

Astragalin Attenuates Allergic Inflammation in a Murine Asthma Model

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Abstract—The present study aimed to determine the protective effects and the underlying mechanisms of astragalin (AG) on ovalbumin (OVA)-induced allergic inflammation in a mouse model of allergic asthma. Our study demonstrated that AG inhibited OVA-induced increases in eosinophil count; IL-4, IL-5, IL-13, and IgE were recovered in bronchoalveolar lavage fluid, and increased IFN- γ level in bronchoalveolar lavage fluid. Histological studies demonstrated that AG substantially inhibited OVA-induced eosinophilia in lung tissue. Western blot analysis demonstrated that AG treatments markedly inhibited OVA-induced SOCS-3 expression and enhancement of SOCS-5 expression in an asthma model. Our findings support the possible use of AG as a therapeutic drug for patients with allergic asthma.

KEY WORDS: astragalin; allergic inflammation; suppressor of cytokine signaling.

INTRODUCTION

Asthma is a chronic inflammatory disease of the lung that is characterized by heightened sensitivity to stimuli that induce bronchoconstriction (airway hyperresponsiveness or AHR), marked tissue eosinophilia, increased mucus production due to the increased differentiation of bronchial epithelium into mucus-producing goblet cells, and prominent structural changes in the airways. These structural changes, which are collectively referred to as airway remodeling, include airway sub-epithelial fibrosis and myofibroblast hyperplasia [1, 2]. Type 1 allergies, which play critical roles in the pathogenesis of asthma, are caused by characteristic immune responses to allergens, primarily mediated by Th2 cells. Th2 cells synthesize high levels of interleukin (IL)-4, IL-5, and IL-13, which leads to the production of allergen-

specific immunoglobulin (Ig) E and the release of mediators from mast cells [3]. Th2 cells produce a distinct set of cytokines that are necessary for the allergic response. These include the cytokines IL-4 and IL-13, which promote IgE production; IL-5 and granulocyte macrophage-colony-stimulating factor (GM-CSF), which promote eosinophil production in the bone marrow; and IL-10, which promotes B cell differentiation into plasma cells. By contrast, Th2 cells do not produce IL-2 and interferon- γ . These cytokines are characteristic of Th1 cells, which reciprocally do not produce Th2-type cytokines. Thus, interventions that inhibit Th2 cytokines via the augmentation of Th1 cytokine production may prove useful in the treatment of allergic asthma.

Suppressor of cytokine signaling (SOCS) is a molecule that functions as a negative regulator of cytokine signaling, and is known to be involved in the pathogenesis of a host of inflammatory diseases [4–6]. The discovery of SOCS proteins has provided novel insights into the cytokine regulation of Th1 and Th2 immune responses. Eight members of the SOCS protein family have been identified thus far: cytokine-inducible SH2 domain-containing protein and SOCS-1 to SOCS-7. Among the SOCS proteins, SOCS-3 is expressed preferentially in Th2 cells and plays a crucial role in the regulation of the onset and maintenance of Th2-mediated allergic immune disease [7]. Serum IgE concentrations are also increased in patients evidencing high SOCS-3 expression. On the other hand, SOCS-5 is

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expressed preferentially in Th1 cells, and its expression can result in a reduction of Th2 differentiation as a consequence of the inhibition of IL-4 signaling [8]. It has been suggested that the inhibition of SOCS-3 expression or enhancement SOCS-5 expression may be a useful therapeutic approach to the treatment of Th2-dominant diseases, including allergic asthma.

Astragalin ($C_{21}H_{20}O_{11}$, Fig. 1) which is extracted from persimmon and *Rosa agrestis* leaves has antiatopic dermatitis and antioxidant activity [9]. It has been reported to possess an *in vitro* inhibitory effect on TNF- α , IL-1 β , and IL-6 production via attenuating the activation of the NF- κ B signaling pathway in the J774A.1 macrophage cell line [10]. In addition, astragalin can attenuate lipopolysaccharide-induced inflammatory responses by downregulating the NF- κ B signaling pathway [11]; however, no experimental study has been found on the therapeutic potential of astragalin for allergic asthma. In this study, we investigated whether treatment with astragalin can attenuate the inflammatory response in an asthma model in BALB/c mice via the regulation of SOCS-3 and SOCS-5 expression.

MATERIALS AND METHODS

Chemicals and Reagents

Astragalin (purity >95 %) was purchased from the National Institutes for Food and Drug Control (Beijing, China). Dexamethasone was purchased from Xiansheng Drug Store (Nanjing, China). Ovalbumin (OVA) was purchased from Sigma Chemical Co. (St. Louis, MO), aluminum hydroxide from Pierce Biotechnology (Rockford,

USA), Wright–Giemsa stain from Nanjing Jiancheng Bio-engineering Institute (Nanjing, China), and enzyme-linked immunosorbent assay (ELISA) kits from R&D (Minneapolis, MN, USA).

Ethics Statement

All animal experiments were performed according to protocols approved by the Shaanxi University of Chinese Medicine Animal Care and Use Committee.

Sensitization, Airway Challenge, and Treatment

Fifty female BALB/c mice (Xianyang, China) were housed in the animal facility at the Shaanxi University of Chinese Medicine. The mice were kept under temperature- and humidity-controlled specific pathogen-free conditions and maintained on a 12-h light–dark cycle. Animals received normal chow containing 18 % protein and 6 % fat (3.1 kcal/g of metabolizable energy) (2918, Harlan Laboratories, Madison, WI). All experimental protocols were approved by the Shaanxi University of Chinese Medicine Animal Care and Use Committee. Mice were divided into five groups (each of ten animals): (1) normal control, (2) asthma-model control, (3) asthma treated with dexamethasone (2 mg/kg, administered by gavage), and (4, 5) asthma treated with AG (0.5 and 1 mg/kg, administered by gavage). Allergic asthma was induced by ovalbumin (grade V) in six of the groups, using the method described by Oh *et al.* [12]. Mice were immunized via intraperitoneal (i.p.) injection with 10 μ g chicken OVA and 2 mg aluminum hydroxide in 200 μ L phosphate-buffered saline (PBS) (pH 7.4), on days 0 and 14. Mice were exposed to a 1 % (w/v) OVA solution in PBS, for 20 min, using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) on days 28, 29, and 30 after initial sensitization. Animals were sacrificed 48 h after the last challenge (thus on day 32) to characterize the suppressive effects of AG. A schematic diagram of the treatment schedule is shown in Fig. 2.

Measurement of Airway Resistance

The forced oscillation technique was used to measure respiratory mechanics. Regular ventilation was interrupted, and a computer-generated volume signal that consisted of waveforms of mutually primed frequencies was delivered to the airway opening. Piston displacement and cylinder pressure were measured. Impedance values were obtained before and after the delivery of increasing concentrations of methacholine aerosols. Prior to the start of the methacholine concentration response curve, two total lung

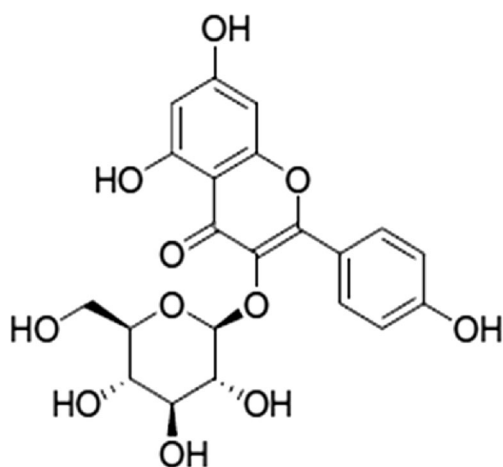


Fig. 1. Chemical structure of AG.

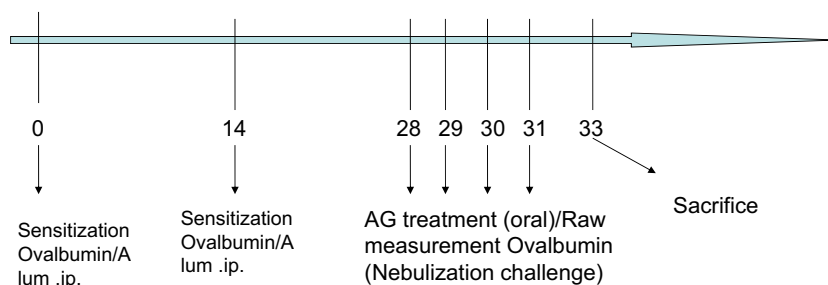


Fig. 2. Sensitization and challenge treatment protocols for the different groups in this study.

capacity breaths were delivered. Methacholine (Sigma) dissolved in saline was given via an ultrasonic nebulizer (Hudson RCI, Teleflex Medical) in increasing concentrations (0.125, 0.25, 0.5, and 1 mg/ml). After delivery of each aerosol, forced oscillations were delivered every 15 s, over a 5-min duration. In between impedance measurements, regular ventilation was resumed. The peak response for each variable was determined.

Collection of Bronchoalveolar Lavage Fluid

Mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 48 h after the last challenge, and tracheotomy was performed. After instilling ice-cold PBS (0.5 ml) into a lung, bronchoalveolar lavage fluid (BALF) fluid was obtained by three successive aspirations (total volume 1.5 ml) via tracheal cannulation [13]. BALF samples were centrifuged at 1500 rpm for 10 min at 4 °C, the supernatants were stored in -80 °C for analysis of cytokine concentrations, and the pellet was resuspended in 100 µl of saline, centrifuged onto slides, and stained for 8 min with Wright–Giemsa stain. The slides were quantified for differential cell count by counting a total of 200 cells/slide at ×40 magnification.

Histological Assessment

Histopathologic evaluations were performed on mice that were not subjected to BALF. Left lungs were removed by dissection and fixed in 4 % paraformaldehyde. Lung tissues were sectioned, embedded in paraffin, and cut at 3 µm. Tissue sections were then stained with hematoxylin and eosin (H&E) for histological assessment. The hematoxylin and eosin staining process was as previously described [14]. Peribronchial cell count based on a five-point scoring system was performed as previously described to estimate the severity of leukocyte infiltration [15]. The scoring system was 0, no cells; 1, a few cells; 2, a ring of cells one cell layer deep; 3, a ring of cells two to four cell

layers deep; and 4, a ring of cells more than four cell layers deep.

Enzyme-Linked Immunosorbent Assay Detection of BALF Cytokines IL-4, IL-5, IL-13, and IFN-γ

BALF levels of IL-4, IL-5, IL-13, and IFN-γ were measured by ELISA according to the manufacturer's instructions (R&D, Minneapolis, MN, USA). All measurements were performed in duplicate. Briefly, the BALF samples were added in duplicate to 96-well plates with 100 µl per well. The appropriate biotin-conjugated antibodies were added to each well. The samples were incubated at room temperature for 2 h. The wells were then aspirated, and each well was washed five times. The substrate solutions were added to each well and were incubated for 30 min at room temperature in the dark. The optical density (O.D.) of each well was determined using a microplate reader (Bio-Rad Model 680, USA) that was set to 450 nm. A standard curve was created of the average of the O.D. duplicate readings. The results were calculated using Excel 2007 (Microsoft Office, USA).

Measurement of OVA-Specific Serum and BALF Levels of IgE

OVA-specific serum and BALF IgE levels were determined by enzyme-linked immunosorbent assay (ELISA) using samples collected 48 h after the last OVA challenge, as described previously [16]. In brief, a 96-well microtiter plate was coated with OVA (10 mg/ml) and then treated with mouse sera followed by biotin-conjugated rat anti-mouse IgE (Pharmingen, San Diego, CA). Then, avidin horseradish peroxidase (HRP) solution was added to each well. Units are optical density readings at 405 nm.

Western Blot Analysis

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease

inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. The samples were loaded to 10 % SDS-PAGE gels and were electrotransferred to nitrocellulose. The blots were incubated with the appropriate concentration of specific antibody. After washing, the blots were incubated with horseradish peroxidase-conjugated second antibody. The membranes were stripped and reblotted with anti-actin antibody (Sigma) to verify the equal loading of protein in each lane.

Densitometric Analysis and Statistics

All immunoreactive signals were analyzed by means of densitometric scanning (Gel Doc XR; Bio-Rad, Hercules, CA). All values were expressed as the mean $\pm$ SD and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test using SPSS version 13.0 software; a p value of less than 0.05 was considered significant and $p < 0.01$ was considered to be statistically very significant.

RESULTS

Effect of AG on Airway Resistance

Measurements of airway resistance (Raw) during acetylcholine-induced constriction are shown in Fig. 3. The Raw value of the OVA-challenged group was significantly higher than that of the control group ($p < 0.05$). The treatment groups showed a significant decrease with acetylcholine 0.5 and 1 mg/ml ($p < 0.05$ and 0.01, compared with the model group respectively).

Effect of AG on OVA-Induced Eosinophilia and BAL Fluid Composition

We examined changes in total cell levels in the BAL fluid to determine the effects of AG on asthma. Suppression of eosinophilia by AG in OVA-challenged mice was estimated by counting of cells recruited to the BAL fluid 48 h after the last challenge. As shown in Fig. 4, OVA induced a marked influx of leukocytes into the BAL fluid in the control group. Eosinophil levels markedly increased in the BAL fluid of OVA-challenged mice. OVA-sensitized and OVA-challenged mice treated with AG displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction.

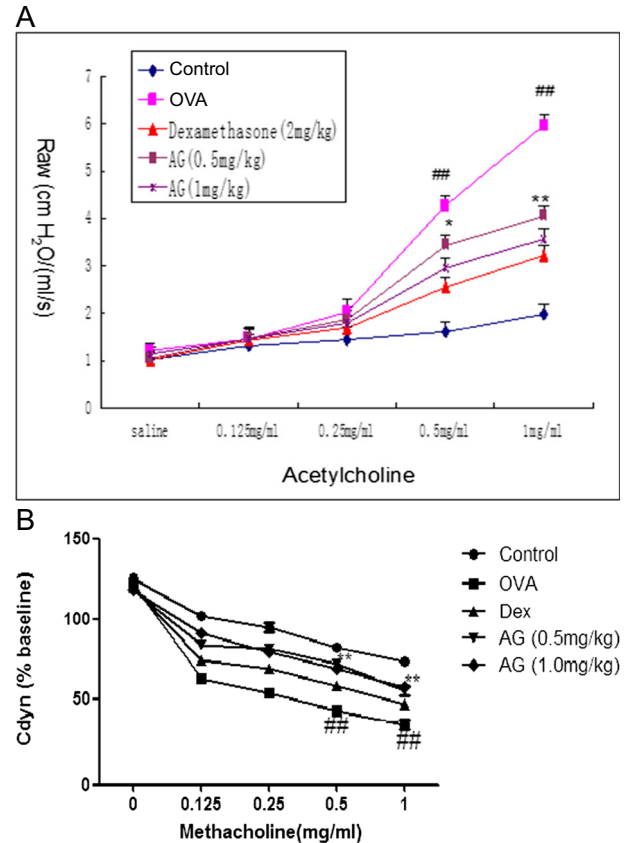


Fig. 3. Effect of AG on Raw (values are expressed as means $\pm$ SDs, compared with control: # $P < 0.05$, ## $P < 0.01$; compared with model: * $P < 0.05$, ** $P < 0.01$. The number of mice (n) = 10).

Effect of AG on OVA-Induced Eosinophilia in Lung Tissue

Because AG inhibits inflammatory cell recruitment into the lung tissue and airway tissue, we next examined the anti-asthmatic effects of the AG. Lung tissue and airway tissue were collected 48 h after the final OVA challenge. In OVA-induced asthmatic lung tissue and airway tissue, we observed marked infiltration of inflammatory cells into the perivascular tissue and airway, peribronchial connective tissues, and airway tissue, compared with the normal tissue. Moreover, most leukocytes were eosinophils. The control group displayed no change in the extent of inflammatory cell infiltration, similar to what was seen in dexamethasone-treated positive control mice. Eosinophilia in OVA-sensitized and challenged mice treated with AG was significantly attenuated compared with the level seen in OVA-challenged mice as shown in Fig. 5a, b.

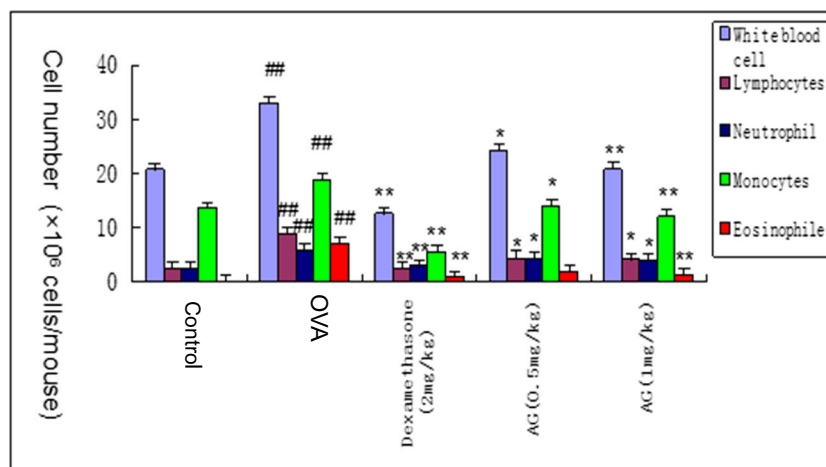


Fig. 4. Result of differential cell counts of BAL fluid (values are expressed as means±SDs, compared with control: # P <0.05, ## P <0.01; compared with model: * P <0.05, ** P <0.01. The number of mice (n)=10).

Effect of AG on Cytokine Levels in BAL Fluid

To determine whether GA influenced cytokine secretion into BAL fluid, IL-4, IL-5, IL-13, and IFN- γ in the BAL fluid were measured by ELISA 24 h after the final challenge. The results are shown in Fig. 6; compared with those of the control group, BALF levels of IL-4, IL-5, and IL-13 in the model group were markedly increased (p <0.05), while, compared with the model group, the treatment groups had markedly enhanced levels of IL-4, IL-5, and IL-13; as for BALF levels of IFN- γ , compared with the control group, levels of IFN- γ in the model group were markedly decreased (p <0.05), while, compared with the model group, treatment groups had markedly enhanced levels of IFN- γ .

Effect of AG on OVA-Specific Serum and BALF Levels of IgE

The cross-linking of allergen-specific IgE on the surfaces of mast cells upon an allergen challenge is relevant to the initiation of the early asthmatic reaction. The serum and BALF levels of OVA-specific immunoglobulins were measured 48 h after the final airway challenge. We determined that sensitization and challenge with OVA resulted in increased serum and BALF levels of OVA-specific IgE, when compared with the control mice. The treatment of sensitized mice with AG resulted in reductions in OVA-specific IgE (Fig. 7). These results support the conclusion that AG suppressed the generation of a Th2-type immune response in this animal asthma model.

Effect of EC Extracts on SOCS-3 and SOCS-5 Expression in Lung Tissues of OVA-Induced Asthma

SOCS-3 and SOCS-5 are expected to be the principal modulators of allergic immune diseases, and they reciprocally inhibit the processes of Th1 and Th2 differentiation. Therefore, we then assessed the expression of SOCS-3 and SOCS-5 in lung tissue via western blot analysis. Western blot analysis showed that the levels of SOCS-3 protein in lung tissues were significantly increased at 48 h after OVA inhalation, as compared with the levels observed following saline inhalation (Fig. 8). The increased level of this protein was reduced as the result of AG administration. Moreover, AG treatment also could augment SOCS-5 expression. These findings indicate that EC extracts could inhibit allergic airway reactions via the inhibition of the recruitment of Th2 cells into the airway, and enhancement of Th1 response.

DISCUSSION

Asthma is associated with a wide range of symptoms that are characterized by airway inflammation, mucus hypersecretion, and airway hyperresponsiveness (AHR). Here, using an OVA-induced mouse model of asthma, we show for the first time that AG can be effective as a therapeutic drug for the treatment of allergic asthma.

Asthma is defined as a variable level of airway obstruction usually accompanied by AHR. Bronchoconstriction attributable to contraction or hypertrophy of airway smooth

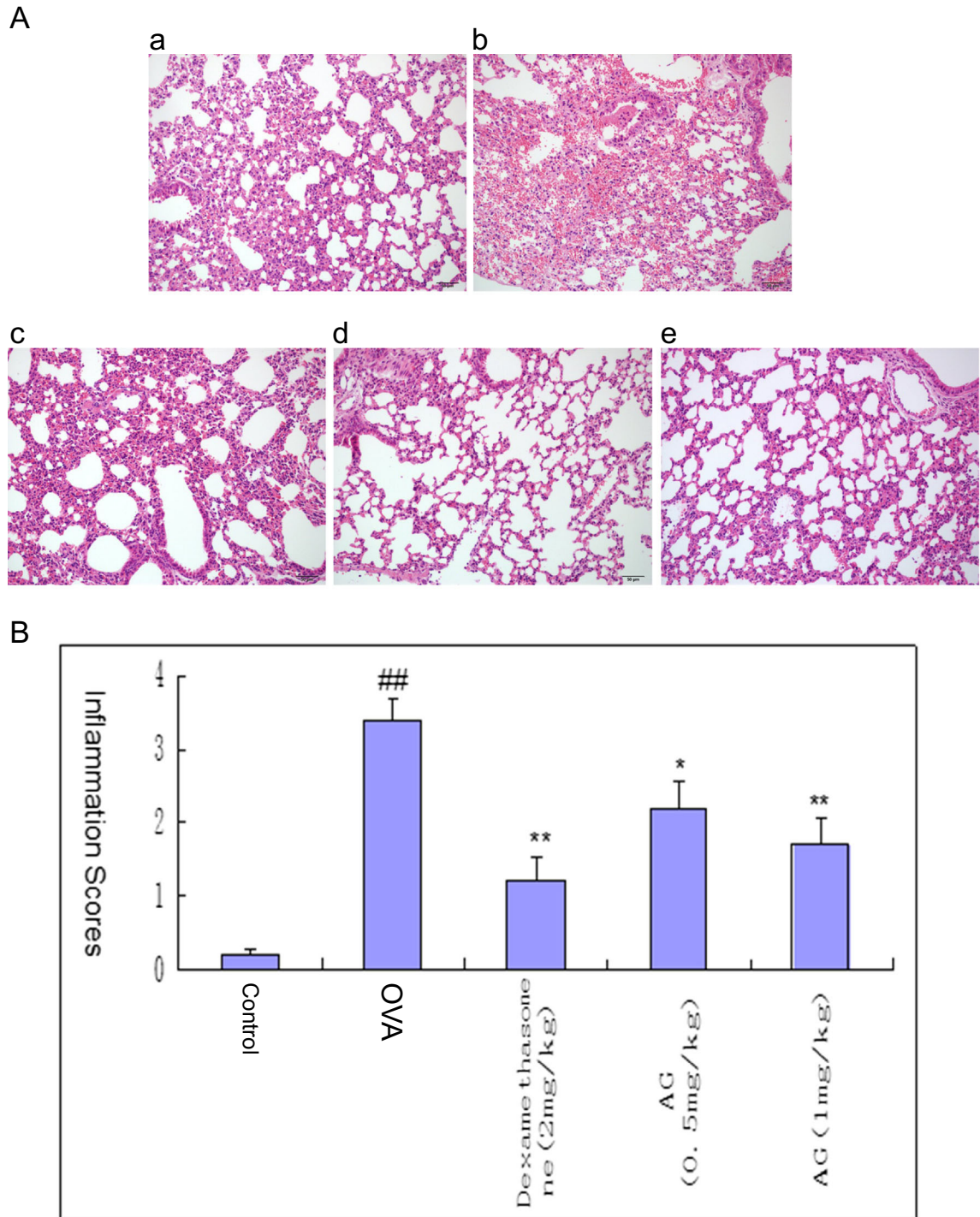


Fig. 5. **a** Pathological changes of lung tissues observed by HE staining (light microscopy, $\times 400$): *A* (control); *B* (model); *C* (dexamethasone, 2 mg/kg); *D* [AG (0.5 mg/kg)]; *E* [AG (1.0 mg/kg)]. **b** Histological scores (values are expressed as means $\pm$ SDs, compared with control: # $P < 0.05$, ## $P < 0.01$; compared with model: * $P < 0.05$, ** $P < 0.01$. The number of mice (n) = 10).

muscle (ASM), and inflammation within the airway, leads to decreased lung function [17]. AHR is a measure of the

bronchial constriction commonly observed in individuals with asthma. We monitored the effects of GA on AHR by

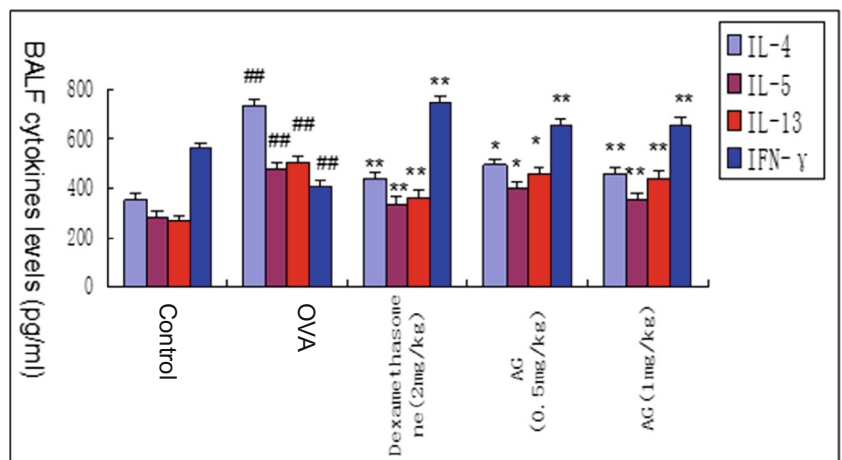


Fig. 6. Cytokine in BALF (values are expressed as means±SDs, compared with control: # P <0.05, ## P <0.01; compared with model: * P <0.05, ** P <0.01. The number of mice (n)=10).

measuring Raw. These experiments disclosed that AG inhibited the OVA-induced AHR in response to inhaled methacholine.

Migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway

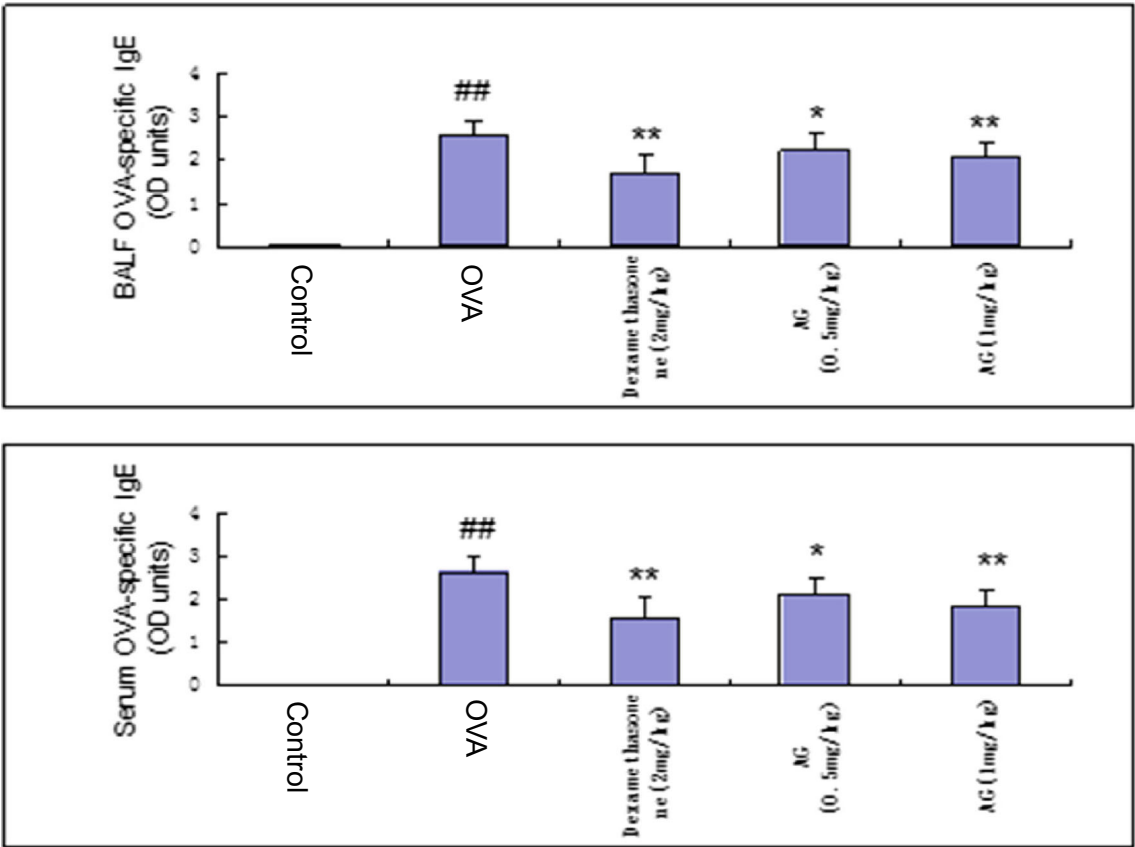


Fig. 7. Effect of GA on OVA-specific serum and BALF levels of IgE (values are expressed as means±SDs, compared with control: # P <0.05, ## P <0.01; compared with model: * P <0.05, ** P <0.01. The number of mice (n)=10).

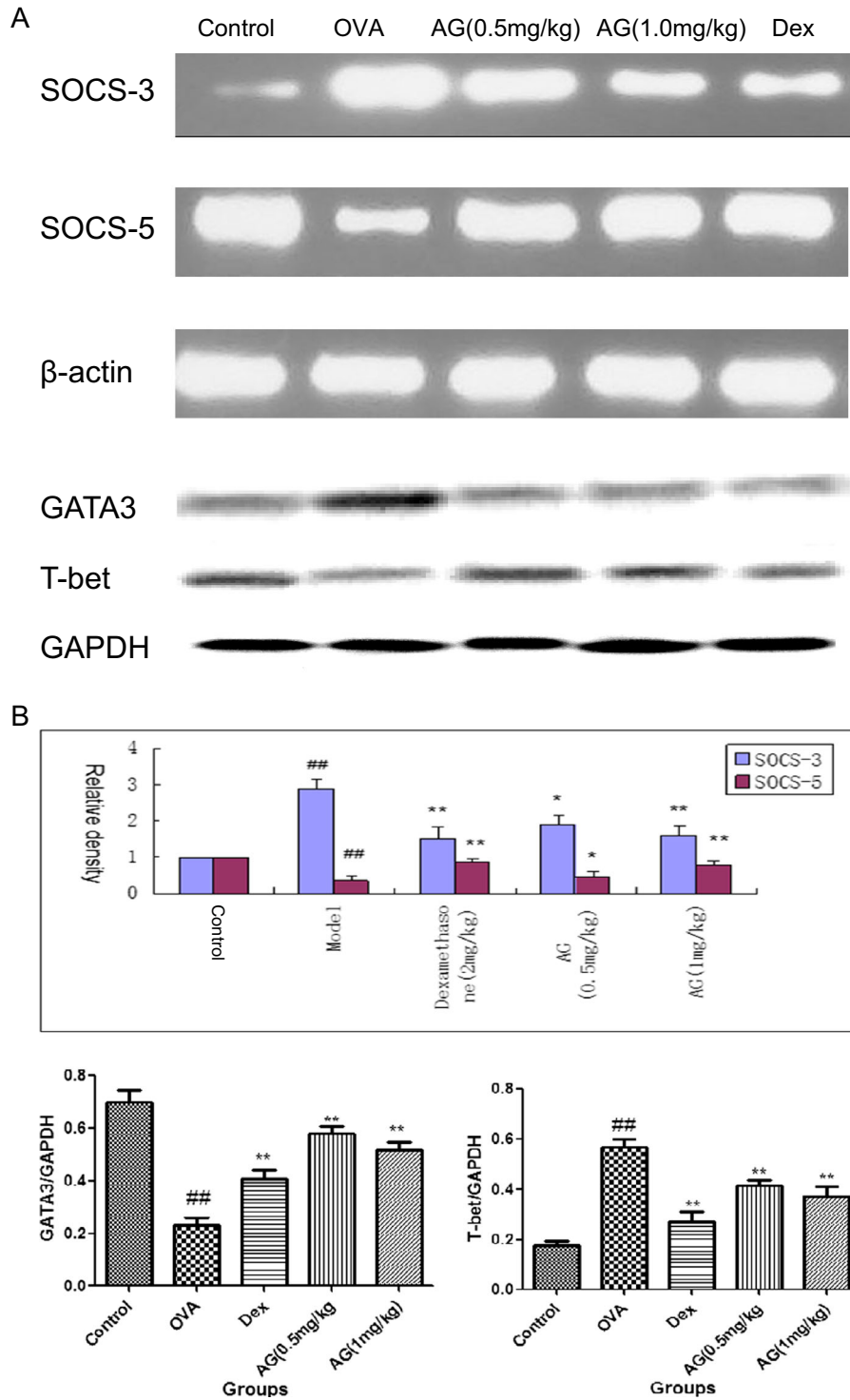


Fig. 8. Effects of AG on SOCS-3 and SOCS-5 expression in lung tissues of OVA-sensitized mice and OVA-challenged mice. **a** Western blotting of SOCS-3 and SOCS-5. **b** Densitometric analyses are presented as the relative ratio of each molecule to actin (values are expressed as means±SDs, compared with control: $P<0.05$, $##P<0.01$; compared with model: $*P<0.05$, $**P<0.01$. The number of mice (n)=10).

inflammation [18]. A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that AG significantly reduces eosinophil numbers in the lung tissue and airway tissue.

Allergic asthma is recognized as a TH2-mediated immune system disease. The induction of allergen-specific TH1 cells can attenuate allergic asthma. Several studies have shown that administration of TH1 cytokines, such as IFN- γ , at the time of sensitization can inhibit the induction of AHR and TH2-driven inflammation [19, 20]. Asthma and inflammation are associated with enhanced production of TH2 cytokines (IL-4, IL-5, and IL-13) and decreased production of TH1 cytokines (IFN- γ). Our data show that AG significantly suppresses IL-4, IL-5, and IL-13 cytokine levels and significantly increases IFN- γ cytokine levels.

Recently, the suppressor of cytokine signaling (SOCS) molecule has been shown to operate as negative regulators for cytokine signaling. The discovery of SOCS proteins has provided novel insights into the cytokine regulation of Th1 and Th2 immune responses [4, 7]. Among the SOCS proteins, SOCS-3 is abundantly expressed in peripheral T cells from human patients suffering from Th2-type diseases such as asthma and dermatitis, and its expression is tightly correlated with disease severity. It has been recently determined that a strong correlation exists between the level of SOCS-3 expression, serum IgE levels in allergic patients, and the severity of asthma and atopic dermatitis. However, SOCS-5 is expressed preferentially in Th1 cells, and its expression can result in a reduction of Th2 differentiation as a consequence of the inhibition of IL-4 signaling. SOCS-3-mediated Th1 inhibition subsequently enhances Th2 development and increases the incidence of allergic airway hyperresponsiveness. Therefore, negative regulators of cytokine signaling, the suppressor of cytokine-signaling proteins, are important cellular factors that regulate the Th1/Th2 balance. Taken together, these findings suggest that SOCS-3 and SOCS-5 may be candidate genes for asthma and also function as regulators of the Th1/Th2 balance. Our data show that AG significantly suppresses SOCS-3 expression and significantly increases SOCS-5 expression. Therefore, AG may be considered as a potential agent for preventing asthma, in the future. Also, further and comprehensive studies are needed before clinical application.

Conflict of Interest. The authors declare that they have no competing interests.

REFERENCES

1. Busse, W.W., and R.F. Lemanske Jr. 2001. Asthma. *New England Journal of Medicine* 344: 350–362.
2. Elias, J.A., et al. 1999. Airway remodeling in asthma. *Journal of Clinical Investigation* 104: 1001–1006.
3. Mosmann, T.R., and K.W. Moore. 1991. The role of IL-10 in cross regulation of TH1 and TH2 responses. *Immunology Today* 12: A49–A53.
4. Inoue, H., and M. Kubo. 2004. SOCS proteins in T helper cell differentiation: implications for allergic disorders? *Expert Reviews in Molecular Medicine* 6: 1–11.
5. Alexander, W.S., and D.J. Hilton. 2004. The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annual Review of Immunology* 22: 503–529.
6. Kubo, M., T. Hanada, and A. Yoshimura. 2003. Suppressors of cytokine signaling and immunity. *Natural Immunity* 4: 1169–1176.
7. Seki, Y., H. Inoue, N. Nagata, K. Hayashi, S. Fukuyama, K. Matsumoto, et al. 2003. SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. *Nature Medicine* 9: 1047–1054.
8. Seki, Y., K. Hayashi, A. Matsumoto, N. Seki, J. Tsukada, J. Ransom, et al. 2002. Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 99: 13003–13008.
9. Kotani, M., M. Matsumoto, A. Fujita, S. Higa, W. Wang, M. Suemura, T. Kishimoto, and T. Tanaka. 2000. Persimmon leaf extract and astragalus inhibit development of dermatitis and IgE elevation in NC/Nga mice. *Journal of Allergy and Clinical Immunology* 106: 159–166.
10. Kim, M.S., and S.H. Kim. 2011. Inhibitory effect of astragalus on expression of lipopolysaccharide-induced inflammatory mediators through NF- κ B in macrophages. *Archives of Pharmacological Research* 34: 2101–2107.
11. Lee, H.B., E.K. Kim, S.J. Park, S.G. Bang, T.G. Kim, and D.W. Chung. 2011. Isolation and antiinflammatory effect of astragalus synthesized by enzymatic hydrolysis of tea seed extract. *Journal of the Science of Food and Agriculture* 91: 2315–2321.
12. Oh, S.R., M.Y. Lee, K. Ahn, B.Y. Park, O.K. Kwon, H. Joung, J. Lee, D.Y. Kim, S. Lee, J.H. Kim, and H.K. Lee. 2006. Suppressive effect of verproside isolated from *Pseudotsuga longifolia* on airway inflammation in a mouse model of allergic asthma. *International Immunopharmacology* 6: 978–986.
13. Djukanović, R., W.R. Roche, J.W. Wilson, C.R. Beasley, O.P. Twentyman, R.H. Howarth, and S.T. Holgate. 1990. Mucosal inflammation in asthma. *American Journal of Respiratory and Critical Care Medicine* 142: 434–457.
14. Valentina, Pinelli, Cinzia L. Marchica, and Mara S. Ludwig. 2009. *Physiology & Neurobiology* 169: 36–43.
15. Duan, W., J.H. Chan, C.H. Wong, B.P. Leung, and W.S. Wong. 2004. Anti inflammatory effects of mitogen-activated protein kinase inhibitor U0126 in an asthma mouse model. *The Journal of Immunology* 172: 7053–7059.
16. Jain, V.V., K. Kitagaki, T. Businga, I. Hussain, C. George, P. O'Shaughnessy, and J.N. Kline. 2002. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *Journal of Allergy and Clinical Immunology* 110: 867–872.

17. Wenzel, S.E. 2006. Asthma: defining of the persistent adult phenotypes. *Lancet* 368: 804–813.
18. Elsner, J., and A. Kapp. 1999. Regulation and modulation of eosinophil effector functions. *Allergy* 54: 15–22.
19. Gavett, S.H., D.J. O’Hearn, X. Li, S.K. Huang, F.D. Finkelman, and M. Wills-Karp. 1995. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *Journal of Experimental Medicine* 182: 1527–1536.
20. Sur, S., J. Lam, P. Bouchard, A. Sigounas, and D. Holbert. 1996. Immunomodulatory effects of IL-12 on allergic lung inflammation depend on timing of doses. *Journal of Immunology* 157: 4173–4180.