

Effect of *Astragalus membranaceus* in Ovalbumin-Induced Allergic Rhinitis Mouse Model

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Abstract

Background: *Astragalus membranaceus* (AM), a traditional Chinese medicine, has been used to treat allergic diseases, but the mechanism for treating allergic rhinitis (AR) remains unclear.

Objective: The purpose of this study was to look at the anti-inflammatory effect of AM on AR and the mechanism of anti-allergy.

Methods: The mouse model of AR was induced by ovalbumin. Allergic symptoms, number of eosinophils in nasal mucosa, and levels of inflammatory cells in nasal lavage fluid were analyzed. We explored the serum immunoglobulin E (IgE), interleukin-4 (IL-4), IL-5, IL-13, interferon- γ (IFN- γ), and IL-10. Besides, the relative mRNA of IL-4, IL-5, and IL-13 was also detected in nasal mucosa tissue. The proportion of CD4⁺ CD25⁺ Foxp3⁺ T cells in the spleen and nasal lymphoid tissue were analyzed. The mRNA levels of nuclear factor-kappa B p65 (NF- κ B p65) and inhibitory kappa B alpha (I κ B α), as well as NF- κ B p65 DNA binding activity, were tested. We also measured the protein levels of NF- κ B p65 and p-NF- κ B p65 in nasal mucosa.

Results: AM could reduce the number of eosinophils in the nasal mucosa and decrease the levels of inflammatory cells in nasal lavage fluid. The serum IgE, IL-4, IL-5, and IL-13 were also decreased, while levels of IFN- γ and IL-10 were increased. The relative mRNA of IL-4, IL-5, and IL-13 was decreased by AM. AM increased the proportion of CD4⁺ CD25⁺ Foxp3⁺ T cells in the spleen and nasal lymphoid tissue. In addition, AM could reduce the activity of NF- κ B by inhibiting the mRNA expression and DNA binding activity of NF- κ B p65. However, AM had no significant effect on mRNA of I κ B α . Above all, AM could reduce the p-NF- κ B p65 protein expression of nasal mucosa.

Conclusions: AM could reduce the secretion of inflammatory cytokines by increasing the level of CD4⁺ CD25⁺ Foxp3⁺ T cells and inhibiting the activation of the NF- κ B.

Keywords

Astragalus membranaceus, allergic rhinitis, mouse, inflammatory cytokine, CD4⁺ CD25⁺ Foxp3 cells, nuclear factor-kappa B

Introduction

Allergic rhinitis (AR) is a common allergic disease caused by a variety of allergens including dust mites, fungi, and cockroaches¹ and often associated with conjunctivitis, otitis media, sinusitis, and nasal polyps, which could cause emotional anxiety and affect physical health. Atopic individuals appear allergic symptoms mainly mediated by immunoglobulin E (IgE) medium release after exposure to allergens.^{2,3} There is a variety of immune active cells and cytokines involved in the nasal mucosa of AR patients. As a chronic allergic disease, AR is typically characterized by chronic inflammation, rhinorrhea, sneezing, nasal itching, nasal obstruction, and eye symptoms.^{4,5} Some patients even

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have lower respiratory symptoms, including chest tightness and difficulty breathing. Drug therapies mainly include corticosteroids, antihistamines, and decongestants, but the long-term efficacy of these drugs is still not satisfactory.

CD4⁺ CD25⁺ Foxp3⁺ T cell has been shown an important role in many immunological diseases, including AR, asthma, and systemic lupus erythematosus.⁶⁻⁸ There is growing evidence that CD4⁺ CD25⁺ Foxp3⁺ T cells could regulate the immune system and maintain autoantigen tolerance by inhibiting abnormal immune activation.⁹ Artemisinin could inhibit the release of inflammatory cytokines in asthma through CD4⁺ CD25⁺ Foxp3⁺ T cells.¹⁰ It has also been reported that fermented fish oil could increase the level of CD4⁺ CD25⁺ Foxp3⁺ T cells and inhibit the symptoms of atopic dermatitis.¹¹

The transcription factor nuclear factor-kappa B (NF-κB) plays a key role in inflammatory diseases.¹² The family members of NF-κB include NF-κB1 (p50), NF-κB2 (p52), c-Rel (p65), and RelB.¹³ When activated, NF-κB transfers to the nucleus in an inactive form and regulates the expression of inflammatory genes. It has been reported that the expression of tumor necrosis factor-α, interleukin (IL)-1b, IL-6, IL-8, and cyclooxygenase 2 are all associated with the activation of the NF-κB signaling.¹⁴ p65 and inhibitory kappa B alpha (IκBα) are key regulators of activation and inhibition in the NF-κB pathway, respectively. NF-κB has been associated with diseases of IgE-dependent diseases.^{12,15,16}

Astragalus membranaceus (AM), a traditional Chinese medicinal herb, contains the major active constituents including polysaccharides, flavonoids, and saponins.¹⁷ Numerous reports have found that AM is antagonistic to many diseases in recent years. The extract of AM increases the expression of transforming growth factor-β (TGF-β1), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) to promote wound healing.¹⁸ In addition, Yang et al. reported AM raising the immune response of the host organism to against tumor activity of liver cancer.¹⁹ Beyond that, although AM has long been used in the treatment of asthma and AR,^{20,21} the mechanism for treating AR remains unclear. Therefore, we hereby investigate the anti-inflammatory effect of AM and whether this effect is associated with the CD4⁺ CD25⁺ Foxp3⁺ T cells and NF-κB pathway.

Materials and Methods

Animals

Six-week-old female BALB/c mice were obtained from Dashuo Laboratory Animal Co., Ltd (Chengdu, Sichuan, China). All procedures were performed

according to the guide for the care and use of laboratory animals and approved by the Laboratory Animal Ethics Committee of Sichuan University (2015060B).

Reagents

AM herb powder, dried root extract, was purchased from the Chengdu University of TCM Affiliated Hospital (Chengdu, China). AM powder production processes were as follows: AM was cut into 2 to 4 mm after being washed and ground into powder after 4 to 6 hours of drying (60°C–80°C); 400 mg AM powder was suspended in 10 mL distilled water and mixed by oscillator. Ovalbumin (OVA), sodium pentobarbital, and aluminum hydroxide were all purchased from Sigma (St. Louis, MO, USA). All the antibodies used in flow cytometric analysis were obtained from Cell Signaling Technology Inc. (Beverly, MA). Reverse transcription system kits were supplied by Toyobo (Osaka, Japan). Nuclear extraction kits and TranAMTM NF-κB p65 Chemi transcription factor assay kits were purchased from Sigma (St. Louis) and Beobioscience (Shenzhen, China). The anti-NF-κB p65 antibody was from Proteintech (Chicago, USA). The antiphosphorylated NF-κB p65 (p-NF-κB) was from Cell Signaling Technology Inc.

The Mouse Sensitization, Challenge, and Treatment

The 60 mice were divided into 6 experimental groups as follows: control group, OVA group, OVA + AM groups (25, 50, and 100 mg/kg/day), and OVA + dexamethasone (DEX) (0.5 mg/kg) group. Ten mice were assigned to each group. The mice were immunized on day 0, 7, and 14 by intraperitoneal injection of 75 μg OVA plus 2 mg aluminum hydroxide with a total volume of 0.2 mL except those in the control group for general sensitization (Figure 1(A)). Normal saline was injected in the control group, respectively, on days 1, 7 and 14. On day 21 to 42, 200 μg OVA added to 20 μL phosphate buffer saline was used to distill the nose. The control group received normal saline instead of OVA. AM and DEX were used to treat the mouse in AM and DEX groups 2 hours before each OVA challenge on days 28 to 42.

Effect of AM in Nasal Rubbing and Sneezing

AR symptoms were evaluated by counting the frequencies of sneezing and nasal rubbing events for 10 minutes in an observation cage, after intranasal instillation.

Nasal Mucosal Tissue Histology

Mice in each group were sacrificed by sodium pentobarbital after the last allergen challenge. The nasal mucosal

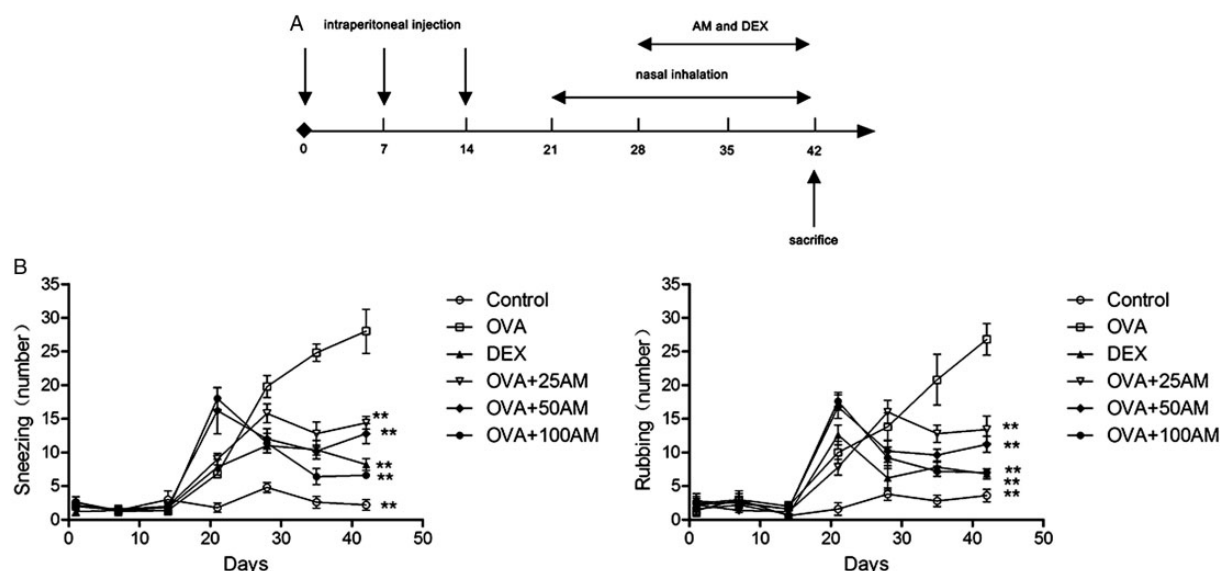


Figure 1. A, Experimental protocol. B, Symptoms of AR. $**P < .01$, vs OVA group. AM: *Astragalus membranaceus*; DEX: dexamethasone; OVA: ovalbumin.

tissue was removed for eosinophils and related signaling assessment. Nasal tissue was decalcified with ethylenediaminetetraacetic acid solution, embedded in paraffin. Serial 5- μ m sections were stained with hematoxylin and eosin for the visualization of eosinophils. Under a light microscope ($\times 400$ magnification), infiltrating eosinophils were counted in 5 different fields for each specimen by a single-blinded observer. The number of eosinophils in the nasal mucosa was calculated as the average of 5 regions.

Evaluation of Antigen-Induced Nasal Inflammation

The nasal lavage fluid was collected and classified by morphology. Briefly, 1 mL of normal saline was injected to the trachea with an empty needle and the nasal cavity was washed 3 times. The collected nasal lavage fluid was washed 3 times with physiological saline after centrifugation (1000 rpm, 2 min, 4°C). Total cell counts were determined by a hemocytometer. Differential cell counts were performed after staining by Wright–Giemsa. To measure levels of IgE, IL-4, IL-5, IL-13, interferon- γ (IFN- γ), and IL-10 in serum, the blood was immediately centrifuged at 2500 rpm for 10 minutes at 4°C. The supernatant from it was collected and stored at -80°C for cytokine investigations by enzyme-linked immunosorbent assay (ELISA). All experimental steps were performed according to the instructions; 100 μ L sample was added to the well of Micro ELISA Plate and incubated at 37°C for 90 minutes; after the liquid was removed, 100 μ L Biotinylated Detection Ab was added and incubated at 37°C for 60 minutes; after washed for 3 times, 100 μ L of the horseradish peroxidase

conjugate was added and incubated at 37°C for 30 minutes; after washed again for 5 times, 90 μ L of the Substrate Reagent was added and incubated at 37°C for 15 minutes; and finally, 50 μ L of the stop solution was added and OD value was measured at the 450 nm immediately.

All the RNA was extracted from the nasal mucosa tissues by microscope, and the quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to evaluate IL-4, IL-5, and IL-13 after reverse transcription as previously described. The IL-4 primer sequences were as follows: forward 5'-GGTCTCAACCCCCAGCTAGT-3' and IL-4 reverse 5'-GCCGATGATCTCTCTCAAGTGAT-3'. The IL-5 primer sequences were as follows: forward 5'-CTCTGTTGACAAGCAATGAGACG-3' and IL-5 reverse 5'-TCTTCAGTATGTCTAGCCCCCTG-3'. The IL-13 primer sequences were as follows: forward 5'-CCTGGCTCTTGCTTGCCTT-3' and IL-13 reverse 5'-GGTCTTGTGTGATGTTGCTCA-3'. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primer sequences were as follows: forward 5'-GGAGCGAGATCCCTCCAAAAT-3' and GAPDH reverse 5'-GGCTGTTGTCATACTTCTCATGG-3'. All steps were carried out according to the instructions. cDNA, 2 $\times$ Real PCR EasyTMMix-SYBR, Forward Primer, Reverse Primer, and DNase-Free ddH₂O were configured into a total of 20 μ L solution. The above indexes were detected by 40 cycles of 95°C predenaturation for 3 minutes, 95°C template denaturation for 10 seconds, and 65°C annealing for 30 seconds on the PCR instrument.

Evaluation of CD4⁺ CD25⁺ Foxp3⁺ Cells Expression

After euthanasia, nasal lymphoid tissue cells and spleen tissue cells were prepared as previously described. Nasal lymphoid tissues were collected by stripping the palate from the incisors teeth side toward the molar teeth side. The spleen tissues were removed from the abdomen of the mice. Nasal lymphoid and spleen tissue cells were obtained by mashing and filtered with a 50-hole screen. Then, cells were analyzed for CD4⁺ CD25⁺ Foxp3⁺ cells expression using a Treg cell staining reagent according to the manufacturer's instructions and analyzed by flow cytometry. The brief steps were as follows: 200 μ L of cell suspension was added to the flow tube, mixed with 1.5 μ L of CD4 and 1.5 μ L of CD25 antibody, and incubated on ice for 25 minutes in the dark; then, phosphate-buffered saline was added for centrifugation at 1500 rpm for 5 minutes; after discard the supernatant, 1 mL fixation/permeabilization was added to incubate for 1 hour at room temperature; when 2 mL of 1 $\times$ permeabilization buffer was added, the supernatant was discarded after centrifugation for 5 minutes at 300 rpm; after added 100 μ L permeabilization Buffer and 1.5 μ L of Foxp3 antibody again, the mixed liquor was incubated at room temperature for 30 minutes in the dark; and finally, 2 mL 1 $\times$ permeabilization buffer was added. After centrifugation at 300 rpm for 5 minutes, the supernatant was discarded again, and 300 μ L of staining buffer was added to detect CD4⁺ CD25⁺ Foxp3⁺ cells by flow cytometry.

Evaluation of NF- κ B p65 and I κ B α mRNA Expression

Total RNA was extracted from the nasal mucosa tissues. The quantitative RT-PCR was performed to evaluate NF- κ B p65 and I κ B α as previously described. The NF- κ B p65 primer sequences were as follows: forward 5'-AGGCTTCTGGGCCTTATGTG-3' and NF- κ B p65 reverse 5'-TGCTTCTCTCGCCAGGAATAC-3'. The I κ B α primer sequences were as follows: forward 5'-TGAAGGACGAGGAGTACGAGC-3' and I κ B α reverse 5'-TTCGTGGATGATTGCCAAGTG-3'.

NF- κ B p65 DNA Binding Activity

Nuclear extraction kit was used to extract nucleoprotein from nasal mucosa tissue for testing its DNA binding activity by measuring the absorbance at 450.

Evaluation of NF- κ B p65 and p-NF- κ B p65 Protein Expression

NF- κ B p65 protein expression and p-NF- κ B p65 protein expression in nasal mucosa were detected by Western blot. The nasal mucosa tissue was fully washed and ground quickly into homogenate and added with lysates

and protease inhibitors at same time. The homogenate was left standing at 4°C for 30 minutes and then centrifuged at low temperature to obtain the supernatant for use. The protein concentration in the sample to be tested was determined by bicinchoninic acid. Each denatured protein was loaded onto 15% polyacrylamide gels for electrophoresis. The sample solution was electrophoresized by SDS-PAGE electrophoresis, and the proteins were transferred onto membranes (polyvinylidene difluoride membrane); the membranes were blocked with the blocking buffer containing 5% skim milk powder for 40 minutes, and then the corresponding first antibodies were, respectively, added onto the membranes and incubated at 4°C overnight. The membrane was washed 3 times in Tris buffered saline-Tween (TBS-T) for 30 minutes, incubated with secondary antibodies (1 : 2000 dilution; in TBS-T) for 2 hours at room temperature, washed 3 times in TBS-T for 30 minutes, and revealed with enhanced chemiluminescence.

Statistical Analysis

SPSS 22.0 statistical software was used to analyze the data and experimental results. All data are expressed as the mean $\pm$ standard deviation. The data of normal distribution were analyzed by Student's *t* test, and the signed-rank test was used to analyze the nonnormal distribution of data. *P* < .05 indicates statistical significance.

Results

Effects of AM on Symptoms in the OVA-Induced Mouse Model

The nasal sneezing and rubbing (Figure 1(B)) symptoms of OVA group (sneezing 27.9 ± 6.9 ; rubbing 26.8 ± 4.9) were significantly elevated versus the control group (sneezing 2.2 ± 1.7 ; rubbing 3.6 ± 2.0) in day 42. The DEX group (sneezing 8.2 ± 1.8 ; rubbing 6.8 ± 1.5) and AM group (sneezing 25 + AM 15.4 ± 2.0 , 50 + AM 12.8 ± 3.1 , and 100 + AM 6.6 ± 0.8 ; rubbing 25 + AM 13.4 ± 4.2 , 50 + AM 11.2 ± 2.5 , and 100 + AM 6.9 ± 0.9) were significantly lower than those in the OVA group.

Effects of AM on Eosinophil Infiltration Into the Nasal Mucosa

The eosinophil number in the AR group (19.8 ± 4.7) was significantly higher than that in control (0.2 ± 0.1), AM (25 + AM 8.9 ± 3.2 ; 50 + AM 5.1 ± 2.0 , and 100 + AM 3.9 ± 1.9), and DEX (3.0 ± 1.2) groups (Figure 2). And the group with 100 mg AM looks no big difference when compared with the DEX group.

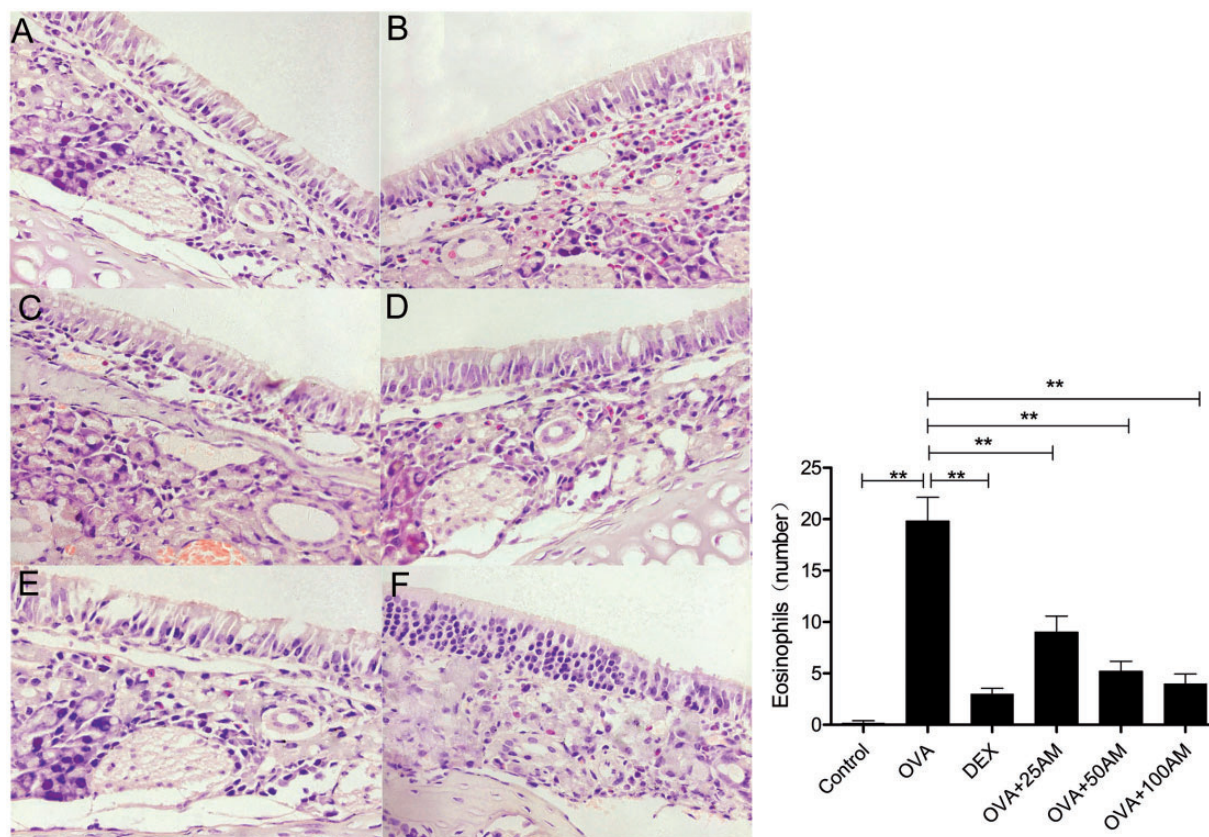


Figure 2. The histological characters of nasal mucosa among 6 groups, $\times 400$. A, Control group. B, OVA group. C, DEX group. D, OVA + 25AM group. E, OVA + 50AM group. F, OVA + 100AM group. * $P < .05$, ** $P < .01$, vs OVA group. AM: *Astragalus membranaceus*; DEX: dexamethasone; OVA: ovalbumin.

Effects of AM on Concentrations of Inflammatory Cytokines

The inflammatory cells including leukocytes, neutrophils, and eosinophils in the nasal lavage fluid were significantly increased after stimulation by OVA (leukocytes 3.9 ± 2.8 ; neutrophils 4.7 ± 1.5 ; eosinophils 4.3 ± 1.0) when compared with control group (leukocytes 0.3 ± 0.2 ; neutrophils 1.3 ± 0.2 ; eosinophils 0.3 ± 0.1). However, these indicators were significantly decreased after DEX (leukocytes 0.2 ± 0.1 ; neutrophils 1.5 ± 0.5 ; eosinophils 1.2 ± 1.0) and AM (25 + AM leukocytes 2.1 ± 0.8 , neutrophils 3.2 ± 0.8 , and eosinophils 2.3 ± 1.3 ; 50 + AM leukocytes 1.5 ± 0.9 , neutrophils 2.7 ± 1.5 , and eosinophils 1.7 ± 1.1 ; 100 + AM leukocytes 0.3 ± 0.1 , neutrophils 1.5 ± 0.1 , and eosinophils 0.4 ± 0.1) treatment (Figure 3(A)).

IgE (Figure 3(B)), IL-4, IL-5, and IL-13 (Figure 3(C)) were elevated significantly in the OVA (IgE 17.8 ± 4.4 ; IL-4 181.3 ± 131.6 ; IL-5 210.8 ± 43.1 ; IL-13 22.9 ± 4.4) group compared with the control (IgE 1.1 ± 0.8 ; IL-4 33.6 ± 22.1 ; IL-5 79.9 ± 19.7 ; IL-13 7.3 ± 2.0), DEX (IgE 3.6 ± 1.5 ; IL-4 58.5 ± 35.6 ; IL-5 77.2 ± 8.3 ; IL-13 7.9 ± 2.3), and AM (IgE 25 + AM 11.5 ± 2.0 , 50 + AM 6.9 ± 1.0 , 100 + AM 5.7 ± 1.4 ; IL-4 25 + AM 92.0 ± 19.3 ,

50 + AM 60.1 ± 23.3 , 100 + AM 43.1 ± 10.6 ; IL-5 25 + AM 158.1 ± 9.4 , 50 + AM 146.3 ± 22.7 , 100 + AM 94.4 ± 9.6 ; IL-13 25 + AM 17.4 ± 1.8 , 50 + AM 15.3 ± 1.9 , 100 + AM 10.1 ± 4.6) groups. With the increase in AM dose, the above inflammatory cytokines showed a decreasing trend. Expressions of IFN- γ and IL-10 were increased significantly in DEX (IFN- γ 1045.6 ± 240.2 ; IL-10 57.9 ± 16.7) and AM groups (IFN- γ 25 + AM 555.9 ± 78.6 , 50 + AM 820.9 ± 249.9 , 100 + AM 1069.9 ± 138.6 ; IL-10 25 + AM 39.4 ± 13.1 , 50 + AM 49.3 ± 26.1 , 100 + AM 58.1 ± 17.0) when compared with the OVA group (IFN- γ 354.1 ± 146.9 ; IL-10 18.8 ± 4.0). However, those indicators in the control group kept at low levels (IFN- γ 732.4 ± 146.9 ; IL-10 48.8 ± 24.3).

The trends of relative mRNA levels for IL-4, IL-5, and IL-13 of nasal mucosa in control group were lower than that in OVA group (IL-4 678%; IL-5 392%; IL-13 322%) (Figure 3(D)). However, DEX (IL-4 161%; IL-5 133%; IL-13 96%) and AM (IL-4 25 + AM 357%, 50 + AM 260%, 100 + AM 165%; IL-5 25 + AM 240%, 50 + AM 145%, 100 + AM 99%; IL-13 25 + AM 217%, 50 + AM 112%, 100 + AM 82%) could significantly increase the levels of these indicators.

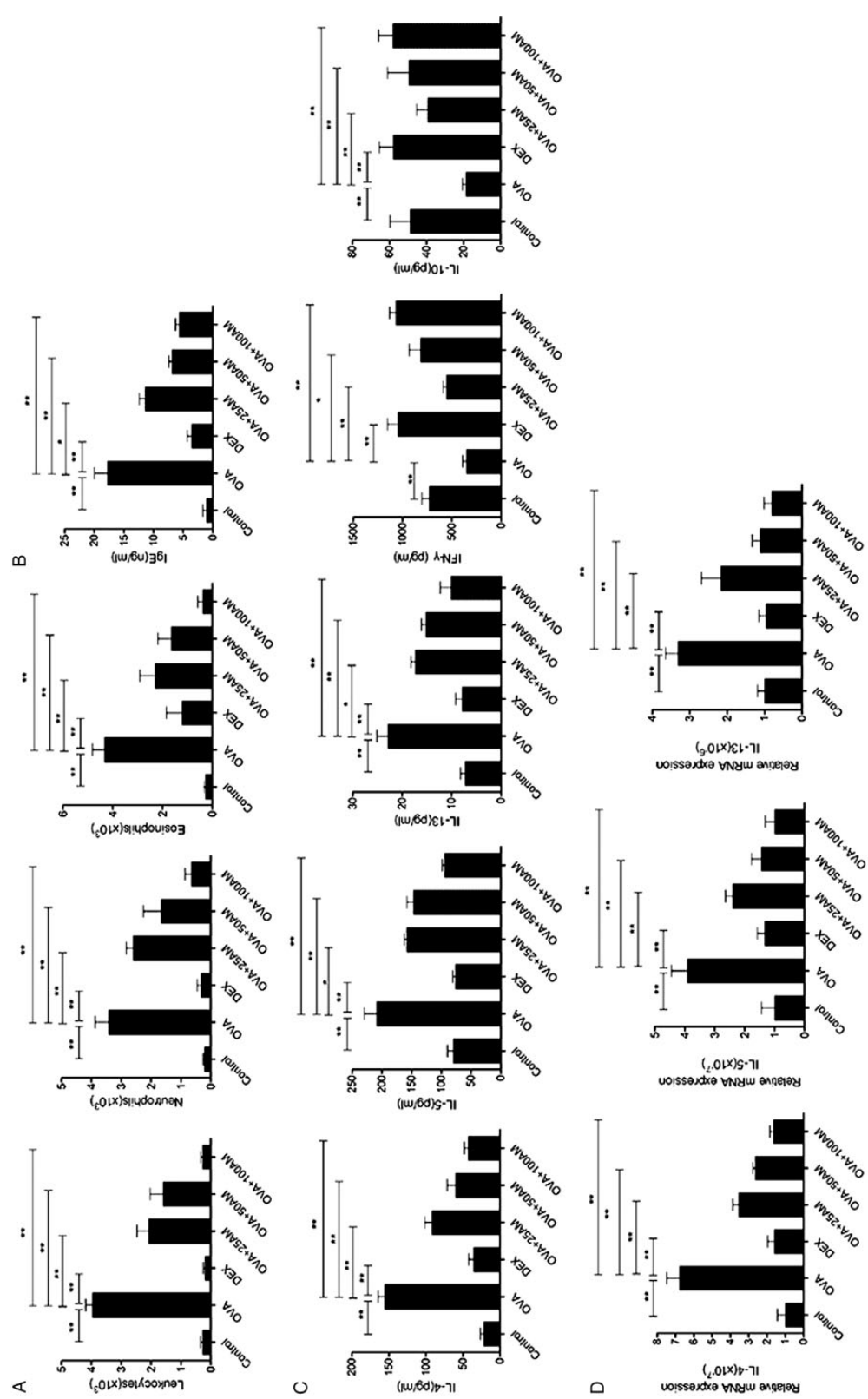


Figure 3. A, Inflammatory cells in nasal lavage fluid. B, OVA-IgE in serum. C, The secretion of cytokine in serum. D, Relative mRNA in nasal lymphoid tissue. * $P < 0.05$, ** $P < 0.01$, vs OVA group. AM: Astragalus membranaceus; DEX: dexamethasone; IFN- γ : interferon- γ ; IgE: immunoglobulin E; IL: interleukin; OVA: ovalbumin.

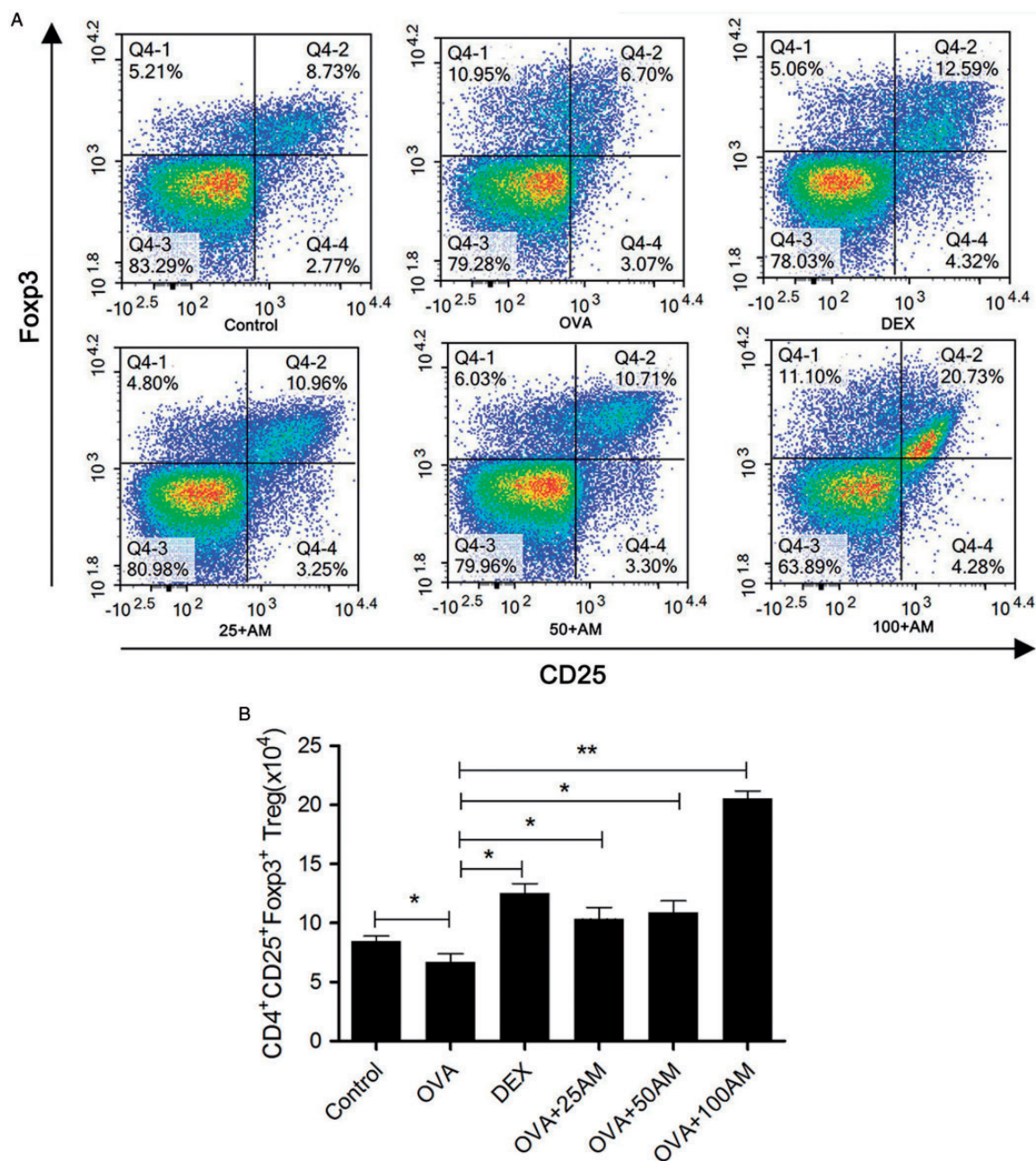


Figure 4. CD4⁺ CD25⁺ Foxp3⁺ T cells in spleen. A, The schematic diagrams of CD4⁺ CD25⁺ Foxp3 cells in spleen. B, The percentage of CD4⁺ CD25⁺ Foxp3⁺ T cells in spleen among the 6 groups. * $P < .05$, ** $P < .01$, vs OVA group. AM: *Astragalus membranaceus*; DEX: dexamethasone; OVA: ovalbumin.

Effects of AM on CD4⁺ CD25⁺ Foxp3⁺ T Cells in Nasal Lymphoid and Spleen

The expression of CD4⁺ CD25⁺ Foxp3⁺ T cells in spleen and nasal lymphoid similar results (Figures 4 and 5). CD4⁺ CD25⁺ Foxp3⁺ T cells were markedly decreased in the OVA group (spleen 6.5 ± 1.3 ; nasal lymphoid 6.1 ± 1.5) than the control group

(spleen 8.4 ± 1.9 ; nasal lymphoid 8.2 ± 1.6). Notably, the oral administration of DEX (spleen 12.6 ± 2.4 ; nasal lymphoid 11.1 ± 2.4) and AM (spleen 25 + AM 10.9 ± 2.2 , 50 + AM 11.2 ± 2.8 , 100 + AM 21.0 ± 1.9 ; nasal lymphoid 25 + AM 9.9 ± 2.6 , 50 + AM 11.0 ± 2.4 , 100 + AM 21.8 ± 5.7) significantly enhanced the frequency of CD4⁺ CD25⁺ Foxp3⁺ T cells compared with the OVA group.

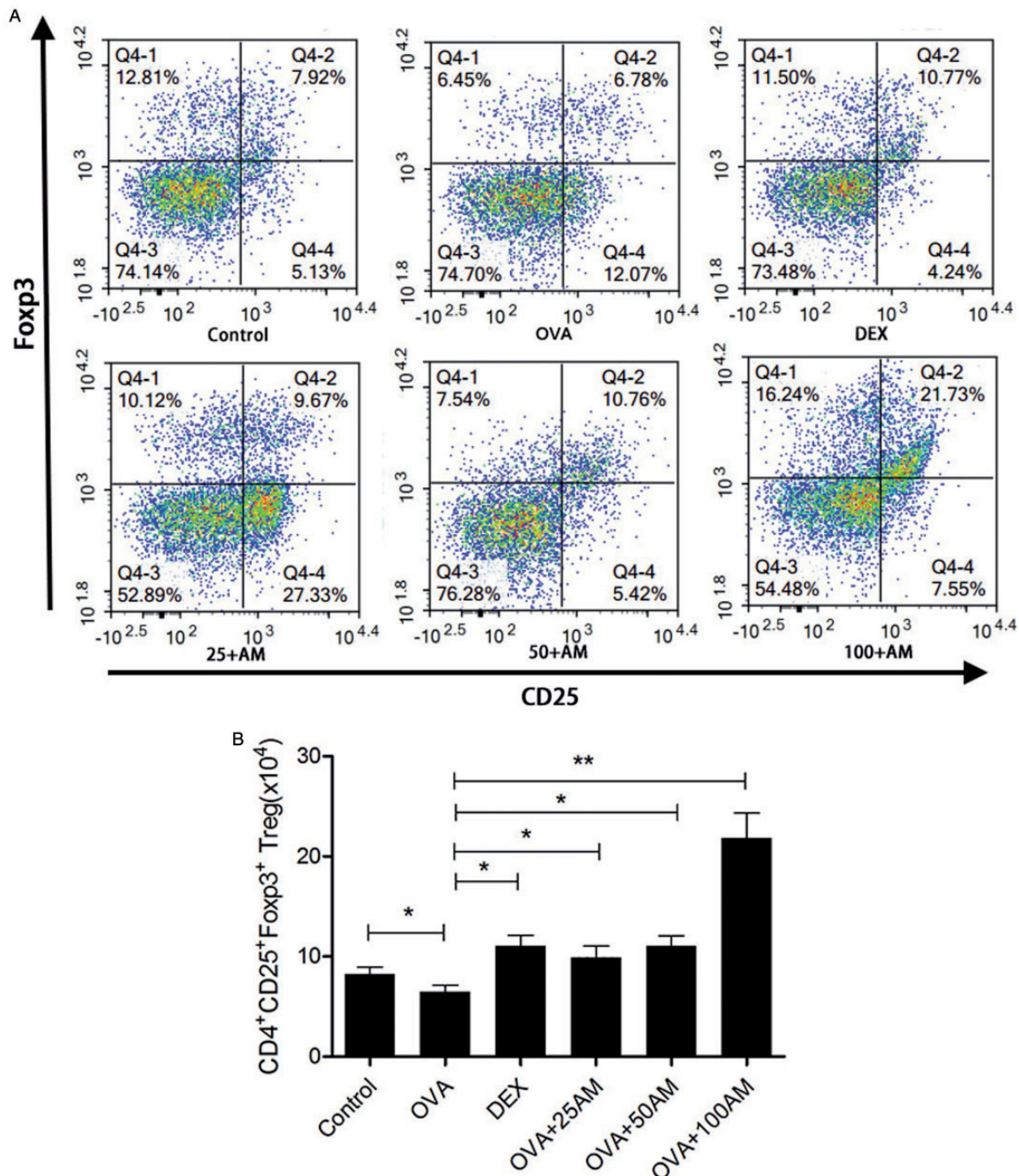


Figure 5. CD4⁺ CD25⁺ Foxp3⁺ T cells in nasal lymphoid tissue. A, The schematic diagrams of CD4⁺ CD25⁺ Foxp3 cells in nasal lymphoid tissue. B, The percentage of CD4⁺ CD25⁺ Foxp3⁺ T cells in nasal lymphoid tissue among the 6 groups. *P < .05, **P < .01, vs OVA group. AM: *Astragalus membranaceus*; DEX: dexamethasone; OVA: ovalbumin.

Effects of AM on NF- κ B p65 and I κ B α mRNA Expression of Nasal Mucosa

Compared with the control, DEX (105%), and the AM group (25+AM 181%, 50+AM 148%, 100+AM 114%), NF- κ B p65 mRNA level in the OVA group

(262%) was significantly increased (Figure 6(A)). With the increase in AM dose, NF- κ B p65 mRNA level gradually decreased. However, there were no significant changes in the levels of I κ B α mRNA in all groups (Figure 6(B)).

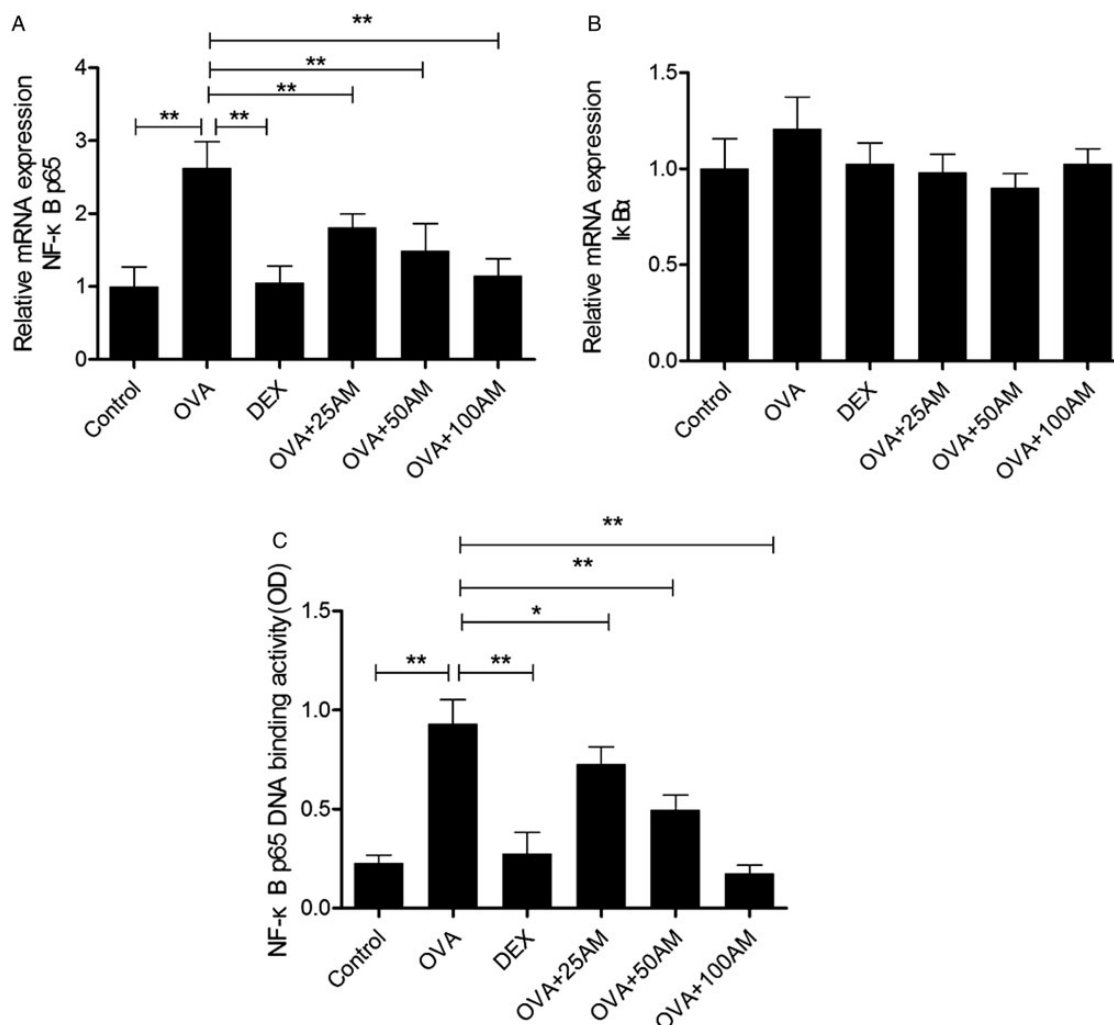


Figure 6. A, NF-κB p65 mRNA expression. B, IκBα mRNA expression. C, NF-κB p65 DNA binding activity. *: $P < .05$, **: $P < .01$, vs OVA group. AM: *Astragalus membranaceus*; DEX: dexamethasone; IκBα: inhibitory kappa B alpha; NF-κB p65: nuclear factor-kappa B p65; OD: optical density; OVA: ovalbumin.

Effects of AM on NF-κB p65 DNA Binding Activity of the Nasal Mucosa

After being sensitized by OVA (0.9 ± 0.3), the DNA binding activity of the nasal mucosa in mice was significantly increased when compared with control group (0.2 ± 0.1), while it showed a downward trend after being given DEX (0.3 ± 0.1) and AM (25 + AM 0.73 ± 0.2 , 50 + AM 0.50 ± 0.2 , and 100 + AM 0.18 ± 0.1) (Figure 6(C)). With the increase in AM concentration, the decreasing trend was more obvious.

Effects of AM on NF-κB p65 and p-NF-κB p65 Protein Expression of Nasal Mucosa

Given the important role of NF-κB signaling pathway in AR, Western blot method was used to investigate whether AM could play an important role in AR through

intervening in the NF-κB p65 signaling pathway. As shown in Figure 7, the p-NF-κB p65 in OVA group was significantly higher than that in the control group. In addition, we found remarkable decrease by DEX and AM. Above all, with the increase in AM dose, p-NF-κB p65 level gradually decreased. However, there was no significant difference in NF-κB p65 among the groups.

Discussion

AM is a traditional Chinese herb with antistress, hypertension, and a wide range of antimicrobial effects. It has been used for thousands of years in the treatment of various diseases. Many studies have found that AM has the obvious therapeutic effect on allergic diseases, such as asthma and AR.^{20–22} Due to its complex therapeutic mechanism, extensive studies and reports are still

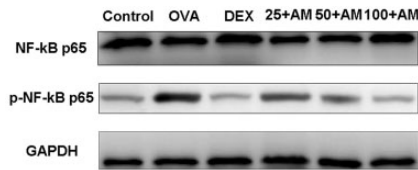


Figure 7. A. NF- κ B p65 and p-NF- κ B p65 protein expression. AM: *Astragalus membranaceus*; DEX: dexamethasone; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; I κ B α : inhibitory kappa B alpha; NF- κ B p65: nuclear factor-kappa B p65; OVA: ovalbumin; p-NF- κ B p65: antiphosphorylated NF- κ B p65.

lacking. Therefore, we mainly studied the role of AM in the mouse model of AR.

AR is a sequence response of the immune system, the noninfectious inflammatory disease of nasal mucosa mainly mediated by IgE after contact with allergens. With the development of social economy, various allergic diseases are increasing.²³ The prevalence of AR has increased significantly, especially in industrialized or developed countries with higher living standards. T-helper type 1 (Th1) and Th2 cytokines interact to participate in the immune response.^{24,25} When Th2 cytokines dominate, it can aggravate the inflammatory response and regulate various immune active cells and cytokines involved in chronic inflammation of the nasal mucosa. The imbalance inflammatory cytokines between Th1 and Th2 causes the allergy symptoms including sneezing, rhinorrhoea, and rhinocnesmus.²⁵ Many drugs have inhibitory effects on the release of inflammatory cytokines in AR but did not reach the satisfactory long-term effects. In recent years, the research on traditional Chinese medicine had become a new direction. In this study, the anti-allergic effect of *Astragalus* on AR had been confirmed.

IgE is an immunoglobulin with the lowest serum content, which leads to the release of a large number of inflammatory mediators and causes local inflammatory reactions in the nasal mucosa.²⁶ Abundant infiltration of eosinophil in the nasal mucosa is another feature of AR. In many studies, inhibition of IL-4, IL-5, and IL-13 could reduce local eosinophilic infiltration and allergy symptoms.²⁷ In the mouse model of AR caused by OVA, we found that the number of inflammatory cells in the nasal irrigation solution, including leukocytes, neutrophils, and eosinophils, was significantly increased. Moreover, the allergic symptoms and the number of eosinophils in the nasal mucosa also had a significant increase. In addition, the blood levels of IgE, IL-4, IL-5, and IL-13 presented a dramatic risen, and the mRNA levels of IL-4, IL-5, and IL-13 in nasal mucosa tissues also had a significant increase, while the level of IFN- γ in serum was decreased. However, these indicators showed an opposite trend after AM was administered by gavage,

and the inhibitory effect became more obvious with the increase in dose.

CD4⁺ CD25⁺ Foxp3⁺ T cell is a kind of T cell subgroup that regulates the autoimmune response, which is closely related to the occurrence of autoimmune diseases.²⁸ The role of CD4⁺ CD25⁺ Foxp3⁺ T cells in regulating allergic diseases has been well-documented. Both artemisinin and berberine have been shown to have significant effects on AR and may act through CD4⁺ CD25⁺ Foxp3⁺ T cells to reduce symptoms of AR by inhibiting the accumulation of inflammatory cells in the nasal mucosa.^{10,29} We found that the proportion of CD4⁺ CD25⁺ Foxp3⁺ T cells in the spleen of AR mouse was significantly increased compared with that before medication. In addition, the proportion of CD4⁺ CD25⁺ Foxp3⁺ T cells in nasal lymphocyte tissue showed the same results. Previous studies have shown that the immunomodulatory function of CD4⁺ CD25⁺ Foxp3⁺ T cells was related to the secretion of IL-10. In our study, the serum IL-10 was significantly improved after AM was given by gastric lavage, which also indicated that the anti-allergic effect of AM was correlated with CD4⁺ CD25⁺ Foxp3⁺ T cells.

As a major regulator of inflammation and immune processes, the activation of NF- κ B could lead to the expression of various inflammatory cytokines.³⁰ The p65 determines the severity of the inflammatory diseases. When the negative regulator, I κ B, is phosphorylated by I κ B, the activity of NF- κ B could be greatly enhanced.¹² Many studies have confirmed the role of NF- κ B in asthma.³¹ In recent years, some reports have also found that NF- κ B is closely related to local inflammation of AR.³⁰ Inhibiting the expression of NF- κ B could alleviate nasal inflammation in mouse with AR. Some studies also showed that herbal decoctions could reduce the release of inflammatory factors in allergic diseases and keratinocytes by inhibiting mitogen-activated protein kinase and NF- κ B pathways.^{32,33} In addition, the decoction composed of AM and angelica sinensis could inhibit the NF- κ B pathway and reduce allergic inflammation of the skin.³⁴ In our study, OVA significantly increased the mRNA expression of p65, while AM significantly decreased this trend. We also found that AM significantly decreased p65 DNA binding activity. In addition, AM could reduce the p-NF- κ B p65 protein expression of nasal mucosa. These pieces of evidence all indicate that AM had an inhibitory effect on the NF- κ B pathway. However, both DEX and AM had no obvious effect on the mRNA of I κ B α , which may require further study in the future.

Although AM has been shown to play an important role in many allergic diseases, the active ingredient remains unclear. Polysaccharides, flavonoids, and saponins were the 3 major active substances of AM.¹⁷ Adesso et al. suggested that an AM extract containing polysaccharides,

flavonoids, and saponins could significantly inhibit the activation of NF- κ B and participate in the process of reducing intestinal inflammation.³⁵ Flavonoids have been shown to play an important role in the anti-inflammatory effects of AM.¹⁷ As important components of flavonoids, formononetin, and calycosin could block the airway remodeling of asthma by inhibiting the TGF- β 1/Smad signaling pathway.³⁶ Astragaloside A, a subtype of saponins, could improve children's asthma by regulating CD4⁺CD25^{high}CD127^{low} Treg cells.³⁷ The inhibitory effect of flavonoids and saponins on local inflammation suggests that the effect of AM on AR may be related to the above 2 active ingredients, but further research is necessary to confirm these suspicions. Although polysaccharides have been reported to have an effect in stimulating the secretion of granulocyte-macrophage colony stimulating factor to restore cyclophosphamide-induced immunosuppression, there were still few reports of its application in allergic diseases.³⁸

Interestingly, previous studies have found that NF- κ B was a key regulator of natural Treg cell development and significantly promotes thymus Treg differentiation.^{39,40} NF- κ B promoted both inflammation and Treg cell development, especially the role of P65.⁴¹ In our study, AM increased the expression of CD4⁺CD25⁺Foxp3⁺ T cells but decreased the production of NF- κ B of the levels in mRNA and protein. This phenomenon may be the result of the interaction of different active components to AM. As a traditional Chinese medicine, although the main active component of AM was isolated and proved to have a strong anti-inflammatory effect, the mechanism and mode of action are still not completely clear. In addition, whether there are different active substances associated with CD4⁺CD25⁺Foxp3⁺ T cells and NF- κ B inflammatory pathways or whether the same substance is at work needs to be confirmed by further experiments.

In conclusion, the present study suggested that AM significantly inhibited the several inflammatory cytokines including IL-4, IL-5, and IL-13 in the mouse model of AR. The results of flow cytometric analysis demonstrated that CD4⁺CD25⁺Foxp3 cells may play a key role in the AM which alleviates AR of mouse. In addition, AM could reduce the release of inflammatory factors by inhibiting the activity of NF- κ B pathway. These findings provide further evidence for the therapeutic effect of AM in AR, it broadens the range of diseases treated by AM and opens up new possibilities for future strategies to treat AR.

Declaration of Conflicting Interests

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