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Original Article

Changes and significance of peripheral blood helper T cell 9 and interleukin-9 in patients with chronic obstructive pulmonary disease

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Abstract: Objective: To observe the changes of peripheral blood helper T cell 9 (Th9) and interleukin-9 (IL-9) in the stable and acute exacerbation phases of chronic obstructive pulmonary disease (COPD) so as to investigate its effects and clinical significance in the pathogenesis of COPD. Methods: A total of 42 patients (22 patients in the acute exacerbation chronic obstructive pulmonary disease (AECOPD) and 20 patients in the stable chronic obstructive pulmonary disease (SCOPD) and 15 healthy controls were enrolled in this study to test the Th9 content by flow cytometry (FCM) and the serum IL-9 level by enzyme linked immunosorbent assay (ELISA). Results: The levels of Th9 and IL-9 in Group AECOPD were significantly higher than those in Group SCOPD (0.91±0.17 vs 0.27±0.13 and 0.28±0.13, $P<0.05$; 3.36±2.59 vs 1.47±0.83 and 1.82±0.62, $P<0.05$), and the IL-9 level and the Th9 ratio in Group AECOPD showed slight positive correlation ($r=0.436$, $P<0.05$). There was no significant difference in the IL-9 level and Th9 ratio in the AECOPD and SCOPD ($P>0.05$). Conclusions: The Th9 cells may be involved in the pathogenesis of AECOPD by secreting IL-9.

Keywords: Chronic obstructive pulmonary disease, Th9, IL-9, pulmonary function

Introduction

Chronic obstructive pulmonary disease (COPD) is a common disease characterized by persistent airflow limitation. The degree of airflow limitation is not completely reversible and progressively develops, and its acute exacerbation and systemic complications may affect the severity and prognosis of the disease [1]. The morbidity and mortality of COPD increases year by year, and now it ranks the third place of global death causes [2]; in 2020, COPD will become the fifth of world's disease-caused economic burden. The pathogenesis of COPD has not been clear yet, and certain scholars thought that COPD may be a smoking triggered autoimmune disease [3]. Previous studies have shown that COPD is characterized by the increase of macrophages, activated neutrophils, and lymphocytes in the peripheral airway, lung parenchyma, or pulmonary vessels. Some patients may also appear the increase of eosinophils, Th2 cells, or type 2 intrinsic lymphocytes (ILC2). The above inflammatory cells, epithelial cells, and other structural cells co-release a variety of inflammatory mediators and thus are involved in the pathogenesis of COPD [4]. Different studies all consider that the immune system plays an important role in the inflammatory response and immune response of COPD. Studies aiming the specific immune function in COPD patients mainly investigate the changes of CD3+T cells, CD4+T cells, CD8+T cells, and CD4+/CD8+T cells, and consider the decreased cell ratio of CD4+/CD8+T as an important indicator of immune function decrease. The helper T cells are a class of CD4+T cell subset that are functionally classified according to different cytokines they secrete. Studies targeting the helper T cells and COPD mainly concentrate on the balance disorder among the Th1/Th2, Th17, and regulatory T cells (Treg). The Th9 cells are a class of CD4+T lymphocyte subset newly discovered in recent years, and mainly secrete IL-9 thus participating in the processes of allergic inflammation, autoimmune diseases, or antitumor [5, 6]. The Th9 cells and IL-9 are significantly increased in the peripheral blood of patients with allergic asthma, and the IL-9 level is negatively correlated with the apoptosis of

Table 1. Three groups in general

Groups	N	General (m/f)	Ages
AECOPD	22	19/3	77.32±9.09
Stable COPD	20	18/2	75.3±9.17
Healthy control	15	11/4	72.5±6.04

Table 2. Three groups Th9 cells and the level of IL-9 ($\bar{x}$±s)

Group	N	Th9 cells (%)	IL-9 (pg/ml)
AECOPD	22	0.91±0.17*	3.36±2.59*
Stable COPD	20	0.27±0.13	1.47±0.83
Healthy control	15	0.28±0.13	1.82±0.62

Note: *Compared with stable COPD group and healthy control group, P<0.05.

neutrophils. The Th9 cells and IL-9 may be involved in allergic asthma-caused chronic airway inflammation in patients with allergic asthma [7], and anti-IL-9 antibodies can reduce airway hyperresponsiveness, goblet cell metaplasia, and other inflammatory responses in rat allergic asthma model. Previously, IL-9 has been investigated the expression from the sputum and exhaled condensate gas in COPD rat model and COPD crowd. The IL-9 levels in smoking-induced COPD rat model, rat alveolar lavage fluid, peripheral blood, and lung tissue are significantly higher than that in normal rats, and positively correlated with the number of white blood cells, macrophages, or neutrophils [8]. Certain studies also reported higher IL-9 in the induced sputum of COPD patients, and positively related to the macrophages and IL-8, so IL-9 may be involved in the COPD airway inflammation and injury through macrophages [9, 10]. There is no report about the relationships of Th9 and IL-9 with COPD. The aim of this study is to investigate the changes of Th9 and IL-9 in the AECOPD and SCOPD patients so as to explore their roles and clinical significance in the pathogenesis of COPD.

Materials and methods

General information

The study subjects were divided into AECOPD, SCOPD, and Control, and all selected from the inpatient or outpatient COPD patients from February to April 2017 in our hospital; the AECOPD and SCOPD met the standards of Global Initiative for Chronic Obstructive Lung Disease (GOLD) in 2017 [3] and had a history of more than 10 years of smoking. Group SCOPD had 20 cases, including 18 males and 2 females, aging (75.3±9.17) years old, with stable condition for 3 months or more; Group AECOPD had 22 cases, including 19 males and 3 females, aging (77.32±9.09) years old. Meanwhile, 15 healthy subjects were also selected as Group Control, including 11 males and 4 females, aging (72.5±6.04) years old, with no history of respiratory infection for nearly 1 month. All the selected subjects should be excluded from the combination of allergic asthma, autoimmune diseases, blood system diseases, tumors, or recent application of hormones or immunosuppressive therapy. The age, sex, smoking history, previous history of illness, and other basic information of all the subjects were collected. This study was approved by the Ethics Committee of Central South Hospital, and all the subjects signed the informed consent.

Comparison of Th9 cell ratio

The human peripheral blood mononuclear lymphocytes were extracted using the human lymphocyte isolation solution (Sigma, St. Louis, USA), cultured in 1640 medium containing phorbol ester PMA (Sigma, St. Louis, USA), monensin (eBioscience, San Diego, USA), and ionomycin (Sigma, St. Louis, USA) for 5 hours; 5 μl of PE-C y7-CD3 (eBioscience, San Diego, USA) and FITC-CD8a (eBioscience, San Diego, USA) were added, respectively, and the mixture was then incubated in darkness at 4°C for 30 min, followed by phosphate buffered saline (PBS) rinsing, two-time centrifugation, 30-min incubation using one fix&perm kit at 4°C, and two-time rinsing and centrifugation using the fix&perm kit. 5 μl of PE-IL9 antibody (eBioscience, San Diego, USA) was then added together with PE-IgG2a K as the isotype control for 30-min incubation at 4°C in darkness; after rinsed using PBS, centrifuged twice, and fixed in 4% paraformaldehyde, the mixture was then preserved in darkness at 4°C for the detection. The Th9 cells were defined as CD9+CD8-IL9+, and detected the ratio by FC500 flow cytometer (Beckman, CA, USA).

Comparison of IL-9

4 ml of fasting peripheral blood was collected from each subject in the morning, and after centrifuged at 2000 r/min for 6 min, the upper

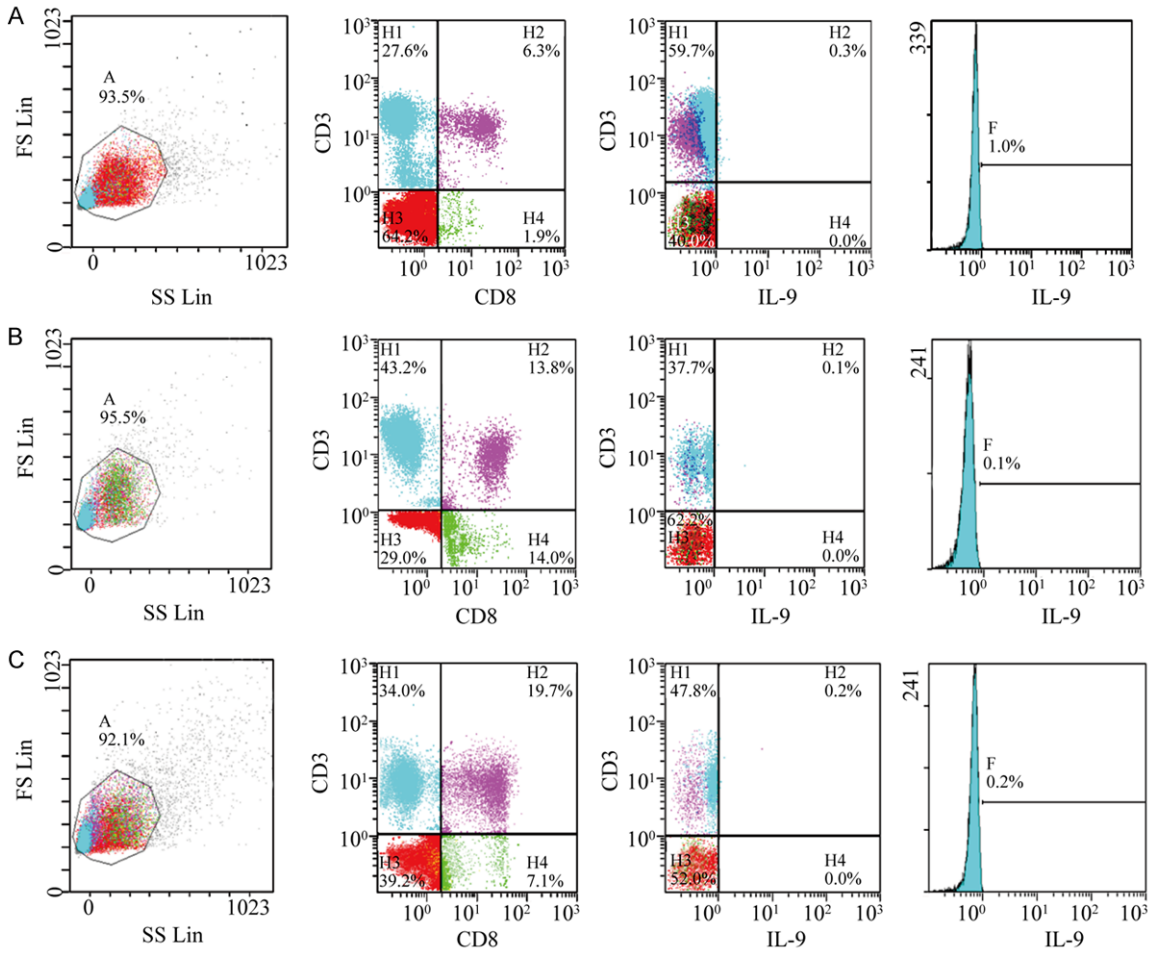

Figure 1. A. Detection of peripheral blood Th9 streaming AECOPD group figure; B. Detection of peripheral blood Th9 streaming SCOPD group figure; C. Controls peripheral blood Th9 flow diagram.

blood serum was tested the serum level of IL-9 by IL-9 ELISA kit (eBioscience, San Diego, USA).

Statistical analysis

The general data such as sex, age, and smoking history of all the subjects were archived using EXCEL and analyzed using SPSS16.0. The data were expressed as mean ± standard deviation ($\bar{x}$±SD), and performed one-way ANOVA after tested the homogeneity of variance. The intragroup data correlation was analyzed using the Pearson correlation analysis, with $P<0.05$ considered as statistical significance.

Results

General information

There was no significant difference in the age and sex among the three groups ($P>0.05$) (**Table 1**).

Comparison of Th9 cell ratio

The Th9 cell ratio in Group AECOPD was significantly higher than those in the other two groups ($P<0.05$), while there was no significant difference in the Th9 cell ratio between Group SCOPD and Control ($P>0.05$) (**Table 2**; **Figure 1**).

Comparison of IL-9

The IL-9 level in Group AECOPD was significantly higher than those in the other two groups ($P<0.05$). There was no significant difference in the IL-9 level and Th9 cell ratio between Group COPD and Control ($P>0.05$) (**Table 2**; **Figure 2**).

Correlation analysis

The IL-9 level and the Th9 cell ratio in Group AECOPD were slightly correlated ($r=0.436$, $P<0.05$). In contrast, there were no relationship between the IL-9 level and the Th9 cell ratio in Group SCOPD and Control group (**Figure 3**).

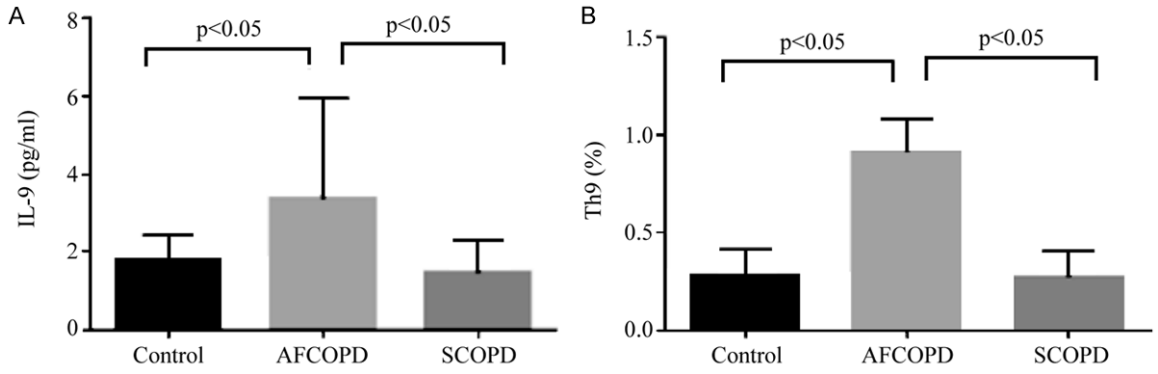

Figure 2. Three groups the comparison of peripheral blood IL-9 levels and Th9 percentage comparison. Contents of peripheral blood Th9 and IL-9 AECOPD up from SCOPD group and Control group significantly (P<0.05).

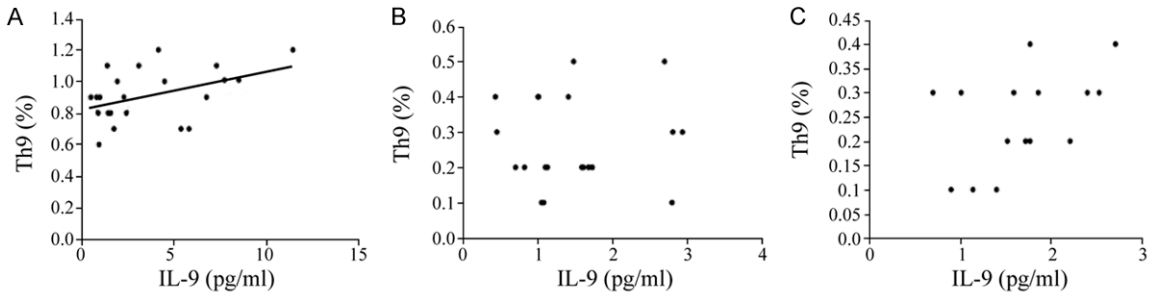

Figure 3. A-C. Respectively present the correlation scatter of IL-9 and Th9 cells in AECOPD, SCOPD and Controls.

Discussion

Chronic airway inflammation and emphysema are the main pathological features of COPD, and many immune cells and inflammatory factors are involved in. Certain studies have shown that COPD patients exist immune dysfunction or disorder, and mainly manifest the imbalance of Th1/Th2 and Th17/Treg cells at the cellular level. The Th1 and Th17 cells secrete proinflammatory cytokines, thus aggravating local inflammatory response and causing airway injury; however, the decrease of Th2 and Treg cell functions will cause the insufficient secretion of inhibitory inflammatory factors, thus resulting in proinflammatory - anti-inflammatory function imbalance, so it's closely related to the severity, disease progression, and prognosis of COPD. The Th9 cells are derived from the initial CD4+T lymphocytes under the co-stimulation of TGF-β and IL-4, which highly secrete IL-9, and IL-9 then combines with IL-9 receptor (IL-9R) and plays its various biological effects, such as participating in the pathogenesis of bronchial asthma, autoimmune diseases, or tumors. IL-9R is widely distributed in the lymphocytes, neutrophils, respiratory epithelial cells and respiratory smooth muscle cells. The Th9 cells and IL-9 level are significantly elevated in the peripheral blood of patients with allergic asthma, and the apoptosis of neutrophils is negatively correlated with the IL-9 level. Th9 and IL-9 may be involved in allergens caused chronic airway inflammation in allergic asthma patients [11]; in rat allergic asthma model, anti-IL-9 antibodies can reduce airway hyperresponsiveness, goblet cell metaplasia, and other inflammatory responses. In the mouse model with experimental autoimmune encephalomyelitis, IL-9 or its receptor deficiency is found to be associated with the severity of this disease [12].

The results of this study show that the Th9 ratio and the IL-9 level in the peripheral blood of Group AECOPD are significantly higher than those in Group SCOPD and Control (**Figure 2A**, **2B**), in contrast, the IL-9 level and the Th9 ratio in Group SCOPD show no statistical significance when compared with Control (**Figure 2A**, **2B**). And the IL-9 level and the Th9 ratio in Group AECOPD show slight positive correlation (**Figure 3A**). However, there were no relationship between the IL-9 level and the Th9 cell ratio in Group SCOPD and Control group (**Figure**

3B, 3C). Previous studies have found that the IL-9 level in the induced sputum of COPD patients is significantly elevated, especially in the cytoplasm of macrophages, and IL-9 may be involved in COPD airway inflammation and injury through the macrophages [13]. IL-9 can promote the proliferation of mast cells in immune response, induce the degranulation of mast cells, and release histamine, IL-13, or IL-1β, thus participating in the body allergic and inflammatory responses [14]. In addition, IL-9 can also stimulate the secretion of IL-17 by stimulating the Th17 cells, thus enhancing inflammatory responses and co-participating in the pathogenesis of autoimmune diseases [15]. The Th9 cells and IL-9 are significantly increased in the peripheral blood of the patients in Group AECOPD, we speculate the possible mechanisms may be: AECOPD may amplify chronic lung inflammation due to acute respiratory infection, which may even develop into systemic inflammatory response; under the stimulation generated by micro-environmental cytokines, the Th9 cells can differentiate, proliferate, and secrete IL-9, and IL-9 can further participate in inflammatory response and airway injury through combining with IL-9R (on the surface of macrophages) or directly through the macrophages. IL-9 can also stimulate the neutrophils and the Th17 cells to secrete proinflammatory cytokines, so that more inflammatory cells and cytokines may gather together, thus enhancing local inflammatory response; when IL-9 stimulates the mast cells to secrete TGF-β and VEGF, it can promote airway remodeling and neovascularization; IL-9 can also promote the proliferation of the mast cells in the immune response and induce the degranulation of mast cells, release histamine, IL-13, IL-1β, and other media, thus participating in the body allergic and inflammatory responses [13]. When IL-9 binds with the receptors on the surface of smooth muscle cells, it can cause airway smooth muscle contraction; IL-9 can also stimulate the Th17 cells to secrete IL-17, thus enhancing inflammatory response and co-participating in the pathology of autoimmune diseases; when IL-9 acts on the airway epithelial cells, it can make it secrete excessive mucus [16]. Asthma-COPD overlap syndrome (ACOS) has both asthma and COPD-related characteristics. Some scholars have suggested that ACOS may be the overlap airway inflammation co-mediated by the eosinophils and neutrophils. Combined with the results of this study, we hypothesize that in such populations, IL-9 may be involved in eosinophil-mediated inflammatory response by promoting the maturation of eosinophils and inhibiting their apoptosis.

Currently, smoking is widely recognized as a risk factor for COPD, but only some smokers eventually develop into COPD, and airway inflammation in most COPD patients continues when they stop smoking; furthermore, the degree of cigarette exposure determines the anti-trypsin antibody concentration in COPD patients [17]. A multi-center longitudinal study in the US and Canada has shown that the decline rate of lung function in COPD patients who discontinue smoking is faster than before [18]. Nunez *et al.* [19] also found that the titers of anti-nuclear antibodies, anti-tissue antibodies, and anti-lung epithelial cell antibodies in nearly 1/3 of patients with stable COPD increase, and 21% of COPD patients exhibit correlations of the titers of peripheral blood anti-tissue antibodies with airway limit severity and injury. Feghali-Bostwick *et al.* [20] reported that the IgG autoantibodies in COPD patients increase and show high affinity to the lung epithelial cells, so they may be involved in regulating the oxidative stress response, and the adaptive immune response of autoimmune response may be involved in the pathological processes of COPD. Autoimmune response may be involved in the pathogenesis of COPD, and the Th9 cells and IL-9 are both upregulated in some autoimmune diseases; the results of this study show that the Th9 and IL-9 levels in Group AECOPD are significantly increased than Group SCOPD and Control, and whether it's involved by Th9 and IL-9-mediated autoimmune response still needs further studies.

The Th9 and IL-9 levels in Group SCOPD and Control show no significant difference. AECOPD mainly appears systemic inflammation and increased expression of IL-32 in the supernatant of lung tissue than in the peripheral blood, suggesting the existence of pulmonary inflammation-based systemic inflammation in COPD, and local inflammation is more severe [21]. Combined with previous studies, we find that the peripheral blood-related to proinflammatory immune cells and cytokines in SCOPD are reduced than those in AECOPD, so we presume that inflammatory responses in SCOPD may be

confined to the lungs, and the lab indexes in the peripheral blood can't fully respond to the real pulmonary inflammatory responses. The relationships among various types of immune cells and cytokines are intertwined and complex, and we'll analyze whether the Th9 cells and IL-9 participate in the pulmonary chronic inflammatory process in SCOPD by multi-layer analysis, such as bronchial lavage fluid, bronchoscopy, or tissue biopsy, when certain conditions are adequate.

Patients with COPD generally suffer from immune system dysfunction or disorder, as well as imbalance of lymphocyte subsets and cytokine network, and these changes are directly or indirectly involved in pulmonary structural damage and disease evolution of COPD. The Th9 cells are one auxiliary T lymphocyte subset newly discovered in recent years, and their differentiation, development, and IL-9 secretion are affected by a variety of factors. This study suggests that the Th9 cells and IL-9 may be involved in the pathogenesis of AECOPD, while the specific pathways and regulatory mechanisms in this disease are not clarified yet. With the deepening of immunological research, more immune cells and inflammatory mediators will be discovered. Exploring the mechanisms among these immune cells and cytokines in diseases, investigating disease-specific biomarkers, and further studying methods to reconstruct the in vivo immune balance and to make the immune system play its normal immune defense function, as well as to block or reduce inflammation, oxidative stress, etc., through the body immune functions and to effectively improve the prognosis and natural progress of COPD, will be our further investigation steps and directions.

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Disclosure of conflict of interest

None.

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