Effect of Liuweibuqi capsules on CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells, helper T cells and lung function in patients with stable chronic obstructive pulmonary disease complicated with lung Qi deficiency

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Background: Chronic obstructive pulmonary disease (COPD) is predicted to become the fifth leading cause of disability and the third leading cause of death around the world by 2020. Though it is potentially treatable and preventable, evidence of brain structural alterations in COPD remains sparse and conflicting. We aim to investigate the effect of Liuweibuqi capsules on CD4<sup>+</sup>CD25<sup>+</sup>Forkhead box protein 3<sup>+</sup> (Foxp3<sup>+</sup>) regulatory T cells (Tregs), helper T cells (Th) and lung function in patients with stable COPD complicated with lung Qi deficiency.

Methods: COPD patients with lung Qi deficiency [458] were assigned into non-smoking COPD (NS-COPD), non-smoking control (NS-control), smoking COPD (S-COPD) and smoking control (S-control) groups, and healthy volunteers [245] into the non-smoking healthy (NSH) and smoking healthy (SH) groups. Levels of inflammatory cytokines were detected by Enzyme-linked immunoassay (ELISA). Contents of inflammatory cells, inflammatory marker, and CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs were measured by flow cytometry. FEV1/FVC (%) and FEV1 (%) were detected by pulmonary function test apparatus. Correlation between FEV1 (%) and Th1, Th2, Th17, Th1/Th2 or CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs was analyzed by Spearman rank correlation test. The related factors affecting treatment efficacy was assessed by logistic analysis.

Results: COPD patients and smoking people showed higher level of INF-γ, IL-4, IL-17, Th1, Th2, Th17 and Th1/Th2 but lower level of CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs. Liuweibuqi capsules could decrease level of inflammatory cells, cytokines, and markers (especially Th17 and IL-17), and increase level of CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs. FEV1 (%) negatively correlated with Th1, Th2, Th17 and Th1/Th2 but positively correlated with CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs, and smoking may strengthen their correlation, but Liuweibuqi capsules may weaken their correlation. Levels of inflammatory cytokines, cells, marker, CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs, FEV1/FVC (%), FEV1 (%), smoking and Liuweibuqi capsules are factors affecting efficacy.

Conclusions: Taken together, our data support the notion that smoking is an important factor to induce and aggravate COPD. Liuweibuqi capsules could stimulate proliferation of CD4<sup>+</sup>CD25<sup>+</sup>Fox3<sup>+</sup>Tregs and decrease Th17 expression to improve the lung function in stable COPD patients with lung Qi deficiency, and it had obvious efficacy for smoking COPD patients.

Keywords: Liuweibuqi capsules; chronic obstructive pulmonary disease (COPD); lung Qi deficiency; regulatory T cells; helper T cells
Introduction

Chronic obstructive pulmonary disease (COPD) is defined as an airflow obstruction with several different but overlapping obstructive syndromes (1). COPD has been thought to be one of major disease prevention goal for public health (2). It was predicted that morbidity of COPD would continue to increase and may be the third leading lethal disease by 2030 (3). The common obstructive syndromes of COPD are chronic bronchitis, emphysema and reversible or irreversible small airways obstruction (4). COPD patients often suffer wheezing, expiratory dyspnea, cough, chest tightness and sputum production (5). Cigarette smoke has been reported as the main cause of COPD, other factors include ambient air pollution, airway hyper-responsiveness and allergy (6,7). Treatment of COPD over the past decades of management includes pharmacological and nonpharmacological interventions with the targets to control symptoms and exacerbations, improve functional performance and quality of life, and decrease risk of hospitalizations, emergency care, and mortality (8,9). However, limitation of clinical practice causes unsatisfactory result as compared with the expectations (10). Qi deficiency and qi-yin deficiency are the most common syndromes, relating to Lung, spleen and kidney (11). And study has shown that the morbidity of lung cancer is implicated in Qi deficiency, which is the internal cause of viscera imbalance (12).

Currently, traditional Chinese medicine (TCM) therapies are widespread in both developing and developed areas (13). Liuweiibuqi capsules, a Chinese patent medicine, consist of Radix Ginseng (Renshen), Radix Astragali Mongolici (Huangqi), Alpiniae Oxyphyllae Fructus (Yizhi), Rhizoma Polygonati Odorati (Yuzhu), Cortex Cinnamomi (Rougui), Pericarpium Citri Reticulatae (Chenpi) (14). Renshen is one of the most popular TCM which could inhibit metastasis of melanoma in the lung (15,16). Astragaloside IV is one of the components of Huangqi which has been found a broad application prospects, especially in cardiovascular diseases and cancers (17). Yizhi is in favor of anti-aging activity and an anti-neoplastic medicine (18,19). Yuzhu is regarded as a traditional medicine which has functions of removing dryness, replenishing vital essence, promoting secretion of fluid and quenching thirst, and has been used to treat many diseases, especially tuberculosis (20). Rougui have been documented that it takes part in a variety of biological activities in vitro, one of its function is to induce apoptosis via reactive oxygen species and anti-inflammatory diseases (21). Chenpi has been commonly used as one of the herbal materials to eliminate phlegm and strengthen spleen, which is also an anti-inflammatory medicine (22). However, few studies investigate the relationship among Liuweiibuqi capsules, inflammatory cells, cytokines and indicator, immune regulatory cells, and lung function in stable COPD patients with lung Qi deficiency. Therefore, from a microcosmic perspective, we conducted this experiment to investigate their relationship with the perspective to provide a clinical reference in treating COPD patients with lung Qi deficiency.

Methods

Ethical statement

This study was approved by ethics committee of the First Affiliated Hospital of Anhui Medical University (2012-006). All subjects voluntarily participated in the experiment were fully informed of gains and risks and signed informed consents. This experiment was in accordance with the Nuremberg criteria and the Helsinki declaration.

Subjects

We randomly selected 458 stable COPD patients with Lung Qi deficiency (11) (with over 3-year disease history) who were admitted into the Respiratory Department of the First Affiliated Hospital of Anhui Medical University from January 2012 to December 2016. Non-smoking COPD patients with Lung Qi deficiency were 230 cases including 126 males and 104 females aged from 39–76 years (mean age of 56.80±9.78 years old), and COPD patients with over 10-year smoking duration were 228 cases including 122 males and 106 females aged from 35–74 years (mean age of 59.32±7.41 years old). We also selected 121 non-smoking healthy volunteers including 62 males and 59 females aged from 31–79 years (mean age of 55.85±10.36 years old) and 124 healthy volunteers with over 10-year smoking duration including 71 males and 53 females aged from 45–67 years (mean age of 56.51±4.45 years old).
Inclusion and exclusion criteria

COPD was diagnosed in accordance with Diagnostic guidelines for chronic obstructive pulmonary disease which was a diagnostic standard of COPD regulated by Chinese Thoracic Society in 2011. The inclusion criteria include COPD patients at stable phase with lung function at I–II grade (50%< FEV1) (23) and lung Qi deficiency syndrome; patients aged 35–76 years old and voluntarily participated and signed informed consents; patients with normal blood and urine routine, normal blood-fat and blood glucose, normal liver function, renal function and electrocardiogram. Exclusion criteria include people with lung disease such as tuberculosis or lung cancer; critical patients with cardiac dysfunction, pulmonary encephalopathy or immunological disease; patients with serious complication such as cardiovascular, hematopoietic, digestive, urinary or endocrine metabolic systems; pregnant woman or suckling period woman; patients with mentally ill.

Therapeutic regimens

Non-smoking COPD patients were randomly grouped into non-smoking COPD patients (NS-COPD group, 114 cases) and non-smoking controls (NS-control group, 116 cases); smoking COPD patients were randomly assigned into smoking COPD patients (S-COPD group, 110 cases) and smoking controls (S-control group, 118 cases). Before experiment, the NS-COPD and NS-control groups were of no significant differences in the baseline characteristics and blood indexes, same as the S-COPD and S-control groups (P>0.05). The NS-COPD and S-COPD groups were dosed with Liuweibuqi capsules (the First Affiliated Hospital of Anhui University of Traditional Chinese Medicine, 0.4 g/capsule), and each patient took three capsules in each morning and evening for two courses of treatment (30 days for one course of treatment); patients in the NS-control and S-control groups were dosed with Roflumilast (Jiangsu Hengrui Medicine Co., Ltd, Lianyungang, Jiangsu, China, 0.5 g/tablet), and each patient took one tablet each time, once a day for two courses of treatment (30 days for one course of treatment). Non-smoking healthy people (NSH group) and smoking healthy people (SH group) were in orthobiosis and in regular check.

Enzyme-linked immunoassay (ELISA)

Venous blood (10 mL) from the elbow was extracted from patients before and after experiment (from healthy people during physical examination). Then the blood was centrifuged for 10 min at a radius of 8 cm and speed of 3,000 rpm. After centrifugation, the serum was separated and stored at −20 °C. ELISA kit (Quantikine, R&D Systems, USA) were used to detect the levels of serum cytokines INF-γ (ab9829, Abcam, Cambridge, UK), IL-4 (ab215089, Abcam, Cambridge, UK) and IL-17 (ab119535, Abcam, Cambridge, UK). The specific experimental steps were as follows: standard samples were diluted with double broth dilution method and added into the blank wells, human serum of the correspondent groups was added to the other wells (100 μL for each well). Each group was set up three duplicated wells which were incubated at 37 °C for 90 min. The plate was washed for 5 times, and the blank wells were added with biotin-labeled antibody diluent. The other wells were added with biotinylated antibody working fluid (100 μL for each well), sealed with sealing adhesive, and incubated at 37 °C for 60 min. After that, the plate was washed for 5 times, the blank wells were added with enzyme combination diluent and the other wells were added with enzyme combination working solution (100 μL for each well), and then sealed with sealing adhesive, incubated at 37 °C for 30 min. The plate was washed for 5 times, the wells were added with chromogenic substrate (100 μL for each well), and then sealed with sealing adhesive, incubated at 37 °C for 15 min. Each well was then added with stop buffer (100 μL), and optical density (OD) value was measured at 450 nm by ELx808™ Absorbance Microplate Reader (BioTek, Winooski, VT, USA). Finally, the standard curve was drawn, and the levels of INF-γ, IL-4 and IL-7 in each group were calculated by standard curve.

Flow cytometry

Venous blood (2 mL) from the elbow was extracted from patients before and after experiment (from healthy people during physical examination). The blood was added with anticoagulant and labeled according to the groups. Then the blood was centrifuged at 3,000 rpm for 10 min with hematocyte removed and cell concentration was adjusted at 10⁶ cells/mL. The cells were packed into a flow tube with three replicates. Then the cells were added with fluorescein isothiocyanate (FITC)-anti-human CD4 antibody (0.25 μg, Abcam Inc., Cambridge, MA, USA) and phycoerythrin (PE)-anti-human CD25 antibody (1.0 μg, Abcam, USA) for incubation for 30 min in the dark. After that, the cells were washed with precooling phosphate buffer saline (PBS, 0.02
M, PH 7.2) for 2 times (5 min for each time), centrifuged at 2,000 g for 5 min with supernatant removed, added with erythrocyte lysate (1 mL) and incubated for 30 min at 4 °C in the dark. After washed with 2 mL Fix & Perm buffer solution for 2 times, each time for 5 min, the cells were centrifuged at 2,000 g for 5 min with supernatant removed, added with 1 mL Fix & Perm, and incubated for 60 min at 4 °C in the dark. Then the cells were centrifuged at 2000 g for 5 min with supernatant removed, and sealed with 3% bovine serum albumin (BSA, 1 mL) in the dark for 15 min. The cells were then added with PE-anti-human Cy5Foxp3 antibody (5 μg, Abcam Inc., Cambridge, MA, USA) for incubation for 30 min at 4 °C in the dark. The cells then washed with 2 mL permeabilization buffer solution for 2 times, centrifuged at 2,000 g for 5 min with supernatant removed. The cells were re-suspended with flow cytometry staining. The percentage of cells was determined by FACSRIA (e140916, BD Biosciences, Franklin Lakes, NJ, USA). The above methods were also used to measure the percentage of Th1, Th2 and Th17 to CD4+Treg.

Measurement of lung function
The forced expiratory volume in one second (FEV1)/forced vital capacity (FVC) (%) and FEV1 (%) of patients before and after treatment and of healthy people during physical examination were detected by pulmonary function test apparatus (AS-507. MINATO, Japan).

Effectiveness evaluation
Markedly effectiveness: patients had obviously relieved or disappeared wheezing symptoms; after activity patients had relieved shortness of breath and patients did not administrate oral and intravenous anti-asthmatic drugs or decreased the dosage of oral and intravenous anti-asthmatic drugs. Effectiveness: patients had relieved wheezing symptoms, shortness of breath, decreased chronic attacks and shortened attack time; patients had decreased dosage of the other anti-asthmatic drugs. Ineffective: patients had no changes in symptoms and dosages compared with that before taking Liuweibuqi capsules.

Statistical analysis
All the data were used SPSS 21 statistical software (IBM Corp., Armonk, NY, USA) for processing, measurement data using form of mean standard ± deviation. Comparison between two groups conducted with the t-test, and comparison among groups with single factor variance analysis. Enumeration data is expressed as a percentage or rate, comparison using χ² test. Analysis of variance (ANOVA) was used to compare multi-group variables with homogeneity test of variance. When the results of ANOVA showed a significant difference, q test were used to compare groups in pairs, and when it showed no significant difference, rank test of non-parametric test were used to compare groups in pairs. Logistic analysis was used to assess the related factors for affecting the efficacy of Liuweibuqi capsules in treating patients with COPD. Text standard was α=0.05, P<0.05 means difference is of statistically significance.

Results
Baseline characteristics of subjects in six groups
Baseline characteristics of all enrolled subjects were collected when they were admitted in the First Affiliated Hospital of Anhui Medical University (Table 1).

Stable COPD patients had higher levels of INF-γ, IL-17 and IL-4
Firstly, levels of inflammatory cytokines (INF-γ, IL-17 and IL-4) before treatment in the NSH, SH, NS-COPD and S-COPD groups were measured, and the results are shown in Figure 1. Compared with the NSH group, serum INF-γ and IL-17 levels increased in the SH group (P<0.05), while IL-4 had no difference in the two groups (P>0.05). Compared with the NSH group, IL-4 had higher level (P<0.05), INF-γ and IL-17 had obvious elevated level (P<0.01) in the NS-COPD and S-COPD groups. Compared with the SH group, INF-γ, IL-17 and IL-4 showed higher level in the NS-COPD group (P<0.05); IL-4 was relative high (P<0.05) but INF-γ, IL-17 are extremely higher (P<0.01) in the S-COPD group. Compared with the NS-COPD group, INF-γ, IL-17 and IL-4 showed higher level in the S-COPD group (P<0.05). The above results indicated that stable COPD patients had higher serum INF-γ, IL-17 and IL-4 levels, and smoking may increase INF-γ, IL-17 and IL-4 levels in both COPD patients and healthy people.

Liuweibuqi capsules lowered levels of INF-γ, IL-17 and IL-4 especially for IL-17
Then levels of inflammatory cytokines (INF-γ, IL-17
**Table 1** Baseline characteristics of all enrolled subjects in six groups

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>NSH</th>
<th>SH</th>
<th>NS-COPD</th>
<th>NS-control</th>
<th>S-COPD</th>
<th>S-control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cases</td>
<td>121</td>
<td>124</td>
<td>114</td>
<td>116</td>
<td>110</td>
<td>118</td>
</tr>
<tr>
<td>Mean ages</td>
<td>55.85±10.36</td>
<td>56.51±4.45</td>
<td>56.95±10.31</td>
<td>56.66±9.28</td>
<td>58.82±7.47</td>
<td>59.78±7.36</td>
</tr>
<tr>
<td>Gender</td>
<td>62/59</td>
<td>71/53</td>
<td>62/52</td>
<td>64/52</td>
<td>59/51</td>
<td>63/55</td>
</tr>
<tr>
<td>Duration of cigarette smoking</td>
<td>–</td>
<td>12.21±3.24</td>
<td>–</td>
<td>–</td>
<td>14.43±4.17</td>
<td>15.09±3.42</td>
</tr>
<tr>
<td>Family medical history</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

NS-COPD, non-smoking COPD; NS-control, non-smoking control; S-COPD, smoking COPD; S-control, smoking control; NSH, non-smoking healthy people; SH, smoking healthy people; COPD, chronic obstructive pulmonary disease.

**Figure 1** Serum levels of INF-γ, IL-17 and IL-4 in the NSH, SH, NS-COPD and S-COPD groups before treatment. *, P<0.05 vs. the NSH group; #, P<0.05 vs. the SH group; & P<0.05 vs. the NCOPD group; number of samples NSH =121, SH =124, NSC =114, SC =110. NS-COPD, non-smoking COPD; S-COPD, smoking COPD; NSH, non-smoking healthy people; SH, smoking healthy people; COPD, chronic obstructive pulmonary disease.

Stable COPD patients had higher levels of Th1, Th2, Th17 and Th1/Th2 and lower level of CD4^+^CD25^+^Fox3^+^Tregs

Next, levels of Th1, Th2, Th17 and Th1/Th2 and CD4^+^CD25^+^Fox3^+^Tregs were determined before treatment (Figure 3). Compared with the NSH group, in the SH group, Th1 and Th1/Th2 showed elevated level (P<0.05) and IL-4) before and after treatment in the NS-control, S-control, NS-COPD and S-COPD groups were detected and the findings are shown in Figure 2. After treatment, the levels of INF-γ and IL-4 were decreased in the S-COPD and S-control groups (P<0.05), IL-17 was obviously decreased (P<0.01); the levels of INF-γ, IL-17 and IL-4 was also decreased in the NS-COPD and NS-control groups (P<0.05). These results showed that Liuweibuqi capsules may lower the levels of INF-γ, IL-17 and IL-4, especially for IL-17 level.

**Figure 2** Comparison of serum levels of INF-γ, IL-17 and IL-4 among the NS-COPD, NS-control, S-COPD and S-control groups before and after treatment. (A) Changes of serum levels of INF-γ before and after treatment; (B) changes of serum levels of IL-17 before and after treatment; (C) changes of serum levels of IL-4 before and after treatment. *, P<0.05 vs. the serum factors before treatment; number of samples: NSC =114, NSCP =116, SC =110, SCP =118. NS-COPD, non-smoking COPD; NS-control, non-smoking control; S-COPD, smoking COPD; S-control, smoking control; COPD, chronic obstructive pulmonary disease.
and Th17 showed significantly higher level (P<0.01), Th2 showed no change (P>0.05), and CD4^+CD25^+Fox3^+Tregs showed lower level (P<0.05) in the NS-COPD group compared with the SH group. Th1, Th2, and Th1/Th2 had higher levels (P<0.05) and Th17 showed significantly higher level (P<0.01) in the S-COPD group compared with the SH group. Th1, Th2, Th17 and Th1/Th2 showed significantly higher levels (P<0.01), and CD4^+CD25^+Fox3^+Tregs showed significantly decreased level (P<0.01) in the NS-COPD group compared with the SH group. The above results indicated that Liuweibuqi capsules may significantly decrease the levels of Th1, Th2, Th17 especially for Th17 level and improve the level of CD4^+CD25^+Fox3^+Tregs.

**COPD patients had lower lung function and smoking may lower lung function**

In the following experiment, lung function indicators FEV1 (%) and FEV1/FVC (%) in the NSH, SH, NS-COPD and S-COPD groups were compared (Figure 5). Compared with the NSH group, the SH group had decreased level of FEV1 (%) and FEV1/FVC (%) (P<0.05); the NS-COPD and S-COPD groups had significant decreased FEV1 (%) and FEV1/FVC (%) (P<0.01). Compared with the SH group, the NS-COPD group had decreased FEV1 (%) and FEV1/FVC (%) (P<0.05) while the S-COPD group had significantly decreased FEV1 (%) and FEV1/FVC (%) (P<0.01). Compared with the NS-COPD group, the S-COPD group had the decreased FEV1 (%) and FEV1/FVC (%) (P<0.05). The above results indicated that the FEV1 (%) and FEV1/FVC (%) in stable COPD patients were decreased, and smoking could further weaken their lung function.

**Liuweibuqi capsules helped COPD patients improve their lung function**

Afterwards, lung function in the NS-COPD, NS-control, S-COPD and S-control groups before and after treatment...
Figure 4  Levels of Th1, Th2, Th17, Th1/Th2 and CD4+CD25+Fox3+Tregs in the NS-COPD, NS-control, S-COPD and S-control groups before and after treatment. (A) Changes of the content of Th1 cells in blood before and after treatment; (B) changes of the content of Th17 cells in blood before and after treatment; (C) changes of the content of Th2 cells in blood before and after treatment; (D) changes of the content of CD4+CD25+Fox3+Tregs cells in blood before and after treatment; (E) changes of the content of Th1/Th2 cells in blood before and after treatment. *, P<0.05 vs. T cells before treatment; number of samples: NSC =114, NSCP =116, SC =110, SCP =118. NS-COPD, non-smoking COPD; NS-control, non-smoking control; S-COPD, smoking COPD; S-control, smoking control; COPD, chronic obstructive pulmonary disease; Tregs, regulatory T cells; Th, helper T cells.

Figure 5  FEV1 (%) and FEV1/FVC (%) in the NSH, SH, NS-COPD and S-COPD groups before treatment. *, P<0.05 vs. the NSH group; #, P<0.05 vs. the SH group; &, P<0.05 vs. the NS-COPD group; number of samples: NSH =121, SH =124, NSC =114, SC =110. NS-COPD, non-smoking COPD; S-COPD, smoking COPD; NSH, non-smoking healthy people; SH, smoking healthy people; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

Liuweibuqi capsules weakened the correlation between lung function and T cells and smoking strengthened it

Spearman rank correlation test was then conducted and the results showed (Table 2) that FEV1 (%) had negative correlation with Th1, Th2, Th17 and Th1/Th2 but had positive correlation with CD4+CD25+Fox3+Tregs in stable COPD patients. Smoking increases the correlation of FEV1 (%) with Th1, Th2, Th17, Th1/Th2 and CD4+CD25+Fox3+Tregs in both stable COPD patients and healthy people. Liuweibuqi capsules may decrease the correlation of FEV1 (%) with Th1, Th2, Th17, Th1/Th2 and CD4+CD25+Fox3+Tregs in smoking and non-smoking
Table 2  Correlation of FEV1 and T cells in each group analyzed by Spearman rank correlation test

<table>
<thead>
<tr>
<th>T cells</th>
<th>NSH (r)</th>
<th>SH (r)</th>
<th>NS-COPD (r)</th>
<th>NS-control (r)</th>
<th>S-COPD (r)</th>
<th>S-control (r)</th>
<th>Before treatment</th>
<th>After treatment</th>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Th1</td>
<td>-0.081</td>
<td>-0.130</td>
<td>-0.882*</td>
<td>-0.507*</td>
<td>-0.449*</td>
<td>-0.195*</td>
<td>-0.528*</td>
<td>-0.411*</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>-0.528*</td>
<td>-0.411*</td>
<td>-0.528*</td>
<td>-0.411*</td>
<td>-0.575*</td>
<td>-0.411*</td>
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<tr>
<td>Th2</td>
<td>-0.134</td>
<td>-0.167</td>
<td>-0.636*</td>
<td>-0.089</td>
<td>-0.482*</td>
<td>-0.147*</td>
<td>-0.443*</td>
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</tr>
<tr>
<td></td>
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<td>-0.443*</td>
<td>-0.050</td>
<td>-0.443*</td>
<td>-0.050</td>
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<td>Th17</td>
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<td>-0.090</td>
<td>-0.575*</td>
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<tr>
<td>CD4⁺CD25⁺Fox3⁺Treg</td>
<td>0.064</td>
<td>0.107</td>
<td>0.924*</td>
<td>0.278*</td>
<td>0.472*</td>
<td>0.300*</td>
<td>0.568*</td>
<td>0.288*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.472*</td>
<td>0.300*</td>
<td>0.472*</td>
<td>0.300*</td>
<td>0.568*</td>
<td>0.288*</td>
</tr>
</tbody>
</table>

*, P<0.05, Spearman rank correlation test; number of samples: NSH =121, SH =124, NSC =114, NSCP =116, SC =110, SCP =118. NS-COPD, non-smoking COPD; NS-control, non-smoking control; S-COPD, smoking COPD; S-control, smoking control; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

COPD patients. The above results provided evidence that Liuweibuqi capsules may weaken the correlation between lung function and T cells, while smoking strengthened the correlation between lung function and T cells.

Level of inflammatory cytokines, cells, marker, pulmonary function indicators, smoking and Liuweibuqi capsules were factors affecting efficacy

Finally, 60 days after treatment, the patients are assigned into effectiveness (n=379) and ineffectiveness (n=79) groups. To establish the Logistic regression analysis, the effectiveness of the inflammatory cells as independent variables, level of inflammatory cytokines (INF-γ, IL-17, IL-4), T cells content (Th1, Th17, Th2, CD4⁺CD25⁺Fox3⁺Tregs, Th1/Th2), pulmonary function indicators [FEV1 (%), FEV1/FVC (%)], smoking and Liuweibuqi capsules as the dependent variables. The results showed that the above factors were factors for affecting efficacy (all P<0.05).

Discussion

As arise of population ages and smoking frequencies,
population with COPD worldwide will increase as predicted (24). Previous studies have proved that herbal medicine relieves COPD symptoms effectively, especially for the stable COPD (25,26). In this study, we investigated how Liuweibuqi capsules act on the stable COPD patients with lung Qi deficiency. We found that Liuweibuqi capsules may relieve COPD patients effectively by stimulating the proliferation of CD4$^{+}$CD25$^{+}$Fox3$^{+}$T regs and increasing the ratio of Th17 so as to balance Th1/Th2 ratio and improve the lung function of COPD patients with lung Qi deficiency.

Firstly, we found that the contents of T cells (Th1, Th2 and Th17), and levels of inflammatory cytokines (INF-$\gamma$, IL-4 and IL-17) and inflammatory indicator Th1/Th2 were elevated, but the level of CD4$^{+}$CD25$^{+}$Fox3$^{+}$T regs was decreased of stable COPD patients. Th17 cells, different from Th1 or Th2 cells, is a subset of T cells produced from IL-17, which regarded as a critical regulator in inflammation and autoimmune diseases (27). Several studies also found that the level of Th1, Th2 and Th17 cells increased in COPD patients (28,29). INF-$\gamma$ is produced by Th1 cells, which takes part in the cell-mediated pro-inflammatory activity (30). IL-4 is an immune response-related cytokine produced by Th2 cells, which are involved in inhibiting anti-tumor immunity (31). IL-17 is found secreted from Th17 cells and neutrophils in pulmonary aspergillosis and systemic histoplasmosis (32). An imbalance of Th1/Th2 status may lead to the development of viral or bacterial infections, allergic disease and autoimmune disorders, and COPD is correlated with Th1/Th2 imbalance (28,33). Increased level of IFN-$\gamma$, IL-4, IL-17 and Th1/Th2 were also found in COPD patients in previous studies, which are consistent with the results in this study (34-37). CD4$^{+}$CD25$^{+}$Fox3$^{+}$Tregs play an important role in creating a regulatory environment that promotes infectious tolerance and it was found to improve prognoses of acute exacerbation in COPD patients (38,39).

Secondly, smoking could increase the level of inflammatory cells, cytokines and indicator, decrease the CD4$^{+}$CD25$^{+}$Fox3$^{+}$T regs levels. T regs have the potential to maintain normal lung function in smokers and may stop the progression of immune responses (40), and the level of Tregs in smokers’ lungs without lung dysfunction is more than that in people without a history of smoking (41). CD8$^{+}$ T cells are inversely correlated with pulmonary function, and previous study indicated that CD8$^{+}$Tregs have reverse correlation with smoking (38). Smoking may directly destroy the balance of pro-inflammatory and anti-inflammatory response via inhibiting CD8$^{+}$T cell/CD8$^{+}$Tregs action (42). Cigarette cessation has been proved to be the most successful and cost-effective regimen for suppressing disease progression (43). Lung functions is generally determined by FEV1 (%), and low FEV1 in early adulthood is regarded as the genesis of COPD (44,45). We found that smoking could strengthen the correlation of FEV1 (%) and inflammatory cells, cytokines, indicator and

| Table 3 Logistic analysis for factors affecting efficacy of Liuweibuqi capsules in treating patients with COPD |
|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Variables       | B   | S.E | Wald | df | P   | Exp (B) | 95% CI          |
| INF-$\gamma$    | 0.145 | 0.045 | 10.622 | 1  | 0.001 | 1.156     | 1.060-1.262     |
| IL-17           | 0.064 | 0.029 | 4.919  | 1  | 0.027 | 1.066     | 1.008-1.129     |
| IL-4            | 0.234 | 0.110 | 4.560  | 1  | 0.033 | 1.263     | 1.019-1.566     |
| Th1             | 2.193 | 1.019 | 6.430  | 1  | 0.031 | 8.961     | 1.216-66.048    |
| Th17            | 0.491 | 0.204 | 5.797  | 1  | 0.016 | 1.635     | 1.096-2.439     |
| Th2             | 1.789 | 0.888 | 4.059  | 1  | 0.044 | 5.984     | 1.050-34.111    |
| CD4$^{+}$CD25$^{+}$Fox3$^{+}$Tregs | 0.466 | 0.162 | 8.301  | 1  | 0.004 | 1.593     | 1.161-2.187     |
| FEV1 (%)        | 0.071 | 0.030 | 5.507  | 1  | 0.011 | 1.074     | 1.012-1.140     |
| FEV1/FVC (%)    | 9.368 | 2.606 | 12.918 | 1  | 0.019 | 11,708.259 | 70.769-11,708.259 |
| Smoking         | 2.435 | 0.859 | 8.046  | 1  | <0.001 | 11.421    | 2.123-61.458    |
| Liuweibuqi capsules | 0.145 | 0.045 | 10.622 | 1  | 0.005 | 1.156     | 1.060-1.262     |

COPD, chronic obstructive pulmonary disease; Tregs, regulatory T cells; Th, helper T cells; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.
CD4+CD25+Fox3+Tregs.

Thirdly, Liuweibuqi capsules may effectively decrease the level of inflammatory cells (Th1, Th2 and Th17), cytokines (INF-γ, IL-4 and IL-17) and indicator (Th1/Th2), and increase the level of CD4+CD25+Fox3+Tregs, indicating markedly effectiveness of treatment with Liuweibuqi capsules. Recent evidence has revealed that systemic inflammation plays vital roles in development of COPD and there are a large number of chemokines and cytokines that have been correlated to the pathogenesis of COPD, including INF-γ, IL-1β, IL-13 and IL-6 (46). The immune mechanism in COPD includes innate immune reaction and adaptive immune reaction (47). Remedium cardinales of Liuweibuqi capsules are Renshen and Huangqi (14). Ginsenosides are major active components of Ren Shen, which positively functions in anticancer, antiaging, and antiviral activities, and it also have anti-inflammatory function via inhibiting TNF receptor-associated factor 6 (48,49). A study suggested that ginsenoside Rg3 combined with acupuncture may reduce IFN-γ and it may also counteract the disorder Th1/Th2 profile (50). In TCM, one of the original causes of COPD is lung deficiency, the regimen of which is treated with supplementary Qi (14). Furthermore, Renshen and Huangqi have been proved as the popular restoratives which mainly used for symptoms due to Qi deficiency (51). Therefore, we may conclude that Liuweibuqi capsules can inhibit COPD-induced inflammatory response, providing markedly effectiveness on treatment of COPD.

Above all, we inferred that in the one hand, Liuweibuqi capsules lower the pro-inflammatory reaction by inhibiting differentiation from Th1 to Th17 and decreasing IL-17, INF-γ and IL-4 levels; in the other hand, Liuweibuqi capsules enhance the immune tolerance of the organism by increasing the contents of CD4+CD25+Fox3+Tregs cells. The study is only related to the adaptive immune, the mechanism of the innate immune is needed further exploration, so as to fulfill mechanism of Liuweibuqi capsules and COPD.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: This study was approved by ethics committee of the First Affiliated Hospital of Anhui Medical University (2012-006). All subjects voluntarily participated in the experiment were fully informed of gains and risks and signed informed consents. This experiment was in accordance with the Nuremberg criteria and the Helsinki declaration.

References


