The expression of Foxp3 and ROR gamma t in lung tissues from normal smokers and chronic obstructive pulmonary disease patients

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A R T I C L E  I N F O

Article history:
Received 3 May 2011
Received in revised form 19 June 2011
Accepted 22 June 2011
Available online 23 July 2011

Keywords:
Forkhead box P3
Retinoic-acid related orphan receptor gamma t
Chronic obstructive pulmonary disease
Emphysema

A B S T R A C T

Foxp3- and ROR gamma t-expressing cells are involved in acquired immune responses. The change in Foxp3 and ROR gamma t expression in lung tissue and their role in emphysema has not been studied for COPD patients and normal smokers. In the present study, Foxp3 and ROR gamma t were assessed using real-time quantitative polymerase chain reaction and western blotting, and the expression and distribution of Foxp3, IL-17, IL-23R and CCR6 were measured by immunohistochemistry in peripheral lung tissue (10 smokers with COPD, 10 smokers and 10 nonsmokers with normal lung function). Foxp3 expression was lower and ROR gamma t expression was higher in COPD patients when compared with smokers and nonsmokers (all P values were less than 0.001). The ratios of Foxp3/ROR gamma t mRNA and protein were positively correlated to FEV1%pred and negatively correlated to the mean alveoli area. Foxp3+ cell numbers were decreased, while the number of IL-17+ cells, IL-23R+ cells and CCR6+ cells were increased in the lung alveolar walls of COPD patients compared with normal smokers and nonsmokers (all P values were less than 0.001). The IL-17+ cell numbers were positively correlated to both CCR6+ and IL-23R+ cells. Our data show a decreased Foxp3 expression and an increased ROR gamma t expression in COPD patients and normal smokers that parallels the aggravation of the disease. The IL-17+ cell-related cytokines receptors CCR6 and IL-23R had an association with the mechanism of IL-17+ cell number increasing, which will provide a new immuno-therapeutic target for COPD.

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1. Introduction

Pulmonary emphysema is one of the pathological hallmarks of chronic obstructive pulmonary disease (COPD), which is characterized by destruction of the lung parenchyma [1–3] and is due mainly to cigarette smoking [1]. The relative extent of emphysema within individual patients can vary widely. Pathological studies have shown that inflammation in COPD occurs in the lung parenchyma [3]. Previous studies have emphasized the potential role of many inflammatory cells in the pathogenesis of COPD, mainly alveolar macrophages and T lymphocytes [1,2,4,5]. Inflammation that is mediated by T cells in the lungs of COPD patients suggests that the immune response is involved in the pathogenesis of COPD.

Intracellular expression of Forkhead box P3 (Foxp3) is currently considered to be the most specific marker for human regulatory T-cells (Tregs), and its activity is the most critical factor for Treg development and function [6]. Tregs have recently been identified as having significant anti-inflammatory and immunomodulatory effects [7,8]. Abnormalities in Tregs regulation have been described in many chronic inflammatory and autoimmune disorders, including atherosclerosis and rheumatoid arthritis [7–10], but their involvement in COPD is unclear. Smyth et al. found that the number of Tregs in bronchoalveolar lavage fluid (BALF) of smokers and COPD patients is increased in relation to those of nonsmokers [11], Barcelo et al. showed that the number of Tregs in the BALF of smokers with normal lung function (normal smokers) was greater than that in both COPD patients and nonsmokers [12]. Isajevs et al. found that Foxp3+ cells were upregulated in large airways in COPD patients compared with normal smokers and nonsmokers (all P values were less than 0.001). Isajevs et al. found that Foxp3+ cells were upregulated in large airways but downregulated in small airways in COPD patients [13]. However, previous studies have emphasized the role of Tregs in airway inflammation instead of in the destruction of the pulmonary alveolus.

Retinoic-acid-related orphan receptor (ROR) gamma t was expressed in T cells that can produce Interleukin-17 (IL-17), and it is the most critical factor for IL-17-producing cell development and function [6,14,15]. The IL-17-producing cell is a recently described effector T cell subset characterized by the production of IL-17A and IL-17F, which have been implicated in the pathogenesis of several inflammatory and autoimmune diseases [14,16]. Unfortunately, the exact role of IL-17-producing cell immune responses in COPD is still not well studied. In animal models of cigarette-smoke-induced emphysema, chronic-smoke-exposed mice have a significantly higher number of IL-17- and Interferon (IFN)-gamma-producing cells in
BALF [17]. In contrast, the level of IL-17 in sputum does not differ between COPD patients and control subjects [18]. The expression of IL-17 and IL-22 was increased in the bronchial mucosa of COPD patients compared with nonsmokers, but it was maintained compared with normal smokers [19,20]. Our previous study found that the level of IL-17 in lung tissue was higher in COPD patients than normal smokers and nonsmokers [21]. However, most previous studies have concentrated on IL-17+ cell-related cytokines, or they have studied pathogenesis in airway inflammation. The role of the IL-17-producing cell itself in pulmonary parenchyma inflammation is unclear.

Although a previous study in 1995 [22] proposed that T-lymphocytes may be implicated in the pathogenesis of emphysema in smokers, the nature and role of T cells (including Th1, Th2, Treg, and Th17 cells) in COPD immunity remains unclear. The Foxp3-expressing cells and ROR gamma t-expressing cells play a role in maintaining the normal immune response, and their distortion may induce a number of autoimmune diseases, malignant tumors, and transplant rejection [23]. A previous study reported that human Treg cells could inhibit the proliferative response of Th17 cells in peripheral blood in COPD patients [24], but the change in Foxp3 and ROR gamma t expression was not confirmed in the pulmonary inflammation of COPD patients and normal smokers. Indeed, the precise involvement of Foxp3 and ROR gamma t in COPD must be further elucidated.

The aim of the present study was to investigate the possible involvement of Foxp3 and gamma t in emphysema. For this purpose, the expression of Foxp3 and ROR gamma t in lung tissue was evaluated, and ROR gamma t-expressing cells expressing the related cytokines IL-17, Interleukin-23 receptor (IL-23R) and CC chemokine receptor 6 (CCR6) were also investigated. We have shown that the ratios of Foxp3/ROR gamma t mRNA and protein in lung tissue were decreased in COPD patients and normal smokers compared to nonsmokers. Furthermore, we demonstrated that this change is involved in the immunopathogenesis of pulmonary inflammation, which may be important in the immunopathogenesis of normal smokers and the onset and progress of emphysema. These findings provide novel insights into immunotherapy for COPD.

2. Materials and methods

2.1. Study subjects

A total of 30 subjects undergoing lung resection for a solitary peripheral carcinoma were obtained from the First Affiliated Hospital of Guangxi Medical University (Guangxi, China) and enrolled in the study. They were subdivided into three groups: 10 subjects were nonsmokers with normal lung ventilation function, 10 subjects were smokers with stable COPD, and 10 subjects were smokers with normal lung ventilation function and 10 were smokers with stable COPD. COPD patients were diagnosed according to the definition of the Global initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [25] upon entry into the study, based on a forced expiratory volume in 1 s (FEV1)/forced vital capacity (FVC) ratio <70%, with a reversibility of less than 15% after inhalation of 200 mg of salbutamol. None of the study subjects had a recent exacerbation, defined as increased dyspnea associated with a change in the quality and quantity of sputum that would have led them to seek medical attention, during the month preceding the study. All subjects were free of acute upper respiratory tract infections, and none had received glucocorticoids, theophylline or antibiotics within the preceding month. All subjects were nonallergic, had negative skin tests for common allergen extracts and had no past history of asthma or allergic rhinitis. The clinical characteristics of the groups are presented in Table 1.

The study was approved by the local ethics committee (the First Affiliated Hospital of Guangxi Medical University ethical committee, Guangxi, China) and conformed to the declaration of Helsinki; written informed consent was obtained from each subject.

Table 1

<table>
<thead>
<tr>
<th>Subject group</th>
<th>NS (n = 10)</th>
<th>COPD (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>61±9</td>
<td>63±9</td>
</tr>
<tr>
<td>FEV1/FVC%</td>
<td>102±13</td>
<td>93±8</td>
</tr>
<tr>
<td>RV% pred</td>
<td>98±12</td>
<td>91±6</td>
</tr>
<tr>
<td>FEV1/FVC%</td>
<td>96±12</td>
<td>86±8</td>
</tr>
<tr>
<td>RV% pred</td>
<td>63±13</td>
<td>77±14</td>
</tr>
<tr>
<td>TLC% pred</td>
<td>60±10</td>
<td>68±12</td>
</tr>
<tr>
<td>RV/TLC</td>
<td>30±4</td>
<td>34±1</td>
</tr>
<tr>
<td>Male/female n</td>
<td>8/2</td>
<td>9/1</td>
</tr>
<tr>
<td>Smoking history pack-yrs</td>
<td>–</td>
<td>55±18</td>
</tr>
<tr>
<td>BMI kg/m²</td>
<td>22.3±3.3</td>
<td>21.6±2.5</td>
</tr>
<tr>
<td>6MWD m</td>
<td>462.42±75.81</td>
<td>428.73±58.46</td>
</tr>
<tr>
<td>BODE</td>
<td>0.01/0.01</td>
<td>1/0.01</td>
</tr>
<tr>
<td>MRC</td>
<td>0.01/0.01</td>
<td>0.01/0.01</td>
</tr>
<tr>
<td>SGRQ</td>
<td>10/3.20</td>
<td>25/1.32</td>
</tr>
</tbody>
</table>

Data are presented as mean±SD or median (range). COPD patients had not received any treatment within the preceding month. NS: nonsmokers; S: smokers with normal lung ventilation function; COPD: COPD patients; FEV1: forced expiratory volume in 1 s; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; BMI: body mass index; 6MWD: 6 minute walking distance; BODE: body-mass index, airflow obstruction, dyspnea and exercise capacity index; MRC: medical research council scale; SGRQ: St-George respiratory questionnaire. a: P<0.05 and b: P<0.01 significantly different from nonsmokers; c: P>0.05 and b: P<0.01 significantly different from smokers.

2.2. Histology and immunohistochemistry

Lung tissue specimens were taken from the subpleural parenchyma of the lobe obtained at surgery as far away as possible from the tumor site; they were then transferred to 10% neutral buffer, formalin fixed and paraffin embedded. Deparaffinized sections (3-μm) were stained with hematoxylin and eosin and analyzed by light microscopy. For immunohistochemistry, formalin-fixed, paraffin-embedded tissue was cut in 3-μm-thick sections. Antigen retrieval was achieved by treatment in a high-temperature pressure cooker heated for 90 s in a citrate buffer, pH 6.0. Sections were incubated in 0.5% H2O2 to quench endogenous peroxidase activity and then blocked with normal goat serum or normal rabbit serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The slides were then incubated with primary monoclonal mouse anti-human antibody against Foxp3 (monoclonal mouse antibody, dilution 1:400, sc-56680; Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-17 (polyclonal rabbit antibody, dilution 1:50, sc-7927; Santa Cruz Biotechnology, Santa Cruz, CA, USA), CCR6 (polyclonal rabbit antibody, dilution 1:600, ab78429; Abcam Inc, Cambridge, MA, USA) or IL-23R (polyclonal goat antibody, dilution 1:250, ab53656; Abcam Inc, Cambridge, MA, USA) for 1 h at room temperature. The resulting slides were incubated in a humid chamber for 10 min each with biotinylated secondary antibody and streptavidin with intervening and subsequent rinses in PBS three times for 5 min. 3’3-diaminobenzidine-tetrahydrochloride was applied as a chromogen for 5 min. Sections were counterstained in haematoxylin for 1 min.

2.3. Image analysis

Analysis of lung parenchyma was performed using a light microscope (Olympus Cor., Tokyo, Japan) connected to a video recorder that was linked to a computerized image system (Image-Pro Plus V6.0, Silver Spring, MD, USA). Foxp3 expression was identified as nuclear immunolocalization. IL-17 expression was identified as cytoplasm immunolocalization, and CCR6 and IL-23R expression were identified as cytomembrane immunolocalization. The cases were coded, and the measurements were made in a blinded fashion by two pathologists without knowledge of clinical data for a given patient specimen. Disagreements were settled by consensus or adjudicated by a third reviewer.
The number of Foxp3⁺, IL-17⁺, CCR6⁺ and IL-23R⁺ cells was calculated in the lung alveolar walls. The cell counts were done as previously described [26]. Briefly, we examined only the alveolar walls with a single layer of cells to avoid bias caused by technical artifacts such as adjacent alveolar walls. At a magnification of 400×, we measured the length of the alveolar walls, and the number of positive cells within these alveolar walls was counted. Ten fields that were randomly distributed across the slide were studied per subject, and the result was expressed as the number of positive cells/mm of alveolar wall. We decided to examine 10 fields per subject because this number of fields was sufficient to obtain a mean value per subject that remained rather constant after further increasing the number of fields examined.

The enlargement of airspace distal to the terminal bronchioles was estimated according to the mean alveoli area. The mean alveoli area was measured as previously reported [21].

### 2.4. Quantitative real-time PCR

Total RNA was extracted from frozen tissue using the TRIzol reagent (Invitrogen, Carlsberg, CA, USA) according to the manufacturer’s instructions and stored at −80 °C until use. The cDNA was synthesized with ReverTra Ace_reverse transcriptase (Toyobo, Osaka, Japan).

A quantitative real-time polymerase chain reaction (PCR) was performed with a Rotor-gene6000 Real Time Thcmal Cycler (Corbett Research Australia) and the SYBR® Premix Ex Taq™ (TaKaRa) in accordance with the manufacturer’s protocol. Each set of experiments was repeated three times. The 25 μl PCR reactions (with 12.5 μl SYBR green, 5× 10⁻⁶ μl M forward and 5× 10⁻⁶ μl M reverse primers, 2 μl cDNA) underwent 30 s at 50 °C, 5 s at 95 °C, then 40 cycles of 30 s at 60 °C. Primers were as follows: human Foxp3 F, 5′-CTGCCCTACTCTCCGTGTAAGTG-3′ and R, 5′-CTGGAGGACTGCTGTAAGTG-3′; human ROR gamma t F, 5′-GTAACGCGGCCTACTCTG-3′ and R, 5′-GTCTTGAC-CACCTGTTCCGTG-3′; β-actin F, 5′-CTCCATCCTGGCCTCGCTGT-3′ and R, 5′-GCTGTCACCTTCACCGTTCC-3′. The threshold cycle number (Ct) was determined for all PCR reactions, and in most cases, the threshold was manually adjusted to lie within the exponential phase using Rotor-Gene Real-Time Analysis Software 6.1.

Levels of mRNA expression for each gene were calculated using the 2⁻ΔΔCt method [27], and the values were expressed as relative arbitrary units.

### 2.5. Western blotting

Tissue samples were directly lysed in Triton buffer (20 mM Hepes and 0.5% Triton X-100, pH 7.6). Aliquots of protein extracts (50 mg) were separated on a 10% SDS-PAGE. Subsequently, the protein was electrothermally transferred onto a PVDF membrane (Bio-Rad). After blocking with TBS-Tween 20 (TBST) containing 5% skim milk, the membranes were incubated with rabbit anti-human polyclonal antibodies against human Foxp3 (dilution 1:600, sc-28705; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rat anti-human ROR gamma (t) Purified antibodies (dilution 1:100, 14–6988; eBioscience, Inc., San Diego, CA, USA) overnight at 4 °C, followed by horseradish peroxidase conjugated goat anti-rabbit or anti-rat antibodies (sc-2030 or sc-2032; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:5000 in TBST for 1 h. Finally, blots were developed with a chemiluminescent reagent (Pierce Biotechnology, Rockford, IL, USA). In order to achieve equal protein loading, blots were re-stained using mouse anti-human GAPDH antibody (dilution 1:10,000, sc-166545, Santa Cruz Biotechnology, CA, USA) as a control.

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**Fig. 1.** Histology of lung tissues. Photomicrograph showing HE (Original magnification: ×100) of the alveolus from (A) nonsmokers; (B) smokers with normal lung ventilation function; (C) COPD patients. (B) Some alveoli expanded and some alveoli were not complete. (C) Many alveoli were expanded and broken.

**Fig. 2.** The expression of Foxp3 mRNA and ROR gamma t mRNA in lung tissues. Expression level of (A) Foxp3 mRNA and (B) ROR gamma t mRNA in lung tissues and (C) the ratio of Treg/Th17 cells in the level of mRNA. N: nonsmokers, S: smokers with normal lung ventilation function, COPD: COPD patients. *: P<0.05, #: P<0.01.
2.6. Statistical analysis

All statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Group data are expressed as mean±Std. Deviation (SD) or median (range). Mean±SD or median (range) were used for functional data and morphological data, and mean±SD were used for others. The pack-yrs of smoking history of smokers with normal lung ventilation function and COPD patients were analyzed using a t-test. Differences between the two groups were analyzed using the one-way analysis of variance (ANOVA) for functional data, the results of western blotting and real-time PCR, and the Kruskal–Wallis test for morphological data, BODE, MRC and SGRQ. When the differences were significant, the one-way ANOVA test was followed by the Student–Newman–Keuls test for FEV1%pred, TLC%pred, RV/TLC, 6MWD and ROR gamma t mRNA expression; the Games–Howell test was used for others, and the Kruskal–Wallis test was followed by the Mann–Whitney U-test for comparison between groups. Correlation coefficients were calculated using Spearman’s rank method. P-values<0.05 were considered to be statistically significant.

3. Results

3.1. Histology findings

Histological analysis showed that many of the alveoli were expanded and broken and that inflammatory cells gathered more obviously in the lung tissue of COPD patients than in smokers with normal lung ventilation function. The mean alveoli area in COPD patients (152,345±24,497 μm²) was larger than that in both smokers (106,551±15,815 μm², P<0.001) and nonsmokers (50,708±14,125 μm², P<0.001) (Fig. 1A–C). The difference in the mean alveoli area between smokers and nonsmokers was also significant (P<0.001) (Fig. 1).

3.2. Expression of Foxp3 and ROR gamma t mRNA

Foxp3 mRNA expression levels were significantly lower in COPD patients (0.431±0.109) than in smokers (0.930±0.413) and nonsmokers (1.391±0.312) (ANOVA P<0.001, COPD patients vs. smokers P=0.010, COPD patients vs. nonsmokers P<0.001, smokers vs. nonsmokers P=0.031) (Fig. 2A).

ROR gamma t mRNA expression levels were significantly increased in COPD patients (10.158±1.574) compared with nonsmokers (2.685±0.886) and smokers (8.564±1.419) (ANOVA P<0.001, COPD patients vs. smokers P<0.05, COPD patients vs. nonsmokers P<0.05, smokers vs. nonsmokers P<0.05) (Fig. 2B).

The ratio of Foxp3/ROR gamma t was significantly lower in COPD patients (0.04±0.01) as compared with smokers (0.11±0.05, P=0.008) and nonsmokers (0.59±0.28, P<0.001). The ratio in smokers was also lower than in nonsmokers (P=0.001) (Fig. 2C).

The ROR gamma t and Foxp3 mRNA expression level was negatively correlated (R=−0.609, P<0.001) (Fig. 6A). The ratio of Foxp3/ROR gamma t mRNA expression level negatively correlated with the mean alveoli area (R=−0.810, P<0.001) (Fig. 6B) and positively correlated with forced expiratory volume in 1 s% predicted (FEV1%pred) (R=0.653, P<0.001) (Fig. 6C).

Fig. 3. Western blotting (A) and quantitation of protein bands (B) showing differentially expressed Foxp3 and ROR gamma t in human lung tissues. Results are representative of those from 10 nonsmokers, 10 smokers with normal lung ventilation function and 10 COPD patients. N: nonsmokers, S: smokers with normal lung ventilation function, COPD: COPD patients. #: P<0.05, #: P<0.01.
3.3. Expression of Foxp3 and ROR gamma t protein

The Foxp3 protein expression level was decreased in COPD patients (0.307±0.051) as compared with both smokers (0.504±0.218) and nonsmokers (0.824±0.180) (ANOVA P<0.001, COPD patients vs. smokers P=0.046, COPD patients vs. nonsmokers P<0.001, smokers vs. nonsmokers P=0.006) (Fig. 3).

The ROR gamma t protein expression level was increased in COPD patients (0.886±0.184) compared with smokers (0.614±0.241) and nonsmokers (0.369±0.094) (ANOVA P<0.001, COPD patients vs. smokers P=0.003, COPD patients vs. nonsmokers P<0.001, smokers vs. nonsmokers P=0.028) (Fig. 3).

The ratio of Foxp3/ROR gamma t was significantly lower in COPD patients (0.37±0.16) as compared with smokers (0.86±0.26, P<0.001) and nonsmokers (2.39±0.85, P<0.001). The ratio in smokers was also lower than in nonsmokers (P=0.001) (Fig. 3).

The protein level of ROR gamma t and Foxp3 was negatively correlated (R=−0.481, P=0.007) (Fig. 6D). The ratio of Foxp3/ROR

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**Fig. 4.** Immunohistochemistry of Foxp3+, IL-17+, CCR6+ and IL-23R+ cells in alveolar walls. Photomicrograph showing (Original magnification: 400×) Foxp3+ cells in alveolar walls (A) nonsmokers, (B) smokers with normal lung ventilation function, (C) COPD patients; IL-17+ cells in alveolar walls (D) nonsmokers, (E) smokers with normal lung ventilation function, (F) COPD patients; CCR6+ cells in alveolar walls (G) nonsmokers, (H) smokers with normal lung ventilation function, (I) COPD patients; IL-23R+ cells in alveolar walls (J) nonsmokers, (K) smokers with normal lung ventilation function, (L) COPD patients. Results are representative of those from 10 nonsmokers, 10 smokers with normal lung ventilation function and 10 COPD patients.
gamma t protein expression level was negatively correlated with the mean alveoli area ($R = -0.807$, $P < 0.001$) (Fig. 6E) and positively correlated with FEV1%pred ($R = 0.658$, $P < 0.001$) (Fig. 6F).

3.4. Expression of Foxp3+, IL-17+, CCR6+ and IL-23R+ cells in alveolar walls

The representative immunohistochemistry staining of different proteins in each group is shown in Fig. 4A–L. The numbers of IL-17+, CCR6+ and IL-23R+ cells were increased, and the number of Foxp3+ cells as well as the ratio of Foxp3+ cell/IL-17+ cell were decreased in the alveolar walls of COPD patients compared to smokers and nonsmokers (all P values were less than 0.01) (Fig. 5).

When all the subjects were considered together, the numbers of Foxp3+ cells and IL-17+ cells were negatively correlated ($R = -0.854$, $P < 0.001$) (Fig. 6G). The ratio of Foxp3+/IL-17+ cells was negatively correlated to the mean alveoli area ($R = -0.782$, $P < 0.001$) (Fig. 6H) and positively correlated to FEV1%pred ($R = 0.702$, $P < 0.001$) (Fig. 6I). The number of IL-17+ cells was positively correlated to CCR6+ cells and IL-23R+ cells ($R = 0.748$, $P < 0.001$; $R = 0.790$, $P < 0.001$, respectively).

4. Discussion

Our present study demonstrates that there is a decreased ratio of Foxp3/ROR gamma t with decreased Foxp3 and increased ROR gamma t in the lung tissue of smokers with COPD and smokers with normal lung ventilation function. This change was in line with the aggravation of COPD. This was also confirmed by the positive correlation between the ratio of Foxp3/ROR gamma t and FEV1%pred and the negative correlation between the ratio and pulmonary alveolus destruction.

Fig. 5. The number of Foxp3+, IL-17+, CCR6+ and IL-23R+ cells in alveolar walls. (A) Foxp3+ cell, (B) IL-17+ cell, (C) CCR6+ cell, (D) IL-23R+ cell, (E) the ratio of Foxp3+ cell/IL-17+ cell. N: nonsmokers, S: smokers with normal lung ventilation function, COPD: COPD patients. *: $P < 0.01$. 

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Our results suggest that the decreased ratio of Foxp3/ROR gamma t should play a role in the progress of COPD in normal smokers and be related to clinical manifestation.

Our results showed that smokers with COPD exhibited a significant increase in ROR gamma t compared with normal smokers and nonsmokers. ROR gamma t was expressed in T cells that can produce IL-17 [14,15]. The IL-17 producing cells response induces pro-inflammatory cytokines and chemokines, such as IL-17, IL-21 and IL-22 [28]. IL-17A and IL-17F could promote neutrophil chemotaxis and stimulate mucin production from the respiratory epithelium in COPD pathogenesis [29,30]. IL-21 is necessary to provide the positive-feedback loop for the expression of IL-23 and IL-23R in Th17 cells and successful amplification of Th17 cells [31]; it is also required for the survival of a range of immune cells such as lymphocytes, NK cells and B-cells. IL-23 induces Th17 cells to produce IL-17A with potential positive feedback loops [32,33]. IL-23R is expressed on IL-17 producing cells [34]. In addition, IL-17-producing cells also express CCR6. CCR6-deficient mice have previously been found to be protected from cigarette-smoke-induced pulmonary inflammation and airspace enlargement [35]. Therefore, our observations indicate that ROR gamma t-expressing cells have an important role in the persistence and development of lung parenchyma inflammation in normal smokers and COPD patients. The present findings confirm these observations and further demonstrate the distribution and activation of ROR gamma t-expressing cells by showing that the numbers of IL-17+ cells, IL-23R+ cells and CCR6+ cells in alveolar walls were increased in COPD patients compared with smokers and nonsmokers.

Our previous study demonstrated that Foxp3+ cells in lung tissues were decreased in the emphysema group compared with controls in rats [36]. The present study confirmed these observations in the human lung by showing that the expression of Foxp3 was significantly lower in COPD patients than in smokers and that it was markedly decreased in smokers compared to nonsmokers. This suggests that...
Foxp3+ cells play a role in the acquired immune response and autoimmune disorders during the onset and development of local inflammation in the lung. Previous studies have described Tregs from airways, BALF and peripheral blood [11–13]. Smyth [11] and Barceló [12] showed that Tregs from COPD patients were significantly different than in the BALF of smokers and nonsmokers but not different in peripheral blood samples. Isajevs et al. found that Tregs were upregulated in large airways but downregulated in small airways in COPD patients [13]. Foxp3+ cell-mediated immune responses in the pathogenesis of COPD may induce tissue inflammation locally rather than systematically. We observed a downregulated expression in COPD patients that indicates Foxp3+ cells are involved in a different pattern of immune response in lung parenchyma inflammation from the airway. Therefore, we confirmed that Foxp3 and ROR gamma t are in a dynamic equilibrium in COPD patients and smokers.

Furthermore, the present study shows that the ratio of Foxp3/ROR gamma t in lung tissues was negatively correlated with pulmonary alveolar destruction in all subjects. From this point, we infer that the Foxp3/ROR gamma t ratio decrease is involved in the alveolar destruction of the lung parenchyma, which then develops into emphysema. For these reasons, the dynamic interaction between Foxp3/ROR gamma t ratio decrease in the pathogenesis of emphysema and symptoms of airway limitation in COPD patients. However, the mechanism of the Foxp3/ROR gamma t ratio decrease in COPD is unclear. A previous study showed that IL-17 production is not simply due to the recruitment or expansion of Tregs in COPD patients with peripheral blood [24]. Therefore, the mechanism of the Foxp3/ROR gamma t ratio decrease is complex, and our conclusion will need to be proven on a larger scale of the population. Ongoing efforts should be made to identify the precise effect and mechanism of the Foxp3/ROR gamma t ratio decrease in the human lungs of COPD patients. A better understanding of the nature, regulation, and function of Foxp3 and ROR gamma t expression in COPD immunity may provide a new target for the treatment of COPD patients and even facilitate the development of a novel and effective immunotherapy for COPD.

Our study, similar to many previous studies of COPD, used peripheral lung tissue from patients undergoing surgery for lung cancer [36–39]. Surgical specimens are the only material available for examination of the pulmonary alveoli in such a study. Any effect of the cancer is ruled out or minimized by taking tissue that is distant from the tumor and using control samples.

In summary, our data demonstrate that a decreased ratio of Foxp3/ROR gamma t exists in patients with COPD and normal smokers, which is in line with the aggravation of the disease. The decreased ratio of Foxp3/ROR gamma t plays an important role in pathogenesis in COPD by immune dysregulation and its participation in persistent and developmental lung inflammation, and it is thus correlated to alveolar destruction in the lung and airway limitation as the disease develops. Interestingly, the chemokine receptors and pro-inflammatory cytokines CCR6 and IL-23R in IL-17-producing cells had an association with the mechanism of the Foxp3/ROR gamma t ratio decrease, which will provide novel avenues in the immunotherapy of COPD.

Acknowledgments

This study was funded by National Natural Science Foundation of China [30862016]. The content of this report does not necessarily reflect the position or policy of the Chinese Government.

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