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Cyanidin-3-*O*-β-glucoside attenuates allergic airway inflammation by modulating the IL-4Rα-STAT6 signaling pathway in a murine asthma model

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ABSTRACT

Cyanidin-3-*O*-β-glucoside (Cy-3-g), a typical and abundant monomer of anthocyanins, exhibits a variety of biological activities, such as anti-atherosclerosis, anti-obesity, and anticancer effects. However, to date little is known about its effects on asthma. This study aimed to investigate the efficacy of dietary Cy-3-g on allergic asthma in an animal model. BALB/c mice were sensitized and challenged with ovalbumin (OVA) to induce allergic asthma. The pathological changes of the lung tissues, type 2 helper (Th2)-associated cytokine production in bronchoalveolar lavage fluid (BALF), and the interleukin 4 receptor alpha (IL-4Rα)-signal transducer and activator of transcription 6 (STAT6) signaling pathway activities were assessed. We found that Cy-3-g significantly inhibited OVA-induced inflammatory cell infiltration and mucus hyper-production in lung tissues, reduced the production of interleukin 4 (IL-4), interleukin 5 (IL-5) and interleukin 13 (IL-13) in BALF. Furthermore, Cy-3-g effectively suppressed OVA-induced up-regulation of the IL-4Rα-STAT6 signaling pathway activity of the lung tissues. These results demonstrated that dietary Cy-3-g could attenuate allergic airway inflammation in a murine asthma model, and Cy-3-g might be used as an agent for asthma prevention and/or treatment in the future.

1. Introduction

Allergic asthma is a chronic inflammatory airway disorder characterized by increased inflammatory eosinophil infiltration, mucus hyperproduction, airway hyperresponsiveness (AHR), and overproduction of multiple pro-inflammatory type 2 helper T cell (Th2)-related cytokines [1]. About 235 million people over the world suffer from allergic asthma nowadays [2]. Bronchodilators and inhaled/oral corticosteroids are suggested to be the most effective pharmaceutical therapies for asthma. But their effectiveness is hampered by side effects after long-term use [3–5] and also depends on the severity of asthma [1]. Selective antibody-based treatments, such as anti-IgE and anti-IL-4 therapies [6], are novel biologic treatments for asthma. However, antibody-based treatments need to identify potentially responsive patients [7] based on specific biomarkers and the cost is high. Thus seeking alternatives safe, effective and affordable treatment for asthma is extremely urgent.

Recent studies highlighted that diet modification could play a role in improving allergic diseases and immune homeostasis [2]. Several clinical trials have indicated that high fat [8] and low fruit and vegetable [9] consumption can lead to exacerbated asthma outcomes. Besides, a Mediterranean diet, including high consumption of fruits,

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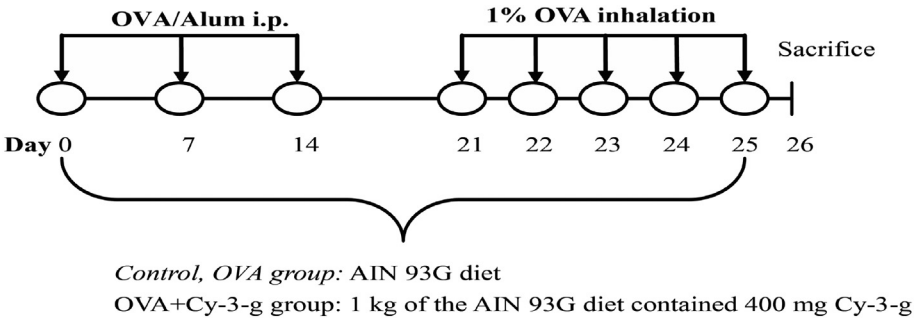

Fig. 1. Experimental protocols for the allergic asthma model and dietary intervention. Mice were sensitized with OVA and alum by intraperitoneal injection on days 0, 7, and 14 and then after a week challenged with aerosolized 1% OVA for 30 min per day on days 21, 22, 23, 24, and 25. Controls were sham-sensitized and challenged with PBS instead of OVA. Mice in control and OVA groups were fed with AIN 93G diet, while mice in OVA plus Cy-3-g group were fed with AIN 93G diet, except for 400 $mg \cdot kg^{-1}$ diet where Cy-3-g was mixed into the AIN 93G diet.

Table 1
Primers used in real-time PCR analyses of cytokine mRNA and transcription factors expression levels.

Gene	Primer forward (5′ → 3′)	Primer reverse (5′ → 3′)
IFN-γ	CTGCTGATGGGAGGAGATGT	TTTGTCATTCGGGTGTAGTCA
T-bet	CGTTTCTACCCCGACCTTCC	ATGCTCACAGCTCGGAACTC
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-5	TCAGGGGCTAGACATACTGAAG	CCAAGGAACTCTTGCAGGTAAT
IL-13	CAGCCTCCCCGATACCAAAA	CTCCTCATTAGAAGGGGCCG
GATA3	AAGCTCAGTATCCGCTGACG	GTTTCCGTAGTAGGACGGGAC
IL-17A	GGCTGACCCCTAAGAAACCC	AAGCAGTTTGGGACCCCTTT
RORc	TCCACTACGGGGTTATCACCT	AGTAGGCCACATTACACTGCT
Foxp3	AGCAGTGTGGACCGTAGATGA	GGCAGGGATTGGAGCACTT
β-Actin	TAGGCGGACTGTTACTGAGC	TGCTCCAACCAACTGCTGTC

vegetables, fish, olive oil and moderate consumption of red wine, may protect against asthma [10,11]. Some studies showed that dietary intervention may help prevent or attenuate asthma by inhibiting Th2 and/or Th17 responses and enhancing Treg activities and the protective effects of diet on asthma can be attributed to some dietary components, such as dietary fibers [12], short-chain fatty acids [13], vitamin D3 [14], and tryptophan [15]. Our previous study also demonstrated that curcumin and its metabolite tetrahydrocurcumin ameliorated allergic airway inflammation in asthmatic mice *via* inhibiting Th2 and Th17 responses [16]. Therefore, dietary components are promising to be new agents to treat or prevent allergic asthma.

Anthocyanins, a group of water-soluble plant pigments, are abundant in colorful fruits, vegetables, grains, and red wine [17]. The health benefits of anthocyanins include but not restrict to anti-atherosclerotic, anti-dementia, anticancer, anti-inflammatory, and antioxidant activities [18]. Epidemiological studies showed that dietary intake of anthocyanins was also associated with reduced lung inflammation [19,20]. Oral administration of cyanidin-3-*O*-β-glucoside (Cy-3-g), the most abundant monomer of anthocyanins, effectively inhibited scratching behaviors in histamine- or compound 48/80-induced mice [21]. In particular, Cy-3-g suppressed Th2 cytokines secretion and GATA3 expression in EL-4 T cells. Therefore, it is possible that Cy-3-g may ameliorate asthma *via* modulating the secretion of Th2-associated cytokines. Although anthocyanins-rich extracts have been found to attenuate lung inflammation in asthmatic mice [22,23], the underlying mechanisms are not yet

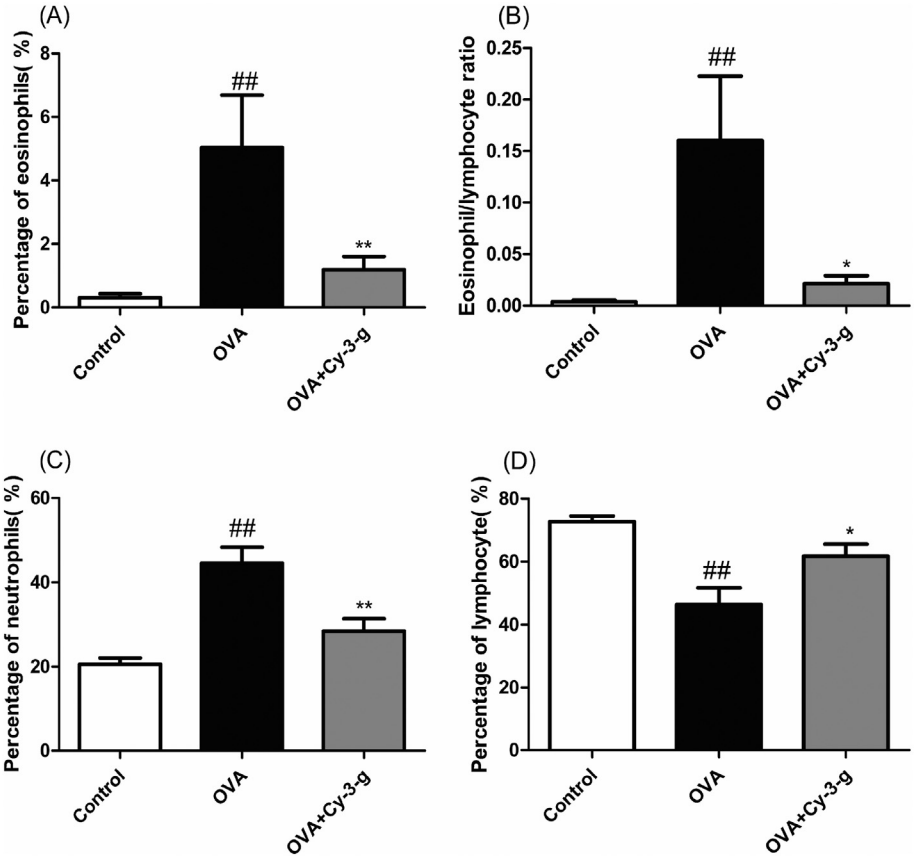

Fig. 2. Effects of Cy-3-g on the peripheral blood cells of OVA-induced mice. Blood eosinophil percentage (A), eosinophil/lymphocyte ratio (ELR) (B), neutrophil percentage (C) and lymphocyte percentage (D) in each group of mice. The values represent the mean ± SEM of three independent experiments (n = 8). $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the OVA group.

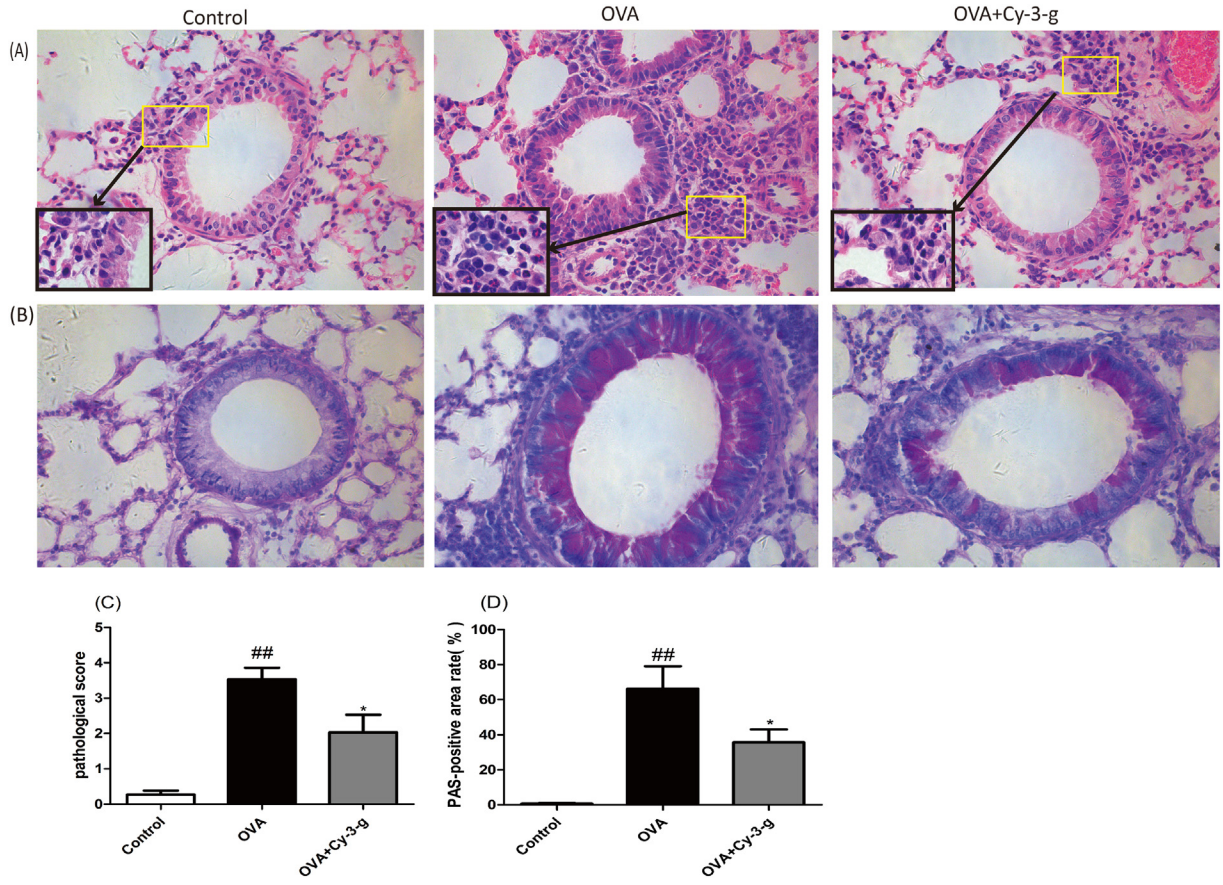

Fig. 3. Effects of Cy-3-g on lung tissues of OVA-induced mice. Histological sections of lung tissues from control, OVA and OVA + Cy-3-g group. Representative pictures of H&E staining (A) and PAS staining (B) of lung tissues for three groups. (A) Lung sections were stained with H&E to analyze the infiltration of inflammatory cells (400× magnification). Amplification areas (1000× magnification) were shown for the indicated areas. (C) Scoring of lung inflammation by pathological evaluation of inflammatory cell infiltration in lung sections; (D) PAS-positive area rate per bronchial was calculated. The values represent the mean ± SEM of three independent experiments (n = 5). $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$ compared to the OVA group.

fully investigated.

In this study, we aimed to investigate whether Cy-3-g would reduce pulmonary eosinophilic infiltration, mucus hypersecretion and production of Th2 cytokines by modulating the IL-4Rα-STAT6 signaling pathway in an OVA-induced allergic asthma model.

2. Materials and methods

2.1. Reagents

The IL-4, IL-5, IL-13, IFN-γ, IL-17A and IL-10 ELISA kits were purchased from eBioscience (San Diego, CA, USA). Rabbit monoclonal antibodies IL-4Rα, Jak1, p-Jak1, STAT6, p-STAT6, GATA3, β-actin and horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Grade V chicken egg OVA was purchased from Sigma-Aldrich (St. Louis, MO, USA). Alum was purchased from Thermo (Rockford, IL, USA).

2.2. Animals

Female BALB/c mice (14–16 g of body weight), 4 weeks of age, were purchased from Guangdong Medical Experimental Animal Center (Guangdong, China). All the mice were kept in the SPF-grade animal room, under 25 °C, with a 12-hour light/dark cycle, and were *ad libitum* accessed to water and chow. Body weight and food intake was recorded weekly. All animal experiments were performed in accordance with the Animal Care and Protection Committee of Sun Yat-Sen University (Certification No. SYXK [Yue] 2017-002).

2.3. Preparation of purified Cy-3-g

Cy-3-g was extracted from the skin of black rice (*Oryza sativa* L. *indica*, Guangdong, China) as previously described [24]. Briefly, the aleurone layer of black rice (about 10% outer layer of whole grain) was infused with 10 vol 60% ethanol (0.1% HCl) for 5 h. Extract was concentrated with a rotary evaporator at 40 °C until alcoholic residues were removed. After defatting with petroleum ether, the extract solution purified with an Amberlite XAD-7HP resin column (Rohmand Haas, Philadelphia, USA), concentrated, and lyophilized. Cy-3-g was purified from the lyophilized anthocyanins powder by middle-pressure liquid chromatography (MPLC, Lisui, Dr Flash-S, China) with C18 adsorption column. Then purified Cy-3-g solution was lyophilized and stored under −20 °C. The purity of extracted Cy-3-g was > 96.5% by high performance liquid chromatography (HPLC) analysis.

2.4. Allergic asthma model and dietary intervention

The allergic asthma model was induced as previously described [25]. Sensitization, challenge, and dietary intervention protocols for three groups are summarized in Fig. 1. Briefly, mice were sensitized intraperitoneally (i.p.) on days 0, 7, and 14 with 20 μg of OVA, adsorbed on 1 mg alum in 200 μL phosphate buffer saline (PBS, pH = 7.3). On days 21, 22, 23, 24, and 25, all mice were exposed to aerosols consisting of 1% of OVA in PBS. Mice were fed with AIN 93G diet (Medicience Ltd., Jiangsu, China) and were randomly divided into 3

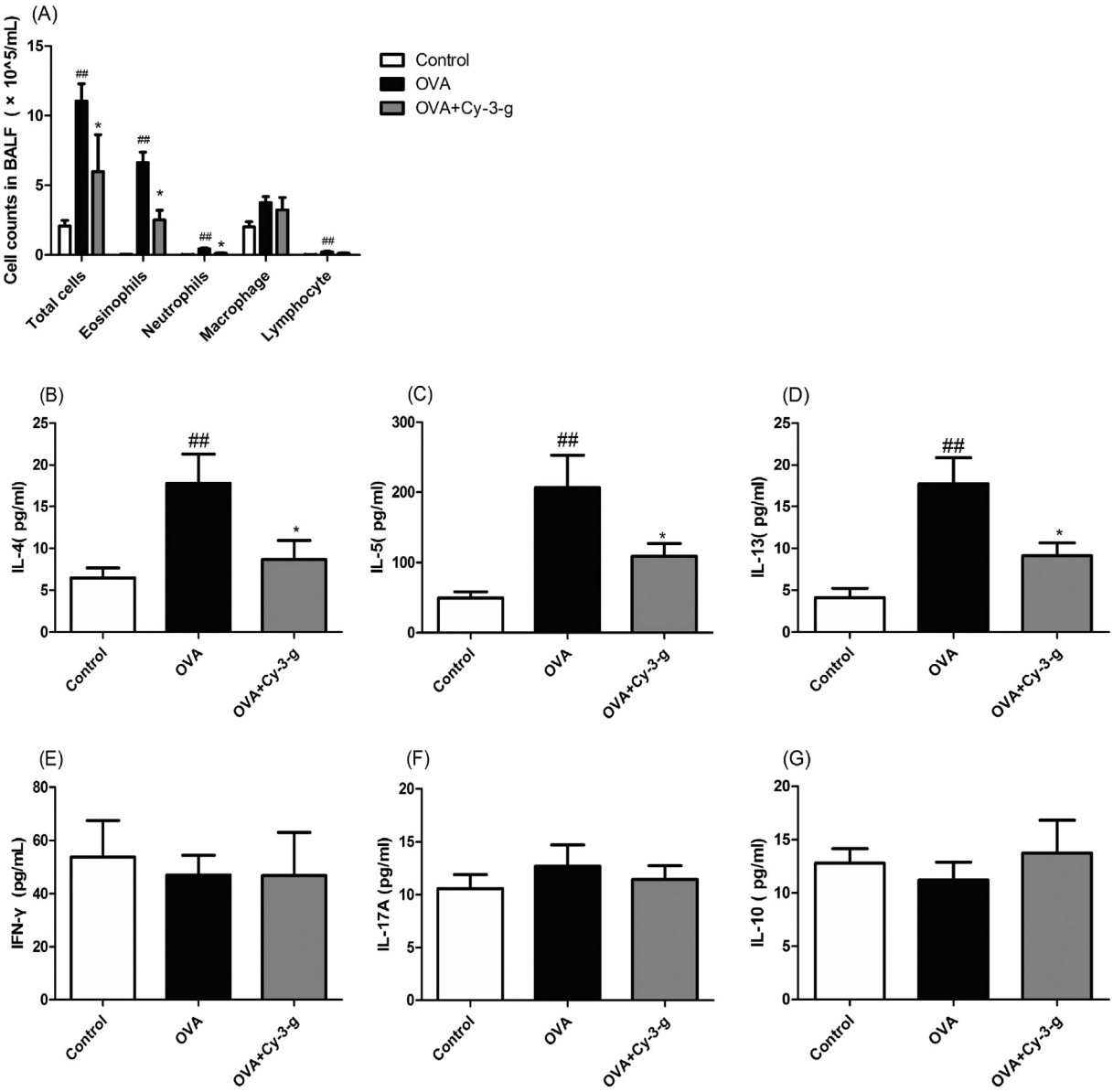

Fig. 4. Effects of Cy-3-g on inflammatory cell infiltration and Th2 cytokine production in BALF. (A) Inflammatory cell counts in BALF. The concentrations of (B) IL-4, (C) IL-5, (D) IL-13, (E) IFN-γ, (F) IL-17A and (G) IL-10 were measured by ELISA in BALF from control, OVA, and OVA + Cy-3-g group. The values represent the mean ± SEM of three independent experiments (n = 8). $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$ compared to the OVA group.

groups: PBS only (vehicle control group), OVA-induced asthma group, OVA-induced asthma plus Cy-3-g group (mice were fed with diet containing 0.4% Cy-3-g. The food intake was approximately 3 g/day (Fig. S1), and Cy-3-g intake was about 1.2 mg per mouse per day, from day 0 to day 25).

2.5. Collection of blood and bronchoalveolar lavage fluid (BALF)

Mice were anesthetized with pentobarbital (20 mg/kg body weight) 24 h after the last OVA challenge. The blood samples were collected carefully by cardiac puncture and then mixed with ACD anticoagulant quickly. Blood cell counts were analyzed using whole blood in an automated hematology analyzer (HEMAVET 950, Drew Scientific, Waterbury, CT). The rest blood was centrifuged with the plasma collected and stored at −80 °C. The lungs were collected, washed twice with PBS *via* trachea cannulation, and centrifuged within PBS. The supernatants were kept as BALF and stored at −80 °C until analysis. PBS was used to resuspend the cell pellets. The numbers of eosinophils, neutrophils, lymphocytes and macrophages in BALF were stained by the Kwik-Diff staining. A minimum of 200 cells were counted per slide.

2.6. Cytokine assay

The concentrations of IL-4, IL-5, IL-13, IFN-γ, IL-17A and IL-10 in the BALF were quantified by ELISA kits according to the manufacturers' protocols. The absorbance of each cytokine was read at 450 nm using a multifunctional microplate detector (Tecan Spark 10M, Tecan, Männedorf, Zürich, Switzerland).

2.7. Histologic analysis of lung tissue

The left lungs were fixed in 10% formalin, embedded in paraffin, and cut into 4 μm-thick sections. The hematoxylin and eosin (H&E) staining was employed to examine inflammatory cell infiltration in the lung sections. The lung inflammation was evaluated by pathological score as previously described [26]. The periodic acid-Schiff (PAS)

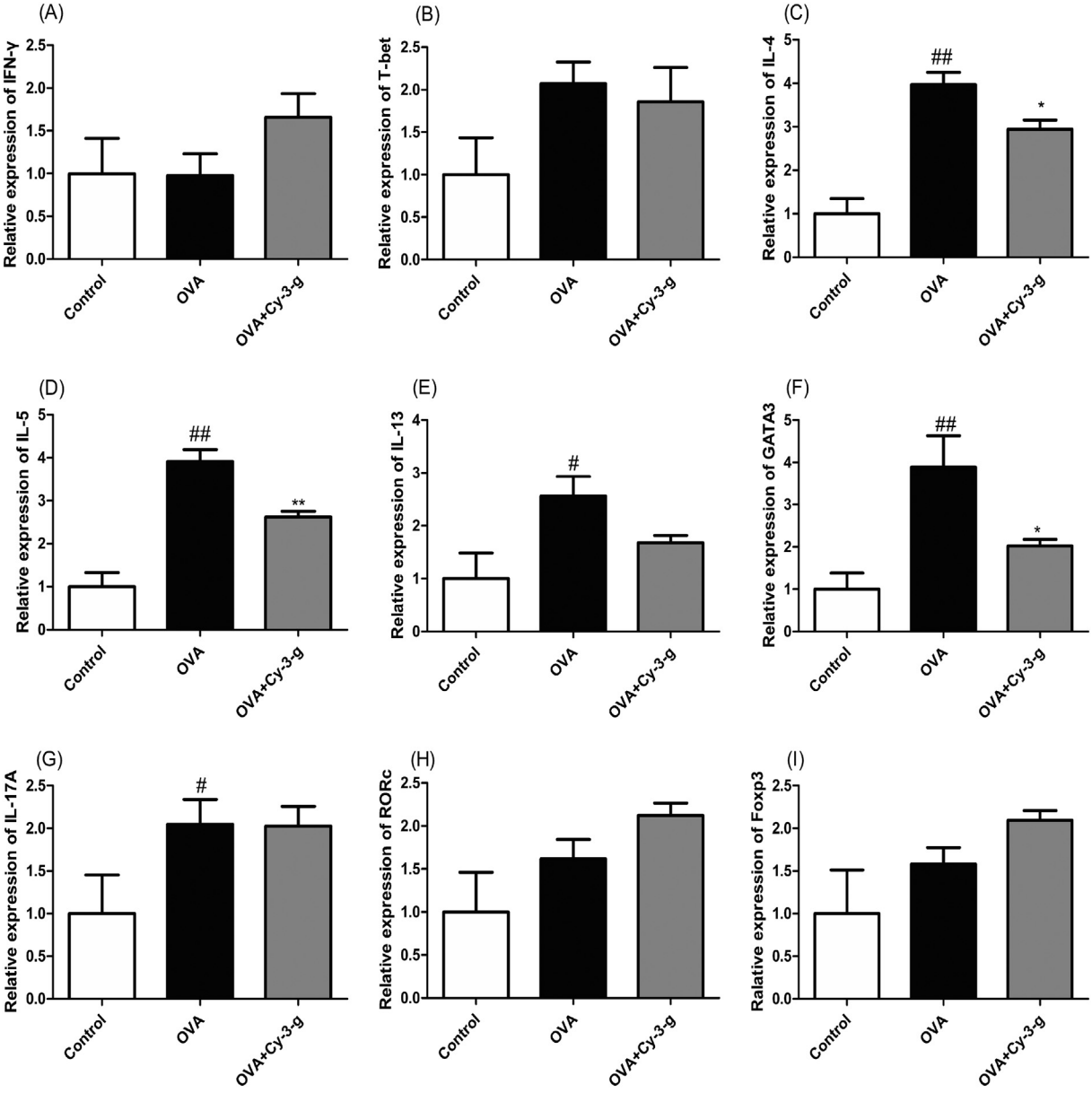

Fig. 5. Effects of Cy-3-g on OVA-induced cytokine mRNA and transcription factors expression in the lungs of experimental mice. Gene expression levels of (A) IFN-γ, (B) T-bet, (C) IL-4, (D) IL-5, (E) IL-13, (F) GATA3, (G) IL-17A, (H) RORc, and (I) Foxp3 were determined by real-time PCR of RNA extracted from lung tissues. The values represent the mean ± SEM of three independent experiments (n = 5). $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$ compared to the OVA group.

staining was adopted to measure goblet cell hyperplasia in the lung sections. The degree of goblet cell hyperproduction was separately quantified as PAS-positive area ratio by measuring 5 randomly selected fields among the epithelium region per lung section for each mouse at 10 × 40 magnification using ImageJ software (version1.47, Media Cybernetics, Rockville, MD, USA).

2.8. Immunohistochemistry

Immunohistochemistry was carried out as described previously [22]. Briefly, lung tissue sections were stained with rabbit anti-mouse IL-4Rα, p-Jak1, or p-STAT6 antibodies and incubated at 4 °C overnight. After washing, the sections were then incubated with DAKO EnVision + System-HRP (Dako, Glostrup, Denmark) at room temperature for 30 min. Signals for immunoreactivity were visualized with diaminobenzidine substrate. Finally, the sections were counterstained with hematoxylin. The percentages of IL-4Rα^{+}, p-Jak1^{+} or p-STAT6^{+} cells of lung sections were determined in 5 randomly selected fields per lung section for each mouse at 10 × 40 magnification by using Image J software.

2.9. Quantitative RT-PCR

Lung tissues were harvested 24 h after the last OVA challenge and total RNA from lung tissues was isolated using mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA). Reverse transcription was carried out using a PrimeScript RT kit (Takara, Osaka, Japan). Real-time PCR was performed on an ABI ViiATM7Dx Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using SYBR Premix Ex Taq (Takara, Osaka, Japan). Relative expression of mRNAs was calculated by the $2^{-\Delta\Delta Ct}$ method using β-actin mRNA for normalization. The sequences of the primers used in this study were listed in Table 1.

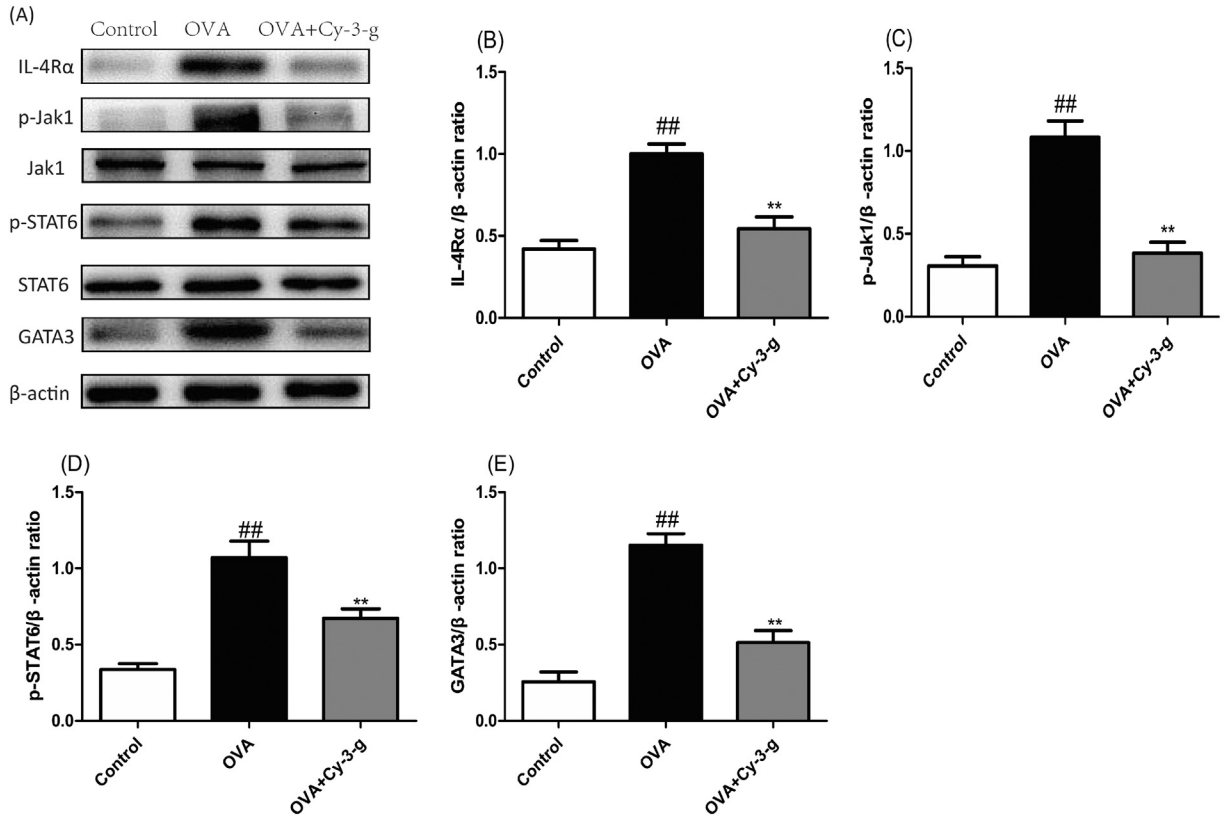

Fig. 6. Effects of Cy-3-g on the IL-4Rα-STAT6 pathway in lungs by western blot. (A) Representative western blot pictures of IL-4Rα, total Jak1, p-Jak1, total STAT6, p-STAT6 and GATA3. Protein expression of IL-4Rα (B), p-Jak1 (C), p-STAT6 (D), and GATA3 were quantified based on the density of the bands. β-Actin was used as an internal control. The values represent the mean ± SEM of three independent experiments (n = 3). $^{\#\#}p < 0.01$ compared to the control group; $^{*}p < 0.05$ compared to the OVA group.

2.10. Western blot analysis

Lung tissues were homogenized with ice-cold RIPA lysis buffer (Beyotime, China) containing protease and phosphatase inhibitors (Cell Signaling Technology, MA, USA) to obtain extracts of lung protein. The samples were loaded to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and were transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were then blocked in tris buffered saline (TBS) buffer containing 5% bovine serum albumin (BSA) for 1.5 h with gentle shaking followed by incubation with the primary antibodies to β-actin (1:1000), IL-4Rα (1:1000), Jak1 (1:1000), p-Jak1 (1:500), STAT6 (1:1000), p-STAT6 (1:500), and GATA3 (1:1000) overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:4000) for 1.5 h at room temperature and washed again. After treatment of the membranes with enhanced chemiluminescence system reagents (Millipore Corporation, Beverly, MA, USA), the binding of specific antibodies was visualized using a Tanon 5200 automatic chemiluminescence image analysis system (Tanon, Shanghai, China) and analyzed by Image J software.

2.11. Statistical analysis

All values are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by the least significant difference test for multiple comparisons of the data with normal distribution and homogeneity of variance. A Kruskal–Wallis rank sum test followed by a Mann–Whitney *U* test was performed for multiple comparisons of the data that do not satisfy the above conditions. Data was considered to be significant when $p < 0.05$. These statistical data were analyzed by using the SPSS 20.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cy-3-g modulated OVA-induced increase in eosinophils in the peripheral blood of experimental mice

Blood eosinophil counts and eosinophil/lymphocyte ratio (ELR) are suggested to be accurate to be predictors of eosinophil asthma phenotype in patients with asthma [27]. Our results showed that white blood cell (WBC), eosinophil and neutrophil counts, and the percentage of blood eosinophil, neutrophil, and ELR were increased in OVA-induced mice compared with controls (Figs. 2A–C & S2A–C; n = 8; $p < 0.05$). Dietary supplementation of Cy-3-g significantly decreased the percentages of eosinophil, neutrophil, and ELR compared with the OVA group (Fig. 2A–C; $p < 0.05$). The percentage of lymphocyte in the peripheral blood was lower in OVA-induced mice, whereas increased in the Cy-3-g group (Fig. 2D; $p < 0.05$). The percentages of monocyte and basophil, or the number of red blood cell (RBC), lymphocyte, monocyte, basophil and platelet was not significantly changed among the three groups (Fig. S2D–J).

3.2. Cy-3-g reduced OVA-induced inflammatory cell infiltration and goblet cell hyperplasia in lungs

The OVA-challenged mice showed a markedly increased infiltration of inflammatory cells into the lung bronchial areas compared with those in the control group (Fig. 3A & C; n = 5; $p < 0.05$). Dietary supplementation of Cy-3-g significantly reduced inflammatory cell infiltration compared to OVA-challenged mice (Fig. 3A & C; $p < 0.05$). Goblet cell hyperplasia was observed using PAS staining. The OVA

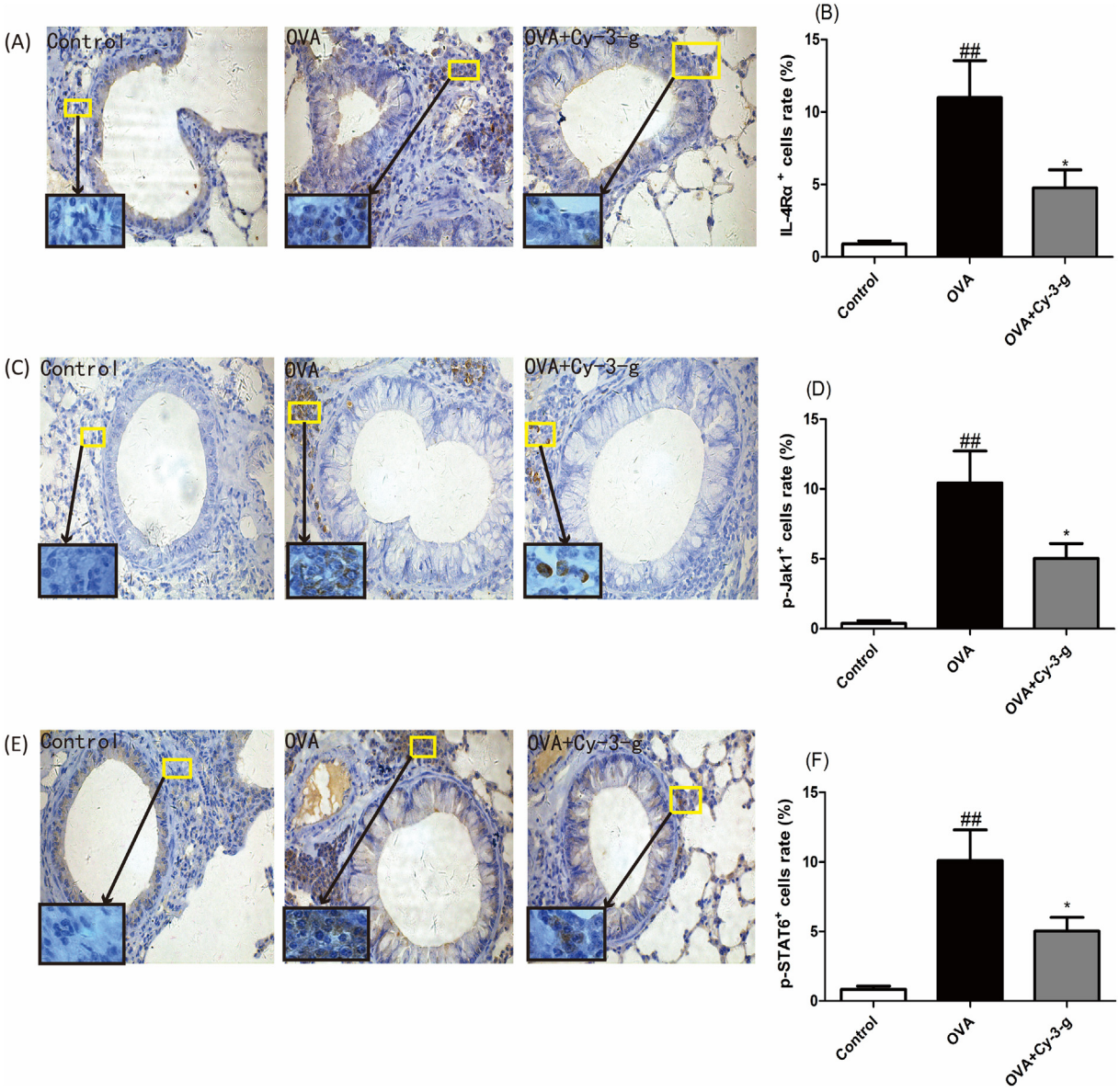

Fig. 7. Effects of Cy-3-g on the IL-4Rα-STAT6 pathway in lungs by immunohistochemistry. Representative immunohistochemistry pictures (400× magnification) for IL-4Rα (A), p-Jak1 (C), and p-STAT6 (E). Amplification areas (1000× magnification) were shown for the indicated areas. The percentages of IL-4Rα$^+$ (B), p-Jak1$^+$ (D), and p-STAT6$^+$ (F) cells around the bronchil were determined. The values represent the mean ± SEM of three independent experiments (n = 5). $^{\#\#}p < 0.01$ compared to the control group; $^*p < 0.05$ compared to the OVA group.

group showed significantly increased mucus production compared with the control ($p < 0.05$), while Cy-3-g significantly reduced mucus production compared to OVA-challenged mice (Fig. 3B & D; $p < 0.05$).

3.3. Cy-3-g alleviated inflammatory cell infiltration and Th2 cytokine production in BALF

Total cells, eosinophils, neutrophils and lymphocytes numbers in BALF were significantly increased in OVA-challenged mice compared to controls (Fig. 4A, n = 5, $p < 0.05$). Cy-3-g treatment significantly reduced the number of total cells, eosinophils and neutrophils ($p < 0.05$). The production of Th2 cytokines IL-4, IL-5, and IL-13 were elevated in BALF of the OVA group compared with controls (Fig. 4B–D, n = 8, $p < 0.05$). Cy-3-g significantly reduced the BALF levels of IL-4, IL-5, and IL-13 compared with the OVA treatment ($p < 0.05$). The production of Th1-signature cytokine IFN-γ, Th17-signature cytokine IL-17A and Treg-signature cytokine IL-10 were not different in the control, asthmatic and Cy-3-g-treated groups (Fig. 4E–G, $p > 0.05$).

3.4. Cy-3-g modulated the mRNA expressions of Th2-associated cytokines and transcription factors in the lung tissues

Real-time PCR was applied to assess the relative mRNA expressions of Th1, Th2, Th17, and Treg-associated cytokines and transcription factors in the murine lung tissues. We found that the OVA challenge substantially increased the mRNA levels of Th2-related cytokine IL-4 and IL-5, as well as GATA3, a Th2 cell transcription factor in the lungs, which were significantly down-regulated by Cy-3-g treatment (Fig. 5C, D, F; $p < 0.05$). However, Cy-3-g administration did not affect the mRNA expressions of IL-13 compared with OVA. The mRNA expressions of Th1-related markers (IFN-γ and T-bet), Th17-related marker RORc, or Treg-related marker Foxp3 was not significantly changed among the three groups (Fig. 5A, B, H, I; $p > 0.05$).

3.5. Cy-3-g inhibited the IL-4Rα-STAT6 pathway in lungs of OVA-challenged mice

IL-4Rα-STAT6 signaling pathway plays a crucial role in the activation and differentiation of Th2 cells [28,29]. The expressions of key proteins in the IL-4Rα-STAT6 signaling pathway including IL-4Rα, total Jak1, p-Jak1, total STAT6 and p-STAT6 as well as Th2 cell transcription factor GATA3 were evaluated by western blot. The relative protein expressions of IL-4Rα, p-Jak1, p-STAT6 and GATA3 in lung tissues of OVA group were significantly elevated than those in the control group (Fig. 6; n = 3; $p < 0.05$). While dietary supplementation of Cy-3-g significantly reduced these protein levels ($p < 0.05$). The distribution of IL-4Rα, p-Jak1, and p-STAT6 in the lungs was evaluated by immunohistochemistry. IL-4Rα, p-JAK1, and p-STAT6 could be observed in leukocytes around the bronchil in the lung sections (Fig. 7A, C, E). The percentage of IL-4Rα$^+$, p-Jak1$^+$, or p-STAT6$^+$ cells around the bronchil were significantly increased in OVA group compared to controls (Fig. 7; n = 5; $p < 0.05$). Dietary supplementation of Cy-3-g significantly reduced the percentage of these cells ($p < 0.05$).

4. Discussion

Previously studies have clearly demonstrated that Cy-3-g, a typical and abundant monomer of anthocyanins, is beneficial for the prevention and treatment of obesity and cardiovascular disease [30–32]. In the present study, we investigated the effects of Cy-3-g on OVA-induced allergic airway inflammation and the underlying mechanism in mice. We found that Cy-3-g could decrease Th2 cytokine secretion, eosinophil infiltration, and mucus production *in vivo*. What's more, our study demonstrated that Cy-3-g protected against asthma by modulating IL-4Rα-STAT6 signaling pathway in the lungs. Therefore, Cy-3-g could be regarded as an alternative dietary therapy for the management of allergic airway inflammation and asthma.

Allergic asthma is a chronic airway inflammatory disease characterized by AHR, mucus hyperproduction, and airway inflammation. The inflammatory response of allergic asthma is dominantly driven by Th2 cells. Activated Th2 cells produce various effector cytokines such as IL-4, IL-5, and IL-13, which in turn induce the differentiation of Th2 cells [28], promote the recruitment, activation and, survival of eosinophils [7], and enhance mucus hypersecretion [33]. Eosinophils contain large amounts of granules, such as major basic protein and eosinophil peroxidase, which can induce or aggravate airway inflammation [34]. The airway mucosa responds to allergic airway inflammation by surface mucous goblet cells [35]. Airway mucus hypersecretion, which produced and secreted from goblet cell, plays an important role in airway obstruction [36]. Cy-3-g, which possesses powerful anti-inflammatory activity both *in vitro* and *in vivo*, turns out to be a promising therapeutic agent for asthma treatment. Cy-3-g treatment effectively inhibited the production of TNF-α and IL-6 in LPS-stimulated THP-1 cells [37], and protected against TNF-α-induced endothelial dysfunction *in vivo* [38]. Furthermore, Cy-3-g ameliorated lipopolysaccharide (LPS)-induced acute lung injury [39] in a mouse model. In this study, our results demonstrated for the first time that Cy-3-g reduced the production of IL-4, IL-5 and IL-13 both in protein and mRNA levels, decreased inflammatory eosinophil infiltration to the lung, suppressed peripheral eosinophil counts, and inhibited goblet cell hyperplasia in OVA-induced asthmatic mice. These results are consistent with previous reports of anthocyanins extract [22,23]. Taken together, these findings indicated that Cy-3-g is effective in treating allergic asthma mice.

Previous researches have shown that the anti-inflammatory effect of Cy-3-g is mediated by inhibiting the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling pathways, reducing inducible nitric oxide synthase (iNOS) activity, and decreasing cyclooxygenase 2 (COX-2) expression [37,40,41]. The IL-4Rα-STAT6 signaling pathway also plays a crucial role in the development of allergic asthma. Once IL-4 and/or IL-13 binds to their receptor IL-4Rα, the relevant janus kinase 1 (Jak1) is activated and phosphorylates the conserved tyrosine-641 on STAT6. The phosphorylated STAT6 then translocates to the nucleus and binds directly to DNA to regulating GATA3 transcription [42]. GATA3 is crucial for directing Th2 cell differentiation and Th2 cytokine production [43]. Previous studies have shown that lymphocytes from STAT6-deficient animals exhibited decreased cell surface expression of IL-4 receptor and MHC class II antigens and failed to proliferate in responding to IL-4 [44]. Deletion of STAT6 also decreases the infiltration of eosinophils and Th2 cells into the lung of mice in response to allergen challenge [45]. STAT6-deficient mice did not develop AHR, and goblet cell hyperplasia [46]. Previous study has showed that Cy-3-g could suppress GATA3 expression in T cells [47]. Consistently, in this study, Cy-3-g reduced the expression of mRNA and protein of GATA3 in lung tissues of OVA-induced mice. And the mRNA levels of Th1-, Th17-, and Treg-related markers (IFN-γ, T-bet, IL-17, RORc, and Foxp3) were not influenced by Cy-3-g treatment. Thus we speculated that Cy-3-g might exhibit the anti-asthmatic effects by modulating the IL-4Rα-STAT6 signaling pathway. In addition, our results demonstrated for the first time that OVA-induced infiltration of IL-4Rα$^+$ cells, p-Jak1$^+$ cells, and p-STAT6$^+$ cells into the lung was strongly suppressed by Cy-3-g, and the protein expressions of IL-4Rα, p-Jak1, and p-STAT6 in lung tissues of OVA-induced mice were down-regulated by Cy-3-g. Therefore, our results suggested that Cy-3-g inhibited asthma inflammation mainly *via* modulating the IL-4Rα-STAT6 signaling pathway in mice.

Previous clinical trials have clearly demonstrated the health benefits of anthocyanins. In a double-blind, randomized, placebo-controlled trial, 160 mg anthocyanins twice daily for 12 weeks significantly increased high-density lipoprotein (HDL)-cholesterol concentrations and decreased low-density lipoprotein (LDL)-cholesterol concentrations in dyslipidemic subjects [48]. In a 12-week intervention trial, 320 mg/day anthocyanins significantly increased brachial artery flow-mediated dilation, plasma cGMP, and HDL-cholesterol concentrations, and significantly decreased serum soluble vascular adhesion molecule-1 and LDL-cholesterol concentrations in hypercholesterolemic individuals [49]. Administration of 250 mL of anthocyanin-rich bayberry juice twice daily (415 mg/day anthocyanins) for 4 weeks was shown to be safe and effective in suppressing the inflammatory response in young adults with non-alcoholic fatty liver [50]. However, the effects of anthocyanins on asthma have not been reported in human studies. In this study, supplemented with 60 mg/kg body weight of Cy-3-g (1 kg of the AIN 93G diet contained 400 mg Cy-3-g) per day for 25 days was effective to significantly inhibit airway inflammation in OVA-induced mice. The dose of Cy-3-g used in this study was equivalent to 400 mg of anthocyanins per day for a 60-kg adult when it was extrapolated from mice by the method of surface area, which is achievable by consuming anthocyanin-abundant foods, such as blueberries, blackcurrants, and mulberries. The bioavailability of anthocyanins also varies dramatically among individuals. Some studies have shown that < 1% of the ingested anthocyanins reaching the circulation [51], while in another study the relative bioavailability of anthocyanins was about 12% [52]. Furthermore, a small portion of anthocyanins could be absorbed into the blood as intact form, whereas most anthocyanins were metabolized by the gut microbiota in the colon and produced a wide range of metabolites [53]. Further studies are needed to clarify whether the gut metabolites of anthocyanins may account for their protective effects on allergic airway inflammation.

In conclusion, our study investigated for the first time the effects of Cy-3-g on allergic asthma in a mouse model. Our results demonstrated that oral administration of Cy-3-g significantly alleviated allergic airway inflammation by reducing eosinophil infiltration, Th2 cytokine production and the IL-4Rα-STAT6 signaling pathway. Cy-3-g is promising as an agent for asthma prevention and/or treatment in the future and further studies are needed to evaluate its clinical effectiveness in humans.

Conflicts of interest

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

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