



Geniposide inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma



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ABSTRACT

Our group recently reported the strong anti-inflammatory effects of geniposide (Gen), a bioactive iridoid glucoside derived from *gardenia jasminoides*, in a mouse acute lung injury model. Herein, we hypothesized that Gen might also have potential therapeutic benefits in treatment of asthma, which was tested in a mouse model of ovalbumin (Ova)-induced allergic airway inflammation. Ova-sensitized and -challenged BALB/c mice, as compared with control animals, displayed airway hyperresponsiveness (AHR), bronchoalveolar lavage eosinophilia, mucus hypersecretion, and increased T help 2 (Th2)-associated cytokine and chemokine amounts, as well as serum Ova-specific immunoglobulin E (IgE) level. Being compared with the Ova-induced hallmarks of asthma, intraperitoneal Gen treatment prevented eosinophilic pulmonary infiltration, attenuated the increases in interleukin (IL)-4, IL-5, and IL-13, and reduced eotaxin and vascular cell adhesion molecule 1 (VCAM-1) expression. Also, Gen significantly ameliorated the Ova-driven airway hyperresponsiveness, mucus hypersecretion, and allergen-specific IgE level, which are the cardinal pathophysiological symptoms in allergic airway diseases. In addition, the efficacy of Gen was comparable to that of dexamethasone (Dex), a currently available anti-asthmatic drug. Collectively, our findings reveal that the development of immunoregulatory strategies based on Gen may be considered as an effective adjuvant therapy for allergic asthma.

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1. Introduction

Cumulative evidence revealed that the worldwide prevalence and severity of allergic asthma have increased substantially over the last decades, especially in industrialized countries [1–3]. Allergic asthma is caused by a T helper 2 (Th2)-type cell-mediated immune response to common environmental allergens and is defined as a chronic inflammatory disease characterized by aggravation of airway inflammation, mucus hypersecretion, airway eosinophilia, elevated IgE levels, and airway hyperresponsiveness (AHR) [4–6]. Although multifactorial in origin, asthma is considered an inflammatory process feature of a relative excess of Th2 cytokines during each immune response to innocuous antigens [7,5]. These Th2 responses are associated with mast cells, B lymphocytes, and eosinophils, as well as an array

of inflammatory cytokines and chemokines, especially interleukin (IL)-4, IL-5, and IL-13 [8]. Furthermore, chemokine such as eotaxin is vital for the delivery of eosinophils to the airways. Accordingly, airway eosinophilia, together with Th2-type cytokines IL-4, IL-5, and IL-13, may ultimately contribute to AHR in asthma [9]. Emerging evidence has revealed that these Th2-type cytokines in turn cause AHR, vascular leakage, inflammatory cell infiltration, mucus hypersecretion, and airway remodeling including goblet cell hyperplasia and airway wall thickening, starting a cycle of events resulting in lung tissue damage which contribute to the development and aggravation of asthma [10,11]. Intriguingly, existing studies support the notion that a possible approach to treatment of asthma has evolved from anti-inflammatory strategies [12,8], although there is limited evidence pointing to therapies for asthma through controlling the process of airway inflammatory reaction.

Despite a growing understanding of the pathophysiology of asthma, its molecular regulatory mechanisms in induction of cytokines expression and activation/recruitment of inflammatory cells in asthma remain elusive [6]. However, there is a need for innovative anti-inflammatory therapeutic protocols and some previous studies

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have shown that asthma is associated with persistent activation of nuclear factor κ B (NF- κ B) [13–15]. Also, inspired by our previous studies [12,16], remedies with anti-inflammatory properties based on modification of NF- κ B signaling pathway may therefore represent a potentially useful alternative. NF- κ B, a pleiotropic transcription factor, is abundant of p50 (NF- κ B1)/p65 (RelA) heterodimer and plays a critical role in immune and inflammatory responses. It has been known to be present in most cell types and many of the inflammatory proteins expressed in asthmatic airway are regulated, at least partially, by NF- κ B [17]. In addition, a previous investigation has reported that eosinophil migration is involved in the inflammatory process of asthma by suppressing the NF- κ B pathway [18]. Therefore, there is growing recognition that NF- κ B has emerged as a promising molecular target for the treatment of asthma. Likewise, growing evidence suggests that numerous components of Chinese medicinal herbs exert excellent anti-inflammatory effects through the negative regulation of NF- κ B signaling pathway [19–21].

Gardenia jasminoides Ellis, which is also called Zhi-Zi in the Chinese pharmacopoeias, has been included in traditional medicine formulations for its anti-inflammatory and antioxidant properties [22,23]. Geniposide (Gen) is the major iridoid glycoside constituent of gardenia herbs and is mainly responsible for its pharmacological activities, such as the inhibition of ovalbumin-induced junction permeability and the recovery of transepithelial electrical resistance in guinea pig tracheas, which highlight the potential of Gen as an anti-asthmatic drug [24]. Our recent report has demonstrated that Gen can strongly protect mice from lipopolysaccharide (LPS)-induced acute lung injury at a dose of 80 mg/kg [19]. Also, Gen can block high glucose-induced cell adhesion through the nuclear factor-kappaB (NF- κ B) signaling pathway in human umbilical vein endothelial cells [25]. There is no available study that evaluates the effectiveness of Gen for reducing airway inflammatory reactions and improving asthma symptoms.

Considering that Gen is an ingredient of many traditional Chinese preparations and has also been widely utilized for food colorants in oriental countries for many years [26]. The potential therapeutic value and possible food intake of Gen may exert its functional benefits in asthma. Herein, we performed this study to examine the effect of Gen on an Ova-induced airway inflammation model in mice and its underlying mechanism, attempting to provide a new potential treatment for allergic asthma from traditional Chinese herbal medicine.

2. Materials and methods

2.1. Animals and materials

Female BALB/c mice, weighing approximately 18 to 20 g, were purchased from Shanghai Jingke Industrial Co., Ltd. (Certificate: SCXK2003-0003; Shanghai, China). Mice were housed in sterile microisolator cages with filtered air and autoclaved bedding, food, and water. Mice were allowed to acclimatize for 1 wk before beginning the experiments. The laboratory temperature was maintained at $24 \pm 1^\circ\text{C}$, and relative humidity was maintained at 40–80%. The experiments were approved by the Ethical Committee on Animal Research of Jilin University. All animal experiments were performed in accordance with the guide for the Care and Use of Laboratory Animals established by the US National Institutes of Health. No mice were dead and no apparent signs of exhaustion were observed during the experimental period.

Geniposide (Gen, purity: >98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ova (Grade δ), Aluminium hydroxide adjuvant and Dexamethasone (Dex, purity: >99.6%) were provided by Sigma-Aldrich Trading Co., Ltd. (St. Louis, MO, USA). Mouse interleukin (IL)-4, IL-5, IL-13, eotaxin, and vascular cell adhesion molecule 1 (VCAM-1) ELISA kits were obtained from Biolegend (San Diego, CA, USA). The ELISA kit for immunoglobulin (Ig) E was purchased from

R&D (Anniston, AL, USA). The primary antibodies including phosphorylated and non-phosphorylated forms of I κ B and nuclear factor-kappaB (NF- κ B) and the horseradish peroxidase-labeled IgG secondary antibodies were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). β -actin was provided by Tianjin Sungene Biotech Co., Ltd (Tianjin, China). Other chemical reagents were obtained from Beijing Dingguo Changsheng Biotech Co., Ltd (Beijing, China). All these reagents were of analytical grade, unless otherwise specified.

2.2. Experimental design

2.2.1. Allergen sensitization/challenge protocol

Mice were randomly assigned to four groups ($n = 8$): (1) Cont group; (2) Ova group; (3) OvaGen group; (4) OvaDex. Hereafter, we used these group abbreviations to clarify the text. Mice were immunized on days 1, 7, and 14 by intraperitoneal (*i.p.*) injection of 20 μ g Ova emulsified in 1 mg aluminium hydroxide adjuvant in a total volume of 0.2 mL [27]. On days 23, 24, 25, and 26 after the initial sensitization, mice were prepared for challenge by anesthetizing with an *i.p.* injection of 0.2 mL of a mixture of ketamine (0.44 mg/mL) and xylazine (6.3 mg/mL) in normal saline. Mice were placed on a board in the supine position. Subsequently, mice were intranasally challenged with 2% (w/v) Ova solution in phosphate-buffered saline (PBS, pH = 7.2), as described previously with minor modifications [28]. Control mice received equivalent volumes of PBS without Ova, *i.p.*, on days 1 and 14 and were challenged with PBS without Ova (w/v) each day from days 23 to 26 on these consecutive days. Airways hyperresponsiveness (AHR) was measured 24 h after the final Ova challenge on day 27 and then mice were sacrificed the following day to characterize the protective effects of Gen. The schematic diagram of the treatment schedule is presented in Fig. 1.

2.2.2. Administration of drugs

The dose of Gen was based on the results of our previous study [19]. We administered Gen to mice at doses of 20, 40, and 80 mg/kg to evaluate the anti-inflammatory properties of Gen using a mouse model of acute lung injury (ALI). Our findings suggest that Gen at the dose of 80 mg/kg exerts strong protective effects against LPS-induced ALI. Thus, in our present study, mice were given an intraperitoneal injection of normal saline or Gen (80 mg/kg) on days 23, 24, 25 and 26, 1 h prior to each corresponding Ova administration [12]. Dex (2 mg/kg), a steroid hormone drug of the glucocorticoid class, was a potent inhibitor of airway inflammation and remodelling [29,16]. Thus, in the present study, Dex was used as a positive control.

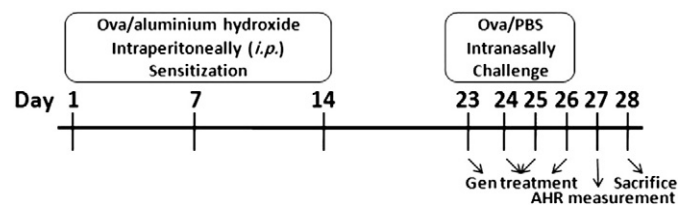


Fig. 1. Experimental protocol for development of allergic asthma and treatment with Gen or Dex using a murine model. Mice were divided into four groups ($n = 8$) and sensitized via an intraperitoneal injection of 20 μ g Ova emulsified in 1 mg aluminium hydroxide in 200 μ L PBS on days 0, 7, and 14, respectively. Subsequently, mice were given an intranasal instillation with 2% (w/v) Ova solution in PBS on days 23, 24, 25, and 26 after the initial sensitization. Mice were given an intraperitoneal injection of Gen (80 mg/kg, dissolved in normal saline) or Dex (2 mg/kg, diluted in normal saline) each day from days 23 to 26 consecutively, 1 h prior to each corresponding Ova challenge. Control mice were sensitized and challenged with equivalent volumes of PBS without drug administration. AHR assay was performed 24 h after the last Ova challenge. Then all mice were sacrificed for further experiments.

2.3. Collection of blood and bronchoalveolar lavage (BAL) fluid and lung tissues separation

Twenty-four hours after the last challenge, mice were anesthetized and bled via the brachial plexus for collection of blood samples used to estimate IgE production. Collection of BAL fluid was performed three times through a tracheal cannula with 0.5 mL of autoclaved phosphate buffered saline (PBS, pH = 7.2) to yield a total volume of 1.3 mL. The recovery ratio of the fluid was about 85%. The collected BAL fluid samples were centrifuged at 700 $\times$ g for 10 min at 4 °C and their supernatants were frozen at –80 °C for enzyme-linked immunosorbent assay (ELISA). Their cell pellets were then treated and further used as samples for inflammatory cells studies. Lung tissues were harvested simultaneously from mice not subjected to BAL fluid collection, then treated or stored at –80 °C for the next experiments.

2.4. Mouse anti-Ova IgE measurement

The concentrations of Ova-specific-IgE in serum were measured using an ELISA method as described previously [12]. Briefly, Microtiter plates were coated with 1% Ova in coating buffer (0.05 M sodium carbonate–bicarbonate, pH 9.6) overnight at 4 °C. After blocking and washing, diluted serum samples (1/20) were added at room temperature for 2 h, then washed and incubated with biotinylated anti-mouse IgE, followed by another multiple washings and incubation for 30 min with extravidin-peroxidase. For colour development, 3,3',5,5'-tetramethylbenzidine (TMB) substrate were used, the reaction was stopped by 2 M H₂SO₄. OD values were read at 450 nm using a Benchmark microplate reader (Bio-Rad, Richmond, CA, USA).

2.5. Bronchoalveolar lavage (BAL) fluid analysis

2.5.1. Differential cell counts

The cell pellets were again resuspended in 1 mL of red blood cell lysis buffer to dismiss red cells. Subsequently, leukocytes were repelleted by centrifugation at 700 $\times$ g for 10 min at 4 °C. Then these white cell pellets were again resuspended in 1.0 mL of PBS (pH = 7.2) and stained with the Kwik-Diff staining kit (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). Next, a total of 200 cells in each sample were taken for differential cell counts, including eosinophils, neutrophils and macrophages, using a hemocytometer.

2.5.2. Measurement of cytokines and chemokines

Concentrations were determined in duplicates for each sample. Levels of the cytokines, including IL-4, IL-5, IL-13, eotaxin, and VCAM-1 in BAL fluid samples, were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the instructions recommended by the manufacture.

2.6. Lung histology

For histopathologic assessment, lungs from mice that did not undergo BAL were removed by dissection and placed in 4% paraformaldehyde for 24 h. Lung tissues were sectioned, embedded in paraffin, and cut into 3 μ m sections. Subsequently, tissue sections were stained with Alcian blue-periodic acid sthiff (AB-PAS) for identifying goblet cells in the epithelium and measuring mucus production [30,31], also, with haematoxylin and eosin (H&E) for infiltration [32]. Photomicrographs were cropped and corrected for brightness and contrast, but not otherwise manipulated. Semi-quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed as previously described [14]. Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were evaluated blind graded on a 5-point scoring system: 0, no cells; 1, a few cells; 2, a ring of cells one cell layer deep; 3, a ring of cells two to four cells deep; 4, a ring of cells of more than four cells deep. To determine the magnitude

of mucus production, goblet cell hyperplasia in the airway epithelium was quantified blind using a 5-point grading system: 0, no goblet cells; 1, 25%; 2, 25 to 50%; 3, 50 to 75%; 4, 75%. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section.

2.7. Measurements of airway hyperresponsiveness

Airway responsiveness of mice to increasing concentrations of aerosolized methacholine was measured 24 h after the last Ova instillation. Mice were anesthetized and tracheotomy was performed as described in detail previously [33]. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Baseline airway resistance (RI) and lung compliance (C_{dyn}), as well as responses to aerosolized saline (0.9% NaCl), were recorded first, followed by responses to the increasing doses of aerosolized methacholine (6.25, 12.5, 25, and 50 mg/mL; Sigma, St Louis, MO) in saline using a whole-body plethysmograph system (Buxco, Sharon, CT) [33]. RI is represented as the main indicator of airflow obstruction. C_{dyn} indicates the distensibility of the lung which is defined as the change in volume of the lung driven by a change in pressure across the lung. Results are expressed as a percentage of the respective basal values in response to saline.

2.8. Western blot analysis

Lung tissues were added to Cell Lysis Buffer for Western and IP (Beyotime, Shanghai, China) and homogenized on ice with protease and phosphatase inhibitors (Boehringer, Mannheim, Germany) following the manufacturers' instructions respectively. The homogenate was centrifuged at 14,000 $\times$ g for 10 min at 4 °C, the supernatant was aliquoted and then stored at –80 °C after removing a small aliquot for protein quantification. Protein concentrations were determined using a BCA protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Equivalent amount of 80 μ g protein was loaded in each lane of the immunoblot, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF). After washing with Tris-buffered saline with 0.1% Tween-20 (TBST), immunoblots were probed with the primary antibodies, including phosphorylated or non-phosphorylated forms of I κ B and NF- κ B (Cell Signaling Technology, Beverly, MA), and β -actin (Sungene, Tianjin, China). With the use of a peroxidase-conjugated secondary anti-mouse or -rabbit antibodies after another three times washings, bound antibodies were developed using an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK). Pre-determined molecular weight standards were used as markers (Beyotime, Shanghai, China).

2.9. Statistical analysis

The values were entered into a database and analyzed using SPSS software (SPSS for Windows version 13.0, Chicago, USA), and expressed as means $\pm$ SEM. Statistically significant differences between groups were determined by ANOVA followed by Student's *t* test. Survival date was presented by the Kaplan-Meier text and comparisons were made by the log rank test. Significance was accepted when *P* < 0.05.

3. Results

3.1. The effect of Gen on Ova-Specific Serum IgE Level

Ova-induced serum specific IgE level was analyzed by a sandwich enzyme-linked immunosorbent assay (ELISA). Ova-sensitized mice showed higher levels of serum anti-Ova IgE antibodies compared to PBS control mice as shown in Fig. 2A (vs. Cont group, **P* < 0.01).

However, a significant reduction in Ova-specific IgE antibodies was observed in mice treated with Gen at dose of 80 mg/kg (vs. Ova group; $^*P < 0.01$). Additionally, the protection of Gen at the dose of 80 mg/kg was comparable with that of Dex treatment at 2 mg/kg (vs. OvaDex group, $P > 0.05$).

3.2. The effect of Gen on the release of eotaxin, VCAM-1, and Th 2-driven cytokines in BAL fluid

As illustrated in Fig. 2B, C, and D, Ova sensitization and challenge induced significant elevation of chemokines and cytokines in the BAL fluid (vs. Cont group, $^{\#}P < 0.01$). Treatment with Gen (80 mg/kg) significantly decreased the concentrations of eotaxin, VCAM-1, IL-4, IL-5, and IL-13 (vs. Ova group; $^*P < 0.01$). In addition, Dex (2 mg/kg) developed a comparable reduction in the levels of these Th2-driven cytokines in BAL fluid (vs. OvaDex group, $P > 0.05$).

3.3. The effect of Gen on inflammatory cell recruitments in BAL fluid

Inflammatory cell levels (i.e., total cells, eosinophils, neutrophils, and macrophages) in BAL fluid were significantly elevated in Ova-challenged mice versus (vs.) PBS control mice as shown in Fig. 2E (vs. Cont group, $^{\#}P < 0.01$). Gen treatment significantly reduced the number of total cells, eosinophils, neutrophils, lymphocytes, and macrophages (vs. Ova group; $^*P < 0.01$). Furthermore, our applied Gen dose (80 mg/kg) for experimental sensitized mice is comparable to effect caused by Dex at the dose of 2 mg/kg with earlier findings (vs. OvaDex group, $P > 0.05$).

3.4. The effect of Gen on airway inflammation, hyperplasia with hypertrophy of goblet cells, and mucus overproduction

To interpret the anti-inflammatory property of Gen, histopathological studies were performed. The inflammatory cells infiltration into peribronchial and perivascular areas was observed in Ova-challenged mice as compared with PBS controls using H&E staining. Treatment with Gen (80 mg/kg) or Dex (2 mg/kg) markedly reduced the magnitude of inflammatory cells infiltration into the peribronchiolar and perivascular connective tissues as compared with Ova group (Fig. 3A). In order to estimate goblet cell hyperplasia, we stained lung sections with AB-PAS staining. Overproduction of mucus, hyperplasia with hypertrophy of goblet cells, and epithelial cells disruption were observed in the bronchial airways of Ova-treated mice (Fig. 3B). Compared with Ova group, Gen or Dex significantly alleviated mucus secretion, goblet cell hyperplasia, and epithelial cell disruption and no obvious differences were observed between Gen- and Dex-treated groups. Semi-quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed as previously described [14], as illustrated in Fig. 3C and D.

3.5. The effect of Gen on airway hyperresponsiveness to methacholine

Attempting to investigate the effect of Gen (80 mg/kg) on airway hyperresponsiveness in response to increasing concentrations of methacholine, we measured airway resistance (RI) and lung compliance (Cdyn) parameters in mechanically ventilated mice. As illustrated in Fig. 4A, Cdyn was markedly decreased in Ova-challenged mice compared to PBS controls (vs. Cont group, $^{\#}P < 0.01$). This down-regulation

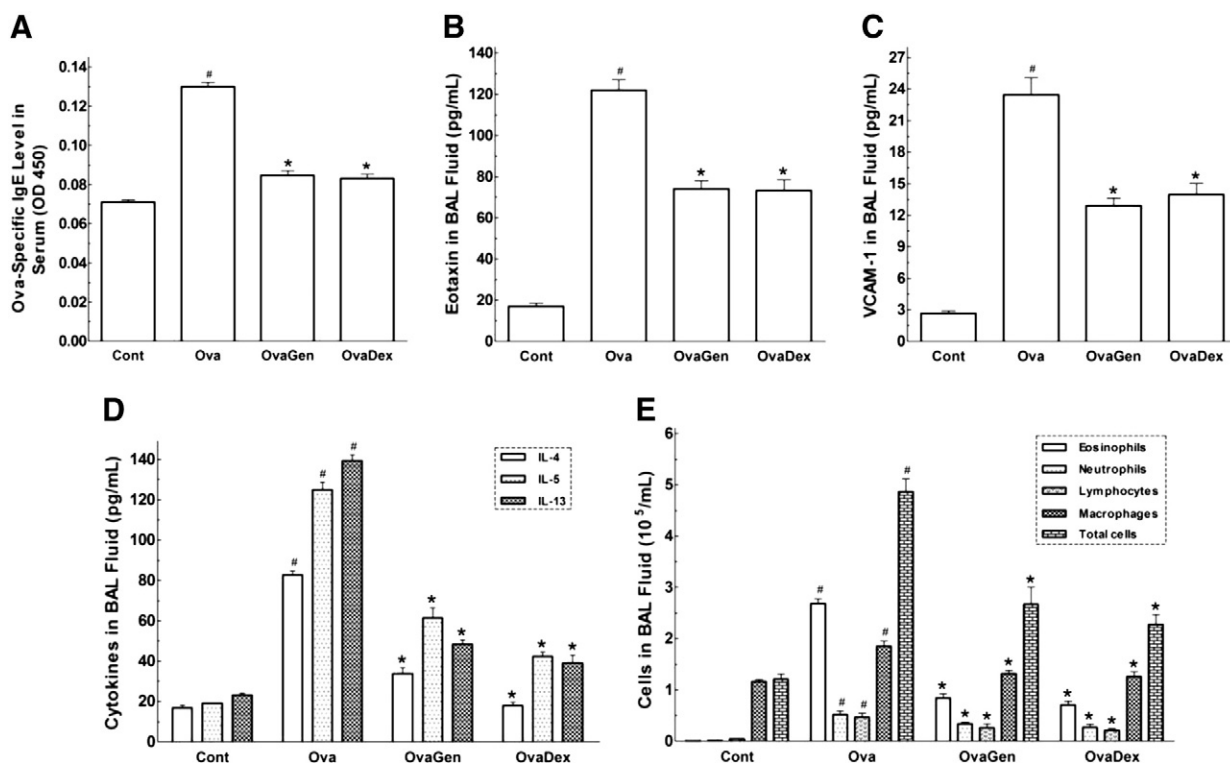


Fig. 2. The effect of Gen on serum and BAL fluid assessments. Samples of serum and BAL fluid were collected 24 h after the last Ova aerosol challenge. Levels of (A) Ova-Specific Serum IgE, (B) eotaxin, (C) VCAM-1, and (D) various cytokines were analyzed using specific ELISAs. (E) Effect of Gen on the recruitment of inflammatory cells in BAL fluid by Kwik-Diff staining. The lavage fluid was centrifuged and red cells were dismissed using its exclusive cell lysis buffer, and then the cell pellets were resuspended in 1 mL of PBS and applied to a slide by cytospinning to obtain differential cell counts. A total of 200 cells were counted per slide. The values presented are the means $\pm$ SEM ($^{\#}P < 0.01$ vs. Cont group; $^*P < 0.01$ vs. Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; $n = 8$).

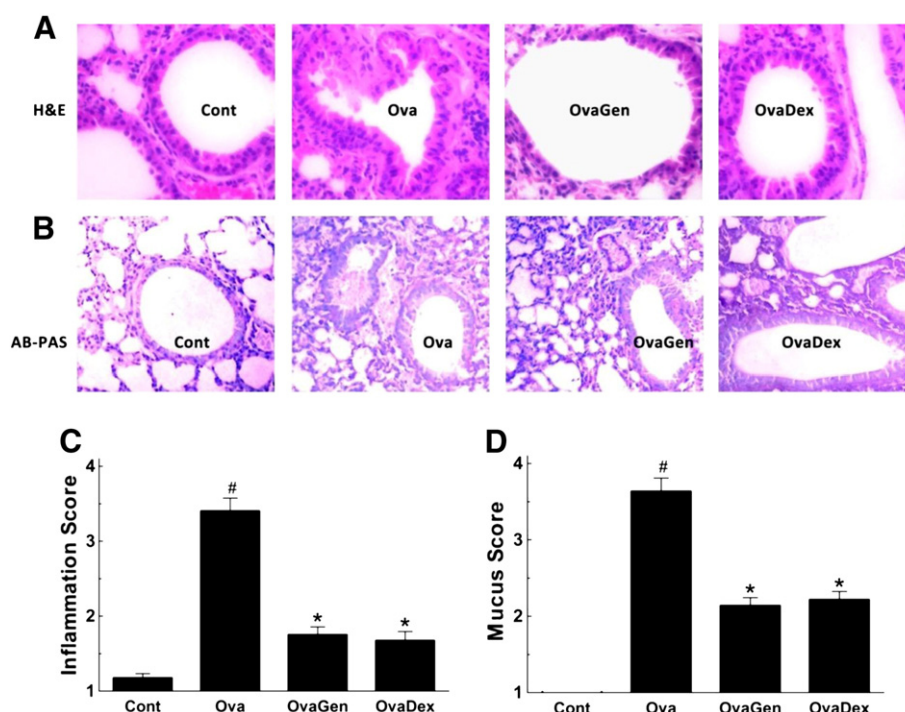


Fig. 3. Effect of Gen on (A) airway inflammation (H&E, magnification $\times 400$) and (B) mucus hypersecretion (AB-PAS, magnification $\times 200$). Exposure to Ova increases airway inflammation and antigen-specific IgE level in sensitized BALB/c mice. Mice were immunized with Ova (Day 1, 7, and 14) and then challenged with Ova on Day 23, 24, 25, and 26 through the experimental protocol of 27 days. Mice were given an intraperitoneal injection of Gen (80 mg/kg) or Dex (2 mg/kg) each day from days 23 to 26 consecutively, 1 h prior to each corresponding Ova challenge. Control mice were sensitized and challenged with equivalent volumes of PBS without Ova and received the same volumes of normal saline. Semi-quantitative analyses of (C) inflammatory cell infiltration and (D) mucus production in lung sections were performed as previously described [14]. Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were evaluated blind graded on a 5-point scoring system: 0, no cells; 1, a few cells; 2, a ring of cells one cell layer deep; 3, a ring of cells two to four cells deep; 4, a ring of cells of more than four cells deep. To determine the magnitude of mucus production, goblet cell hyperplasia in the airway epithelium was quantified blind using a 5-point grading system: 0, no goblet cells; 1, 25%; 2, 25 to 50%; 3, 50 to 75%; 4, 75%. The scoring data of inflammatory cells and goblet cells were assessed in at least three different fields for each lung section. The values presented are the means $\pm$ SEM ($^{\#}P < 0.01$ vs. Cont group; $^*P < 0.01$ vs. Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; $n = 8$).

was also effectively prevented by Gen administration (vs. Ova group, $^*P < 0.01$). Also, as shown in Fig. 4B, the RI of Ova-challenged mice were dramatically increased by methacholine challenge compared to that of negative control mice (vs. Cont group, $^{\#}P < 0.01$). However, this up-regulation of RI in Ova-sensitized mice was effectively reduced by Gen (80 mg/kg) pretreatment (vs. Ova group, $^*P < 0.01$). Furthermore, the regulative effects of Gen on RI and Cdyn values at the dose of 80 mg/kg was comparable as those of Dex treatment at 2 mg/kg (vs. OvaDex group, $P > 0.05$).

3.6. The effect of Gen on NF- κ B signaling pathway activation

Within the asthmatic lung, the transcription factor NF- κ B has been implicated in the regulation of airway inflammation, Th2 cell differentiation and recruitment, and elevation of Ova-Specific Serum IgE secretion [34]. In our present study, Western blot analysis revealed that Gen can modify NF- κ B activity. But to achieve this general result we need more research to verify the hypothesized Gen might also have potential therapeutic benefits in treatment of asthma associating with suppression of NF- κ B signaling pathway (figures of Western blot analysis not shown).

4. Discussion

Geniposide (Gen), which is a bioactive iridoid glucoside derived from *Gardenia jasminoides*, has anti-inflammatory effects [23,25]. Nevertheless, the effects of Gen on airway inflammation are still incompletely understood. Our present study was designed to explore whether the negative regulation of NF- κ B by Gen would affect Ova-challenged

inflammatory responses in the airways. To this end, mice were given an intraperitoneal injection of Gen at dose of 80 mg/kg 1 h prior to each corresponding Ova administration. The results show that treatment with Gen before allergen sensitization attenuates the development of AHR with significantly affecting the severity of pulmonary allergic inflammation (BAL fluid eosinophilia, elevated Th2-driven cytokine and serum IgE levels, mucus hypersecretion, and pathological changes). In agreement herewith, Fleming and coworkers demonstrated that differences in NF- κ B activation following Ova challenge were not directly associated with alterations in AHR [35]. However, changes of Th2-associated cytokines in BAL fluid were detected, suggesting the underlying mechanisms for attenuation of AHR. Although data from our present study showed that Gen has a potent protective effect on an allergic airway inflammation model, its exact molecular mechanisms are still not well established.

It is well-known that allergic airway inflammation process and AHR development involve multiple inflammatory cells and a wide array of mediators [14]. Likewise, the overall effects of Gen on inflammation are mediated primarily by down-regulating the synthesis of a number of cytokines. In our experiments, the concentrations of IL-4, IL-5, and IL-13 were significantly elevated by airway exposure to Ova, but these levels of Th2-type cytokines were markedly inhibited by Gen administration as compared with those of the Ova-induced group. With regard to IL-4, Lee and colleagues have shown that it also plays critical roles in eosinophil immigration into lung tissue and stimulates mucus hypersecretion and AHR response [15]. IL-5, a product of Th2 cells, has been shown to provide an essential signal for the survival and mobilization of eosinophils from the bone marrow into the lung after allergen challenge as well as development of

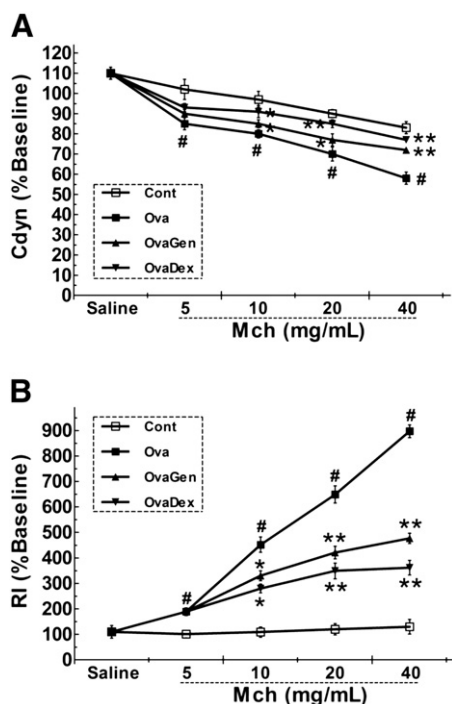


Fig. 4. Effect of Gen on AHR in response to increasing concentrations of aerosolized methacholine (5, 10, 20, and 40 mg/mL) or normal saline was analyzed 24 h after the last Ova instillation in mechanically ventilated mice. Airway responsiveness was assessed by percentage change from the baseline level of (A) Cdyn, defined as the change in volume of the lung produced by a change in pressure across the lung and (B) RI, viewed as the pressure driving respiration divided by flow. The values presented are the means $\pm$ SEM (* $P < 0.01$ vs. Cont group; * $P < 0.05$ and ** $P < 0.01$ vs. Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; $n = 8$).

AHR [1]. Specifically, IL-13 is by far the most potent inducer of eosinophil infiltration into lung tissue [7,14]. Importantly, Gen induced a reduction in the levels of IL-4, IL-5, and IL-13 was consistent with the inflammatory cell count in BAL fluid. Furthermore, the recruitment of eosinophils into the airways is also regulated by the expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) by endothelial cells [1]. In agreement herewith, our results clearly show that Ova-induced VCAM-1 and ICAM-1 expression is reduced by Gen. Given that eosinophilic infiltration of the airway is a characteristic feature of allergic asthma, it is tempting to speculate that Gen alleviates airway inflammation by decreasing the severity of eosinophilia.

Previous studies demonstrated that multiple inflammatory cells play a central role in the pathogenesis of allergic inflammation [14,36,37]. Choi and coworkers demonstrated that eosinophils, neutrophils, and macrophages can participate in the development of allergic asthma by generating reactive oxygen species (ROS) [15]. Since eosinophilic infiltration of the airway is an important facet for asthma and allergic disease, we were interested to examine whether the beneficial effects could be potentiated by decreasing the severity of eosinophilia. As expected, our present results showed that Gen substantially prevented inflammatory cell infiltration into the airways as shown by a significant decrease in differential cell count in BAL fluid, which consists of eosinophil, neutrophil, macrophage, and total cell count. Moreover, these data are consistent with evaluations of hematoxylin and eosin (H&E)-stained lung sections. Also, it is believed that leukocyte transmigration into the airways is orchestrated by cytokines, such as IL-4, IL-5, and IL-13, and coordinated by specific chemokines, such as eotaxin and VCAM-1 [14,38]. Our results clearly show that Gen attenuates the overproduction of Th2-associated cytokines and down-regulates the expression of eotaxin and VCAM-1. Based on these findings, we propose that Gen interferes with the

recruitment of inflammatory cells by reducing the levels of chemokines and Th2-driven cytokines, providing a possible mechanistic explanation for the observed effects of Gen on the eosinophilic inflammation in a mouse model of asthma.

Histopathological alterations, such as goblet cell hyperplasia, mucus hypersecretion, and infiltration of inflammatory cells in lung tissue, are major characteristic features of asthma [15], which are also loosely related to the Th2 cytokines [39]. According to previous studies [40,41], these biological cytokines play critical roles in directing B-cell growth, differentiation, and secretion of IgE, which triggers mucus hypersecretion and goblet cell hyperplasia. Also, elevated serum Ova-specific IgE level is served as a hallmark of the Th2-driven immune response [14]. Klemm and coworkers and Galli and colleagues have shown that the action of IgE is implicated in its interaction with the FcεRI on mast cells and basophils and further cross-linking of FcεRI initiates multiple signaling cascades leading to NF-κB activation and production of lipid mediators, cytokines, and chemokines [42,43]. In concert with these findings, our present results showed that serum levels of Ova-specific IgE were substantially reduced by Gen in Ova-sensitized mice. These data are consistent with our measurements of Th2 cytokines that Gen induced a reduction in the levels of IL-4, IL-5, and IL-13. Thus, the inhibitory effect of Gen on elevated IgE levels appears to result from its suppression on Th2 cytokines, which are also known to induce AHR [44].

Airway hyperresponsiveness (AHR) is a major characteristic feature of asthma and the pathophysiologic consequence of the effect of inflammation process [8]. Likewise, we demonstrated that Gen effectively inhibited Ova challenge-induced AHR to methacholine. A possible explanation for this finding may be that Gen could modulate the AHR response via down-regulation of Th2 responses. Thus, we favor the likely possibility that alterations in cytokine milieu by Gen could account for its effect on AHR.

Persistent NF-κB activation plays an essential role in the immune and inflammatory process, including those of asthma [15]. Antigen receptor activation in T and B lymphocytes and mast cells culminates in NF-κB activation, resulting in abnormal overtranscription of a variety of inflammatory genes in asthma models. In addition, NF-κB is a critical transcription factor for Th2 cell differentiation and serves as a controller of Th2 cytokines in the pathogenesis of the allergic airway inflammation [14]. It is interesting that Choi and colleagues and Birrell and coworkers have provided insights into novel therapeutic strategies targeted at the NF-κB signaling pathway, such as p65-specific antisense oligonucleotide and IKKβ-selective small molecule inhibitor [45,46]. Inspired by these previous studies, we considered that a possible approach to the treatment of allergic asthma had evolved from remedies modifying NF-κB signaling pathway accompanied by alleviation of inflammatory reaction in airways. Growing experimental evidence suggests that numerous components of Chinese medicinal herbs exert excellent anti-inflammatory properties. To some extent, our results confirmed that Gen can modify NF-κB activity, possibly related to controlling the Ova-induced overproduction of pro-inflammatory mediators in the asthmatic lung tissue. However, more research is needed to explore its precise mechanism of NF-κB signaling pathway (figures of Western blot analysis not shown).

In brief, our data clearly show that exposure of mice to Ova developed the asthma-like phenotype. Prior treatment of Gen before antigen challenge suppressed Th2-type cytokines and serum IgE levels, attenuated excess eosinophilia and mucin overproduction, and improved the functional airway response to methacholine. The present findings suggest that the protective effect of Gen is associated with the modulation of inflammatory response and hyperresponsiveness, which provides novel insights into the disease mechanism underlying asthma. However, further studies should be carried out to assess the therapeutic effect of Gen administration before using it as a promising candidate in clinical practice.

Disclosure

All authors declare that they have no conflicts of interest.

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