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Ginsenoside regulates Treg/Th17 cell ratio and inhibits inflammation to treat COPD

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Objective: Several studies have suggested an involvement of the immune system in the occurrence and development of chronic obstructive pulmonary disease (COPD), but the mechanism is still unclear. The aim of this study was to explore the mechanism of ginsenoside in inhibiting inflammation by regulating FOXP3 in COPD. *Methods*: Eighty COPD patients were selected and 35 healthy people were enrolled in the study to determine clinical efficacy, observation index, and SGRQ scores. Percentage of Treg and Th17 cells were detected by flow cytometry; HE staining was used to detect the effect of ginsenoside therapy on pathological changes of COPD in mice. Additionally, we transfected FOXP3 inhibitor; RT-PCR and western blot were used to detect the inflammation related genes and proteins. *Results:* The basic information of the patients were comparable. The clinical outcome in the treatment group was better than that in the control group, which indicated that ginsenoside has a certain therapeutic effect on COPD patients. The lung function and 6MWT distance results indicated that ginsenoside could stabilize the clinical symptoms of COPD patients and improve their quality of life. Flow cytometry results showed that ginsenoside can increase Treg expression while reducing Th17 cell expression. RT-PCR and western blot results showed that the expression of TNF-α and IL-17 in the model group was significantly increased after treatment, obviously caused by an increased expression of FOXP3. *Conclusion:* Ginsenoside can inhibit inflammation in COPD by up-regulating FOXP3.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a group of lung diseases characterized by airflow limitation. Airflow limitation is not completely reversible and shows a progressive development (Brandsma et al., 2017; Hillas et al. 2016). Chronic inflammation of the airways, lung parenchyma, and pulmonary blood vessels are typically involved in COPD (Barnes 2017). Inflammatory cells such as neutrophils, macrophages, and T lymphocytes all contribute to the pathogenesis of COPD. The activation and aggregation of neutrophils are an important step in the inflammation process of COPD (Scoditti et al. 2019; Szucs et al. 2019). The release of neutrophil elastase, neutrophil cathepsin G, neutrophil protease 3 and matrix metalloproteinase causes chronic mucus hypersecretion and destroys the lung parenchyma. Therefore, in COPD treatment, it is an effective way to suppress the occurrence and development of inflammation.

Both Treg and Th17 are derived from the common initial $CD4^+$T cells. Th17 cells have the effect of promoting inflammation, while Treg cells are antagonizing the inflammatory response (Mathur et al. 2019; Tahmasebinia and Pourgholaminejad 2017). The dynamic balance of the two in terms of number and role is to maintain the body's immune normal dynamics. Balance is of great significance and is an important prerequisite for the body to maintain immune homeostasis (Faschinget al. 2017). Th17/Treg imbalance plays an important role in lung immune dysfunction. There are enough Tregs in the lungs of smokers with normal lung function, which can inhibit the destruction of $CD8^+$T cells. Even smoking does not cause the disease, indicating that the normality of regulatory T cells is one of the keys to COPD (Zhou et al. 2019). Treg expression is elevated in COPD tissues, and its role may be to control local inflammation. Studies have shown that the number of Treg cells and FOXP3 mRNA are reduced in patients with COPD (Ito et al. 2019), and FOXP3 expression is increased in the airway of smokers with normal lung function and COPD patients.

In our study, we planned to use patients with stable chronic obstructive pulmonary disease, and randomly assign COPD patients with signed informed consent to a treatment group and a control group, with 40 cases in each group. The control group was recommended to use combined inhalation of tiotropium bromide, salmeterol ticasone powder inhalation and salmeterol inhalation. The treatment group was given ginsenoside treatment and given the same treatment after 3 mouths. We observed patients' FOXP3 mRNA, IL-17, lung function, quality of life (CAT), 6-minute walking distance and other indicators. The purpose of this study was to observe the clinical effect of ginsenosides on COPD patients in the stable phase, and to explore the role of ginsenosides in regulating Treg cells and Th17 cells by inhibiting inflammation to improve the immune function of COPD patients and reduce the acute onset of disease.

2. Investigations and results

2.1. Basic information of patients

The 80 patients with COPD in the study were divided into two groups according to random number table method; control group (n=40) was composed of 25 males and 15 females (Table 1). The average age was 40.94±3.13 years; the average course of disease was 7.59±2.45 years. In the treatment group, there were 29 males and 11 females; the average age was 41.08±3.3 years; the average course of disease was 7.56±2.78 years. No obvious difference was observed between the groups.

Table 1: Basic information about the patients

Groups	Blank	Treatment	Control
Males/females	25/10	29/11	25/15
Average age	41.78±3.26	41.08±3.37	40.94±3.13
Average course	-	7.56±2.78	7.59±2.45

2.2. Clinical efficacy

In order to verify the therapeutic effect of ginsenosides on patients with COPD, we judged the therapeutic effect by the clinical symptoms and signs of the patients. The results show that there was a statistically significant difference in the comparison of the total effective rate between the two groups before and after treatment (P<0.05). The clinical outcome in the treatment group was better than that in the control group (Table 2). This proves that ginsenoside has a certain therapeutic effect on COPD patients.

Table 2: Comparison of clinical efficacy between the two groups after treatment

Groups	Clinical control	Marked effect	Effective	Invalid	Total efficiency (%)
Treatment (n=40)	33 (82.5)	3 (7.5)	3 (7.5)	1 (2.5)	39(97.5)
Control (n=40)	25 (62.5)	4 (0.1)	4 (0.1)	7(17.5)	33(82.5)

2.3. Comparison of lung function and 6MWT distance before and after treatment in two groups

In order to verify the effect of ginsenosides on lung function in COPD patients, the MASTERSCREEN spirometer was used to measure the forced expiratory volume (FEV1) and forced vital capacity (FVC) at the first second before and after treatment, and the FEV1/FVC value was calculated. It can be seen from Table 3, that FEV1, FVC, FEV1/FVC and 6MWT were increased after treatment compared to control, and the differences were statistically significant (P<0.05). The improvement of lung function indexes and the distance of 6MWT were significantly better than those of the control group (P<0.05). The above results indicate that ginsenoside treatment can help relieve the clinical symptoms of COPD patients in the stable phase and improve lung function.

Table 3: Comparison of observation index between two groups of patients before and after treatment (x±s)

Groups	FEV_1(L)	FVC(L)	FEV_1/ FVC(%)	6MWT(m)
Treatment (n=40)				
Before treatment	0.85±0.62	1.79±0.82	48.48±10.12	223.68±82.64
After treatment	1.36±0.45$^{*\#}$	2.42±0.87$^{*\#}$	57.56±8.16$^{*\#}$	328.64±86.73$^{*\#}$
Control (n=40)				
Before treatment	0.81±0.57	1.80±0.79	48.59±9.87	230.32±102.53
After treatment	1.01±0.46*	2.02±0.80*	53.63±9.94*	267.29±116.18*

Compared with before treatment, $^{*}P$<0.05; compared with control group, $^{\#}P$<0.05

2.4. Comparison of SGRQ scores between 2 groups before and after treatment

Improving the quality of life is the ultimate goal of the treatment method. SGRQ is the authoritative scale for evaluating the quality of life of COPD patients. Its reliability and validity have been unanimously recognized by domestic and foreign scholars. From Table 4, it can be seen that the clinical symptoms, activity ability, psychological impact score and total score were lower than before treatment in this group, and the treatment group was lower than the control group, the difference was statistically significant (P<0.05). In this study, the treatment group SGRQ scale scores in all dimensions were significantly lower than the control group (P<0.05), indicating that ginsenoside treatment can help improve the quality of life of patients.

Table 4: Comparison of SGRQ score between the two groups before and after treatment (x±s)

Groups	Clinical symptoms	Activity ability	Psychological influence	Total score
Treatment (n=40)				
Before treatment	22.18±1.45	23.74±4.42	9.65±1.17	55.57±10.04
After treatment	11.25±3.57$^{*\#}$	12.65±3.31$^{*\#}$	6.71±1.61$^{*\#}$	30.61±8.49$^{*\#}$
Control (n=40)				
Before treatment	22.87±4.76	23.98±5.62	9.29±1.62	56.14±12.00
After treatment	13.87±4.86*	16.63±5.90*	7.76±1.80*	38.26±12.56*

Compared with before treatment, $^{*}P$<0.05; compared with control group, $^{\#}P$<0.05

2.5. Ginsenosides treat COPD by increasing the ratio of Treg to Th17

Compared with the model group, the proportion of Treg in the control group and treatment group after treatment was significantly increased (P<0.01); compared with the model group, the proportion of Th17 cells in the control group and treatment group after treatment was significantly reduced (P<0.01); and the ratio of Treg to Th17 increased significantly (P<0.05, P<0.01) (Table 5). Ginsenoside can increase Treg while reducing Th17 cell expression, and it can treat COPD by increasing the ratio of Treg to Th17.

Table 5: Percentage of Treg and Th17 cells in each group(x±s)

Groups	Treg cells proportion/%	Th17 cells proportion/%	Treg/Th17 ratio
Treatment (n=40)			
Before treatment	1.881±0.695**	2.375±0.362**	0.803±0.307*
After treatment	3.413±1.618$^{\#\#}$	1.075±0.385$^{\#\#}$	3.536±1.837$^{\#\#}$
Control (n=40)			
Before treatment	1.874±0.597**	2.298±0.402**	0.812±0.312*
After treatment	2.963±1.010$^{\#}$	1.213±0.629$^{\#}$	2.945±1.471$^{\#}$
Blank (n=35)	3.488±1.106	1.038±0.478	3.846±1.937

Compared with before treatment, $^{*}P$<0.05, $^{**}P$<0.01; compared with control group, $^{\#}P$<0.05, $^{\#\#}P$<0.01

2.6. Ginsenoside can alleviate the pathological damage of lung in COPD mice

In this study, the 28-day smoking method was used to construct the COPD model, and the lung tissues of mice in the control group, model group and ginsenoside group were HE stained. As shown in Fig. 1, the alveolar structure of the lung tissue of the control group of mice was clear, the alveolar wall was intact, there was no breakage, fusion, and only a few infiltrating inflammatory cells were seen. A large number of inflammatory cells infiltrated the lung tissue of the COPD smoked model group, the structure of the alveolar wall was destroyed and broken, and the adjacent alveolar cavity merged and expanded to form a typical emphysema. This smoked mouse model has the typical pathological features

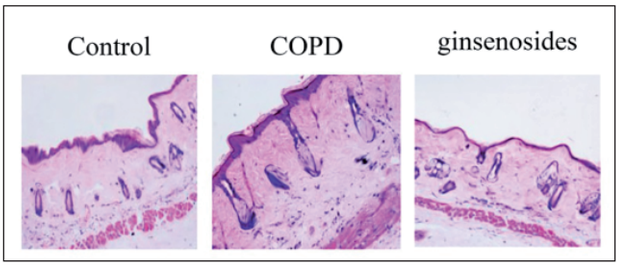

Fig. 1: Effect of ginsenoside therapy on pathological changes in COPD mice.

of COPD, including pulmonary parenchymal inflammation and emphysema. The lung tissue damage of mice was significantly improved after the second administration of ginsenosides, what indicated that ginsenosides have a therapeutic effect on COPD.

2.7. Ginsenoside can upregulate FOXP3 and inhibit the expression of inflammatory factors and related proteins in clinical samples

In order to verify the regulation of ginsenosides on inflammatory factors and related proteins, we detected the expression of related genes and proteins by RT-PCR and Western blot, respectively. Compared with the blank group, the expression of TNF-α and IL-17 mRNA in the control group were increased, and the expression of FOXP3 mRNA was decreased. Compared with the control group, ginsenoside treatment can significantly inhibit the expression of TNF-α and IL-17, and at the same time, it can upregulate the expression of FOXP3 (Fig. 2A). Compared with the blank group, the expression of TNF-α and IL-17 protein was increased and the expression of FOXP3 protein was decreased in the control group and the treatment group. Compared with the control group, ginsenoside treatment can significantly inhibit TNF-α. However, whether ginsenosides inhibit inflammation regulated by FOXP3 needs further study (Fig. 2B).

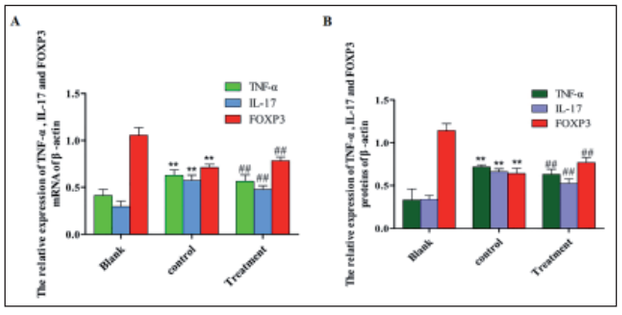

Fig. 2: Ginsenoside up-regulates FOXP3 and inhibits the expression of inflammatory factors. (A, B) mRNAs and proteins expression level was detected by density method semi-quantitatively, and β-actin was used as internal reference; ** P<0.01 vs. blank; $^{\#\#}$ P<0.01 vs. control.

2.8. Ginsenoside inhibits inflammation by upregulating FOXP3

Through the above experiments, we have verified that ginsenoside can inhibit inflammation and upregulate FOXP3, but the specific anti-inflammatory mechanism is still unknown, so this part of the experiment introduced FOXP3 inhibitors to explore the specific mechanism of ginsenoside treatment of COPD by inhibiting inflammation.

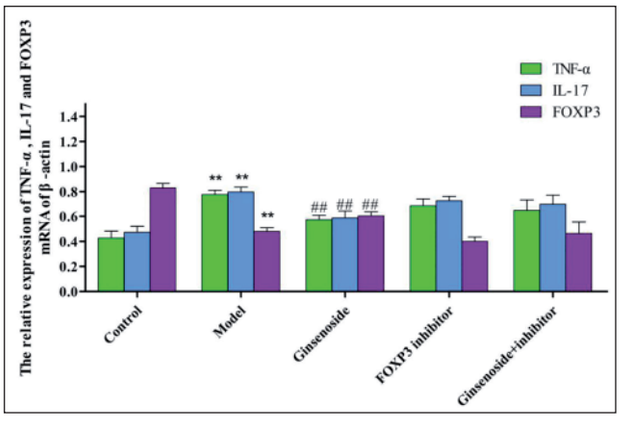

Fig. 3: RT-PCR detection of target gene expression on FOXP3. RT-PCR method was used to detect the expression of TNF-α, IL-17, foxp3 mRNA; ** P <0.01 vs. control; $^{\#\#}$ P <0.01 vs. model.

We divided the mice into control group, model group, ginsenoside group, FOXP3 inhibitor group, ginsenoside+inhibitor group, and detected the expression of inflammation-related factors and proteins by RT-PCR and Western blot. control group. Compared with the control group, the expression of TNF-a and IL-17 mRNA in the model group was significantly increased, and the expression of FOXP3 was significantly reduced after treatment. Next, we used FOXP3 inhibitors to reduce the expression of FOXP3. Low expression will lead to high expression of TNF-α and IL-17, and prevent the therapeutic effect of ginsenoside on COPD (Fig. 3).

Western blot results showed that (Fig. 4) the expression of TNF-α and IL-17 in the model group was significantly increased, and the expression of ginsenoside was significantly reduced after treatment compared with the control group. At the same time, ginsenoside treatment can increase the expression of FOXP3. A FOXP3 inhibitor completely inhibits the expression of FOXP3, which leads to high expression of TNF-α and IL-17, and prevents the therapeutic effect of ginsenoside on COPD. The above results indicate that ginsenoside can inhibit inflammation by upregulating FOXP3.

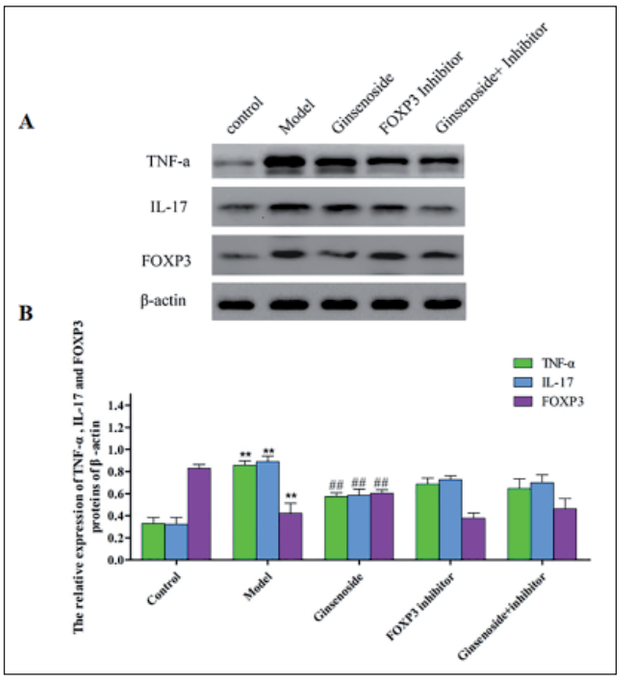

Fig. 4: Western Blot detection of target protein expression on FOXP3. A: Western Blot method strip chart. B: Western Blot method to detect the expression of TNF-α, IL-17, FOXP3 proteins; ** P <0.01 vs. control; $^{\#\#}$ P <0.01 vs. model.

3. Discussion

In recent years, with regard to the study of the pathogenesis of COPD, the immunological mechanism has become a hot spot at home and abroad. Smoking is a clear and most important environmental risk factor affecting the occurrence and development of COPD (Mouronte-Roibas et al. 2019). Some studies have found that cigarette smoke causes the body's immune function to be hyperactive. Long-term smoke stimulation will damage airway epithelial cells and release a large amount of cellular mediators. COPD patients are in a state of hypoxia for a long time, affecting the normal metabolism of cells and inhibiting the normal function of immune cells. Infiltration of inflammatory cells caused by smoking (such as macrophages, neutrophils, DC cells, T lymphocytes and B lymphocytes) is considered to be the most important mechanism leading to COPD (Balbi et al. 2019). At present, some studies tend to involve immunological mechanisms in the occurrence and development of COPD, and even some domestic and foreign experts have suggested that COPD is an autoimmune disease. Studies have reported that smoking can induce high

expression of Treg and Th17 cells in the peripheral blood of smokers, thereby inducing COPD or accelerating its progress.

Regulatory cells (Treg) are a subset of T cells that control autoimmunity in the body. Treg cells have a negative immune regulation function and participate in the development of autoimmune diseases (Sun et al. 2019). Fork-head transcription factor 3 (Foxp3) is a specific transcriptional regulator of Treg. It regulates the transformation of naive CD4 cells into a subset of Treg cells. If the Foxp3 gene is mutated, it will affect the development of Treg cells (Wang et al. 2019). Th17 cells are a subset of T cells with immunoregulatory functions. They are differentiated from natural T cell precursors and have independent differentiation and regulation mechanisms. They specifically produce interleukin-17 (IL-17) effector, IL-17 exerts its biological role by recruiting and activating neutrophils, and plays an important role in autoimmune diseases and allergen-specific reactions. Both Treg and Th17 are derived from the common initial $CD4^{+}$T cells. Th17 cells have the role of promoting inflammation, while Treg cells have the role of antagonizing the inflammatory response (Fitch et al. 2009). The dynamic balance of the two in terms of number and role is to maintain the body's immune normal dynamics Balance is of great significance and is an important prerequisite for the body to maintain immune homeostasis.

We analyzed Th17/Treg immune balance by analyzing the changes of the above immune cells and cytokines in peripheral blood before and after ginsenoside treatment. The percentage of Th17/Treg and the content of IL-17 and TNF-a in peripheral blood of the observation group after treatment were lower than those of the control group. This result showed that conventional combined inhalation of tiotropium bromide, salmeterol ticasone powder inhalation and salmeterol inhalation treatment can simultaneously inhibit the function of Th17 and Treg and the inhibition of Th17 is more significant, shifting the balance of Th17/Treg to Treg. Adding ginsenosides on the basis of normal treatment can further correct improve the function and balance of Th17 and Treg. Next, we detected the expression of inflammation-related factors and proteins by RT-PCR and Western blot. The results showed that compared with the control group, the expression of TNF-α and IL-17 in the model group was significantly increased, and the expression of FOXP3 was significantly reduced after treatment. At the same time, ginsenoside treatment can increase the expression of FOXP3, which leads to high expression of TNF-α and IL-17, and prevents the therapeutic effect of ginsenoside on COPD. The above results indicated that ginsenoside can inhibit inflammation in COPD by upregulating FOXP3.

In summary, ginsenoside can effectively support treatment of patients with stable COPD, relieving clinical symptoms, improving lung function, improving quality of life. It is contributing to significant improvements in various inflammatory response factors in peripheral blood of COPD patients, most probably upregulating FOXP3.

4. Experimental

4.1. Patients and groups

This experiment was carried out in Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine from December 2018 to December 2019. 80 chronic obstructive pulmonary disease (COPD) patients were selected and 35 healthy people were admitted to our study. In the blank group, there were 25 males and 10 females, and the average age was 41.78±3.26 years. Patients with stable COPD were randomly divided into a treatment group and a control group, with 40 cases in each group. The control group received tiotropium bromide [Boehringer Ingelheim Pharma GmbH & Co.KG (Germany), approval number H20140933], 18 μg·once a day. On this basis, patients in the observation group were given salmeterol ticasone powder inhalation (Glaxo Wellcome Production, National Medicine Zhunzi H20150324) inhalation treatment, twice a day, according to the COPD Global Initiative guidelines plus salmeterol/ticasone inhalation. The treatment group was given ginsenoside treatment on the basis of combination therapy for three months. In the treatment group, there were 29 males and 11 females, and the average age was 41.08±3.37 years and the average course of disease was 7.56±2.78 years. In the control group, there were 25 males and 15 females, and the average age was 40.94±3.13 years and the average course of disease was 7.59±2.45 years. The general data of the two groups did not show statistically significant differences (P>0.05), which indicated that the three groups were comparable. This study was approved by the ethics committee of Jiangsu Province Hospital of Chinese Medicine, Affiliated Hospital of Nanjing University of Chinese Medicine. From all subjects, venous blood was collected before treatment and set aside.

4.2. Diagnostic criteria

We took the Guidelines for Primary Diagnosis and Treatment of Chronic Obstructive Pulmonary Disease (2018) as a reference. If the patient is treated with bronchodilators, FEV1 estimates < 80%, FEV1/FVC < 70%, and clinical symptoms such as cough, shortness of breath, and wheezing gradually increase, and the sputum mucus produced by the patient becomes purulent with fever. Level I: FEV1 estimated value exceeds 80%; Level II: FEV1 estimated value is below 80% and above 50%; Level III: FEV1 estimated value is below 50% and above 30%; Level IV: FEV1 is estimated at 50% below or below 30%, with respiratory failure. Syndrome types refer to Diagnostic Criteria of Traditional Chinese Medicine Syndrome of Chronic Obstructive Pulmonary Disease (2011 Edition). Phlegm, stasis and lungs: The patient coughs with asthma, wheezing, chest tightness and shortness of breath, sputum between the larynx, yellow sputum, multiple spitting, and dark purple face. Lips and nails are bluish-purple, the tongue is dark purple or there is ecchymosis under the tongue, and the veins of the lower tongue are tortuous (Sorinoet al. 2017).

Inclusion criteria: (1) meet the diagnostic criteria; (2) age 45-70 years; (3) lung function grade □-□; (4) Patient informed consent (Bohannon and Crouch 2017).

Exclusion criteria: (1) Malignant tumors; (2) Patients with severe liver and kidney function disorders, unable to carry out drug metabolism normally; (3) Allergic to the drugs used in this study (Murphy et al. 2017).

4.3. Efficacy standard

For clinical efficacy, we referred to the Clinical Guiding Principles of New Chinese Medicines (Trial). Clinical control: clinical symptoms and signs disappeared or basically disappeared, syndrome scores decreased by ≥ 95%; significant effect: clinical symptoms and signs improved significantly, syndrome scores decreased by ≥ 70%; effective: clinical symptoms and signs improved, syndrome points decrease ≥ 30%; Invalid: No obvious improvement or even worsening of clinical symptoms and signs, reduction of syndrome score < 30%. Clinical symptoms and signs: We compared the changes of clinical main symptoms and signs before and after treatment in the two groups, mainly with respect to cough, expectoration, wheezing, wheezing, and scores 0, 2, 4, and 6 according to rising severity (Daubin et al. 2018).

4.4. Observation index

Pulmonary function: The forced expiratory volume (FEV1) and forced vital capacity (FVC) of the first second were measured with the MASTERSCREEN spirometer of the German JAECER company before and after treatment, and the FEV1/FVC value was calculated.

Six min walking test (6MWT) distance: according to a literature method (Reychler et al. 2018).

Quality of life: St George's respiratory questionnaire (SGRQ)(Calzetta et al. 2017) was used for evaluation, including three major areas of clinical symptoms, mobility, and psychological impact. A higher score indicates a more serious impact on quality of life.

4.5. Flow cytometry detects the ratio of Treg and Th17 cells in the sample

We adjusted the blood cell concentration (1×10^{6} cells/mL), stimulated the cells with interleukin stimulant (only when Th17 was detected), blocked with FcR blocking agent, stained the cell surface (CD4 and CD25), fixed, broke the membrane. After cleaning; intracellular staining (Foxp3 and Foxp3 isotype control, IL-17 and IL-17 isotype control), membrane rupture, washing, up-flow cytometry detection, data were analyzed (Zheng et al. 2018).

4.6. Animals and groups

We used 50 C57BL/6 mice with SPF grade, 8 weeks old, with average body weight of 22.7±2.4 g. The culture room temperature was 22–25 °C, humidity 50%~70%, free feeding, free drinking water. 40 mice were randomly separated into 4 groups, model (smoking method), ginsenoside (ginsenoside processed), inhibitor (transfect FOXP3 inhibitor) and ginsenoside+inhibitor group. Ten C57BL/6 mice were used as control group (abbreviated as normal group). The model group and the normal group were raised normally every day.

4.7. HE staining

After anesthesia, the mice were injected with 10 mL each of 1×PBS and 4% paraformaldehyde into the heart. The lung was taken out and placed in a 30% sucrose solution overnight. After the tissue was sunk to the bottom, it was embedded at -80 °C. Continuous coronal sectioning was performed by a freezing slicer. Sections were fixed with 70%, 80% and 90% alcohol for 5 s, hematoxylin staining was used for 15 s, 1% hydrochloric acid alcohol for 5 s, 0.5% ammonia for 10 s, and eosin staining. After staining for 5 s, each step needs to be washed with 1×PBS, and then dehydrated with 70%, 80%, 90% alcohol for 5 s, and sealed with neutral gum. The final section was observed under a microscope (Zou et al. 2018).

4.8. RT-PCR analysis

The total RNA was isolated from mice and blood in each group using TRizol reagent (Invitrogen, Carlsbad, CA, USA) and converted cDNA by OneScript Reverse Transcriptase OneScript cDNA Synthesis Kit (Abm). 25μL Dream Taq PCR Master Mix (Abm), 1.5 μL forward and reverse primer (Ribobio, Guangzhou, China), 2 μL cDNA and 20 μL water nuclease free in amplification reaction mixture (50 μL), and the

PCR condition were as follows: 95 °C (2 min, a cycle), 95 °C (30 s), 58 °C (30 s), 72 °C (1 min), 35 cycles in total (Lu et al. 2017), finally, 72 °C (10 min, a cycle). Glyceraldehyde 3-phosphate dehydrogenase (β-actin) served as the control of APP, the primer sequences were as follows: β-actin, F: 5'-TCACCATCT TCCAGGAG-CGAG-3', R: 5'-TGTCGCTGTTGAAGTCAGAG-3'; Foxp3, F: 5'-CATCCGCCA-CAACCTGAGTCTG-3', R: 5'-CCTGTTCGTCCATCCTCCTTTCCT-3'; IL-17, F: 5'-GCCGAGGCCAATAACTTTCT-3', R: 5'-GAGTCCAGGGTGAAGTGGAA3'; TNF-α, F: 5'-GGAAAGCATGATCCGAGATG-3, R: 5'-CGAGCAGGAATGAGAA-GAGG-3'.

4.9. Western blot

Western blot was used to detect the expression of the relative protein. After extracting the total protein of the submandibular gland tissue, 10–25 μL were added to each well for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (60V, when the bromophenol blue distance was about 1 cm from the bottom of the separation gel electrophoresis was stopped). 120 mA (2–3 h) wet to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA), 5% skim milk powder sealed at room temperature for 1 h, TBST rinse 3 times. The membrane was incubated with anti-foxp3, TNF-α, IL-17 and anti-β-actin (Abcam, Cambridge, MA, USA) at 4 °C overnight, TBST rinse 3 times, secondary anti-light room temperature incubate for 2 h, rinse TBST 3 times in the dark, and Bands were visualized with electrochemiluminescence (ECL) (Pierce, Rockford, IL, USA)(Bewley et al. 2017).

4.10. Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 22.0 software (IBM, Armonk, NY, USA). Data were represented as mean±SD. Differences between two groups were analyzed by using the Student's t-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post Hoc Test (Least Significant Difference). $P<0.05$, $P<0.01$ were indicated the significant difference.

Authors' contributions: SUO-FANG SHI conceived and designed the experiments; WEN LV and HONG-JUAN WU performed the experiments; YAN-QIU XU analyzed the data and wrote the paper.

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