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Restoring Th17/Treg balance via modulation of STAT3 and STAT5 activation contributes to the amelioration of chronic obstructive pulmonary disease by Bufei Yishen formula

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ABSTRACT

Ethnopharmacology relevance: Bufei Yishen formula (BYF), a Traditional Chinese Medicine (TCM), has been extensively applied in clinical treatment of chronic obstructive pulmonary disease (COPD) and provides an effective treatment strategy for the syndrome of lung-kidney qi deficiency in COPD patients. Here, we investigated its anti-COPD mechanism in COPD rats in relation to the balance between T helper (Th) 17 cells and regulatory T (Treg) cells.

Methods: Rat model of cigarette smoke- and bacterial infection-induced COPD was established, and orally treated with BYF for 12 consecutive weeks. Then, the rats were sacrificed, their lung tissues were removed for histological analysis, and spleens and mesenteric lymph nodes (MLNs) were collected to evaluate the Th17 and Treg cells.

Results: Oral treatment of BYF markedly suppressed the disease progression and alleviated the pathological changes of COPD. It also decreased the bronchoalveolar lavage fluid (BALF) levels of pro-inflammatory cytokines, including IL-1β, IL-6, TNF-α and Th17-related IL-17A, and induced a significant increase in Treg-related IL-10. Furthermore, BYF treatment obviously decreased the proportion of $CD4^+ROR\gamma t^+$ T (Th17) cell and increased the proportion of $CD4^+CD25^+Foxp3^+$ T (Treg) cell, leading to restore the Th17/Treg balance. BYF treated groups also decreased RORγt and increased Foxp3 expression in the spleens and MLNs. BYF further inhibited the phosphorylation of signal transducer and activator of transcription-3 (STAT3) and boosted the phosphorylation of STAT5, that were critical transcription factors for TH17 and Treg differentiation.

Conclusion: these results demonstrated that BYF exerted its anti-COPD efficacy by restoring Th17/Treg balance via reciprocally modulating the activities of STAT3 and STAT5 in COPD rats, which may help to elucidate the underlying immunomodulatory mode of BYF on COPD treatment.

1. Introduction

Chronic obstructive pulmonary disease is a heterogeneous syndrome associated with abnormal inflammatory immune responses of the lung to noxious particles and gases, which affects more than 200 million people worldwide and is the fourth leading cause of death (Guan et al., 2016; Vogelmeier et al., 2017). Although the exact pathogenesis of COPD remains unclear, an abnormal immune response is regarded to be a vital inducer of alveolar destruction and inflammation (Eppert et al., 2013; Wouters et al., 2009). Many studies have provided extensive evidence suggesting that T cell-mediated adaptive immunity is deeply involved in the pathogenesis and progression of COPD (Brusselle et al., 2011; Zhang et al., 2016). Th17 cells, a distinct lineage of activated $CD4^+$ T cells, are raised in the airways and lungs of COPD patients, upregulating tissue inflammation and exacerbate alveolar destruction by producing IL-17, an important pathogenic factor (Brusselle et al., 2011; Eppert et al., 2013; Zhang et al., 2016). Th17 cells also exert direct influence on epithelial cells, smooth muscle cells and airway fibroblasts to induce neutrophil chemokine secretion (Curtis et al., 2007; Jones and Chan, 2002). In contrast, Treg cells, subsets of

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Table 1
The compositions and chemical compounds of Bufei Yishen formula.

No.	Herbal drug	Latin scientific name	plant part(s)	Chemical compounds	Amount (g)
1	Ginseng Radix et Rhizoma	*Panax ginseng* C.A. Mey	Radix et Rhizoma	ginsenoside Rb1, ginsenoside Rd, pseudoginsenoside	9
2	Astragali Radix	*Astragalus tibetanus* Bunge	Radix	Astragaloside, calycosin, formononetin	15
3	Corni Fructus	*Cornus officinalis* Siebold & Zucc	Fructus	ursolic acid, Loganin	12
4	Lycii Fructus	*Lycium barbarum* L.	Fructus	Cyanin, glycitein, fucosterol,	12
5	Schisandrae Chinensis Fructus	*Schisandra arisanensis* Hayata	Fructus	deoxyschizandrin Schisandrin Schisandrin B	9
6	Fritillariae Thunbergii Bulbus	*Fritillaria thunbergii* Miq	Bulbus	Peiminoside, peimisine, pelargonidin	9
7	Perillae Fructus	*Perilla frutescens* (L.) Britton	Fructus	Luteolin, spinasterol, obtusifoliol	9
8	Citri Reticulatae Pericarpium	*Citrus sinensis* (L.) Osbeck	Pericarpium	nobiletin Hesperidin	9
9	Epimedii Folium	*Epimedium acuminatum* Franch	Folium	Epimedin A Epimedin B Epimedin C icariside I Hyperin	9
10	Paeoniae Rubra Radix	*Paeonia anomala* L	Radix	paeoniflorin	9
11	Pheretima	*Pheretima aspergillum* (E. Perrier)		Lumbrofebrine, lumbritin	12
12	Ardisiae Japonicae Herba	*Ardisia japonica*(Thumb.) Blume	Herba	bergenin Kaempferol	15

$CD4^+$ T cells with immunoregulatory functions, maintenance of immune homeostasis by inhibiting aberrant immune responses and suppressing inflammation which are harmful to COPD patients (Brusselle et al., 2011). Furthermore, Foxp3 + Tregs could improve prognoses in cases of acute exacerbation of COPD (Xiong et al., 2008). Several studies on the immune responsibility of Tregs indicate levels of peripheral Tregs are significantly decreased in COPD patients compared with healthy subjects (Yang et al., 2011). Therefore, the balance between Th17 and Treg cells is critical for the maintenance of immune homeostasis, which may be served as a potential approach to control COPD.

COPD is referred to as lung distention (Feizhang disease) in TCM (Wang et al., 2015). Lung-kidney qi deficiency is one of the most important syndromes in COPD, which included panting, shortness of breath, lassitude and spontaneous sweating, weakness in the lower back and knees, tinnitus, vertigo, frequent nycturie, soreness and weakness of the waist and knees (Professional Committee of Pulmonary Disease of Internal Medicine Branch, 2012). Bufei Yishen formula (patent: ZL.201110117578.1), including twelve medicinal herbs, is specially prescribed for COPD patients with lung-kidney qi deficiency syndrome (Li et al., 2012a, b). Previous studies demonstrated that BYF have the potential to alleviate COPD symptoms by reducing the exacerbation frequency, delaying acute exacerbation, and improving pulmonary function and exercise capacity in COPD patients (Li et al., 2012a, b; Li et al., 2014). System biology analysis of anti-COPD mechanisms of BYF showed that BYF achieved its ameliorative effect over COPD probably by regulating immune response, inflammatory response, lipid metabolism, etc. (Li et al., 2016a, b; Li et al., 2015; Tian et al., 2016; Li et al., 2016a, b). However, the regulatory effect of BYF on Th17 and Treg cells in COPD remains poorly understood.

In the present work, we demonstrated that BYF exerted an immunomodulatory activity. It ameliorated cigarette smoke- and bacterial infection-induced COPD in rats by regulating cytokine profile in $CD4^+$ T cells and signaling pathways of STAT3 and STAT5, and thus provided a potential therapeutic approach for the treatment of COPD.

2. Materials and methods

2.1. Chemicals and animals

Klebsiella pneumoniae (strain ID: 46114) was purchased from the National Center for Medical Culture Collection (Beijing, China). Tobacco was obtained from Henan Tobacco Industry (Hongqi Canal® Filter tip cigarette; tobacco type, tar: 10 mg; nicotine content: 1.0 mg; carbon monoxide: 12 mg, Zhengzhou, China). Aminophylline was obtained from Shandong Xinhua Pharmaceutical Co., LTD. (Shandong, China). Antibodies for rat CD4, IL-17A, CD25, and Foxp3 were purchased from eBioscience, Inc. (Affymetrix, CA, USA). Rat IL-1β, TNF-α, IL-6, IFN-γ, IL-4, IL-17A, and IL-10 ELISA kits were purchased from Boster Biological Engineering (Wuhan, China). Antibodies against Foxp3, RORγ, p-STAT3 (Tyr705), p-STAT5 (Tyr694), STAT3, STAT5 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The RNeasy kit was obtained from Qiagen (Valencia, CA, USA). SYBR Green master mix was purchased from Vazyme Biotech Co., Ltd (Nanjing, China).

Sixty-two Sprague-Dawley rats (31 male and 31 female; 200 ± 20 g) were purchased from the Experimental Animal Center of Henan Province (Zhengzhou, China). The rats were maintained in SPF facilities and housed in filter-top cages under standard conditions of humidity (50 ± 10%), temperature (25 ± 2 °C), and light (12 h light/12 h dark cycle).

2.2. BYF preparation

BYF consists of 12 herbs: Ginseng Radix et Rhizoma 9 g, Astragali Radix 15 g, Corni Fructus 12 g, Lycii Fructus 12 g, Schisandrae Chinensis Fructus 9 g, Epimedii Herba 9 g, Fritillariae Thunbergii Bulbus 9 g, Paeoniae Rubra Radix 9 g, Pheretima 12 g, Perillae Fructus 9 g, Ardisiae Japonicae Herba 15 g, Citri Reticulatae Pericarpium 9 g (Table 1).

The herbal drugs were identified and prepared in dry extract. Briefly, Ginseng Radix et Rhizoma, Corni Fructus , Fritillariae Thunbergii Bulbus , Perillae Fructus , Schisandrae Chinensis Fructus , Citri Reticulatae Pericarpium were extracted with ethanol. Astragali Radix , Lycii Fructus , Epimedii Herba , Paeoniae Rubra Radix , Pheretima , Ardisiae Japonicae Herba were boiled with water. Then, the two extracts were filtered, condensed, and spray-dried. In addition, high performance liquid chromatography (HPLC) fingerprint was performed to identify the main chemical constituents in BYF. Fingerprints of BYF from ten batches were identified (Fig. 1A), and 20 chemical constituents of BYF were identified according to the spectrograms and retention times of their standard substances using mass spectrometry (Fig. 1B).

2.3. COPD rat model and drug administration

COPD rat model was prepared as described in the previous study (Li

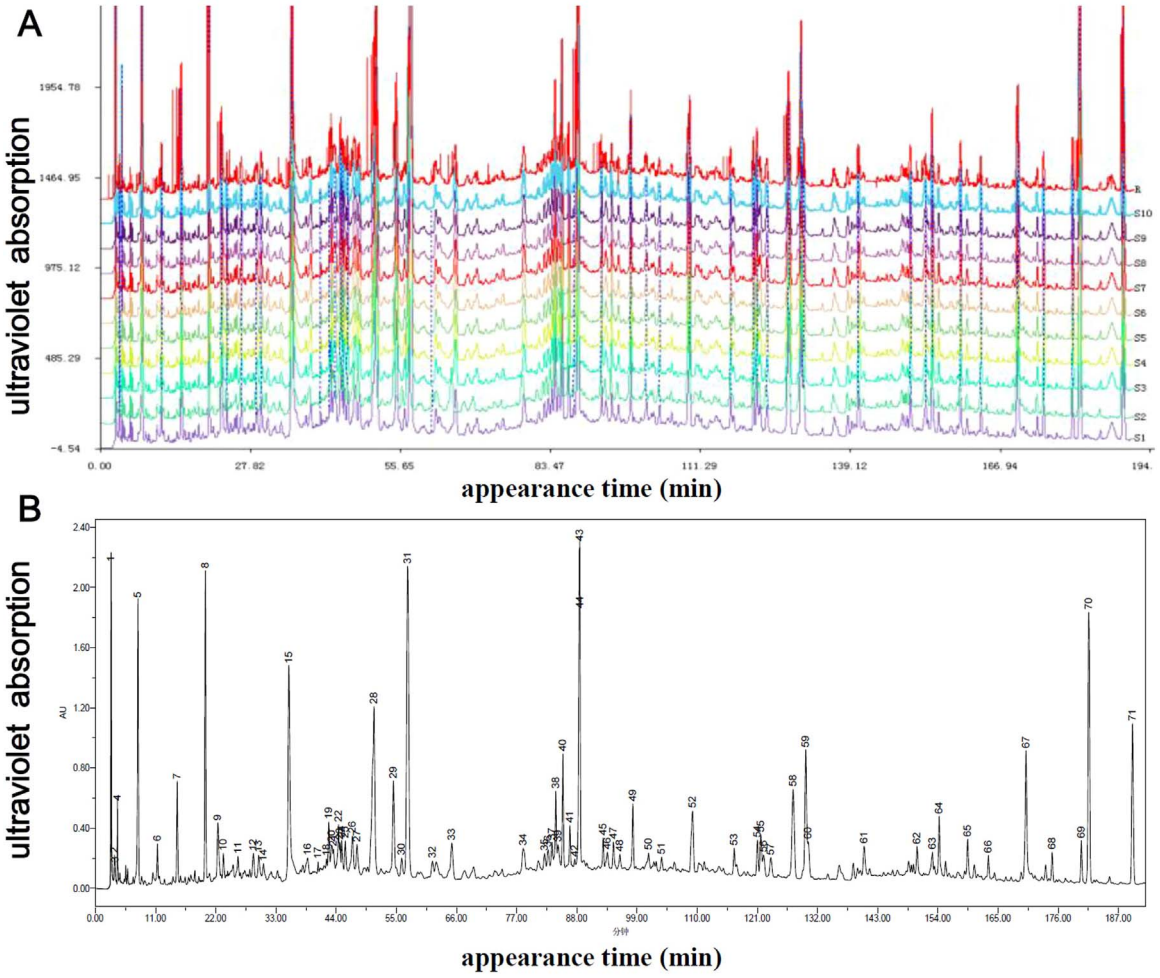

Fig. 1. The fingerprint of Bufei Yishen Formula (BYF). (A) fingerprints of BYF from ten batches. (B) Peak number and identity, 7: ursolic acid (PubChem CID: 64945); 8: bergenin (PubChem CID: 66065); 12: Loganin (PubChem CID: 87691) ; 15: paeoniflorin (PubChem CID: 442534); 17: nobiletin (PubChem CID: 72344); 19: Calycosin-7-glucoside (PubChem CID: 71571502); 25: hyperin (PubChem CID: 5281643); 31: Hesperidin (PubChem CID: 10621); 39: Epimedin A (PubChem CID: 44259065); 40: Epimedin B (PubChem CID: 5748393); 41: Epimedin C (PubChem CID:5748394); 43: icariin (PubChem CID:5318997); 45: Kaempferol (PubChem CID: 5280863); 49: deoxyschizandrin (PubChem CID:155256); 50: ginsenoside Rb1 (PubChem CID: 9898279); 52: ginsenoside Rd (PubChem CID: 24721561); 58: Schisandrin (PubChem CID:23915); 68: Schisandrin B (PubChem CID:108130); 70: Peimine (PubChem CID:131900);71: Peiminine (PubChem CID:167691).

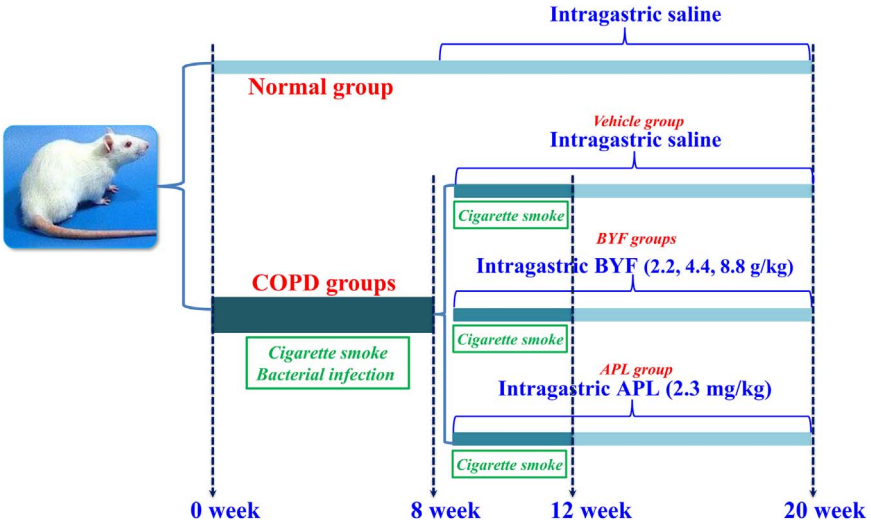

Fig. 2. The flow chart explaining the chronic obstructive pulmonary disease (COPD) induction and Bufei Yishen formula (BYF) and aminophylline (APL) administration regime.

et al., 2015; Li et al., 2012a, 2012b). As shown in Fig. 2, fifty-two rats were maintained in a chamber, then treated with cigarette smoke twice per day for two weeks and then thrice per day for next ten weeks. *Klebsiella pneumonia* suspension (6×10^8 CFU/mL, 100 μL) was dropped into the nasal cavities of rats every 5 days for the first eight weeks. On week 8, pathological analysis of lung tissues obtained from two COPD rats were conducted to confirm that this rat model was successful.

On week 9, fifty COPD rats were randomly divided into five groups. BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (2.3 mg/kg) was given orally twice per day for 12 weeks. Normal rats in control group were intragastrically administrated normal saline (2 mL). All rats were killed at week 20, and then lung tissues, BALF, spleen and MLNs were collected. All experimental procedures were examined and approved by Experimental Animal Care and Ethics Committee of the First Affiliated Hospital, Henan University of Chinese Medicine. The medium dose of BYF (4.4 g/kg) was calculated by the formula: $D_{rat} = D_{human} \times (I_{rat}/I_{human}) \times (W_{rat}/W_{human})^{2/3}$. D, dose; I, body shape index; W, body weight.

2.4. Pulmonary function analysis

Measurement of lung function was performed with unrestrained pulmonary function testing plethysmography (Buxco Inc., Wilmington, NC, USA). Buxco air flow transducers were connected to chambers with a reference chamber to compensate for pressure changes. Tidal volume (TV), peak expiratory flow (PEF), and maximum minute ventilation (MMV) were evaluated.

2.5. Histopathology

The left lower lobe was fixed in 10% formalin, embedded in paraffin, then cut into 4-μm sections. For histological examination, the tissues were stained with Mayer's hematoxylin and 1% eosin (H&E staining). Then, the morphological lesions and changes in lung tissues were evaluated by a light microscope.

2.6. ELISA assay for cytokines

The levels of IL-1β, TNF-α, IL-6, IFN-γ, IL-4, IL-17A and IL-10 in BALF of rat were determined by ELISA kits according to the manufacturer's instructions. All determinations were performed in triplicate.

2.7. Flow cytometry

T cell suspensions from rat spleen and MLNs were counted, prepared into 10^6 cells/mL and stained with FITC-anti-CD4, APC-anti-CD25 antibodies for 30 min followed by fixation and permeabilization, and then stained with PE-anti-Foxp3, PE-anti-IL-17A antibodies for 1 h. The T cells were analyzed on a FACS CantoTM II (BD Biosciences, San Jose, CA, USA) and the results were analyzed with the FlowJo7.6.1 software (Tree Star, USA).

2.8. Quantitative real-time PCR

Total RNA was extracted from spleen and MLNs using an RNeasy kit, and then reverse transcriptase reactions were performed using the HiScript II Reverse Transcriptase (Vazyme, Nanjing, China). Real time quantitative PCR was performed using real-time RT–PCR (Applied Biosystems, CA, USA) based on general fluorescence detection by SYBR Green. To normalize the amount of total RNA in each reaction, the reference gene, GAPDH, was used as an internal standard.

2.9. Western blot assay

The proteins were extracted from spleen and MLNs using radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio life sciences, Beijing, China). Samples containing 20 μg of protein were mixed with SDS sample buffer and boiled, then separated on a 10% SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked for 2 h at room temperature with 5% nonfat-milk and then incubated with the corresponding primary antibodies: RORγt (1:4000 diluted), Foxp3 (1:2000 diluted), STAT3 (1:2000 diluted), STAT5 (1:2000 diluted), p-STAT3 (1:1000 diluted), p-STAT5 (1:1000 diluted), and GAPDH (1:5000 diluted) at 4 °C overnight. The membranes then were incubated with HRP-conjugated secondary antibodies (1:5000 diluted) for 2 h. Finally, the bands were visualized by film exposure with ECL reagent.

2.10. Statistical analysis

All values were expressed as means ± standard errors of the means (S.E.M.). Statistical differences were assessed by one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test. Values of $P < 0.05$ were considered a significant difference.

3. Results

3.1. BYF markedly reduces the severity of COPD in rats

To verify therapeutic effect of BYF on COPD in rats, BYF was orally administered to the COPD rats for 12 consecutive weeks, and aminophylline was used as a positive control. Histopathological analysis of pulmonary tissue using HE staining revealed that BYF obviously ameliorated the COPD symptoms, yielding reductions in the lung injury scores (in 4.4, 8.8 g/kg BYF, $P < 0.0001$, n = 6, one-way ANOVA, a post hoc Tukey's test), bronchiole stenosis (in 2.2 g/kg BYF, $P < 0.05$; in 4.4, 8.8 g/kg BYF, $P < 0.0001$, n = 6, one-way ANOVA, a post hoc Tukey's test), bronchiole wall thickness (in 8.8 g/kg BYF, $P < 0.0001$, n = 6, one-way ANOVA, a post hoc Tukey's test), small pulmonary vessels wall thickness (in 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test), and alveolar diameter (in 4.4, 8.8 g/kg BYF, $P < 0.05$, n = 6, one-way ANOVA, a post hoc Tukey's test), and increase in alveolar number (in 4.4, 8.8 g/kg BYF, $P < 0.05$, n = 6, one-way ANOVA, a post hoc Tukey's test) (Fig. 3A-G). Respiratory function analysis showed that the TV, PEF, and MMV decreased in COPD rat on weeks 20, whereas, BYF treatment resulted in the increase of TV (in 4.4 g/kg BYF, $P < 0.05$; in 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test), PEF and MMV (in 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) in the COPD rats (Fig. 3H-J). These results suggested that BYF markedly ameliorated COPD in rats.

3.2. BYF downregulates pro-inflammatory cytokines but upregulates anti-inflammatory cytokine IL-10 in BALF of COPD rats

Pro-inflammatory cytokines are recognized as critical mediators that initiate and maintain chronic inflammation in COPD. Thus, we evaluated the effect of BYF on the levels of pro-inflammatory cytokines in BALF of rat. As shown in Fig. 4A-C, BYF significantly reduced the levels of IL-1β (in 2.2 g/kg BYF, $P < 0.01$; in 4.4, 8.8 g/kg BYF, $P < 0.05$, n = 6, one-way ANOVA, a post hoc Tukey's test), TNF-a (in 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) and IL-6 (in 2.2, 4.4 g/kg BYF, $P < 0.01$; in 8.8 g/kg BYF, $P < 0.0001$, n = 6, one-way ANOVA, a post hoc Tukey's test), indicating an apparent systemic attenuation of the pro-inflammatory cytokine response. Furthermore, to determine whether BYF treatment could modulate the dysregulated effector T cell responses involved in COPD, we evaluated the effect of BYF on the BALF levels of related cytokines. The results showed that BYF markedly reduced the levels of IL-17A (in 2.2 g/kg BYF, $P < 0.05$; in 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test), but increased the level of IL-10 (in 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a

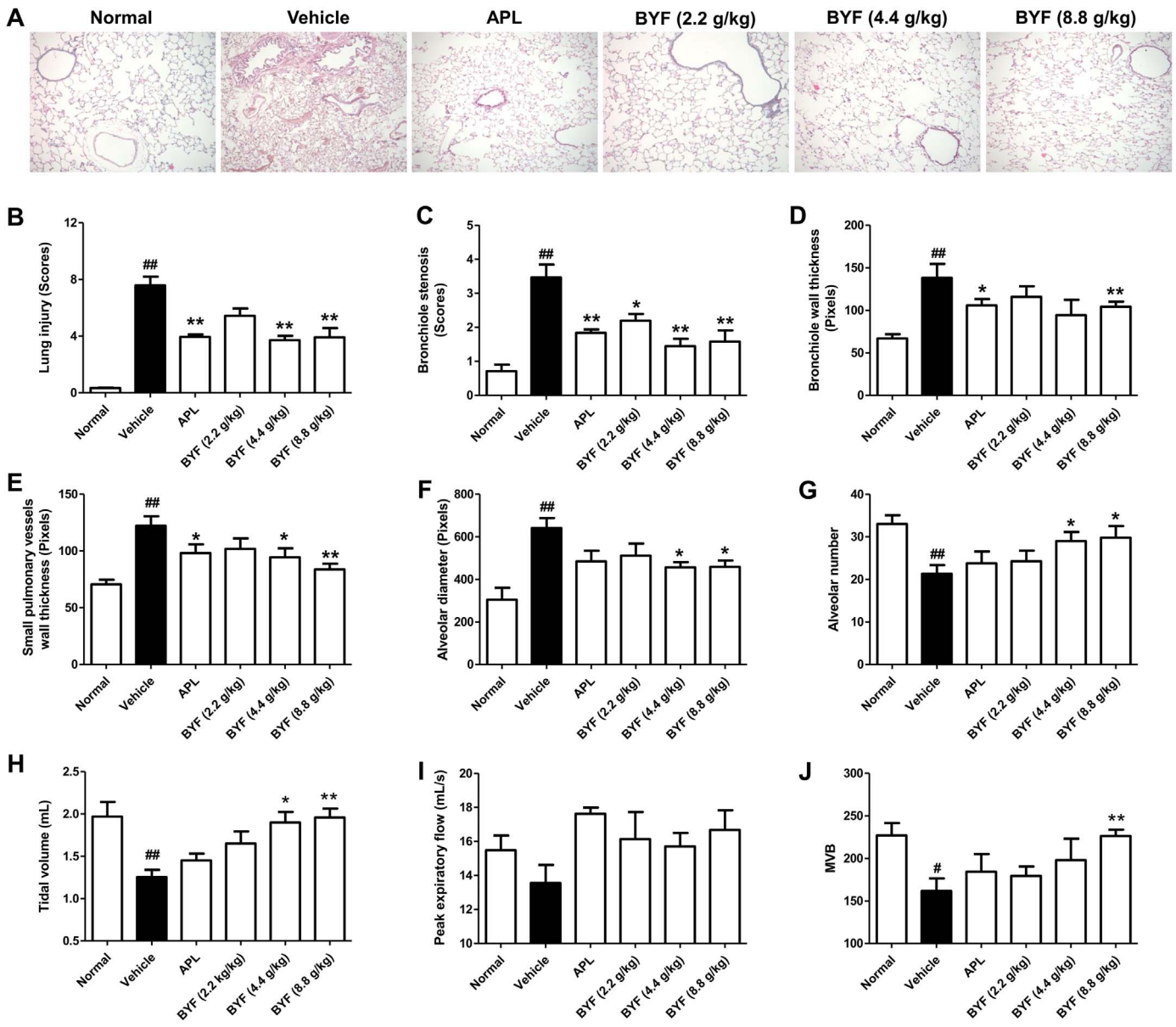

Fig. 3. Bufei Yishen formula (BYF) ameliorated cigarette smoke- and bacterial infection-induced chronic obstructive pulmonary disease (COPD) in rats. COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. Changes were examined as described in Materials and methods. (A) Histopathological changes of lung tissues of each group (HE staining, magnification, × 100). (B) Statistics of lung injury scores, (C)bronchiole stenosis, (D) bronchial wall thickness, (E) Small pulmonary vessels wall thickness, (F) alveolar diameter, (G) alveolar number, (H) Tidal volume, (I) Peak expiratory flow, (J) Maximum minute ventilation. All data are presented as mean ± SEM. n = 6 for each group. *P < 0.05, **P < 0.01 vs. model group, ##P < 0.01 vs. Control.

post hoc Tukey's test) (Fig. 4D, E). However, it did not significantly affect the BALF levels of IL-4 and IFN-γ (Fig. 4F, G). These data suggested that BYF treatment could suppress of the inflammation and Th17-related cytokine levels, and up-regulation of the Treg-related cytokine levels.

3.3. BYF restored the Th17/Treg balance in spleen and MLNs of COPD rats

To identify how BYF mediates its therapeutic effects by regulated the dysregulated effector T cell responses in COPD rats, we explored the effect of BYF treatment on the imbalance between Th17 and Treg cells in the rat spleens and MLNs. As shown in Figs. 5 and 6, COPD rats showed markedly higher percentages of $CD4^+$ $IL\text{-}17A^+$ T cells, but not significant change in the proportion of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in rat spleens and MLNs. BYF treatment obviously decreased the percentage of $CD4^+IL\text{-}17^+$ T cells (Fig. 5A, C, in 4.4, 8.8 g/kg BYF, P < 0.01; Fig. 6A, C, in 4.4 g/kg BYF, P < 0.05 in 8.8 g/kg BYF, P < 0.01, n = 6, one-way ANOVA, a post hoc Tukey's test) and increased the percentage of $CD4^+CD25^+Foxp3^+$ T cells (Fig. 5B, D in 8.8 g/kg BYF, P < 0.05; Fig. 6B, D, in 4.4 g/kg BYF, P < 0.05 in 4.4, 8.8 g/kg BYF, P < 0.05, n = 6, one-way ANOVA, a post hoc Tukey's test). Noteworthy, in the spleen of rats, the ratio of Th17/Treg cells was 0.11 in the normal group, and it increased to 0.29 in the COPD group. BYF (2.2, 4.4, 8.8 g/kg) reduced the ratio of Th17/Treg cells to 0.22, 0.12 and 0.11, respectively. Similarly, BYF also reduced the ration of Th17/Treg in MLNs of COPD rats. Taken together, BYF treatment alleviated COPD in rats likely by restoring the balance of Th17/Treg cells.

3.4. BYF regulated Th17 and Treg transcription factor expression

Transcription factors RORγt and Foxp3 play the critical roles in differentiation and function of Th17 and Treg cell, respectively (Huh

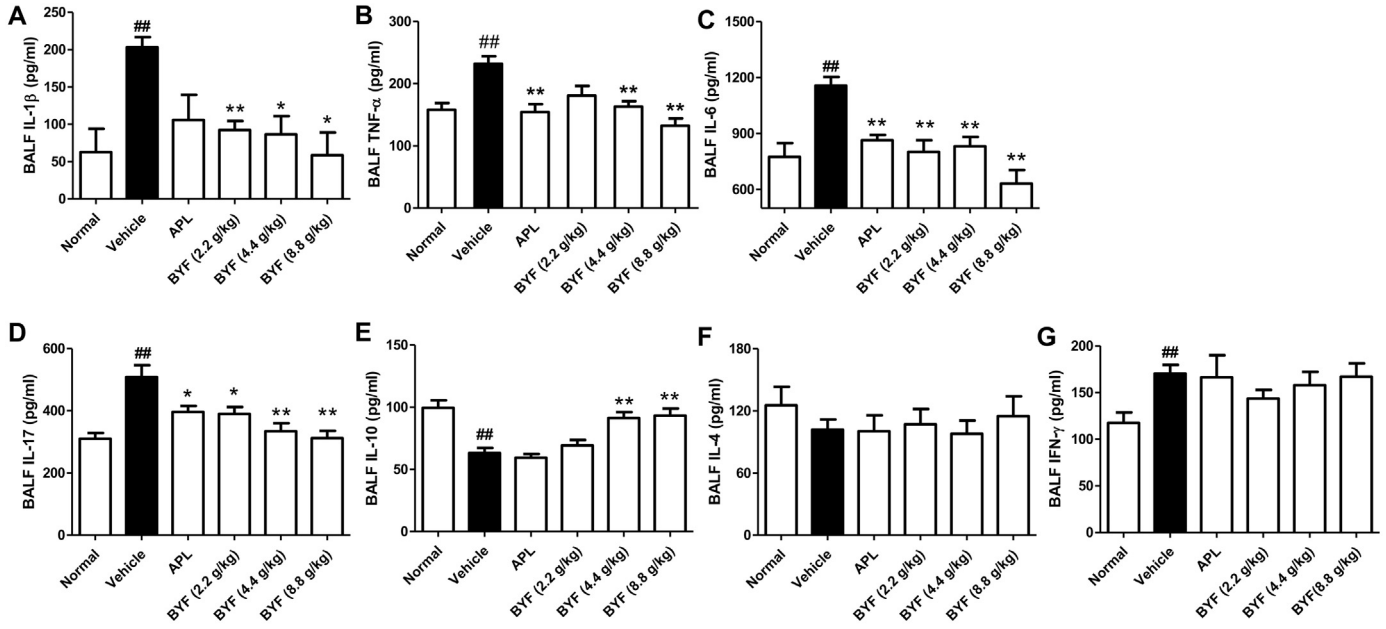

Fig. 4. Bufei Yishen formula (BYF) regulated cytokine profiles in BALF of rats with chronic obstructive pulmonary disease (COPD). COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. Cytokine levels of f IL-1β, TNF-a, IL-6, IL-17A, IL-4, IFN-γ, and IL-10 in BALF of rats were assessed by ELISA. The results were independently replicated. The values are presented as the means ± SEM (n = 6 mice per group). $^{\#\#}P < 0.01$ vs. control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. model group.

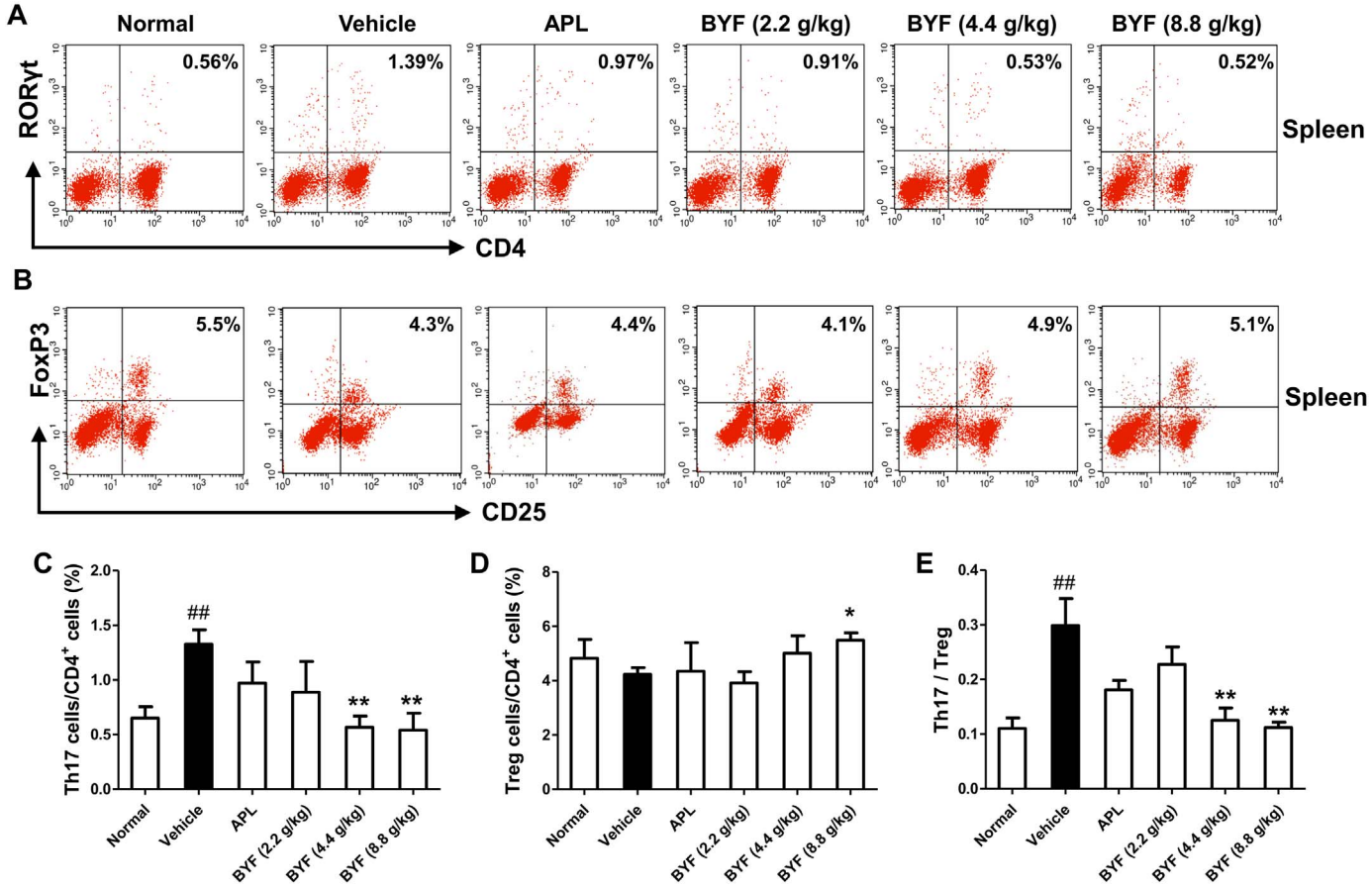

Fig. 5. Bufei Yishen formula (BYF) regulated the imbalance between the Th17 and Treg cells in spleens of chronic obstructive pulmonary disease (COPD). COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. Representative flow cytometry data for the T cell subsets gated on the CD4 + T cells from the spleens; the proportion of Th17 (A) and Treg cells (B) was determined. Then, statistics of the proportion of Th17 (C) and Treg cells (D) of each group. The ratio of Th17 /Treg also were analyzed (E). The values are presented as the means ± SEM (n = 6 mice per group). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Model group.

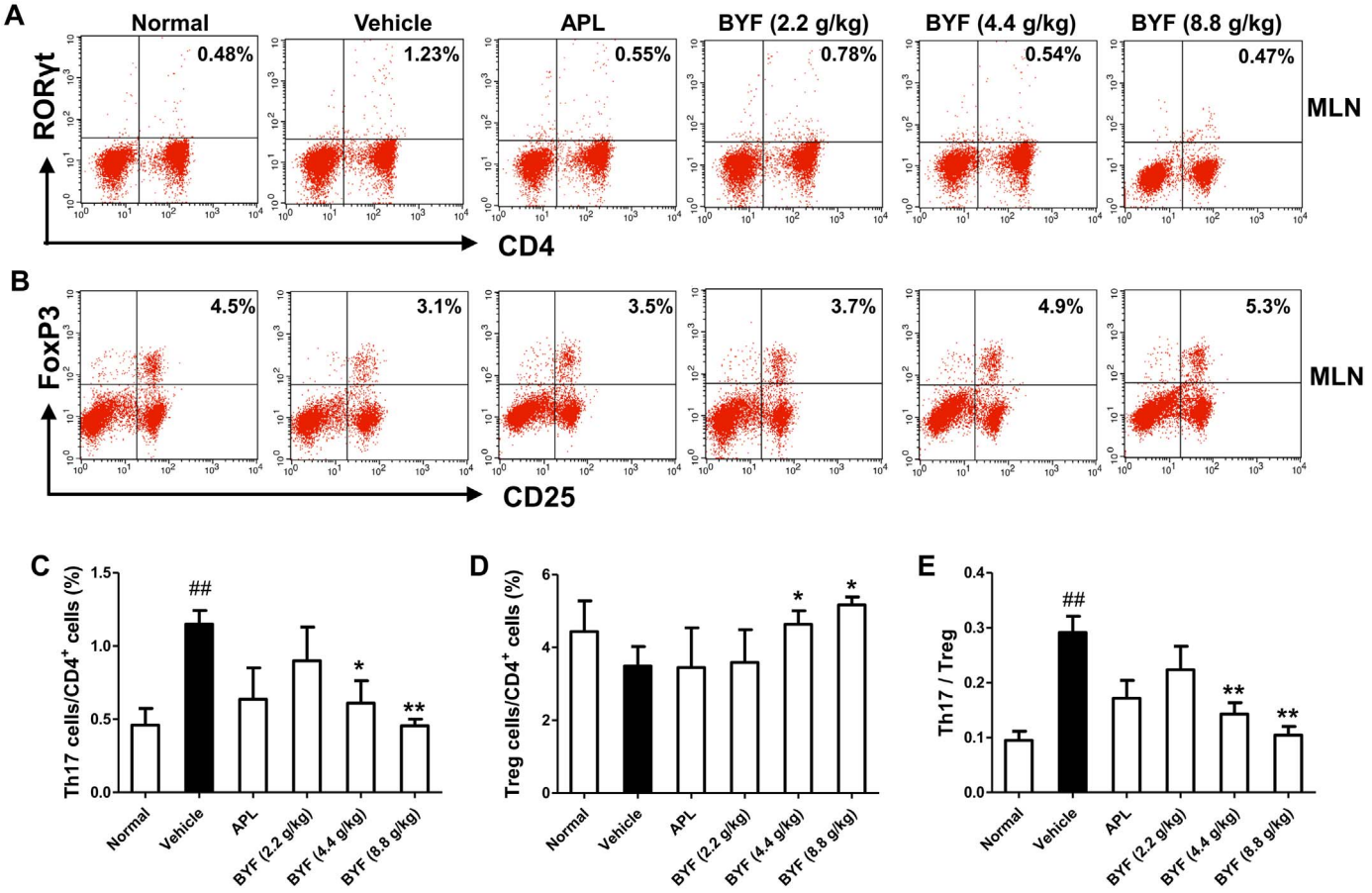

Fig. 6. Bufei Yishen formula (BYF) restored the Th17/Treg balance in mesenteric lymph nodes (MLNs) of chronic obstructive pulmonary disease (COPD). COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. The proportion of Th17 (A) and Treg cells (B) in MLNs was determined by flow cytometry. Then, statistics of the proportion of Th17 (C) and Treg cells (D) of each group. The ratio of Th17 /Treg also were analyzed (E). The values are presented as the means ± SEM (n = 6 mice per group). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Model group.

and Littman, 2012; Ohkura et al., 2013). To determine the effect of BYF on RORγt and Foxp3 expression, we evaluated the mRNA and protein levels of RORγt and Foxp3 in the spleens and MLNs of rats. As shown in Figs. 7 and 8, mRNA and protein levels of RORγt were markedly increased in COPD rats, and BYF treatment significantly inhibited the expression of the RORγt mRNA (Fig. 7A, C, in 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) and protein (Fig. 8A, in 2.2, 4.4, 8.8 g/kg BYF, $P < 0.01$, Fig. 8C, in 2.2 g/kg BYF, $P < 0.05$, in 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test). Additionally, BYF administration resulted in a dramatic increase in Foxp3 mRNA (Fig. 7B, in 4.4 g/kg BYF, $P < 0.05$, in 8.8 g/kg BYF, $P < 0.01$; Fig. 7D, in 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) and protein expression (Fig. 8B, D, in 2.2, 4.4, 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) in the spleens and MLNs. These results indicated that BYF treatment restored the balance of Th17/Treg cells probably through regulating the differentiation and function of Th17 and Treg cell.

3.5. BYF regulated the STAT3 and STAT5 pathway

STAT signaling pathways play the critical roles in the induction of T cell activation and Th cell differentiation. For instance, STAT3 is essential transcription factor for Th17 differentiation and Treg inhibition. Conversely, STAT5 activation is crucial with regard to the differentiation and maintenance of Treg cells, and suppressing the differentiation of Th17 cell (O'Shea and Paul, 2010; Sheng et al., 2014). Therefore, the protein levels of STAT3, p-STAT3, STAT5 and p-STAT5 in the spleens and MLNs of rats with COPD were analyzed. We found that BYF treatment significantly reduced the phosphorylation of STAT3 (Fig. 9A, in 4.4, 8.8 g/kg BYF, $P < 0.01$; Fig. 9B, in 2.2 g/kg BYF, $P < 0.05$; in 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) and boosted the phosphorylation of STAT5 (Fig. 9C, in 4.4, 8.8 g/kg BYF, $P < 0.01$; Fig. 9D, in 2.2, 4.4 g/kg BYF, $P < 0.05$; in 8.8 g/kg BYF, $P < 0.01$, n = 6, one-way ANOVA, a post hoc Tukey's test) without the significant effect on the protein levels of STAT3/5 in spleens and MLNs. Together, the results suggested that BYF could suppress the phosphorylation of STAT3 and enhance the phosphorylation of STAT5, which might contribute to its modulation effect of the Th17/Treg balance.

4. Discussion

COPD is characterized by a progressive and partially reversible airflow limitation associated with abnormal inflammatory responses and permanent enlargement of the pulmonary airspace (Brusselle et al., 2011). Current treatments for COPD, including bronchodilators (β2-agonists and muscarinic antagonists) and steroids, have been proved effective in improving airflow limitation and suppressing the inflammatory responses. However, they cannot significantly modulate disease progression and mortality and also have potential side effects including steroid dependence (Alsaeedi et al., 2002; Calverley et al., 2007). Therefore, novel strategies are urgently needed. In present study, oral administration of BYF obviously recovered the respiratory function and improved pathological changes including lung injury. Moreover, it exhibited a regulatory effect on cytokine profiles as it

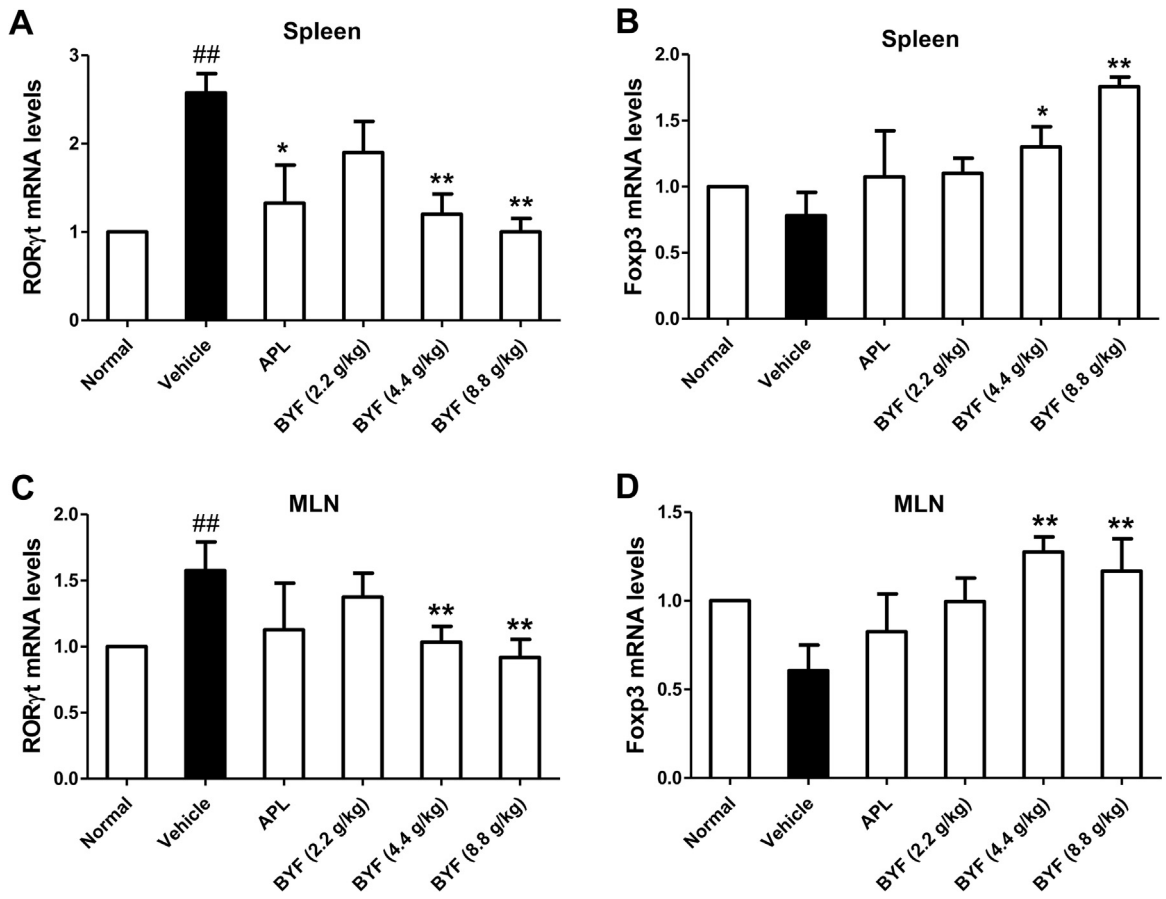

Fig. 7. Bufei Yishen formula (BYF) modulated the mRNA levels transcription factor RORγt and Foxp3 in the spleens and mesenteric lymph nodes (MLNs) of chronic obstructive pulmonary disease (COPD). COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. The mRNA levels for the transcription factors RORγt and Foxp3 were measured in the spleens and MLNs by a quantitative PCR assay. The values are presented as the means ± SEM (n = 6 mice per group). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Model group.

reduced the levels of pro-inflammatory cytokines and up-regulated the levels of the anti-inflammatory factor IL-10 in the BALF of COPD rats. BYF treatment was also showed to inhibit the expansion of the Th17 cells and enhanced the expansion of the Treg cells in the spleens and MLNs, as well as modulated their related transcription factors including STAT3 and STAT5. All these data demonstrated that BYF was a beneficial agent for the treatment of COPD.

It is well known that the pathology of COPD involves imbalance of pro-inflammatory and anti-inflammatory cytokines (Brusselle et al., 2011; Sinden and Stockley, 2010). In this study, significant reduction in proinflammatory cytokines (TNF-α, IL-6, IL-1β, and IL-17A) and increase in anti-inflammatory cytokine (IL-10) were observed in the BALF of BYF-treated COPD rats. IL-17, primarily generated by Th17 cells to activate responder T cells, is a typical proinflammatory cytokine inducing the expression of IL-6, IL-1 and TNF-α (Caramori et al., 2014; Jones and Chan, 2002). While, IL-10, primarily produced by Treg cells, can inhibit Th17 cell differentiation in the $CD4^+$ T cell population. However, BYF treatment did not obviously suppressed the levels of the Th2-associated IL-4 and Th1 cytokine (IFN-γ).

Th17 cell plays a potent proinflammatory role in autoimmune and lung diseases in animal and clinical studies by producing the signature cytokine IL-17A (Halwani et al., 2013). In contrast, Treg cell and their effector molecules such as IL-10 have been identified as vital immune modulators that efficiently maintain immune homeostasis, avoiding unnecessary reaction (Brusselle et al., 2011; Curtis et al., 2007; Zhang et al., 2016). Furthermore, Th17/Treg imbalance has a significant role in the induction and maintenance of chronic inflammation in COPD (Curtis et al., 2007; Zhang et al., 2016). Several previous studies have demonstrated that significantly increased levels of Th17 and decreased levels of Treg cells in COPD patients compared with healthy subjects (Vargas-Rojas et al., 2011; Yang et al., 2011). In this study, we found that the percentage of Th17 cells was significantly elevated in COPD rat group, whereas the percentage of Treg cells slightly decreased in COPD rat group compared with control group. Consistent with BYF's modulation effect on the cytokine profile, it considerably decreased the proportion of Th17 cells, and increased the percentage of Treg cells, then restored the balance of Th17/Treg.

RORγt, the crucial transcription factor, promotes the differentiation of Th17 cells, and facilitates expression of genes encoding IL-17A (Huh and Littman, 2012; O'Shea et al., 2009). BYF treatment significantly decreased the expression levels of RORγt, while, increased the Treg associated transcription factor Foxp3 expression in spleens and MLNs, which supporting the possibility that BYF regulated the Th17/Treg balance in COPD rat.

STAT3, activated by Th17-induced IL-6, IL-23, and IL-21, can induce RORγt gene expression, then operate in Th17 cell differentiation processes. While, STAT5 can directly bind the *Foxp3* gene to induce development and maintenance of Treg cells, and also directly binds the *Il17* gene to suppress Th17-related gene transcription (Sheng et al., 2014; Zhou et al., 2008; Zorn et al., 2006). In this study, BYF treatment declined phosphorylation level of STAT3 and increased the STAT5 phosphorylation level.

Moreover, many studies have shown that between 50% and 70% of patients diagnosed with non-small cell lung cancer had the important comorbidity, COPD (Hashimoto et al., 2014; Omote et al., 2017; Osuka et al., 2015). Similarly, the risk of lung cancer is also increased in COPD patients, and the prevalence of COPD in lung cancer ranges from 8% to 50% (Kurishima et al., 2001). Serial reports show that respiratory

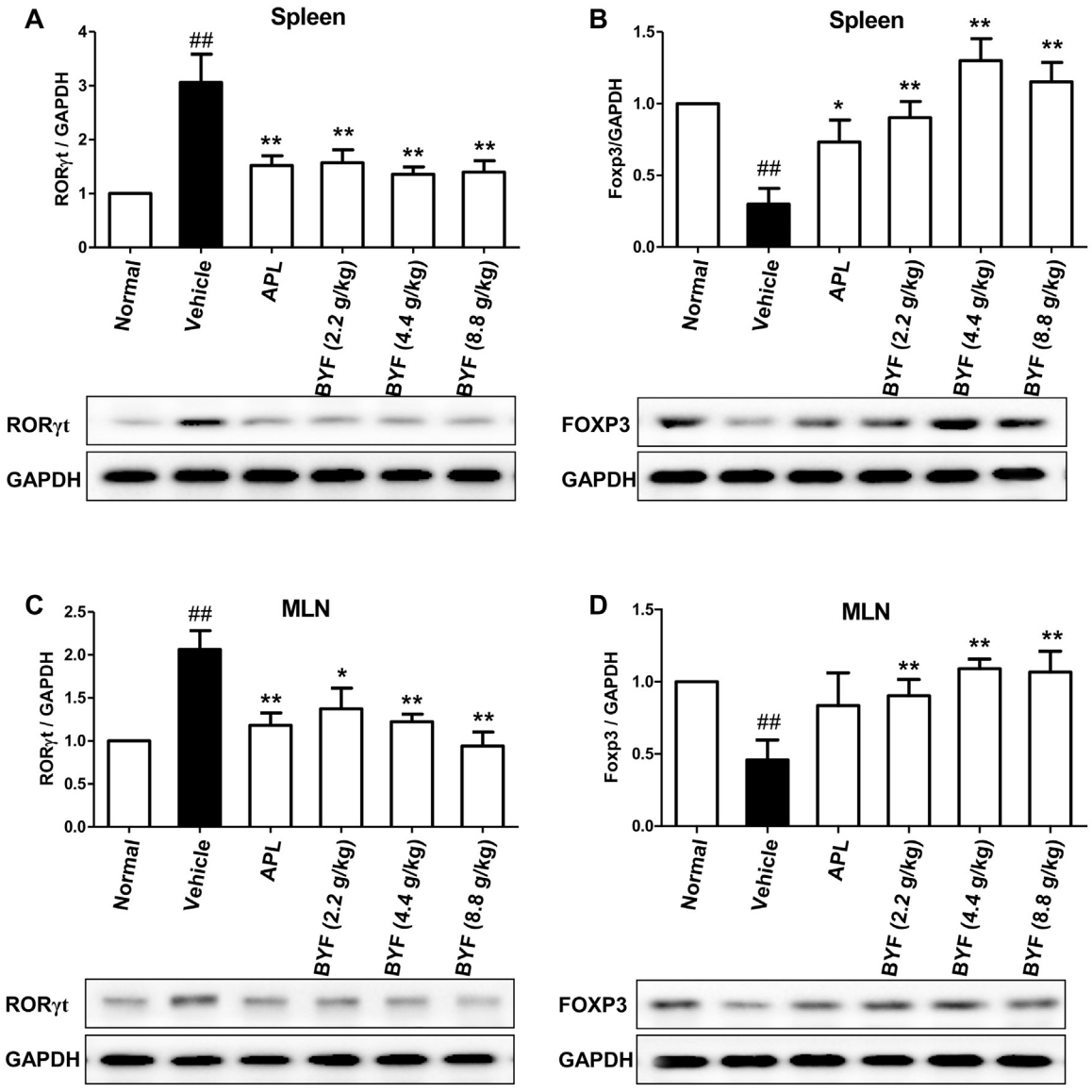

Fig. 8. Bufei Yishen formula (BYF) suppressed transcription factor RORγt and augmented Foxp3 protein levels in the spleens and mesenteric lymph nodes (MLNs) of chronic obstructive pulmonary disease (COPD). COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. The protein levels of RORγt and Foxp3 were analyzed and quantified. Data was showed as the means ± SEM (n = 6 mice per group). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Model group.

failure is the primary cause of death in patients with severe COPD, however, the predominant causes of death in mild to moderate COPD are lung cancer and cardiovascular diseases (Sin et al., 2006). In the mechanism linking between COPD and lung cancer, previous studies found lung tumor carcinogenesis and progression can be initiated by inflammatory cytokine/chemokine production and their-related pathways in COPD (Adams et al., 2017). For instance, protumorgenic effects of NF-κB and STAT3 activation in immune cells play the critical roles in regulating the production of pro-inflammatory cytokines in the inflammatory microenvironment of tumor and COPD (Adams et al., 2017; Omote et al., 2017; Sekine et al., 2014). BYF could inhibit the production of inflammatory cytokine, and the activation of NF-κB and STAT3, which might serve as prevention and treatment for COPD patients with lung cancer (Li et al., 2016a, b; Li et al., 2015; Li et al., 2014a, b).

Taken together, this study clarified BYF exerted beneficial effects on COPD rat by improving the lung functions and attenuating inflammatory conditions in COPD rat. The mechanism of BYF's effect involved reduction of the proportion of Th17 cells and elevation of the percentage of Treg cells, as well as regulation of their respective cytokine levels. Moreover, suppression of activation of STAT3 and enhancement of STAT5 activation contributed to the effect of BYF on Th17/Treg imbalance. These results collectively suggested that BYF could ameliorate COPD by regulating Th17/Treg imbalance via modulation of the activation of STAT3 and STAT5.

Acknowledgments

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Authors' contributions

J-SL and PZ designed the outline of the study. PZ, Y-GT and S-XF performed experiments, conceived the study, draft and revised the manuscript. JM, X-FL, J-ZL and Q-QB were involved performing experiments, acquisition of data and statistical analysis. H-GJ and L-XZ contributed to the data analysis and interpretation. All authors

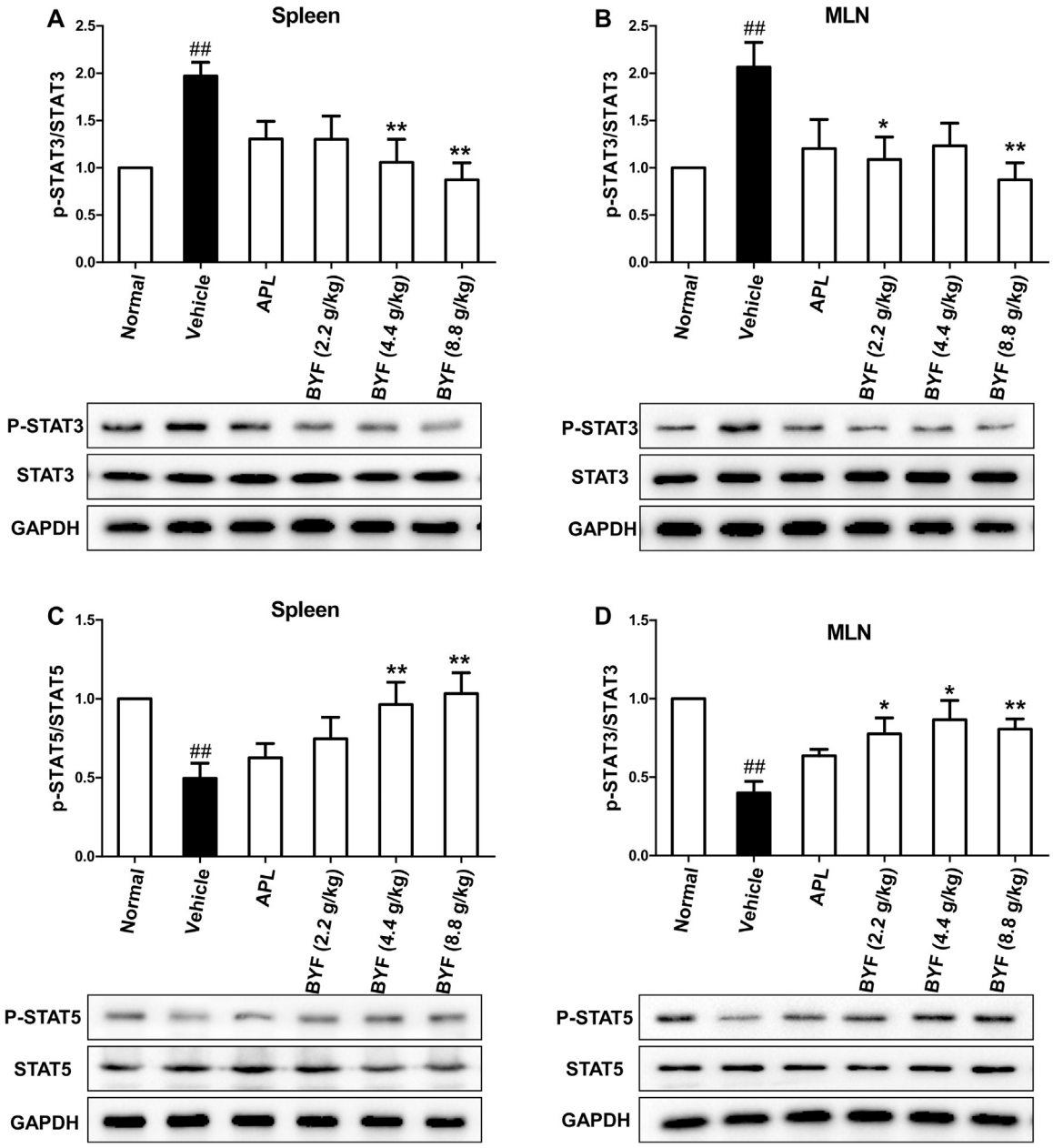

Fig. 9. Bufei Yishen formula (BYF) regulated the phosphorylation of STAT3 and STAT5 in the spleens and mesenteric lymph nodes (MLNs) of chronic obstructive pulmonary disease (COPD). COPD rats were orally administrated with BYF (2.2, 4.4, 8.8 g/kg) and aminophylline (APL, 2.3 mg/kg) from week 9–20. Expression of p-STAT3, STAT3, p-STAT5 and STAT5 in spleen and MLN protein was examined by western blot. Band intensity of western blot was normalized to actin and quantitated by densitometry. Data was showed as the means ± SEM (n = 6 mice per group). $^{\#\#}P < 0.01$ vs. the control group; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Model group.

contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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