Dendrobium nobile protects against ovalbumin-induced allergic rhinitis by regulating intestinal flora and suppressing lung inflammation

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[ABSTRACT] Antibiotic exposure-induced dysbiosis of the intestinal flora increases the risk of developing allergic rhinitis. Hence, regulating the balance of intestinal flora may be useful for preventing and treating allergic rhinitis. However, the underlying mechanism is unclear. Dendrobium nobile (Shihu) exhibits anti-inflammatory and immune activities. Hence, in this study, we investigated the mechanism via which Shihu may improve allergic rhinitis. Mouse models of allergic rhinitis with intestinal flora dysbiosis (Model-D, antibiotics induce intestinal flora dysbiosis with ovalbumin-induced allergy) and normal intestinal flora with allergic rhinitis (Model-N, ovalbumin-induced allergy) were established. The effect of Shihu on intestinal flora and inflammation caused during allergic rhinitis were analyzed. Allergic symptoms, infiltration of hematoxylin and eosin in the lungs and nose, and the release of various factors [interleukin (IL)-2, IL-4, IFN-γ, IL-6, IL-10, and IL-17] in the lungs were evaluated. The results indicate that intestinal flora dysbiosis exacerbated lung and nose inflammation in allergic rhinitis. However, treatment with the Shihu extract effectively reversed these symptoms. Besides, the Shihu extract inhibited the PI3K/AKT/mTOR pathway and increased the level of Forkhead box protein in the lungs. Additionally, the Shihu extract reversed intestinal flora dysbiosis at the phylum and genus levels and improved regulator T cell differentiation. Furthermore, in the Model-D group, the Shihu extract inhibited the decrease in the diversity and abundance of the intestinal flora. Screening was performed to determine which intestinal flora was positively correlated with Treg differentiation using Spearman’s correlation analysis. In conclusion, we showed that Shihu extract restored the balance in intestinal flora and ameliorated inflammation in the lungs of allergic rhinitis mice and predicted a therapeutic new approach using Traditional Chinese Medicine to improve allergic rhinitis.

[KEY WORDS] Dendrobium nobile (Shihu); Allergic rhinitis; Intestinal flora; Regulator T cell; Inflammation

Introduction

Allergic rhinitis (AR) is caused by chronic non-infectious inflammation of the upper respiratory tract and is char-

actertized by nasal congestion, rhinorrhea, sneezing, and nasal itching. In addition, AR induces systemic allergic reactions and is closely related to allergic asthma. Allergen exposure induces type 2 helper T cell (Th2) polarization, which triggers IgE-mediated inflammation, subsequently activating mast cells, eosinophils, and lymphocytes and the release of inflammatory factors. Allergen-specific immunotherapy is the primary treatment for AR, which induces tolerance to foreign allergens.

The hygiene hypothesis, developed in the 1980s, suggests that microbial exposure decreases the type 2 immune response. This has led scientists to speculate that the establishment of the intestinal flora during infancy is key for im-
mune tolerance to environmental antigens \[4\], which was confirmed in subsequent research. Bisgaard found that the intestinal flora diversity at 1 and 12 months after birth was inversely associated with the risk of AR \[5\]. Early antibiotic exposure has deleterious effects on the infant microbiome, which can be associated with a wide range of diseases, including asthma, allergies, obesity, and inflammatory bowel disease \[5\]. The overuse of antibiotics is the main reason for reducing intestinal flora diversity, which is remarkably associated with the development of asthma and AR \[9\]. However, the underlying mechanism is not well understood.

Intestinal flora is an essential part of the intestinal mucosal immune system, and intestinal immune tolerance is induced by naïve T lymphocyte differentiation into Tregs \[10\]. Intestinal flora directly or indirectly modulates the differentiation and function of Tregs, which may be associated with the development of allergic, inflammatory, and autoimmune diseases \[11\]. Tregs maintain immune homeostasis in the body by inhibiting the function and activation of other leukocytes (Th2 and type 17 helper T cells) \[12\]. Forkhead box protein P3 (FOXP3) is a critical transcription factor that controls Treg development and function. Recent studies showed that Tregs with defective Foxp3 cannot suppress immune responses to foreign antigens and self-antigens \[13\]. The activation of the PI3K/AKT/mTOR pathway prevents the transfer of FOXO to the nucleus to initiate Foxp3 expression, thereby inhibiting Treg differentiation \[14\]. The PI3K/AKT/mTOR pathway has been demonstrated to play an essential role in inflammatory processes and the immune response. The suppression of mTOR expression can induce the differentiation of Tregs and inhibit the proliferation of Th2 cells, thereby alleviating the pathogenesis of allergic asthma \[15\]. Therefore, the PI3K/AKT/mTOR pathway plays an integral role in inflammation and the immune response in the lungs in allergic diseases.

*Dendrobium nobile* Lindl. (Shihu) is a traditional and valuable plant in China belonging to the orchidaceae family with medicinal and food properties \[16\]. Shihu was first recorded in Shennong Bencao Jing (Shennong’s Classic of Materia Medica) and has been shown to exert the following functions: “Sheng Jin Zhi Ke” creates clear fluid and has an anti-tussive function, “Run Fei Yi Wei” moistens the lungs and benefits the stomach, “Hou Chang Wei” has positive effects on gastrointestinal function, and “Ming mu qiang shen ” brightens the eyes and strengthens the body \[17, 18\]. Recent studies showed that Shihu possesses pharmacological properties, such as anti-inflammatory, antioxidant, and immunomodulatory properties \[19, 20, 21\]. Polysaccharide, one of the main active ingredients of Shihu \[22\], regulates immunity and intestinal flora. As mentioned above, intestinal flora dysbiosis plays a vital role in AR development, and Shihu shows anti-inflammatory, immunomodulatory, and potentially flora-regulating effects. However, whether Shihu can improve AR is unclear. Because of its efficacy as a traditional medicine for treating various health conditions and its pharmacological properties, we established a mouse model of intestinal flora dysbiosis with AR by administering antibiotics during early growth in this study. We investigated whether Shihu can improve lung inflammation in mice with AR by antagonizing antibiotic-induced disorder of the intestinal flora. Our research can help to explain the underlying theories of traditional Chinese medicine (TCM) using modern pharmacological methods and reveal a potential therapeutic method for AR.

**Materials and Methods**

**Preparation of shihu extract**

Shihu was obtained from Guangzhou Lingnan Traditional Chinese Medicine Beverage Co. (lot: 190601, Guangzhou, China). The raw materials were identified by the deputy director of TCM, LI Yi-Sheng (Pharmacy of Traditional Chinese Medicine, Laboratory for Research and Development of new TCM, Longgang ENT Hospital), and the experimental specimens were sent to the Laboratory for Research and Development of new TCM of Longgang ENT Hospital. Two hundred grams Shihu was soaked in 2000 mL water for 30 min, and then heated and refluxed for 1 h; after filtration, the mixture was heated to reflux the residue with 1600 mL water for 1 h. The two filtrates were combined under reduced pressure and dried in vacuum to obtain the extracts (yield of the water extract of Shihu was 16.8%). The total polysaccharide content of the Shihu extract was determined using the anthrone-sulfuric acid method. The dendrophenol content in the Shihu extract was determined using high-performance liquid chromatography. The detailed experimental methods are provided in the Supplementary Information. The total polysaccharide and dendrophenol contents in the *Dendrobium* extract were 198.9 mg g\(^{-1}\) and 189 μg g\(^{-1}\), respectively.

**Animals and experimental design**

The animal experimental procedures were reviewed and approved by the Ethics Committee of ZSSOM on Laboratory Animal Care (No. 2020-0256).

Forty-two healthy three-week-old female BALB/c mice were obtained from the center of Guangdong’s laboratory animal science (quality exequatur number, SCXK 20180002). The animals were housed under standard temperature (20–25 °C), humidity (50%–60%), and light conditions (12-h light/dark cycle) with free access to food and pure water. The animals were randomly divided into seven groups: control, model group (flora normal, Model-N), model group (flora dysbiosis, Model-D), positive control group treated with loratadine (flora dysbiosis, loratadine; Bayer Pharmaceuticals, Shanghai, China), Shihu low-dose group (flora dysbiosis, 20 mg kg\(^{-1}\)d\(^{-1}\)), Shihu middle-dose group (flora dysbiosis, 40 mg kg\(^{-1}\)d\(^{-1}\)), and Shihu high-dose group (flora dysbiosis, 80 mg kg\(^{-1}\)d\(^{-1}\)).

To construct an AR mouse model (Fig. 1), after one week of adaptive rearing, the control and Model-N groups were administered 0.2 mL of 0.5% sodium carboxymethyl-cellulose, whereas the other groups were administered an an-
tibiotic cocktail [55.2 mg kg\(^{-1}\) d\(^{-1}\) cefixime dispersible tablets, Guangzhou Baiyunshan Pharmaceutical, Guangzhou, China; 460 mg kg\(^{-1}\) d\(^{-1}\) metronidazole tablets, Guangdong Huanan Pharmaceutical Group Co., Ltd., Guangzhou, China] for two weeks. During the following two weeks (4–5 weeks), except the control group, the other groups were intraperitoneally injected three times with 100 μg ovalbumin (OVA) and 1 mg alun (Fig. 1) to induce sensitization. At 6–9 weeks, except the control group, the other groups were challenged with 20 μL of 15 mg mL\(^{-1}\) OVA solution via each nostril. After 4–9 weeks, 200 μL of loratadine (3 mg kg\(^{-1}\) d\(^{-1}\)) and the Shi-hu extract (low-dose group: 20 mg kg\(^{-1}\) d\(^{-1}\), middle-dose group: 40 mg kg\(^{-1}\) d\(^{-1}\), and high-dose group: 80 mg kg\(^{-1}\) d\(^{-1}\)) were administered via oral gavage. The control group was not sensitized, challenged, or treated with antibiotics (only treated with 0.5% sodium carboxymethylcellulose). The mice were sacrificed 24 h after the last challenge, and the blood and nasal samples and the lung and spleen tissues were collected for further analyses.

Evaluation of symptoms of allergic rhinitis

Nasal rubbing and sneezing were evaluated after the last nasal challenge by counting the frequency of these events in 15 min. These frequencies were counted by blinded reviewers who were unaware of the grouping.

Collection of serum and bronchoalveolar lavage fluid

The mice were anesthetized with diethyl ether after the last challenge; blood was harvested from the orbital venous varix and centrifuged (3000 r min\(^{-1}\), 10 min, 4 °C) to collect the serum, which was stored at −80 °C. The neck was dissected, and the trachea exposed for intubation; next, 0.8 mL glucose-phosphate-buffered saline was used to perfuse the lungs, and the bronchoalveolar lavage fluid (BALF) was obtained. The levels of OVA-specific IgE (OVA-sIgE) in the serum and interleukin (IL)-2, IL-4, IL-6, IL-10, and IL-17 and IFN-γ in the BALF were determined using enzyme-linked immunosorbent assay kits (Thermo Fisher Scientific, Waltham, MA, USA).

Histopathology of lung and nasal tissue

The nasal mucosa and middle lobe of the right lung were collected and fixed overnight with 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin; subsequently, 4-μm tissue sections were prepared using a rotary microtome and stained with hematoxylin and eosin. The images of the lung and nasal tissue sections stained with hematoxylin and eosin were captured using a microscope (Nikon Eclipse CI-U, Tokyo, Japan). Epithelium thickness and mononuclear cell infiltration of nasal mucosa were counted as described in Piao et al. and Hu et al. Inflammation score of lung tissue was evaluated as described in Dong et al.

Flow cytometry

Splenic lymphocytes were obtained as described by Chen et al. The spleen was dissected and placed on a nylon mesh with a 70-μm pore size and gently ground with a syringe plunger. The filtered cells were added to 4 mL lymphocyte isolate solution (DAKEWE, Shenzhen, China) and centrifuged in a density gradient for 30 min at 800 × g. The lymphocyte layer was gently aspirated and washed twice with Roswell Park Memorial Institute-1640 culture medium (Hyclone, Logan, UT, USA) at 300 × g for 5 min. The collected cells were cultured in Roswell Park Memorial Institute-1640 culture medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) in a humidified chamber in the presence of 5% CO\(_2\) at 37 °C. The cells were fixed and permeabilized with BD Cytofix/Cytoperm Plus kit (BD Biosciences, Franklin Lakes, NJ, USA) following the manufacturer’s instructions and then incubated with anti-mouse CD4-FITC, anti-CD25-APC, and anti-Foxp3-PE monoclonal antibodies (BioLegend, San Diego, CA, USA) for 1 h at 4 °C. Data were acquired using a flow cytometer (BD Biosciences).

Western Blotting

Lungs were collected and homogenized in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) containing a protease and phosphatase inhibitor cocktail. The equal amounts of protein (40 μg) were separated on 8% sodium dodecyl sulfate-polyacrylamide gels at 120 V for 1.5 h and then transferred to polyvinylidene fluoride membranes with a 0.45-μm pore size at 300 mA for 70 min. The membranes were blocked with QuickBlock blocking buffer (Beyotime) at room temperature for 20 min and then incubated with primary antibodies (p-mTOR, mTOR, PI3k, Akt, phosphorylated-AKT, hypoxia inducible factor (HIF)-1α, FOXP3, and FOXO1; all purchased from Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. The next day, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Abcam, Cambridge, UK) at room temperature for 2 h. The intensity of the protein band was scanned using iBright Imaging Systems (Thermo Fisher Scientific) and quantified based on the level of GAPDH (internal control).

16S rDNA sequencing and analysis

Bacterial DNA was extracted from mouse feces using the E.Z.N.A.® soil DNA kit (Omega Bio-tek, GA, USA) according to the manufacturer’s instructions. The sequences of the
Shihu extract markedly reversed the pathological symptoms. Cell infiltration in the nose and lung tissues were observed in Fig. 2B and 2C. Considerable epithelium thickness, mononuclear cell infiltration in the nasal mucosa and inflammatory score in lung tissue significantly increased inflammatory cell infiltration in the nose and lung tissues were observed in the Model-N and Model-D groups. The treatment with the Shihu extract markedly reversed the pathological symptoms.

These results for Model-N and Model-D revealed that intestinal flora dysbiosis exacerbates nasal and pulmonary inflammation in mice with AR.

**Shihu modulated levels of cytokines associated with allergic inflammation in serum and BALF**

The serum levels of OVA-sIgE were measured to evaluate whether the Shihu extract affected OVA-sIgE levels in mice with AR. As shown in Fig. 3A, the OVA-sIgE level in the serum increased significantly in mice with AR (Model-N and Model-D); the OVA-sIgE level in the Model-D group was higher than that in the Model-N group. In contrast, treatment with the Shihu extract reduced the OVA-sIgE concentration in the serum. As inflammation between upper and lower airways is related, we evaluated the levels of inflammatory cytokines in BALF, including those of IFN-γ (Fig. 3E), IL-2 (Fig. 3F), IL-4 (Fig. 3B), IL-6 (Fig. 3C), IL-10 (Fig. 3G), and IL-17 (Fig. 3D). In the Model-N and Model-D groups, the levels of IL-4, IL-6, and IL-17 increased significantly (P < 0.05 vs control), whereas the levels of IL-2, IFN-γ, and IL-10 decreased in the BALF (P < 0.05 vs control). However, the Shihu extract decreased the secretion of IL-4, IL-6, and IL-17, and IL-2, IFN-γ, and IL-10 levels were higher in the middle- and high-dose groups than in the Model-D group (P < 0.05). A similar effect on inflammatory cytokines was observed in the BALF of the low-dose group. However, the level of IFN-γ and IL-10 did not differ significantly between the low-dose and model-D groups.

**Shihu extract promoted FOXP3 expression by inhibiting the PI3K/AKT/mTOR pathway**

Tregs have been shown to reduce airway inflammation by secreting IL-10 and attenuating allergen-induced respiratory inflammation. mTOR is a critical signal that regulates Treg activation, differentiation, and function. To investigate the mechanism via which the Shihu extract regulates mTOR-mediated Treg differentiation, we assessed the expression of various members of the PI3K/AKT/mTOR pathway after treatment with the Shihu extract. As shown in Fig. 4, p-mTOR, PI3K, and HIF-1α levels were significantly higher in the Model-N and Model-D groups than in the control group, although AKT expression was unaffected; however, AKT phosphorylation increased significantly. In contrast, FOXO-1 and FOXP3 were significantly downregulated. These results indicate that OVA-induced AR activated the PI3K/AKT/mTOR pathway in the lung and promoted the phosphorylation of AKT, which caused HIF-1α overexpression and suppressed the expression of FOXO-1 and FOXP3, thereby inhibiting CD4+ CD25+ Treg cell differentiation and function. Interestingly, the above AR-induced changes were exacerbated by intestinal dysbiosis in the model-D group compared to those in the model-N group. However, the treatment of Model-D group with the Shihu extract significantly inhibited the activation of the PI3K/AKT/mTOR pathway in the lung and upregulated the expression of FOXO-1 and FOXP3, which protected against OVA-induced impairment of CD4+ CD25+ Treg function. Among the different doses of...
Fig. 2 Effects of Shihu on nasal symptoms (A), nasal mucosa inflammation (B), and lung inflammation (C) in AR mice. The model-N group was established with an OVA sensitization method, the model-D group was established with antibiotic exposure and OVA sensitization method. Shihu extract was administrated at doses of 20, 40, and 80 mg·kg\(^{-1}\)·d\(^{-1}\). A: Nasal symptoms in mice with allergic rhinitis decreased significantly after administering loratadine and Shihu. B: Shihu extract decreased nasal mucosa inflammation in mice with allergic rhinitis and intestinal flora dysbiosis (magnification: × 200). C: Shihu extract decreased lung inflammation in mice with allergic rhinitis and intestinal flora dysbiosis (magnification: × 400). Data are shown as the mean ± SD, n = 6; data were analyzed using one-way ANOVA with Tukey’s multiple comparison test. \(**P < 0.01, ****P < 0.001\), model-N and model-D groups were compared to the control group; \(^*P < 0.05\), model-N group was compared to model-D group; \(^{**}P < 0.01\), Shihu treatment group was compared to Model-D group.
the Shihu extract, the middle dose exhibited the best modulatory effect on the Model-D group.

**Shihu extract promoted Treg differentiation in mouse spleen**

Treg cells act as negative regulators of immunity, suppressing the activity of self and allergen-reactive T cells to maintain intestinal immune tolerance, inhibiting Th17 overproduction, and suppressing Th2 cell immune responses [25]. We investigated the CD4⁺ CD25⁺ FOXP3⁺ T cell population in the spleen CD4⁺ T cells and observed that the populations of Treg cells were decreased dramatically in the Model-N (1.79%, \( P < 0.01 \)) and Model-D groups (1.23%, \( P < 0.01 \)) compared to those in the control group (8.71%) and that the different doses (20, 40, and 80 mg·kg⁻¹·d⁻¹) of the Shihu extract significantly upregulated the population of Treg cells by 7.18%, 13.3%, and 9.26% compared to that in the model-D group (Fig. 5). Our results indicate that the Shihu extract ameliorates the symptoms of AR by promoting Treg proliferation.

**Shihu extract affected intestinal flora composition**

Intestinal flora affects systemic immunity, which further influences the respiratory and lower respiratory immune response [26]; however, how intestinal flora affects allergic diseases (AR and allergic asthma) is not well understood. Therefore, we constructed a mouse model of AR with intestinal flora dysbiosis to investigate the mechanism via which the Shihu extract affects the flora to improve AR. As shown in
Variability in the flora among different groups is shown in Fig. 8. Compared to that in the control group, the population of Bacteroidota and Proteobacteria increased significantly ($P < 0.05$), whereas that of Firmicutes and Desulfovibrio decreased significantly ($P < 0.05$) in the Model-D group. However, the Shihu extract remarkably reduced the population of Bacteroidota and Proteobacteria ($P > 0.05$) and increased that of Firmicutes and Desulfovibrio ($P > 0.05$) (Fig. 8A–D). At the genus level, a significant decrease in Alloprevotella and Faecalibaculum ($P < 0.05$) and an increase in Bacteroides, Parabacteroides, Hungatella,
**Fig. 5** Effects of Shihu extract on the population of CD4+ CD25+ FOXP3 cells in the spleen. The model-N group was established with an OVA sensitization method and the model-D group was established through antibiotic exposure and an OVA sensitization method. Shihu extract was administrated at doses of 20, 40, and 80 mg kg⁻¹ d⁻¹. A: Proportion of CD4+ T cells. B: Proportion of CD 25+ FOXP3+ Treg cells in CD 4+ T cells of control group, Model-N group, Model-D group, low-dose group, middle-dose group, and high-dose group. C: Percentage of CD4+ CD25+ FOXP3+ cells in CD 4+ T cells. Data are shown as the mean ± SD, n = 6. Data were analyzed using one-way ANOVA with Tukey’s multiple comparison test. "**P < 0.01, model-N and model-D groups were compared to the control group; "***P < 0.001, Shihu treatment group was compared to the model-D group.

**Fig. 6** Comparison of α diversity indices of microbial community in different groups. A: Shannon index; B: Simpson index. Data are shown as the mean ± SD, n = 5–6; data points were analyzed using Student’s t-test. *P < 0.05, model-D group was compared to the control group.

*Clostridium_innocuum* group, and *Parasutterella* (P < 0.05) was observed in the Model-D group. The Shihu treatment significantly reduced the abundance of *Hungatella* and *Clostridium_innocuum* group (P < 0.05), increased that of *Alloprevotella* and *Faecalibaculum* (P < 0.05), and remarkably reduced that of *Bacteroides, Parabacteroides*, and *Parasutterella* (P > 0.05) (Fig. 8E–K). As described above, intestinal flora dysbiosis in the Model-D group caused by antibiotic and OVA treatment was restored to some extent by the Shihu extract.

To further evaluate the changes in the fecal microbial community composition, linear discriminant analysis effect size multi-level species difference discriminant analysis was used to identify the microbial community with the greatest contribution to each group. As shown in Fig. 9A, in the control group, *Firmicutes* was enriched at the phylum level, whereas *Lactobacillales, Alistipes, Lachnospiraceae\_NK4A136*, and *Prevotellaceae_UCG-014*, and *Prevotellaceae_UCG-001* were enriched at the genus level. In contrast, the phylum *Bacteroidota* (class, *Bacteroidia*; order, *Bacteroidales*; family, *Bacteroidaceae*; genus, *Bacteroides*) and genera *Parabacteroides, Blautia*, and *Hungatella* were abundant in the model-D group. *Alloprevotella* and *Lachnoclostridium* were the most abundant genera in the Shihu treatment group. In addition, the *Alloprevotella* population was significantly higher in the Shihu-
Fig. 7 Effects of Shihu on intestinal flora composition. (A, B) Abundance and variation in the composition of the microbial community at the phylum and genus levels. (C, E) Principal coordinate analysis of microbial community at the phylum and genus levels. (D, F) Partial least squares discriminant analysis of the microbial community at the phylum and genus levels.

Shihu Extract Increased Abundance of Bacteria Associated with Treg Induction and Decreased that of Bacteria Associated with OVA-sIgE Induction

Tregs (CD4^+ CD25^+ T) play crucial roles in maintaining self-tolerance and preventing the development of autoimmunity. IL-10 secreted by Treg cells inhibits the activation of Th2 cells, an important pathogenic mechanism in allergic diseases. Furthermore, the serum level of OVA-sIgE is an important indicator of AR. Correlation heat map plots were used to assess the correlation between microbial taxa and clinical variables. The correlation heat map plots among microbial taxa at the genus level and Treg and OVA-sIgE level are shown in Fig. 10; Alloprevotella (r = 0.784, P < 0.01) showed a significant positive correlation with Treg differentiation; Anaerotruncus (r = 0.493, P = 0.052), Faecalibaculum (r = 0.462, P = 0.072), Rikenellaceae_RC9_gut_group (r = 0.422, P = 0.104), and Paludicola (r = 0.388, P = 0.137) showed a positive correlation with Treg differentiation. Candidatus_Saccharimonas (r = −0.853, P <
Fig. 8 Shihu extract reversed intestinal flora dysbiosis at the phylum and genus levels in mice with allergic rhinitis. (A–D) Phylum level: A: Firmicutes; B: Bacteroidota; C: Proteobacteria; D: Desulfobacterota; (E–K) Genus level: E: Bacteroides; F: Parabacteroides; G: Alloprevotella; H: Faecalibaculum; I: Hungatella; J: Clostridium_innocuum_group; K: Parasutterella. Data are shown as the mean ± SD, n = 5–6; Data were analyzed using one-way ANOVA with Tukey’s multiple comparison test. *P < 0.05, **P < 0.01, ***P < 0.001, model-D group was compared to the control group; *P < 0.05, **P < 0.01, ***P < 0.001, Shihu treatment group was compared to the model-D group

0.01), Odoribacter (r = −0.843, P < 0.01), Intestimononas (r = −0.843, P < 0.01), Lachnospiraceae_UCG-006 (r = −0.836, P < 0.01), and Oscillibacter (r = −0.809, P < 0.01) showed a significant negative correlation with the serum OVA-sIgE levels. This is consistent with the results of microbial community composition analysis showing that the relative abundance of Alloprevotella was significantly high and was the greatest contributor to the microbial flora of the Shihu treatment group.

Discussion

AR is a chronic airway disease that is thought to be affected by the intestinal microbial composition. In this study, we investigated the relation between the intestinal microbiota and AR pathology and whether Shihu, a traditional Chinese medicine, can improve the immune response in AR by regulating the gut microbiome composition. We administered antibiotics orally to mice before constructing a model of OVA-induced AR and investigated the effect of intestinal flora disorders on AR. In addition, the effects of pretreatment with the Shihu extract were investigated in this mouse model of AR with intestinal flora dysbiosis. The results showed that intestinal flora dysbiosis aggravated the extent of inflammation in mice with AR and that the Shihu extract effectively inhibited inflammation in the lungs and nose of these mice.

From an anatomical and physiological perspective, the nose positioned in the upper respiratory tract and lungs in the lower respiratory tract are physiologically interrelated. In TCM, the term “lungs” encompasses the nose, airway, larynx, and lungs [39]. Modern pharmacological studies consider mucosal immunity as an integrated system. The intestinal, nasal, and pulmonary mucosal immune responses interact with each other, and changes in the intestinal flora can influence the development of lung diseases [36]. The TCM theory suggests that “the lung and the large intestine are interior-exterior related”; in other words, a physiological and pathological interaction exists between the lungs and large intestine, and the lungs are critical points of interaction between the large intestine and nose. Hence, the regulation of intestinal physiology can improve lung function [30].

Most TCM are obtained from plants and are rich in polysaccharides. Polysaccharides possess immune-regulatory, antioxidant, and anti-inflammatory properties [75, 38]. The intestinal flora can also influence the intestinal microenvironment and regulate intestinal immune function. In addition to sesquiterpenes, alkaloids, and phenols, Shihu is rich in polysaccharides, which are responsible for its pharmacological properties such as anti-inflammatory, antioxidant, and immunomodulatory activities [39]. In this study, antibiotic exposure led to a worsened inflammatory response in the lungs and nose of AR mice, resulting in increased levels of pro-inflammatory factors and decreased levels of anti-inflammatory factors in the BALF. However, Shihu treatment effectively reversed the inflammatory responses by promoting the Th1 cell response and inhibiting the Th2 cell response. Th2 cells play an essential role in AR pathology, which is character-
Fig. 9  Most differential flora from the phylum to the genus levels screened using linear discriminant analysis effect size in mice with allergic rhinitis. A: Linear discriminant analysis score distribution among control, model-D, and Shihu extract-treated groups (middle dose). B: Cladogram of the most differentially abundant taxa in different groups.
ized by the generation of an inflammatory infiltrate consisting of mast cells and eosinophils, and release of multiple mediators, chemokines, and cytokines. IFN-γ secreted by Th1 cells suppresses the Th2 type response by inhibiting the production of IgE from mast cells. Th17-type cytokine (IL-17) plays a pivotal role in allergic inflammation. The overproduction of IL-17 leads to chronic and autoimmune diseases and is closely related to the allergic response in the lungs and airway, as it regulates neutrophilic, macrophagic, and eosinophilic inflammation. Tregs secrete IL-10 for immunosuppressive functions and inhibit the differentiation and migration of Th1, Th2, and Th17 cells. Increase in Treg cell population can suppress Th2-driven allergic responses in the local mucosa, lung, and gastrointestinal tract of a mouse model of allergy. Furthermore, Treg cells are essential for maintaining immune tolerance, including airway immune tolerance, in the body.

Treg induction is one of the key approaches used to treat allergic diseases, such as allergen-specific immunotherapy. In this study, the number of Tregs decreased considerably in mice with AR and intestinal flora dysbiosis. However, administration of the Shihu extract reversed this effect. In addition, the Shihu extract effectively reversed the reduction in FOXP3 expression in the lungs of mice with AR and intestinal flora dysbiosis. FOXP3 is among the key transcription factors controlling the development and function of Tregs. To determine the mechanism via which FOXP3 is upregulated in the lung tissue, we analyzed the expression of proteins involved in the PI3K/AKT/mTOR pathway. The results showed that intestinal flora dysbiosis led to the activation of the PI3K/AKT/mTOR pathway and the upregulation of HIF-α expression in the lungs of mice with AR, which may have been the main cause of FOXP3 downregulation. The PI3K/AKT/mTOR signaling pathway is involved in Treg differentiation and function. The
inhibition of PI3K, promoted Treg differentiation. Furthermore, the production of Th1, Th2, and Th17 cells decreased significantly in mTOR-deficient mice, whereas Treg production increased \(^{[49]}\). FOXO1 and HIF-1α, transcription factors acting downstream of the PI3K/AKT/mTOR pathway, play opposite roles in regulating Treg differentiation, with FOXO1 acting as a key factor in Treg differentiation and HIF-1α inhibiting FOXP3 expression \(^{[14]}\). Based on these findings, Shihu may function via the PI3K/AKT/mTOR pathway to induce Treg differentiation, thereby improving AR, providing a direction for our further studies.

The intestine is one of the most important sites for the induction of Treg differentiation. Indeed, the intestinal flora has been found to regulate immune cell differentiation (Treg and Th17) \(^{[47]}\). A substantial body of evidence has shown that a decrease in intestinal microbiota diversity is correlated with AR. Hence, remodeling of the intestinal flora and restoration of intestinal immune homeostasis have become new therapeutic directions for AR. Treatment with *Bifidobacterium longum* IM55 and *Lactobacillus plantarum* IM76 reshapes the intestinal flora and restores the Th2/Th17 immune balance, improving the symptoms of AR \(^{[1]}\). In this study, we established a model of intestinal flora dysbiosis in mice that developed AR in early life and treated them with the Shihu extract to alleviate intestinal flora dysbiosis. Although the Shihu extract did not significantly improve the proportions of Firmicutes and Desulfovibacterota and decreased the population of Bacteroidota and Proteobacteria at the phylum level, it significantly increased the populations of *Alloprevotella* and *Faecalibaculum* and decreased those of *Hungatella* and *Clostridium innocuum* group at the genus level. *Anaerotruncus*, *Robinsoniella*, *Paludicola*, *Alloprevotella*, and *Faecalibaculum* showed positive correlations with Treg differentiation. *Anaerotruncus*, *Alloprevotella*, and *Faecalibaculum* are butyrate-producing bacteria \(^{[48, 49]}\), and *Robinsoniella* may be associated with the protection of the intestinal mucosal barrier and the stimulation of immune response \(^{[50]}\). Hence, the Shihu extract improved AR possibly by reshaping intestinal flora via an increase in Treg differentiation and induction of the systemic immune response. Intestinal flora can modulate the host immune response by metabolizing dietary fiber and polysaccharides from food and drugs into short-chain fatty acids (acetate, propionate, and butyrate) \(^{[51]}\). Short-chain fatty acids enter the intestinal lymph nodes and through G-protein coupled receptors (GPRs) GPR41, GPR43, and GPR109A, induce IL-10 production in dendritic cells and promote the expression of Forkhead box P3 (FoxP3), which in turn regulates Treg cell differentiation and proliferation \(^{[52]}\). Recent research showed that the immunomodulatory molecule polysaccharide A, secreted by *Bacteroides fragilis*, can be recognized by dendritic cells via TLR2, which promotes the differentiation of naïve CD4+ T cells to Foxp3+ Treg cells and initiates an efficient immune response \(^{[53]}\).

Our study had some limitations. Because of the complex composition of herbal medicines and their numerous targets, some indicators in this paper did not exhibit dose-dependent effects among the three groups; moreover, the narrow treatment window of Chinese herbal medicines is also responsible for this result. We explored the effect of intestinal dysbiosis on AR through antibiotic exposure. However, the strong inhibitory effect of antibiotics on the flora may have led to irreversible flora dysbiosis. This may be the main reason why the regulatory impact of Shihu on the intestinal flora at the phylum level was not significant. However, its role in restoring the balance of intestinal flora at the species level was evident. We also found that the intestinal flora disorder caused by antibiotics aggravated pulmonary inflammation and the AR reaction. After the administration of the Shihu extract, pulmonary inflammation and the AR reaction were effectively suppressed. However, the mechanism of these effects remains unclear. Presently, the immunological link between the nose and lung is not clear and further studies are needed to determine the mechanism of action. Chinese medicine theory recognizes that "the lung opens into the nose." In anatomical terms, the nose and lungs are in the same airway. Additionally, the hypothesis of "one airway one disease" suggests that the nose and lungs are functionally related \(^{[34]}\). The mucosal immune system includes the lymphoid tissue, which is widely distributed in the respiratory tract, urinary tract, and gastrointestinal tract and is the leading site for local specific immune responses. Several studies have suggested that stimulation in one part of the mucosal immune system can result in immune responses in other areas. The immune connection between the nose and lungs is likely the mucosal immune system, which will be evaluated in our future research.

**Conclusions**

Our results indicate that dysbiosis of the intestinal flora leads to further deterioration of nasal and pulmonary inflammation in a mouse model of AR and that administration of the Shihu extract mitigates this deterioration. Additionally, the PI3K/AKT/mTOR pathway was activated in the lungs of mice with AR, which was further aggravated by intestinal flora dysbiosis. Shihu treatment significantly inhibited activation of the PI3K/AKT/mTOR pathway, induced Treg differentiation, and suppressed the development of lung inflammation; these effects may lead to restoration of the balance of the intestinal flora caused by Shihu. Our results suggest that AR can be improved by restoring the balance of the intestinal flora. Overall, this study provides a basis for the TCM theory that states that “the lung and the large intestine are interior-exterior related”.

**Abbreviations**

AR, Allergic rhinitis; Treg, regulatory T cell; OVA, ovalbumin; H&E, hematoxylin and eosin; FOXP3, Forkhead box protein; Th2, type 2 helper T cell; Th1, type 1 helper T cell; Th17, type 17 helper T cell; mTOR, mammalian target of rapamycin; TCM, Traditional Chinese Medicine; BALF, bronchial alveolar lavage fluid
Supplementary Material

Supplementary materials are available as Supporting Information, and can be requested by sending E-mail to the corresponding author.

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