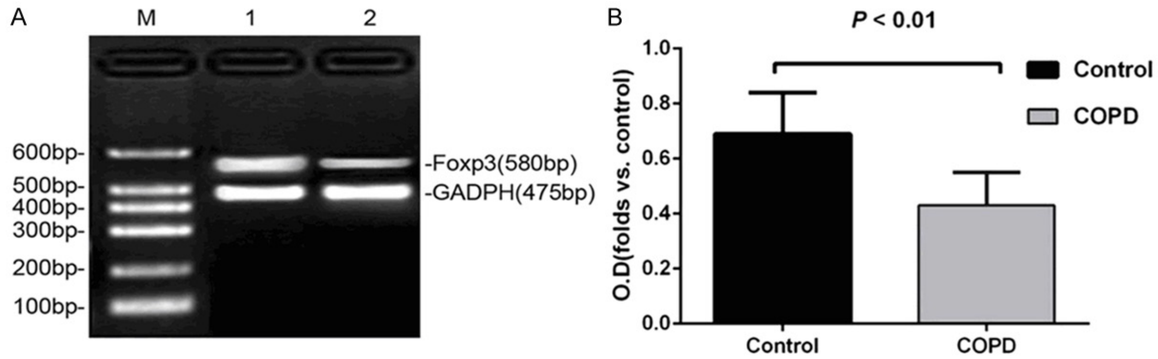

Figure 2. Detection by transcriptase-polymerase chain reaction (RT-PCR) 1, Healthy controls; 2, chronic obstructive pulmonary disease (COPD) patients.

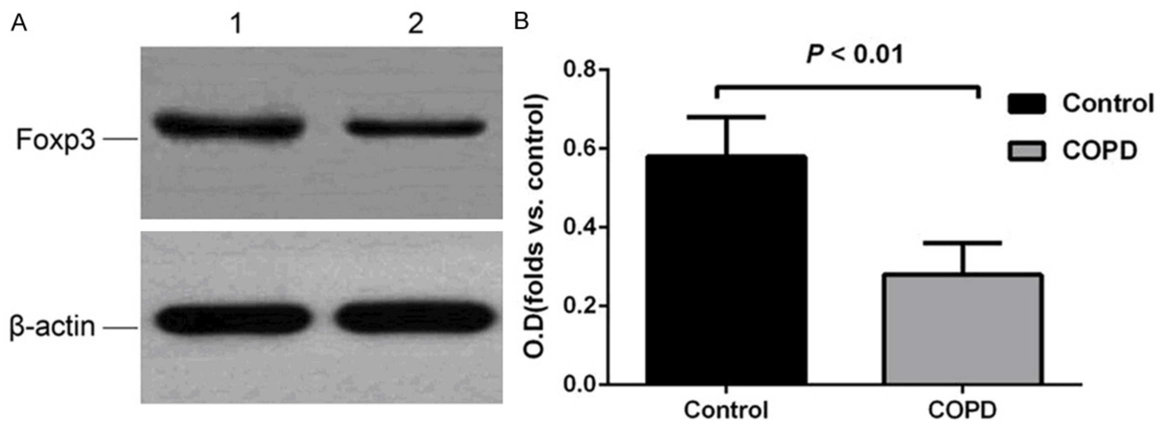

Figure 3. Detection by Western blot; 1, Healthy controls; 2, Chronic obstructive pulmonary disease (COPD) patients.

(including denaturation at 95°C, 30 s, annealing at 60°C, 30 s, extension at 68°C, 90 s), extension at 68°C, 10 min again. Semi-quantitative PCR reaction products run electrophoresis at 100 V, 30 min and the scanning analysis was performed by GDS8000 automatic gel imaging analysis system (UVP, UK). The relative expression level of *Foxp*3 gene was measured by the ratio of the gray value of *Foxp3* to that of GADPH.

Detection by Western blot: PBMC were washed by PBS and dissolved in lysis buffer. With mouse anti-human β-actin as the primary antibody and goat anti-mouse IgG labeled by horse radish peroxidase (HPR) as the second antibody, with the color treated and then exposed to X-ray film. After scanning and being treated using ScnImage analysis system, a ratio of optical density of target protein brand to that of β-actin brand was considered as the relative content of target protein.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 software package. All values were expressed as means ± standard deviation ($\bar{x}$±s). For comparison between groups, t-test was used; for enumeration data, chi-square test was applied. $P < 0.5$ was considered as statistical significant.

Results

General information

When compared with healthy controls, not only PEV1 was significantly decreased in COPD patients (71.9±8.9 vs 97.8±8.2, $P < 0.01$), but also PEV1/FVC was significantly reduced (**Table 1**). There were no statistical difference in age, gender, smoking history, FVC and PEF in both of COPD patients and healthy controls (all $P >$ 0.05, respectively).

Comparison of Treg subset between COPD patients and healthy controls

Comparing with healthy controls, in COPD patients, both the expression of CD4+ Treg (28.65±3.53 vs 30.18±4.43, $P < 0.01$) and the expression of CD4+CD25+ Treg (2.91±0.97 vs 3.42±1.12, $P < 0.01$) were significantly decreased. Moreover, the percentage of CD4+ CD25+ Treg in CD4+ Treg of COPD patients was lower than that of healthy controls (10.27±3.63 vs 11.69±4.60, $P < 0.05$) (**Table 2**; **Figure 1**).

Comparison of serum cytokine level between COPD patients and healthy controls

The concentration of serum IL-10 of COPD patients was lower than that of healthy controls (30.53±9.45 vs 33.77±10.25, $P < 0.05$) and when compared with healthy controls, the concentration of serum TGF-β was lower as well (165.26±35.53 vs 178.31±43.19, $P < 0.05$). Besides, the concentration of both serum IL-4 and IFN-γ of COPD patients was lower than that of healthy controls and the concentration of IFN-α was higher. However, the difference was not of statistical significance (**Table 3**).

Differential expression of Foxp3 mRNA and protein between COPD patients and healthy controls

The expression of *Foxp3* mRNA in PBMC of COPD patients and healthy controls (**Figure 2**): comparing with healthy controls, the expression level of *Foxp3* mRNA in PBMC of COPD patients was significantly lower (0.43±0.12 vs 0.69±0.15, $P < 0.01$).

The expression of *Foxp3* protein in PBMC of COPD patients and healthy controls (**Figure 3**): **Figure 3** showed that in both of COPD patients and healthy controls, the expression tendency of *Foxp3* protein was consistent with that of *Foxp3* mRNA, but when compared with healthy control, the expression level of *Foxp3* protein in PBMC of COPD patients was significantly lower (0.28±0.10 vs 0.58±0.08, $P < 0.01$).

Discussion

In recent years, lots of scholars have conducted researches about immune function of COPD patient. More and more evidences indicated that there was close correlation of occurrence and development of COPD with changes in immune function [4, 11]. With immune anergy and suppressive immunity, CD4+CD25+ Treg cells produce memory cells in the presence of infection signal and inhibit the occurrence of pathological immune response. Our study showed that comparing with healthy controls, the expression of CD4+ Treg and CD4+CD25+ Treg was significantly decreased and the percentage of CD4+CD25+ Treg in CD4+ Treg was far lower. This suggested that the depletion in the population of Treg cells functioning suppressive immunity broke immune homeostasis, which might promote occurrence and development of COPD. A study consistent with our view reported significantly lower proportion of CD4+CD25 high T cells as well as CD4+CD25+ Treg cells in peripheral blood of patients with mild/moderate COPD than in healthy controls [12]. However, multiple studies inconsistent with our findings have showed that the population of CD4+CD25+ Treg cells was increased in COPD patients when compared with healthy controls [1, 11]. Lee et al. also has pointed out a significant increasing of Tregs in peripheral blood and lungs of COPD patients and emphysema patients. This finding has demonstrated that the increasing of CD4+CD25+ Treg cells in COPD patients suppressed the cytotoxicity of NK cell mediate so as to suppress antitumor immune response of host and promote the occurrence and development of tumor.

Our study also showed that comparing with healthy controls, the concentration of IL-10 and TGF-β of COPD patients was reduced, which revealed that in COPD patients existed disorder in specific cells and cytokines. Previous study found that IL-10 was known as an important proinflammatory cytokine that can induce acute inflammatory reaction and impair vascular endothelial cells [13]. Besides that, TGF-β could induce not only IL-10 synthesis but also vascular endothelial cells apoptosis [14]. Hence, the decreasing concentration of IL-10 and TGF-β weakened the inhibition of organism on inflammatory response. With suppressing other T-cells response, Treg performed the inhibition of inflammatory response, and this function of Treg was mainly realized through its differentiation which relied on joint-participation of antigen, regulatory factor and co-stimulating factor [11]. Treg cells exerted suppressive function effectively by releasing cytokines and other cells (like neutrophil, macrophage, etc.) which

secreted a large amount of inflammatory cytokines. Otherwise, once the expression of Treg cells is too poor, Treg cells cannot regulate their own function so that they are incapable of suppressing secretion of inflammatory cytokines. Consequently, that leads to continuous occurrence of inflammation [8].

Furthermore, this study reported the significantly decreasing in the expression of Foxp3 mRNA and *Foxp3* protein in COPD patients when compared with healthy controls. Besides continuously expressing in CD4+CD25+ Treg, Foxp3 also poorly expressed in activated effector T cells, which suggested there was a significant correlation of the expression of Foxp3 with functions of Treg cells including immune regulation and suppressing inflammatory response [1]. A study, which was in line with our findings, has demonstrated that in COPD patients, the less CD4+CD25+ Treg cells, the poorer expression of Foxp3 [1]. In addition, a study has also showed that with the decreasing expression of CD4+CD25+ Treg cells in peripheral blood of model rat with COPD, the expression of Foxp3 decreased, which was consistent with findings of our study that both the population of CD4+CD25+ Treg cells and the expression of Foxp3 mRNA and Foxp3 protein were reduced [1]. Here, the decreasing expression of Foxp3 weakens the immune regulation of Treg cells, which leads to fail in suppressing inflammation stimulus to the organism. Consequently, the increasing inflammatory response in lungs and small airway eventually leads to the occurrence of COPD.

To sum up, for COPD patients, both the population of CD4+CD25+ Treg cells and serum concentration were decreased, as well as the expression of Foxp3, which indicated immune dysfunction of COPD patients might relate to the abnormal expression of CD4+CD25+ Treg and Foxp3. In this regards, our study provided a reference for prevention and treatment of COPD in cell and gene levels.

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Disclosure of conflict of interest

None.

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