Imbalance of Th17/Treg cells in mice with chronic cigarette smoke exposure

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Background: Recent studies have revealed that autoimmune responses mediated by CD4+ T cells may contribute to the development of chronic obstructive pulmonary disease (COPD). Meanwhile, imbalance of Th17/Treg has been reported to play a key role in the pathogenesis of autoimmune diseases. However, information on Th17/Treg balance in COPD is relatively limited.

Method: We established a mouse model of COPD induced by chronic cigarette smoke (CS) exposure. Th17 and Treg in lung tissue and peripheral blood were quantified by flow cytometry. The level of the specific transcription factors of both T cell subsets in lung tissue was determined by real-time PCR. The expressions of Th17- and Treg-related cytokines in serum and bronchoalveolar lavage fluid (BALF) were measured by enzyme-linked immunosorbent assay (ELISA).

Results: We found that mice with chronic CS exposure showed significant increase in lung Th17 prevalence, retinoic acid orphan receptor (ROR)-γt mRNA and Th17-related cytokines (IL-17A, IL-6 and IL-23). Meanwhile, there was obvious decrease in Treg cell prevalence, Forkhead box (Fox) p3 mRNA and Treg-related cytokine IL-10, as compared to mice underwent sub-acute CS exposure and air-exposure. Similar tendency was also found for the Th17/Treg ratio in peripheral blood.

Conclusions: Our study thus reveals that the Th17/Treg imbalance exists in mice with chronic CS exposure, suggesting its potential role in the breakdown of immune self-tolerance in COPD. Further research on regulation of Th17/Treg balance may provide insights into the development of new therapeutic targets for this disease.

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1. Introduction

Chronic obstructive pulmonary disease (COPD) is a disease of the airways and lungs characterized by a progressive airflow limitation that is not fully reversible [1]. It is one of major causes of morbidity and mortality throughout the world and projected to become the third cause of death worldwide by 2030 [2]. Long-term exposure to cigarette smoke (CS) is the predominant risk factor [3]. However, the molecular mechanisms involved in the pathogenesis of COPD are poorly defined. Attention has traditionally been centered on the roles of neutrophils and macrophages [4]. Recently, lymphocytes have received increased attention, and there is accumulating evidence indicating that T lymphocytes play important roles in the development and progression of COPD [5,6]. Although the exact role of T cell subsets in COPD patients has not been well studied, some reports have demonstrated that patients with COPD exhibit many of the classical signs of T cell-mediated autoimmunity, such as the presence of auto-antibodies and auto-reactive T cells in the patient’s peripheral blood [7,8]. These results indicate that autoimmune responses mediated by CD4+ T cells may play an important role in the pathogenesis of COPD.

Recent studies have defined a previously unknown arm of the CD4+ helper T cells, the Th17 lineage, which promises to change our understanding of immune regulation, immune pathogenesis and host defense [9]. Th17 cells are characterized by the production of IL-17A, IL-17F and IL-22, which have been implicated in the progression of several T cell driven autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis [10–13]. Remarkably, Th17 development depends on the pleiotropic cytokine TGF-β, which is also linked to CD4+CD25+Foxp3+ regulatory T (Treg) cell development and function, providing a unique mechanism for matching CD4+ T cell effector and regulatory lineage specification [14]. Reduced generation or deficient function of Treg cells has been found in a number of different autoimmune diseases. For instance, ablation of Treg in mice could lead to fatal autoimmune disease [15]. Taking all this evidence together, it is reasonable to hypothesize that the fine balance between Th17 and Treg is crucial for maintenance of immune
homeostasis and an imbalance of Th17/Treg might exist and play a role in the pathogenesis of autoimmunity in COPD.

In this study, we first established a mouse model of COPD induced by chronic smoke exposure, using sub-acute CS exposure and air exposure mice as the controls. To measure the degree of airway inflammation of mice induced by CS exposure, we counted the cell number and analyzed the different cell types in bronchoalveolar lavage fluid (BALF). Flow cytometry was employed to determine the ratio of Th17 and Treg cells in the peripheral blood and lung tissue in mice with chronic CS exposure. Specific transcription factors of both T cell subsets in lung tissue were determined by real-time PCR. The corresponding cytokine profiles (such as IL-17A, IL-6, IL-23, IL-10 and TGF-β) were also measured using enzyme-linked immunosorbent assay (ELISA). Our results clearly show that the imbalance of Th17/Treg, related cytokines and transcription factors was observed in mice with chronic CS exposure, indicating that Th17/Treg imbalance may play a vital role in the pathogenesis of COPD.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice, 6 to 8 weeks old (weigh 20–22 g) and free of specific pathogens, were purchased from Shanghai Experimental Animal Center of Chinese Academy of Sciences (Shanghai, China). All experimental procedures were performed according to protocols approved by the Animal Studies Committee of Ningbo University.

2.2. Smoke exposure

Mice (n = 20 per group) were exposed whole body to cigarette smoke (CS) as described previously [16]. Briefly, twenty mice were exposed to the CS of five cigarettes (Marlboro® Lights, whose toxicity is similar to the 1R4F reference cigarette from University of Kentucky, Lexington, KY [17]) four times a day with 30-minute smoke-free intervals. The CS exposure animals were divided into two subgroups that endured 5 days per week for 4 weeks (sub-acute exposure, initiate CS exposure at 26 to 28 weeks old) or 24 weeks (chronic exposure, initiate CS exposure at 6 to 8 weeks old). An optimal smoke:air ratio of 1:6 was obtained. The control group animals were exposed to air. The mice of each group were 30–32 weeks old when the CS or air exposure finished. All mice tolerated smoke exposure without discernible toxicity.

2.3. Bronchoalveolar lavage

Twenty-four hours after the last smoke exposure, mice were anesthetized by intraperitoneal (i.p.) injection of phenobarbital (40 mg/kg) (Henrui Corp., Jiangsu Province, China). Blood samples were collected and peripheral blood mononuclear cells (PBMCs) were isolated by standard density centrifugation (Biochrom, Berlin, Germany). A tracheal cannula was inserted into the upper cervical trachea through a tracheotomy. The right lung was lavaged six times with 1 mL D-Hang’s solution. Total BALF cells were counted from a 0.05-mL aliquot. The remaining fluid was centrifuged (1000 g) and analyzed the different cell types in bronchoalveolar lavage fluid (BALF). Flow cytometry was employed to determine the ratio of Th17 and Treg cells in the peripheral blood and lung tissue in mice with chronic CS exposure. Specific transcription factors of both T cell subsets in lung tissue were determined by real-time PCR. The corresponding cytokine profiles (such as IL-17A, IL-6, IL-23, IL-10 and TGF-β) were also measured using enzyme-linked immunosorbent assay (ELISA). Our results clearly show that the imbalance of Th17/Treg, related cytokines and transcription factors was observed in mice with chronic CS exposure, indicating that Th17/Treg imbalance may play a vital role in the pathogenesis of COPD.

![Fig. 1. CS exposure induces inflammatory cell infiltration in airway. The number of total inflammatory cells, including neutrophils, lymphocytes, and macrophages was determined 24 h after the final CS exposure (A). The percent of each type cell in total cells was analyzed (B). Values were shown as mean ± SEM of twenty mice per group. *P<0.05 and **P<0.01 versus control group; #P<0.05 and ##P<0.01 versus sub-acute CS exposure group.](image)

2.4. Preparation of lung lymphocytes

Mouse lung lymphocytes from either CS-exposed or control mice were isolated as previously described [6]. Firstly, the lungs were voided of any remaining circulating lymphocytes by perfusion through the right ventricle with 10 mL PBS containing 0.6 mM EDTA. Then, the left lung was removed from the chest cavity, washed with PBS, diced into pieces, and digested in 5 mL RPMI 1640 medium (Invitrogen, USA) containing 175 U/mL collagenase I-A (Sigma-Aldrich, USA), 0.2 U/mL pancreatic elastase (Sigma-Aldrich, USA), 35 U/mL hyaluronidase (Sigma-Aldrich, USA), 20 kU/mL DNase I (Sigma-Aldrich, USA), 10% FCS, 100 U/mL penicillin (Lukang Corp, Shandong Province, China), and 100 μg/mL streptomycin (Lukang Corp, Shandong Province, China) for 1 h at 37 °C on an orbital shaker (60 rpm). After digestion, lungs were sheared through 19- and 21-gauge needles, and filtered through 40 μm cell strainers (BD Biosciences, USA) to obtain a single-cell suspension. Residual RBCs from lungs were lysed with RBC Lysis Solution (Qiagen, Germany). After a final resuspension, samples were resuspended in RPMI 1640 medium containing 40% Percoll (density 1.124 g/dL; Amersham Biosciences, UK). This cell suspension was overlaid onto 70% Percoll and centrifuged for 40 min at 750 g. Viable cells at the 40/70% interface were collected for intracellular and extracellular staining and flow cytometry analyses.

2.5. Flow cytometry

FITC-labeled anti-mouse CD4 (Isotype rat IgG2b; eBioscience, San Diego, USA) and PE-labeled anti-mouse IL-17A (Isotype rat IgG1; eBioscience, San Diego, USA) were used to detect Th17 cells. FITC-labeled anti-mouse CD4 (Isotype rat IgG2b; eBioscience, San Diego,
USA), PE-Cy5-labeled anti-mouse CD25 (Isotype rat IgG1; eBioscience, San Diego, USA) and PE-labeled anti-mouse Foxp3 (Isotype rat IgG2a, κ, eBioscience, San Diego, USA) were used to detect CD4⁺CD25⁺Foxp3⁺ Treg cells. Rat IgG of the corresponding class (eBioscience, San Diego, USA) was used as an isotype control. For Th17 cell analysis, PBMCs and cells (1×10⁶/mL) from the lung tissue were stimulated for 4 h with PMA, ionomycin and BFA for 4 h, and then stained with labeled antibodies as described in Materials and methods.

Fig. 2. Th17 prevalence in lung tissue and peripheral blood increased in mice with sub-acute and chronic CS exposure. PBMCs and lymphocytes from lung tissue from mice with sub-acute, chronic CS exposure and control mice were stimulated with PMA, ionomycin and BFA for 4 h, and then stained with labeled antibodies as described in Materials and methods. A: Representative FACS pictures from a single mouse in each group (lymphocytes from lung tissue). B: Representative FACS pictures from a single mouse in each group (PBMCs). (a) Lymphocytes were gated by flow cytometry (P1); (b) CD4⁺ T subsets were gated by flow cytometry. Plots in intern box (P2) represented CD4⁺ T cells; (c, d and e) representative IL-17A expression in CD4⁺ T cells from control mice, sub-acute CS exposure mice and chronic CS exposure mice, respectively; C: collective analyses of result from all three groups. Values were shown as mean±SEM of twenty mice per group. *P<0.05 and **P<0.01 versus control group; #P<0.05 and ##P<0.01 versus sub-acute CS exposure group.
50 ng/mL phorbol 12-myristate 13-acetate (PMA, ENZO Alexis-Biomol, USA) and 500 ng/mL ionomycin (ENZO Alexis-Biomol, USA) in the presence of 2.5 μg/mL brefeldin A (BFA, ENZO Alexis-Biomol, USA). Cells were harvested, washed and were surface stained for anti-CD4 at 4 °C for 30 min and treated with the Fix and Perm Reagent (Invitrogen, USA), followed by staining the cell with PE-labeled anti-IL-17A intracellularly. For analysis of Treg cells, PBMCs and cells (1 × 10^9/mL) from the lung tissue were aliquoted into tubes without PMA and ionomycin stimulation and surface-labeled with CD4-FITC and CD25-PE-Cy5 followed by fixation and permeabilization and intracellular staining with Foxp3-PE according to the manufacturer's instructions (eBioscience). After washing, the stained cells were analyzed by flow cytometry with a FACSCalibur (BD FACSCanto™ II, USA), and the results were analyzed with CellQuest software (BD FACSDiva, USA).

2.6. Quantitative real-time RT-PCR analysis

Total RNA was extracted from the lung tissue by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was prepared by reverse transcription with oligo(dT) from total RNA extraction. Real-time PCR for Foxp3, retinoic acid orphan receptor (ROR)-γt and a reference gene (β-Actin) was performed using an ABI 7500 Sequence Detection System (Applied Biosystems, USA) with the SYBR green mastermix kit (Takara, β′-Actin) was performed using an ABI 7500 Sequence Detection System (Applied Biosystems, USA) with the SYBR green mastermix kit (Takara, β′-Actin) was performed using an ABI 7500 Sequence Detection System (Applied Biosystems, USA) with the SYBR green mastermix kit (Takara, β′-Actin) was performed using an ABI 7500 Sequence Detection System (Applied Biosystems, USA) with the SYBR green mastermix kit (Takara, β′-Actin) was performed using an ABI 7500 Sequence Detection System (Applied Biosystems, USA) with the SYBR green mastermix kit (Takara). The primer sequences for RORγt were 5′-GCC TGT ATT CCC CTG ACC G-3′ (forward primer) and 5′-CCA GGT AAC ATC ATG T-3′ (reverse primer). The Ct values of Foxp3 and RORγt were normalized against those of β-Actin. Data are presented relative to the normalized Foxp3 and RORγt levels.

2.7. Cytokine ELISA

The concentrations of IL-6, IL-17A, IL-23, TGF-β and IL-10 in BALF supernatant and serum were measured by standardized sandwich ELISA according to the manufacturer’s protocol. All kits were purchased from Bender Corporation (USA).

2.8. Statistical analysis

All data were expressed as mean ± SEM. Differences between groups were examined for statistical significance by one-way analysis of variance using SPSS 11.0 software (SPSS Inc., Chicago, USA). A P value of <0.05 denoted the presence of a statistically significant difference.

3. Results

3.1. CS induced inflammatory cell infiltration into the airway

Throughout the experiments, the body weight of all mice was monitored. No differences were observed among different groups (data not shown), suggesting that CS exposure has no significant systematic toxicity on those mice.

To assess the effect of CS exposure on lung inflammation, BALF was collected from mice with chronic CS exposure (24 weeks), sub-acute CS exposure (4 weeks), and the control mice. Total number of alveolar inflammatory cells was dramatically increased in mice with chronic CS exposure. Total number of cells was also increased in mice with sub-acute CS exposure, but it was significantly lower than that of chronic CS exposure group (P<0.01) (Fig. 1A).

To further evaluate the effect of CS exposure on different inflammatory cell types, differential cell count was performed (Fig. 1B). As expected, there was a significant increase of all inflammatory cells in alveolar space after sub-acute and chronic CS exposure, with the highest increment in neutrophil population (P<0.01). Number of lymphocytes, and macrophages was also significantly increased after CS exposure (P<0.01), but the percent of macrophages in total cells was significantly decreased (P<0.01), while the percentage of lymphocytes, however, remained largely unchanged (Fig. 1B).

3.2. Th17 prevalence were increased in lung tissue and peripheral blood of mice with chronic CS exposure

To assess the effect of CS exposure on Th17 cell expansion, peripheral blood and lung tissue were collected, and then PBMCs and lymphocytes were isolated for flow cytometry analysis. Prevalence of Th17 cells refers to the ratio of CD4^+ IL-17A^+ cells to the total amount of CD4^+ T lymphocytes in peripheral blood or lung tissue.

As shown in Fig. 2, the prevalence of Th17 (CD4^+ IL-17A^-/CD4^+ T cells) in lung tissue was markedly higher in mice with sub-acute CS exposure (2.60±0.90%) and chronic CS exposure (6.80±1.30%) than those with air exposure (2.52±0.62%) (both P<0.01), but the percent of macrophages in total cells was significantly decreased (P<0.01), while the percentage of lymphocytes, however, remained largely unchanged (Fig. 1B).

3.3. CD4^+CD25^-Foxp3^+ Treg prevalence were decreased in lung tissue and peripheral blood of mice with chronic CS exposure

Threg prevalence was also detected by flow cytometry analysis. Prevalence of Treg refer to the ratio of CD4^+ CD25^- Foxp3^+ cells to the total amount of CD4^+ T lymphocytes. As shown in Fig. 2, the prevalence of Treg (CD4^+ CD25^- Foxp3^-/ CD4^+ T cells) in lung tissue was markedly higher in mice with sub-acute CS exposure (5.24±0.86%) and then significantly decreased in mice with chronic CS exposure (1.83±0.39%) than those with air exposure (2.52±0.62%) (both P<0.01), and there was also obvious difference between the sub-acute CS exposure and chronic CS exposure group (P<0.01). The same tendency of Th17 cells was also observed in peripheral blood.

3.4. Th17/Treg imbalance were observed in mice with chronic CS exposure

We observed that a remarkable imbalance of the mean ratio of Th17 to Treg existed in lung tissue of mice with chronic CS exposure. As shown in Fig. 4, the ratio of Th17/Treg was decreased in mice with...
sub-acute CS exposure (0.51 ± 0.20), and then markedly increased in mice with chronic CS exposure (3.81 ± 0.83) compared with control mice with air-exposure (0.87 ± 0.35) (both \( P < 0.01 \)). However, the ratio of Th17/Treg in peripheral blood was significantly elevated in chronic CS exposure mice (2.94 ± 0.71) compared with control (0.60 ± 0.19) and sub-acute CS exposure mice (0.65 ± 0.21) (\( P < 0.01 \)). Statistical
difference of this ratio was not found between sub-acute CS exposure and control groups ($P>0.05$).

### 3.5. Increased RORγt while decreased Foxp3 mRNA expression in lung tissue from chronic CS exposure mice

In order to confirm the above observations, we also determined the specific transcription factor of both T subsets by real-time PCR. As shown in Fig. 5A, increased mRNA expression of Treg-specific transcription factor Foxp3 was observed in sub-acute CS exposure mice and decreased mRNA expression of Foxp3 was observed in chronic CS exposure mice compared with air-exposure control mice (both $P<0.05$). Furthermore, there was also significant difference between sub-acute and chronic CS exposure groups ($P<0.05$). As for Th17-specific transcription factor, RORγt mRNA expression was significantly higher in chronic CS exposure mice than those in sub-acute CS exposure and air-exposure control mice (both $P<0.05$), while there was no significant difference between sub-acute and chronic CS exposure groups ($P>0.05$) (Fig. 5B). These results were consistent with flow cytometric analysis of Th17 cells and Treg cells.

### 3.6. Cytokine levels in BALF supernatant and serum

In order to determine the changes of Th17 and Treg related cytokine profile, serum and BALF supernatant from sub-acute, chronic CS exposure mice and control mice were collected. Th17 and Treg cells related cytokines such as IL-17A, IL-6, IL-23, TGF-β and IL-10 were measured by ELISA according to the manufacturer’s protocol. As demonstrated in Table 1, the levels of IL-17A, IL-6, IL-23, and TGF-β in serum were significantly higher in chronic CS exposure mice than those in control and sub-acute CS exposure mice ($P<0.01$). In the contrast, the level of IL-10 in serum was significantly decreased in chronic CS exposure mice when it was increased in sub-acute CS exposure mice than those in control mice ($P<0.01$ and $P=0.003$, respectively). There was also obvious difference between the sub-acute CS exposure and chronic CS exposure group for each

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**Fig. 3.** CD4$^+$CD25$^+$Foxp3$^+$ Treg prevalence in lung tissue and peripheral blood increased in mice with sub-acute CS exposure and decreased in mice with chronic CS exposure. PBMCs and lymphocytes from lung tissue from mice with sub-acute, chronic CS exposure and control mice were stained with labeled antibodies as described in Materials and methods. A: Representative FACS pictures from a single mouse in each group (lymphocytes from lung tissue). B: Representative FACS pictures from a single mouse in each group (PBMCs). (a) Lymphocytes were gated by flow cytometry (P1); (b) CD4$^+$ T subsets were gated by flow cytometry. Plots in internal box (P2) represented CD4$^+$ T cells; (c, d and e) representative CD25 and Foxp3 expression in CD4$^+$ subsets from control mice, sub-acute CS exposure mice and chronic CS exposure mice; C: collective analyses of result from all three groups. Values were shown as mean±SEM of twenty mice per group. *$P<0.05$ and **$P<0.01$ versus control group; $^*$P$<0.05$ and **P$<0.01$ versus sub-acute CS exposure group.

**Fig. 4.** The ratio of Th17/Treg in lung tissue and peripheral blood. There was a significant increase in the ratio of Th17/Treg in lung tissue and peripheral blood in mice with chronic CS exposure compared with control and sub-acute CS exposure mice. Values were shown as mean±SEM of twenty mice per group. *$P<0.05$ and **$P<0.01$ versus control group; $^*$P$<0.05$ and **P$<0.01$ versus sub-acute CS exposure group.

**Fig. 5.** The expression of Foxp3 mRNA and RORγt mRNA in lung tissues. There was a significant increase in the expression of RORγt and decrease of Foxp3 in mice with chronic CS exposure compared with control and sub-acute CS exposure mice. Expression level of mRNA was measured by real-time quantitative PCR. The result was normalized relative to $β$-actin, and data are presented relative to the normalized Foxp3 (A) and RORγt (B) levels. *$P<0.05$ and **$P<0.01$ versus control group; $^*$P$<0.05$ and **P$<0.01$ versus sub-acute CS exposure group.
type of cytokine ($P<0.01$). The results were consistent with the increased prevalence of Th17 and decreased Treg cells in PBMCs of chronic CS exposure mice. The levels of IL-17A, IL-6, IL-23, TGF-β and IL-10 in BALF supernatant also showed the same change trend as those in the serum (Table 2). The results were also consistent with the increased prevalence of Th17 and decreased Treg cells in lung tissue of mice with chronic CS exposure.

4. Discussion

The effect of chronic CS exposure on the adaptive immunity, especially T lymphocytes, is not well understood. In our present study, we aim to provide the first evidence of CD4$^+$ T cell differentiation and related cytokine profile in a mouse model of COPD. We reported here a significant increase of Th17 cells and Th17-related cytokines (IL-17A, IL-6 and IL-23) in the peripheral blood and lung tissue of mice with chronic CS exposure compared with sub-acute CS exposure and air-exposure mice. On the other hand, the number of Foxp3$^+$ Treg cells and Treg related cytokine IL-10 in the peripheral blood and lung tissue of mice with chronic CS exposure was decreased. Consistently, there was significant increase of expression level of RORγt, the specific transcription factor of Th17; while the expression level of Foxp3, the specific transcription factor of Treg significantly decreased in lung tissue from chronic CS exposure mice. These results suggest that an imbalance of Th17/Treg in peripheral blood and lung tissue may be involved in the pathogenesis of COPD.

Apart from the preventive steps of smoking cessation, there are no other specific treatments for COPD that are able to reverse the lung function. A better understanding of the pathogenesis might lead to new therapeutic strategies. However, studying the molecular pathways or immune mechanisms in patients is restricted to morphological and molecular assessment of lung tissues obtained at surgery at one single time point and at relatively late stage of the disease [18].

There is in dire need of in vivo animal models to examine more closely the molecular mechanisms in the process of pathogenesis, which may lead to more effective therapeutic strategies. It is well known that an important feature of COPD is the ongoing chronic inflammatory process in the airways [19]. There are differences between COPD and asthma: the major inflammatory cell types in COPD are neutrophils and macrophages, while eosinophils and mast cells are the prominent cell types in allergic asthma [20–22]. However, the role of T lymphocytes in COPD is less well defined. Therefore, we tried to focus on the changes of lymphocytes, in particular the balance of T lymphocytes in COPD.

CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cell (Treg) produced in the thymus (naturally occurring Treg (nTreg)) constitutes 3–6% of total CD4$^+$ T cells [15]. Treg and their effector molecules such as IL-10 have been demonstrated to control autoimmune disease efficiently [23]. Although COPD has been considered as a kind of autoimmune disease recently, reports on Treg cell number changes are controversial [24–27]. The lack of consensus may arise from the different experimental models used in various studies. Generally, Treg may attempt to resolve inflammation through IL-10 production. IL-10 is a potent anti-inflammatory cytokine that inhibits the synthesis of many inflammatory proteins, including several cytokines like tumor necrosis factor (TNF-α), IL-1β, granulocyte-macrophage colony-stimulating factor (GM-CSF), chemokines, as well as MMPs, such as MMP-9, that are overexpressed in COPD [28]. Previous studies have shown that the concentrations of IL-10 in sputum and lung tissue are reduced in patients with COPD [29,30]. In this study, one interesting finding is that IL-10 level in serum and BALF, as well as the Treg-specific transcription factor (Foxp3) mRNA level in lung, increased significantly from mice with sub-acute CS exposure and decreased markedly in mice with chronic CS exposure compared with the control mice. Such results suggest that the immune system may attempt to control the inflammatory response at the initiation phase via Treg cells, as part of the protective response from the organism. However, when the CS exposure persists, the ability of Treg cells and IL-10 in inhibiting the inflammatory response could not be sustained, thus leading to enhanced inflammatory response in mice with chronic CS exposure. Inconsistent with our study, an earlier report has shown that the number of Treg was increased in peripheral blood of COPD patients and smokers compared to healthy subjects [31]. The reason for this discrepancy is possible that limited scale of COPD subjects, difference between patients and animal models, the course of the disease and methods for detecting Treg partly contribute to this discrepancy.

Th17 cells are regulated by the transcription factor, ROR-γt [10] and known to play an important role in inflammatory and autoimmune diseases [32]. At present, little is known about the role of Th17 in COPD, but an increase of Th17 in peripheral blood and bronchial biopsies was observed in COPD patients compared with healthy subjects [31,33]. Meanwhile, Chen K et al. found that CS is a potent Th17 adjuvant and IL-17RA signaling is required for chemokine expression necessary for MMP12 induction and tissue emphysema [34] and Harrison OJ et al. found that Th1 and Th17 type response may play an important role in the pathogenesis of cigarette smoke induced autoimmunity in a mouse model [35]. In our study, the number of Th17 cells in lung tissue and peripheral blood, and Th17-specific transcription factor (ROR-γt) mRNA level in lung tissue were significantly increased in chronic CS exposure mice. The results indicate the possibility that Th17-mediated immune response is involved in the pathogenesis of COPD. IL-17 is the predominant product and key effector molecule of Th17 cells. The levels of IL-17 were increased in the sputum of individuals with COPD [36]. In contrast, Barczyk et al. has reported that the level of IL-17 in sputum is not different between patients with COPD and control subjects [37]. We found that the changes of IL-17A were consistent with the change tendency of Th17 cells in peripheral blood and local lung tissue, which indicated that IL-17 secreted by Th17 cells may play a vital role in the development of inflammatory response in COPD. IL-17 may stimulate chemokine production such as CXCL5, CXCL6, and CXCL8 [38–41] and promote the role of neutrophils and macrophages in COPD pathogenesis [42]. It may partly explain the reason of the increase of neutrophils and macrophages in number in BALF from mice with chronic CS exposure in our study. As far as we know, the differentiation and development of Th17 cell are determined by the microenvironment such as cytokine profile. IL-6, IL-1β, TGF-β, and IL-23 are all involved in the differentiation of human Th17 cells but IL-1β is not needed in mouse [43,44]. Interestingly, IL-23 that appears to be essential to expand and maintain Th17 cells plays a pivotal role in the establishment and maintenance of inflammatory autoimmune diseases when IL-6 and TGF-β are important to differentiate and develop Th17 cells. The role of IL-23 in COPD has not been well investigated although there is a report that describes increased expression of IL-23 in the lung tissues of patients with COPD [45]. From our results, we found that the concentrations of IL-6, IL-23 and TGF-β in serum and BALF supernatant of mice with chronic CS exposure were significantly increased compared with those of mice with sub-acute CS exposure and control mice, which indicates that chronic CS exposure can induce inflammatory cell infiltration and produces pro-inflammatory cytokines such as IL-6, IL-23 and TGF-β, and then intrigue Th17 cell differentiation, development and maintenance. As is well known that TGF-β is also a critical differentiation factor for the generation of Treg cells, Th17 and Treg cells arise in a mutually exclusive fashion, depending on whether they are activated in the presence of TGF-β or TGF-β plus IL-6 [46]. In our study, both TGF-β and IL-6 in serum and BALF supernatant in mice with chronic CS exposure were increased, which may suppress the generation of TGF-β-induced Treg cells and induce a pro-inflammatory T-cell response predominated by Th17 cells.
Table 1
Cytokines in serum in three groups.

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Control</th>
<th>Sub-acute CS exposure</th>
<th>Chronic CS exposure</th>
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<tr>
<td>IL-17A (pg/ml)</td>
<td>7.34±1.60</td>
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<td>22.12±12.07****</td>
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<tr>
<td>IL-6 (pg/ml)</td>
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<td>56.47±19.41****</td>
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<tr>
<td>IL-23 (pg/ml)</td>
<td>14.55±3.52</td>
<td>17.13±5.64</td>
<td>50.41±10.00***</td>
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<tr>
<td>TGF-β (ng/ml)</td>
<td>18.14±13.00</td>
<td>23.32±8.32</td>
<td>144.22±43.19***</td>
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<tr>
<td>IL-10 (pg/ml)</td>
<td>8.26±2.02</td>
<td>10.42±2.45**</td>
<td>4.04±2.57***</td>
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</tbody>
</table>

Values were shown as mean±SEM of twenty mice per group. *P<0.05 and **P<0.01 versus control group; *P<0.05 and ***P<0.01 versus sub-acute CS exposure group.

However, there is limited information on the balance between Th17 and Treg cells in COPD patients or animal models of COPD. As expected, our study found increased Th17 cell prevalence and decreased Th17 cell proliferation, both in peripheral blood and lung tissue of chronic CS exposure mice compared with those sub-acute CS exposure and control mice. Th17-specific transcription factor (ROR-γt) and Treg-specific transcription factor (Foxp3) mRNA level in lung tissue also showed the similar change tendency. We calculated the Th17/Treg ratio for each sample. Mice with sub-acute CS exposure and air-exposure exhibited low Th17/Treg ratios, while mice with chronic CS exposure have a high Th17/Treg ratio, which indicated that the balance between Th17 and Treg characterized by low Th17/Treg ratio may be broken in COPD mouse model induced by chronic CS exposure. Such Th17/Treg imbalance may play a pivotal role in the pathogenesis of COPD because predominant Th17 cells can exacerbate pro-inflammatory effects by producing IL-17.

5. Conclusion

In summary, the prevalence of Th17 and Th17-specific transcription factor, ROR-γt mRNA were increased continuously but Treg cell prevalence and Treg-specific factor, Foxp3 mRNA were decreased gradually after an initial increment in the early stage of disease development, therefore leading to a Th17/Treg imbalance in the mouse model of COPD induced by CS exposure. The imbalance may partly be due to the serum and lung tissue cytokine microenvironment. Our results suggest a potential role of Th17/Treg imbalance and related cytokine profile changes in the development and progression of COPD. Further study on the underlying mechanism regulating the Th17/Treg balance may lead to a novel therapeutic strategy for COPD.

Abbreviations

COPD chronic obstructive pulmonary disease
CS cigarette exposure
Th T helper type
IFN-γ interferon-γ
IL interleukin
IgE immunoglobulin E
PBMC peripheral blood mononuclear cell
MMP matrix metalloproteinase
BALF bronchoalveolar lavage fluid
Foxp3 forkhead box P3 (the Treg-defining transcription factor)

Table 2
Cytokines in BALF supernatant in three groups.

<table>
<thead>
<tr>
<th>Cytokines (pg/ml)</th>
<th>Control</th>
<th>Sub-acute CS exposure</th>
<th>Chronic CS exposure</th>
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</thead>
<tbody>
<tr>
<td>IL-17A</td>
<td>45.83±13.61</td>
<td>52.25±13.92</td>
<td>114.58±17.92****</td>
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<tr>
<td>IL-6</td>
<td>4.84±1.67</td>
<td>5.25±4.35</td>
<td>14.65±4.24****</td>
</tr>
<tr>
<td>IL-23</td>
<td>19.08±6.04</td>
<td>21.89±8.50</td>
<td>32.69±13.82***</td>
</tr>
<tr>
<td>TGF-β</td>
<td>46.54±11.22</td>
<td>50.82±15.24</td>
<td>95.66±15.02**</td>
</tr>
<tr>
<td>IL-10</td>
<td>33.42±13.70</td>
<td>40.20±13.48</td>
<td>18.80±6.00***</td>
</tr>
</tbody>
</table>

Values were shown as mean±SEM of twenty mice per group. *P<0.05 and **P<0.01 versus control group; *P<0.05 and ***P<0.01 versus sub-acute CS exposure group.

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Authors’ contributions: HW carried out the animal study, participated in the flow cytometry analysis, ELISA measurement and drafted the manuscript. WP carried out the real-time PCR. YW participated in the flow cytometry analysis. HY carried out the real-time PCR. HL carried out the animal study, participated in the flow cytometry analysis. DX participated in the design of the study and performed the statistical analysis. WY conceived of the study and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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