



Immunoregulatory effects of Glycyrrhizic acid exerts anti-asthmatic effects via modulation of Th1/Th2 cytokines and enhancement of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in ovalbumin-sensitized mice



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ABSTRACT

Ethnopharmacological relevance: Glycyrrhizic acid (GA) is the main bioactive ingredient of licorice (*Glycyrrhiza glabra*), and has been found to be associated with multiple therapeutic properties.

Aim of the study: In this study, we investigated immunoregulatory effects of glycyrrhizic acid on anti-asthmatic effects and underlying mechanisms.

Materials and methods: Asthma model was established by ovalbumin-induced. A total of 60 mice were randomly assigned to six experimental groups: control, model, dexamethasone (2 mg/kg) and GA (10 mg/kg, 20 mg/kg, 40 mg/kg). Airway resistance (Raw) were measured by the forced oscillation technique, histological studies were evaluated by The hematoxylin and eosin (HE) staining, Th1/Th2 and Th17 cytokines were evaluated by enzyme-linked immunosorbent assay (ELISA), and CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) was evaluated by Flow Cytometry (FCM), the forkhead/winged helix transcription factor (Foxp3) was evaluated by western blotting.

Results: Our study demonstrated that, compared with model group, GA inhibited OVA-induced increases in Raw and eosinophil count; interleukin (IL)-4, IL-5, IL-13 levels were recovered in bronchoalveolar lavage fluid compared; increased IFN- γ level in bronchoalveolar lavage fluid; histological studies demonstrated that GA substantially inhibited OVA-induced eosinophilia in lung tissue and airway tissue compared with model group. Flow cytometry studies demonstrated that GA substantially enhanced Tregs compared with model group.

Conclusion: These findings suggest that GA may effectively ameliorate the progression of asthma and could be used as a therapy for patients with allergic asthma.

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1. Introduction

As a resource of drug, traditional Chinese herbs have attracted attention as health beneficial foods and herbal medicines. Licorice (*Glycyrrhiza glabra* L.) is often used to treat a variety of illnesses ranging from the common cold to liver disease for several centuries; it is specially used in respiratory disease, such as cough. The active ingredient from licorice had been extracted and purified, such as glycyrrhizinic acid, glycyrrhetic acid, and

liquoric flavour. Glycyrrhizic acid (Fig.1.) extracted from licorice root, is often used as a sweetening and flavoring agent due to its extreme sweetness (more than 50 times as sweet as sucrose). Glycyrrhizic acid has a corticosteroid-like structure, and has been shown to possess several beneficial pharmacological activities, such as an antiulcerative effect (Doll et al., 1962), anti-inflammatory activity (Ohuchi et al., 1981), antiviral activity (Pompei et al., 1979) and antihepatitis effect (Kiso et al., 1984).

Asthma is one of the most common chronic lung diseases in children and adults and yet it is a disease that we are currently unable to prevent or cure. Asthma, that is defined as a chronic inflammation of the bronchial airways and characterized by reversible airway obstruction, increased mucus production and infiltration of the airway with eosinophils, neutrophils, mast cells and T-lymphocytes (Busse and Lemanske, 2001). Allergen-specific CD4⁺T cells are believed to play a pivotal role in the development of asthma (Romagnani, 2000). Type 1 allergies are caused by

Abbreviations: (GA), glycyrrhizic acid; (ELISA), enzyme-linked immunosorbent assay; (HE) staining, hematoxylin and eosin; (Tregs), CD4⁺CD25⁺Foxp3⁺ regulatory T cells; (Foxp3), forkhead/winged helix transcription factor; (Dex), dexamethasone.

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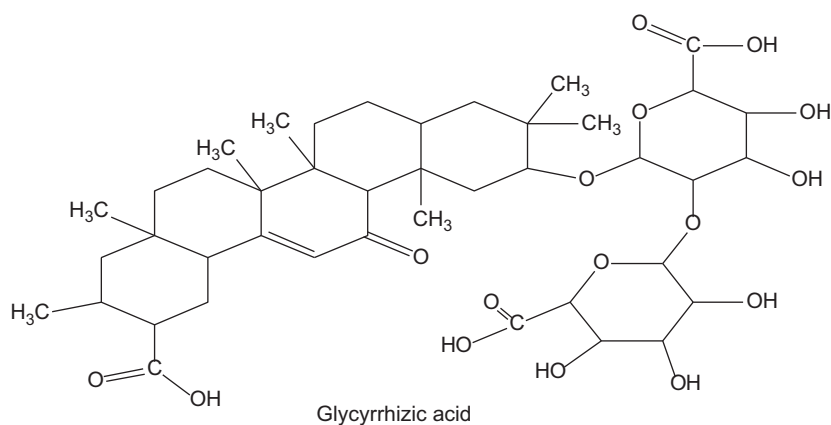


Fig. 1. Chemical structure of GA.

characteristic immuneresponses 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 (Mosmann and Moore, 1991). Th1 cells suppress Th2 immune responses by secreting interferon IFN- γ . IL-4 induces class switching in IgG1 and IgE, where as IFN- γ is involved in IgG2a class switching. Accordingly, estimation of IgE production and Th1/Th2 cytokine balance is an important tool in the evaluation of asthma (Brewer, et al., 1999).

Recently, regulatory T cells (Treg cells) have been described as two distinct subsets from Th1 and Th2 cells. Treg cells expressing the forkhead/winged helix transcription factor (Foxp3) have an anti-inflammatory role and maintain tolerance to self-components through direct contact with cells or by releasing anti-inflammatory cytokines such as IL-10 and transforming growth factor- β 1 (TGF- β 1) (Sakaguchi et al., 2006). CD4⁺CD25⁺ regulatory T cells (Treg) play a critical role in asthma.

Hence, we hypothesized that GA, which have protective effects in ovalbumin-sensitized asthma *in vivo*, might also be associated with the inhibition of inflammation and immunoregulatory. The present study was undertaken to evaluate the protective effects of GA and to elucidate the mechanism underlying these protective effects in mice. Our results indicate that GA pretreatment significantly protected against ovalbumin-sensitized asthma.

2. Materials and methods

2.1. Materials

GA powder (pure:98.7%) was purchased from National Institutes for Food and Drug Control (Beijing, China) and GA solution (4 mg/ml) was prepared in distilled water. Dexamethasone was purchased from Xiansheng drug Store (Nanjing, China). ovalbumin (OVA) (Sigma Chemical Co., St Louis, MO), aluminium hydroxide (Pierce Biotechnology, Rockford, USA), Wright–Giemsa staining (Nanjing Jiancheng Bioengineering institute, Nanjin, China). ELISA kits ((R&D, Minneapolis, MN, USA), RPMI1640 media (Gibco, USA), Cell disrupter (Beckman, USA), fluorescein isothiocyanate (FITC) anti-mouse Foxp3 (eBioscience).

2.2. Animals

Specific pathogen-free female BALB/c mice (2013839), aged 6–8 weeks, which were routinely screened serologically for relevant respiratory pathogens, were purchased from Experimental Animal Center of China Pharmaceutical University (Nanjing, China).

Animal experiment was carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University China Pharmaceutical University. A total of 60 female BALB/c mice obtained from Experimental Animal Center of China Pharmaceutical University (Nanjing, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided with water and standard chow *ad libitum*. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China.

2.3. Sensitization, airway challenge and treatment

Mice were divided into six groups (each of 10 animals): (1) normal control, (2) asthma-model control, (3) asthma treated with dexamethasone (2 mg/kg, administered by gavage) (4–6) asthma treated with GA (10, 20, 40 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. (2006). Mice were immunized via intraperitoneal (i.p.) injection with 10 μ g chicken OVA and 2 mg aluminium 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 GA. A schematic diagram of the treatment schedule is shown in Fig. 2.

2.4. Measurement of airway resistance (Raw)

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 capacity breaths were delivered. Methacholine (Sigma) dissolved in saline was given via an ultrasonic nebuliser (Hudson RCI, Teleflex Medical) in increasing concentrations (0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, 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.

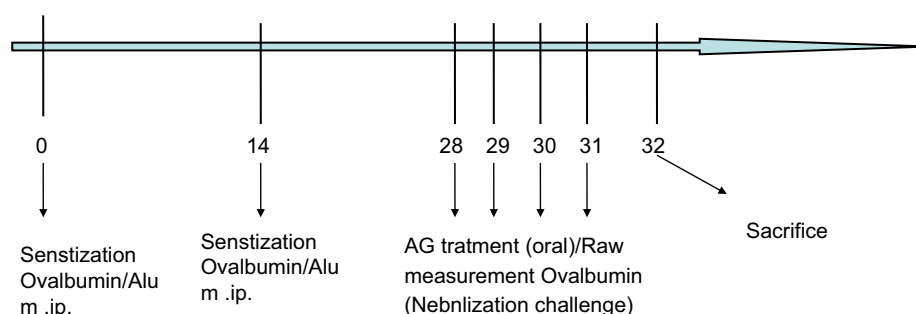


Fig. 2. Sensitization, challenge treatment protocols for the different groups in this study.

2.5. Collection of bronchoalveolar lavage (BAL) fluid

Mice were sacrificed using an overdose of 50 mg/kg of pento-barbital 48 h after the last challenge, and tracheotomy was performed. After instilling ice-cold PBS (0.5 ml) into a lung, BAL fluid was obtained by three successive aspirations (total volume 1.5 ml) via tracheal cannulation (Djukanović et al., 1990). BAL fluid (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 staining. The slides were quantified for differential cell count by counting a total of 200 cells/slide at 40 magnification.

2.6. Histological assessment

Lung tissues and airway tissues were detached from the mice and fixed with Carnoy's solution overnight at 4°. The fixed tissues were embedded in paraffin and cut into 4-µm sections with amicrotome (Leica, Nussloch, Germany). The sections were placed on slide glasses, deparaffinized, and stained with hematoxylin and eosin (Sigma, Korea) in order to examine the cells that had infiltrated into the peribronchial connective tissues and airway tissues. Peribronchial and airway cells count based on a five point scoring system was performed as previously described to estimate the severity of leukocyte infiltration (Duan et al., 2004). The scoring system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cell layers deep; and 4, a ring of cells more than 4 cell layers deep.

2.7. 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 biotinconjugated 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 5 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).

2.8. Measurement of OVA-specific serum and BALF levels of IgE

OVA-specific serum and BALF IgE levels were determined by enzymelinked immunosorbent assay (ELISA) using samples

collected 48 h after the last OVA challenge, as described previously (Jain et al., 2002). In brief, a 96-well microtitre 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, avidinehorseradish peroxidase (HRP) solution was added to each well. Units are optical density readings at 405 nm.

2.9. Flow cytometric analysis of CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs)

The spleen was removed from each sacrificed mouse and placed in a tube containing RPMI1640 media (Gibco). A single cell suspension was prepared by disrupting the spleen using Cell disrupter (Beckman), cells were equally distributed into tubes and washed once in phosphate-buffered saline (PBS). For Treg analysis, cells were incubated with fluorescein isothiocyanate (FITC) anti-mouse CD4 and phycoerythrin (PE) anti-mouse CD25 at 4 °C for 30 min. Following surface staining, cells were fixed and permeabilized according to the manufacturer's instructions, and then then stained with APC anti-mouse Foxp3 (eBioscience) for Tregs detection.

2.10. Western blotting of Foxp3

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 electro-transferred 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.

2.11. Statistical analysis

All values were expressed as the mean ± S.D. 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.

3. Results

3.1. Effect of GA on airway resistance (Raw)

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

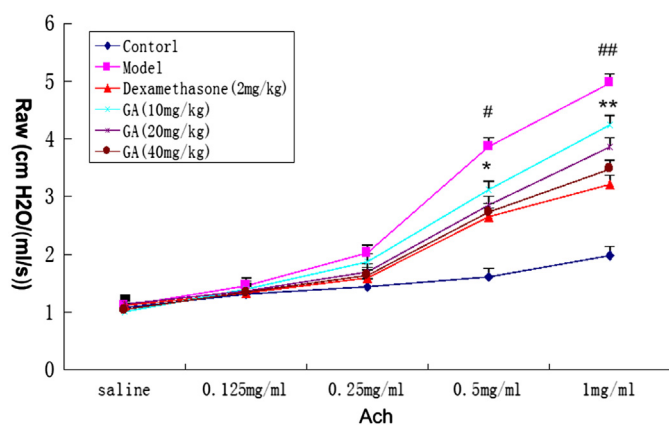


Fig. 3. Effect of GA on airway Resistance (Raw). Values are expressed as means $\pm$ SDs. Compared with control: # $P < 0.05$, ## $P < 0.01$; Compared with model: * $P < 0.05$, ** $P < 0.01$. Columns, mean of six independent experiments; bars, SD.

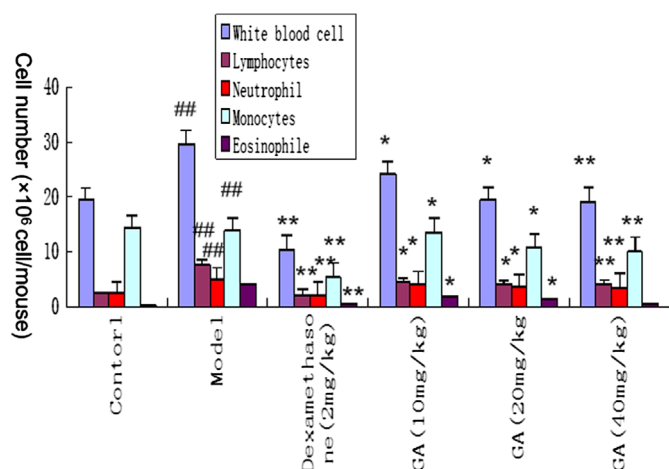


Fig. 4. Effect of GA on OVA-induced eosinophilia and BAL fluid composition. Values are expressed as means $\pm$ SDs. Compared with control: # $P < 0.05$, ## $P < 0.01$; Compared with model: * $P < 0.05$, ** $P < 0.01$. Columns, mean of six independent experiments; bars, SD.

decrease with acetylcholine 0.5 mg/ml and 1 mg/ml ($P < 0.05$ and 0.01, compared with model group, respectively).

3.2. Effect of GA on OVA-induced eosinophilia and BAL fluid composition

We examined changes in total cell levels in the BAL fluid to determine the effects of GA on asthma. Suppression of eosinophilia by GA 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 BAL fluid in the control group. Eosinophil levels markedly increased in the BAL fluid of OVA-challenged mice. OVA-sensitized and-challenged mice treated with GA displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction.

3.3. Effect of GA on OVA-induced eosinophilia in lung tissue and airway tissue

Because GA inhibits inflammatory cell recruitment into the lung tissue and airway tissue, we next examined the anti-asthmatic effects of the GA. 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 perivascular 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 GA was significantly attenuated compared with the level seen in OVA-challenged mice as shown in Fig. 5a–d.

3.4. Effect of GA 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), from (Fig. 6), as for BALF levels of IL-4, IL-5 and IL-13, compared with control group, levels of IL-4, IL-5 and IL-13 in model group was markedly increased ($P < 0.05$), while, compared with model group, the treatment groups can markedly enhance levels of IL-4, IL-5 and IL-13, especial GA group (40 mg/kg) ($P < 0.01$); as for BALF levels of IFN- γ , compared with control group, levels of IFN- γ in model group was markedly decreased ($P < 0.05$), while, compared with model group, treatment groups can markedly enhance levels of IFN- γ , especial GA group (40 mg/kg) ($P < 0.01$).

3.5. Effect of GA on OVA-specific serum and BALF levels of IgE

The cross-linking of allergen-specific IgE on the surfaces of mast cells upon 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 GA resulted in reductions in OVA-specific IgE Fig. 7. These results support the conclusion that GA suppressed the generation of a Th2-type immune response in this animal asthma model.

3.6. Effect of GA on Tregs

As shown in Figs. 8 and 9, the frequencies of Treg in model group ($1.33 \pm 0.40\%$) were lower than those in the normal group ($3.33 \pm 0.12\%$), while in dexamethasone group and treatment groups were significantly higher than those in model group ($P < 0.05$).

3.7. Effect of GA on Foxp3

The expression of inflammation-related protein Foxp3 was changed by OVA in lung. As shown in Fig. 10 compared with the control group the protein level of Foxp3 in model group was significantly decreased ($P < 0.05$). In GA, the protein level of Foxp3 was significantly increased in a dose-dependent manner compared to the model group, respectively ($P < 0.05$).

4. Discussion

Asthma, a chronic inflammatory disease of the airways, affects many individuals worldwide. The disease may cause severe morbidity, and even mortality if exacerbated. To date, a variety of drugs have been developed to treat asthma; these include steroids, leukotriene inhibitors, mast cell stabilizers, and β_2 adrenergic agonists. Many herbal medicines, including GA, were

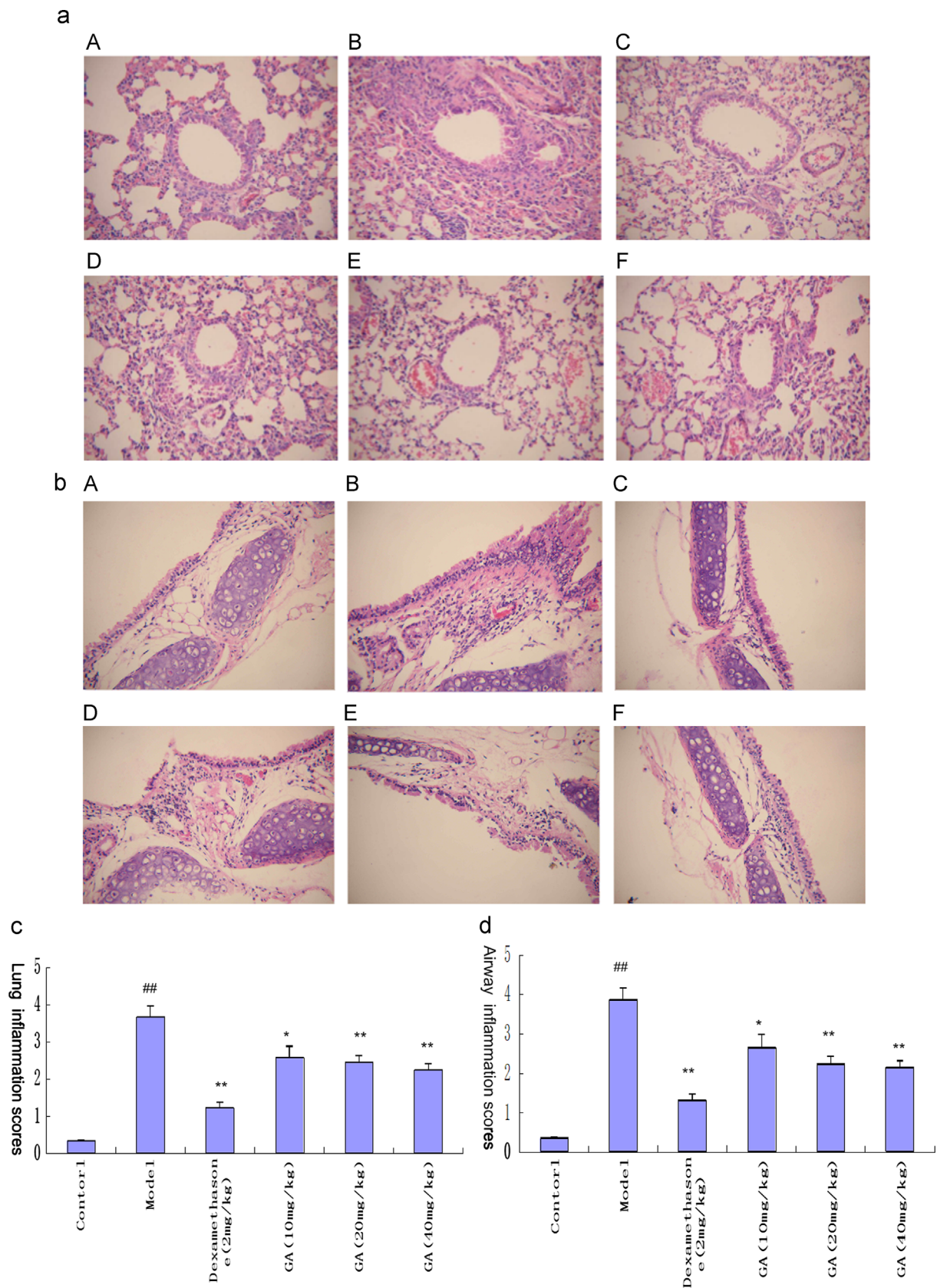


Fig. 5. **a** Pathological changes of lung tissues observed by HE staining (Light microscopy, $\times 200$): A (control); B (model); C (dexamethasone, 2 mg/kg); D [GA(10 mg/kg)]; E [GA(20 mg/kg)]; F [GA(40 mg/kg)] **b** Pathological changes of airway tissues observed by HE staining (Light microscopy, $\times 200$): A (control); B (model); C (dexamethasone, 2 mg/kg); D [GA(10 mg/kg)]; E [GA(20 mg/kg)]; F [GA(40 mg/kg)] **c** Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; Compared with model: * $P < 0.05$, ** $P < 0.01$. Columns, mean of six independent experiments; bars, SD. **d** Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in airway sections. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; Compared with model: * $P < 0.05$, ** $P < 0.01$. Columns, mean of six independent experiments; bars, SD.

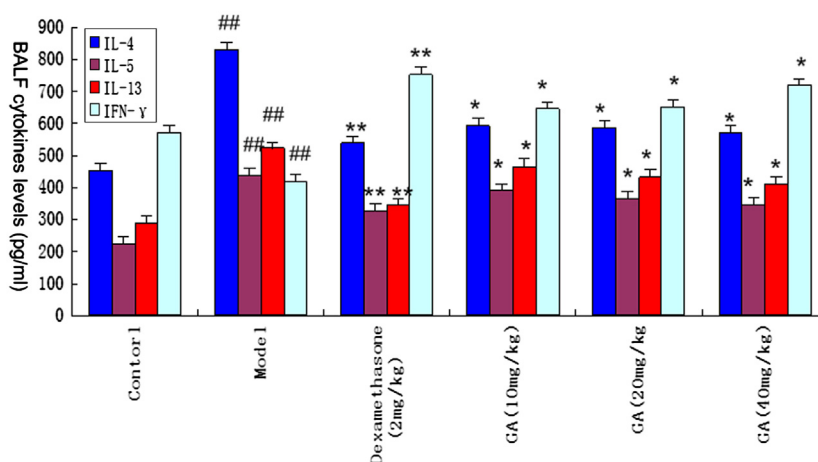


Fig. 6. Effect of GA on cytokine levels in BAL fluid. Values are expressed as means $\pm$ SDs. Compared with control: $^{\#} P < 0.05$, $^{##} P < 0.01$; Compared with model: $^{*} P < 0.05$, $^{**} P < 0.01$. Columns, mean of six independent experiments; bars, SD.

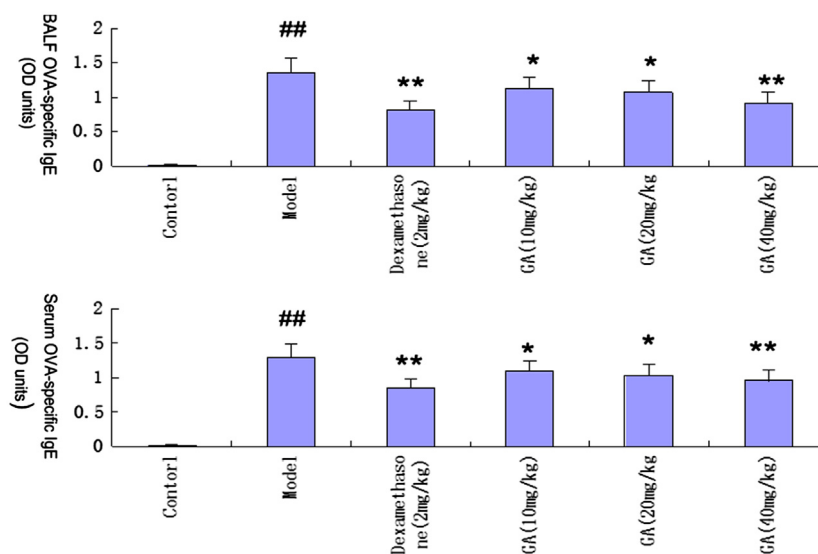


Fig. 7. Effect of GA on OVA-specific serum and BALF levels of IgE. Values are expressed as means $\pm$ SDs. Compared with control: $^{\#} P < 0.05$, $^{##} P < 0.01$; Compared with model: $^{*} P < 0.05$, $^{**} P < 0.01$. Columns, mean of six independent experiments; bars, SD.

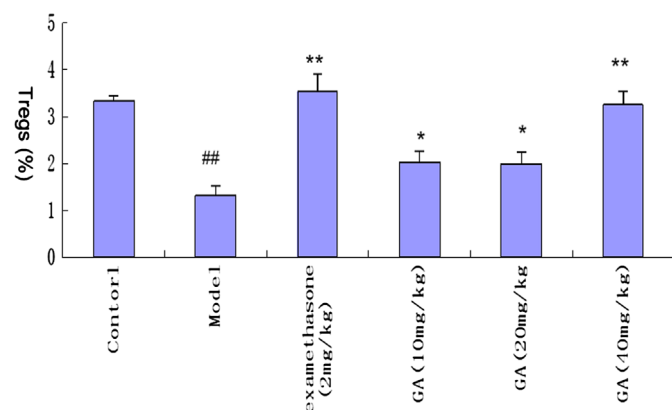


Fig. 8. Effect of GA on Tregs. Values are expressed as means $\pm$ SDs. Compared with control: $^{\#} P < 0.05$, $^{##} P < 0.01$; Compared with model: $^{*} P < 0.05$, $^{**} P < 0.01$. Columns, mean of six independent experiments; bars, SD.

commonly used to treat asthma, but the therapeutic efficacies and modes of action of such medicines are currently unclear. Here, using an OVA-induced mouse model of asthma, we show for the

first time that GA, a famous classical formula, 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 muscle (ASM), and inflammation within the airway, leads to decreased lung function (Wenzel, 2006). AHR is a measure of the bronchial constriction commonly observed in individuals with asthma. We monitored the effects of GA on AHR by measuring Raw. These experiments disclosed that GA inhibited the OVA-induced AHR in response to inhaled methacholine.

The mechanisms of recruitment of inflammatory cells associated with and presumably causing AHR have been well-studied. Migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation (Elsner and Kapp, 1999). A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that GA 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

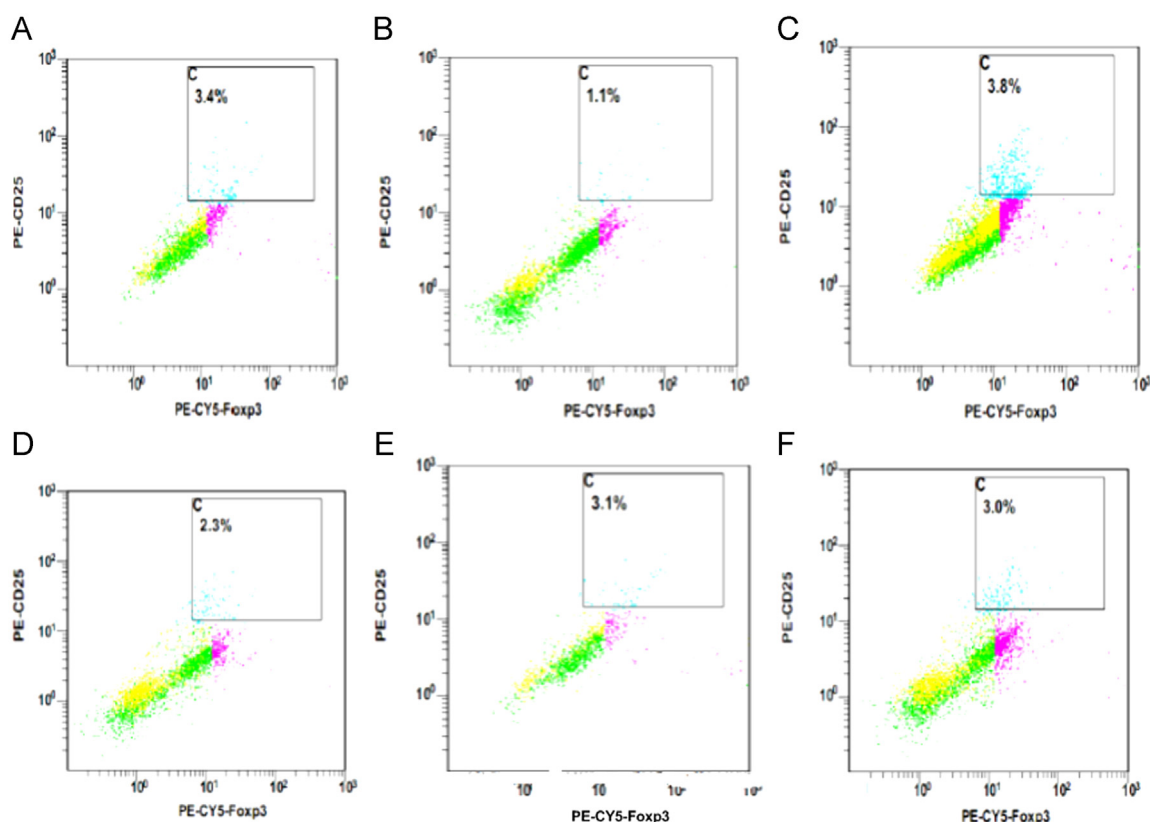


Fig. 9. Effect of GA on Tregs. A (control); B (model); C (dexamethasone, 2 mg/kg); D [GA(10 mg/kg)]; E [GA(20 mg/kg)]; F [GA(40 mg/kg)].

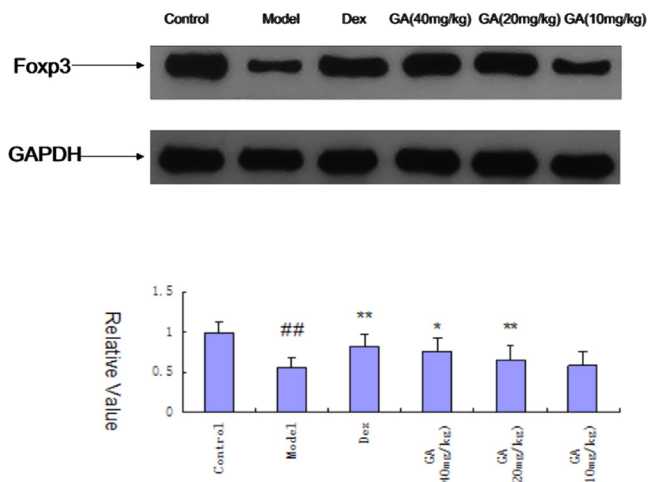


Fig. 10. Effect of GA on Foxp3. A (control); B (model); C (dexamethasone, 2 mg/kg); D [GA(10 mg/kg)]; E [GA(20 mg/kg)]; F [GA(40 mg/kg)].

administration of TH1 cytokines, such as IFN- γ , at the time of sensitization can inhibit the induction of AHR and TH2-driven inflammation (Gavett et al., 1995; Sur et al., 1996). 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 GA significantly suppresses IL-4, IL-5 and IL-13 cytokine levels; and significantly increases IFN- γ cytokine levels.

CD4⁺ CD25⁺ regulatory T (Treg) cells has recently been described as one distinct subsets which are different from Th1 and Th2 cells. Treg cells expressing the forkhead/winged helix transcription factor (Foxp3) have anti-inflammatory properties and maintain tolerance

against self components by contact dependent suppression or releasing anti-inflammatory cytokines, such as interleukin (IL)-10. The Tregs plays an important role in regulating the immune response. Tregs can reduce inflammation and allergic reactions through their inhibitory functions. Using a murine model of asthma (Doganci et al., 2005) provided evidence that the diminished levels of Tregs resulted in increased airway hyperreactivity, thus linking Tregs to airway physiology (Seroogy and Gern, 2005). Given the capacity of Tregs to suppress disease-promoting immune responses, enhancing Treg function in patients represents an attractive therapeutic strategy for treating allergic diseases, including asthma. In this study, our data show that GA significantly increased Tregs.

5. Conclusion

In this study, GA effectively suppressed IL-4, IL-5 and IL-13, improved IFN- γ and inhibited both the recruitment of eosinophils and mucus overproduction, in mice with OVA-induced asthma. Airway inflammation and bronchial hyperresponsiveness were attenuated in the presence of GA. Moreover, GA effectively increased Tregs. Our findings support the possible use of GA as a therapeutic drug for patients with allergic asthma.

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