

Gallic acid attenuates allergic airway inflammation via suppressed interleukin-33 and group 2 innate lymphoid cells in ovalbumin-induced asthma in mice

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Background: Asthma is an inflammatory disease characterized by airway hyperresponsiveness. Gallic acid is a powerful anti-inflammatory agent. In this study we aimed to investigate the efficacy of gallic acid in asthma treatment and its mechanisms.

Methods: An ovalbumin-induced asthma mouse model was generated. Pro-inflammatory cell infiltration and T helper (Th2)-associated cytokine release in the bronchoalveolar lavage fluid (BALF) were analyzed to reflect the severity of asthma in mice. An interleukin-33 (IL-33)-induced asthma mouse model was also generated to study the mechanism by which gallic acid could improve asthma. Group 2 lymphoid cells (ILC2s) were identified using flow cytometry. Proteins were detected using Western blotting.

Results: Ovalbumin significantly increased the infiltration of pro-inflammatory cells, including eosinophils, macrophages, lymphocytes, and neutrophils, accompanied by enhanced airway hyperresponsiveness. Gallic acid reduced pro-inflammatory cell infiltration and improved

airway hyperresponsiveness. Meanwhile, gallic acid reduced IL-5 and IL-13 levels in BALF and decreased expression of IL-33 in the lungs. Mechanistically, gallic acid inhibited MyD88 expression and downregulated nuclear factor (NF)- κ B signaling to decrease IL-33 expression.

Conclusions: Gallic acid can mollify ovalbumin-induced asthma in mice, possibly by inhibiting IL-33-mediated ILC2 activation and subsequent Th2 cytokine release via downregulation of the MyD88/NF- κ B signaling pathway. ©2018 ARSAAOA, LLC. © 2018 ARS-AAOA, LLC.

Key Words:

asthma; ILC2; IL-33; MyD88; gallic acid

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Asthma is a common disease characterized by bronchial hyperresponsiveness, lung infiltration of inflammatory cells, and airway remodeling.¹ Around 300 million patients suffer from asthma on an annual basis, and the incidence has been increasing in the past 2 decades.² Therefore, asthma has become a serious public health issue and carries a major economic burden. Currently, the pathogenesis of asthma is associated with type 2 helper T (Th2)

cells, group 2 innate lymphoid cells (ILC2s),³ and the Th2-related cytokines released by Th2 cells and ILC2s. In addition, interleukin-33 (IL-33) can promote production of Th2-related cytokines, enhancing the Th2-type immune response leading to the development of asthma.⁴ In response to environmental stimuli, IL-33 secreted by epithelial cells combines with ST2,⁵ recruiting the myeloid differentiation primary response gene (MyD88), tumor necrosis factor receptor-associated factor 6 (TRAF6), and IL-1-receptor-associated kinase (IRAK).⁶ This activates mitogen-activated protein kinase kinase (MAPKK),⁷ and also activates activin-1 (AP-1) via c-Jun N-terminal kinase (JNK2). Meanwhile, TRAF6 activates nuclear factor (NF)- κ B and induces expression of Th2-related cytokine genes.⁸

Gallic acid, which is derived from many plants, is an effective anti-inflammatory agent and has been tested in a number of inflammatory conditions.⁹⁻¹¹ Mechanistically, gallic acid has been reported to act on various kinds of cells and alter the secretion of many cytokines.^{12,13} Given

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these characteristics, it is believed that gallic acid could be beneficial for asthma treatment. However, its role in asthma is largely unknown. In this study, we utilized a mouse model to assess the efficacy of gallic acid in the treatment of asthma. We observed inflammation and airway responsiveness in asthmatic mice with or without gallic acid treatment, and analyzed the relevant cytokines released. We also investigated the target of gallic acid and the mechanisms by which this agent demonstrates anti-asthma effects.

Materials and methods

Animal models

The animal experimentation protocols in our study were reviewed and approved by the ethics committee of The Affiliated Wuxi No. 2 People's Hospital of Nanjing Medical University. Specific pathogen-free mice were maintained in an animal facility with suitable animal welfare. Ovalbumin (OVA) was obtained from Sigma-Aldrich (St. Louis, MO). An OVA-induced asthma model with pathogen-free female BALB/c mice (age, 6–8 weeks; weight, 18–20 g) was used. On days 1, 7, and 14, the mice were intraperitoneally treated with 20 μ g of OVA emulsified with 1 mg aluminum hydroxide in 200 μ L of phosphate-buffered saline (PBS). On days 22–25, the mice were again challenged through intranasal inhalations with 100 μ g OVA in 20 μ L PBS per day. Gallic acid (Sigma-Aldrich) was dissolved in absolute ethanol (50 mg/mL), filtered through 0.45- μ m membranes (Minisart; Sartorius Stedim Biotech, Germany), and diluted using 4 mg/mL sterile PBS solution (pH 7.4). The gallic acid was then intragastrically administered at a dose of 100 mg/kg body weight (within a safe range in rats) once daily.¹⁴ The mice in the control group were administered an equal volume of PBS. Mice were then euthanized at 24 hours after the last OVA challenge (Fig. 1). An IL-33-induced asthmatic mouse model was established according to a previously described method.¹⁵ Mice were treated daily with 1 μ g IL-33 (BioLegend, San Diego, CA) in 25 μ L of normal saline for a total of 4 days, and then euthanized 24 hours after the final treatment. In another group, both gallic acid and IL-33 were administered as described and treated in the same manner and at the same time of day (Fig. 1).

Evaluation of airway hyperresponsiveness

We evaluated airway hyperresponsiveness (AHR) in unrestrained and conscious mice at 24 hours after the last challenge with OVA. Mice were placed in a barometric plethysmographic chamber and maintained for 3 minutes to record baseline readings. The enhanced pause (P_{enh} value) was calculated according to the formula: [(expiratory time / relaxation time – 1) $\times$ (peak expiratory flow / peak inspiratory flow)]. The P_{enh} value was used to reflect airway resistance to methacholine. Results were expressed as relative increase

in P_{enh} value after challenge with different concentrations of methacholine (0, 5, 10, 25, and 50 mg/mL).

Immunoblotting

Immunoblotting was performed as described previously.¹⁶ In brief, total proteins were extracted from whole-lung homogenates using RIPA lysis buffer (ThermoFisher Scientific, Waltham, MA). Protein quantification was conducted using a bicinchoninic acid assay (ThermoFisher). Gel electrophoresis was performed to separate the proteins, which were then transferred to nitrocellulose membranes. The membranes were first blocked with nonfat milk for 1 hour, and then incubated with specific primary antibodies overnight. Anti-mouse or anti-rabbit immunoglobulin G (IgG) secondary antibodies were then used as appropriate for 1 hour. Electrochemiluminescence (ECL) Western blotting substrate (ThermoFisher) was used to detect the bands. All primary antibodies were purchased from Cell Signaling Technology (Danvers, MA). Photodensity of the bands was quantified using Image J software (National Institutes of Health, Bethesda, MD), and the relative expression of certain proteins was calculated by normalization into the internal controls.

Analysis of bronchoalveolar lavage fluid

After mice were anesthetized, the tracheas were cannulated and lavaged with 2 aliquots (0.8 mL) of ice-cold PBS. The bronchoalveolar lavage fluid (BALF) samples were collected and immediately centrifuged. The supernatants were stored at –80°C until being used for enzyme-linked immunosorbent assay (ELISA) to determine the level of cytokines. The cell pellets were resuspended in PBS, and the total and certain types of cells were counted using a hemocytometer (total cells) or using cytospin preparations stained with a Kwik-Diff (ThermoFisher) staining set (eosinophils, neutrophils, macrophages, and lymphocytes).

Flow cytometry

Single-cell suspensions of lung tissue were prepared by digesting the right lobes of the lung in 2 U/mL liberase TL (Roche) and 80 U/mL DNase (Life Technologies, Pleasanton, CA) at 37°C with agitation for 35 minutes. Draining mediastinal lymph nodes and digested lung tissue were filtered through 70-mm cell strainers. ILC2s were characterized as lineage[–] (CD3/CD19/CD4/CD5/CD8a/TcRb/CD49b/CD11b/CD11c) and inducible T cell costimulator (ICOS⁺). Antibodies were purchased from BioLegend (San Diego, CA) or BD Biosciences (San Jose, CA). For intracellular cytokine staining, cells were stimulated for 4 hours at 37°C with 500 ng/mL phorbol myristate acetate, 1 mg/mL ionomycin, and 10 mg/mL Brefeldin A (Sigma-Aldrich). Cells were surface-stained, then permeabilized using a fixation/permeabilization kit (BD Biosciences) before staining for IL-5 and IL-13. Live/dead fixable yellow or aqua dyes (Invitrogen) were used to exclude dead cells. Flow

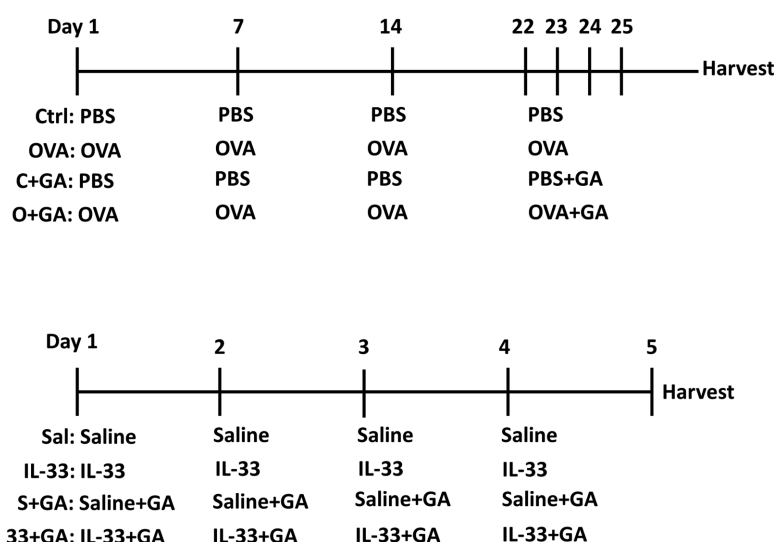


FIGURE 1. Schematic diagram of the experimental protocol. GA = gallic acid; IL = interleukin; OVA = ovalbumin; PBS = phosphate-buffered saline.

cytometry was performed on a flow cytometer (Canto II; BD Biosciences).

Statistical analysis

All data are expressed as mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using SPSS version 18 (IBM SPSS, Inc, Armonk, NY). Data were compared using Student's *t* test. $p < 0.05$ was considered statistically significant.

Results

Gallic acid in amelioration of inflammatory cell infiltration and OVA-induced airway hyperresponsiveness

We first tested the possible role of gallic acid in asthma treatment by analyzing its anti-inflammatory ability, as asthma is an inflammation-related disease. We detected increased numbers of total cells in the BALF of OVA mice (Fig. 2A). In particular, the number of eosinophils, neutrophils, macrophages, and lymphocytes were all upregulated in BALF (Fig. 2B-E). Noticeably, use of gallic acid significantly decreased the number of these cells in BALF, suggesting that it could inhibit inflammatory cell infiltration. We then investigated the extent of AHR using the P_{enh} value. As expected, P_{enh} gradually increased with inhalation of methacholine in the OVA-challenged mice; however, gallic acid significantly delayed the increase in P_{enh} value (Fig. 2F).

Gallic acid in reduction of Th2 cytokine level in BALF and IL-33 expression in lungs

Increased levels of IL-5 and IL-13 secreted by Th2 are notable characteristics of asthma, which were also observed in the BALF of our mouse model (Fig. 3A and B). Gallic acid, however, largely reduced the levels of IL-5 and IL-13.

In addition, IL-33, which is capable of enhancing Th2 immune reaction, was found to be overexpressed in the lungs of OVA-challenged mice (Fig. 3C and D). Although gallic acid inhibited IL-33 expression to a great extent, we thus assumed that its effect on asthma may have been due to reduced IL-33.

Gallic acid in reduction of IL-33-induced inflammatory cell infiltration in BALF

To further confirm our hypothesis, we used IL-33 instead of OVA to induce asthma-related inflammation in the lungs. IL-33 was sufficient to induce infiltration of total cells and eosinophils (Fig. 4A and B), and also enhanced the secretion of IL-5 and IL-13 (Fig. 4C and D), the two Th2 cytokines. When gallic acid was administered to the mice, however, the number of inflammatory cells and the concentrations of IL-5 and IL-13 in the BALF decreased significantly (Fig. 4A-D).

Gallic acid in inactivation of ILC2s in mice

ILC2s can secrete Th2 cytokines and were recently found to play a major role in the development of asthma. IL-33 was reported to activate ILC2s in the presence of allergen, which means the ILC2 is a target of IL-33. Therefore, we tested whether gallic acid could reduce the number and activity of ILC2s. Indeed, IL-33 and OVA both extensively increased the number of ICOS⁺lineage⁻ ILC2s, whereas gallic acid largely decreased ILC2 cell number in both models (Fig. 5A and B). In parallel, the number of IL-13-expressing ILC2s was upregulated by IL-33 and OVA treatment, and was downregulated in case of gallic acid challenge (Fig. 5C and D).

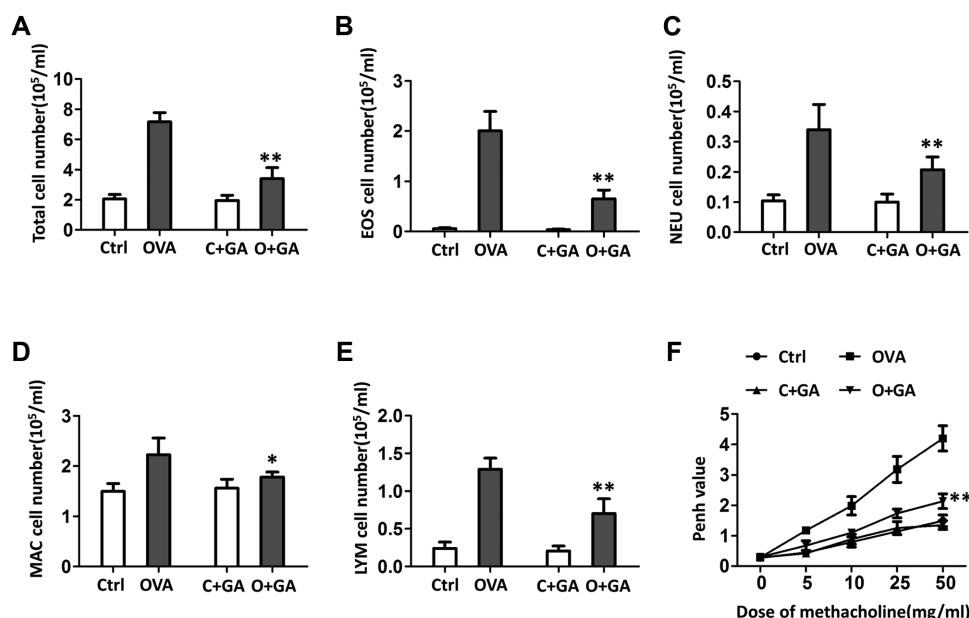


FIGURE 2. The effects of gallic acid on recruitment of inflammatory cells in the BALF and airway hyperresponsiveness in the OVA-induced asthma mouse model. Twenty-four hours after the last challenge (A-E), cells collected from BALF were counted and airway hyperresponsiveness was assessed (F) using whole-body barometric plethysmography. Data are presented as mean $\pm$ standard error of the mean (N = 7). * p < 0.5, ** p < 0.01 vs OVA group. BALF = bronchoalveolar lavage fluid; Ctrl = control; GA = gallic acid; OVA = ovalbumin; EOS = eosinophils; NEU = neutrophils; MAC = macrophages; LYM = lymphocytes.

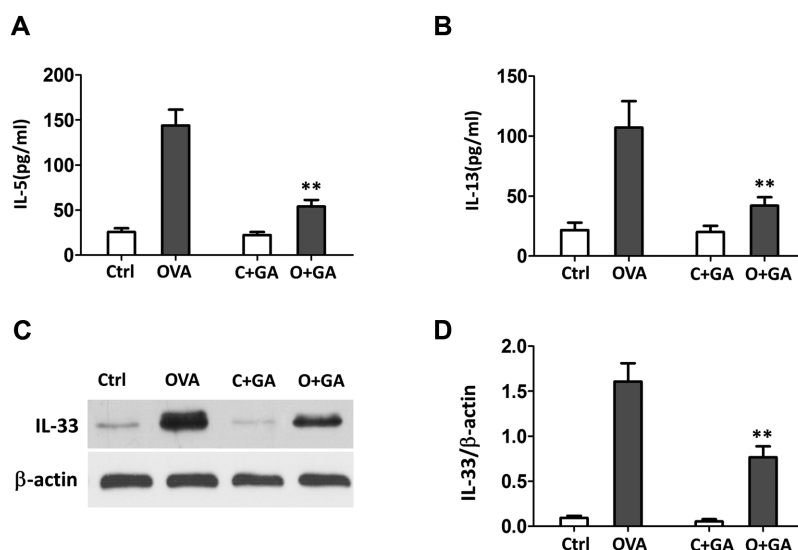


FIGURE 3. Effects of gallic acid on levels of IL-5, IL-13, and IL-33 in OVA mice. The BALF and lung homogenates were collected 24 hours after the last challenge. (A, B) Levels of IL-5 and IL-13 in the BALF were determined using ELISA. (C, D) Immunoblotting was performed to test the IL-33 expression in the lung homogenates. Data are presented as mean $\pm$ standard error of the mean (N = 7). ** p < 0.01 vs OVA group. BALF = bronchoalveolar lavage fluid; Ctrl = control; ELISA = enzyme-linked immunoassay; IL = interleukin; OVA = ovalbumin.

Gallic acid in blockage of IL-33-induced NF- κ B activation by inhibition of myeloid differentiation primary response gene

Classic inflammation signaling was activated in the OVA-treated mice, demonstrating overexpression of toll-like receptor 4 (TLR4), myeloid differentiation primary response gene (MyD88), and p65 (Fig. 6A-D). When gallic acid was present, expression of MyD88 and p65

decreased significantly, yet TLR4 expression remained unchanged.

Discussion

It is widely known that asthma is an inflammation-related disease, and anti-inflammation strategies have been proven to be effective in treating this intractable condition. Thus,

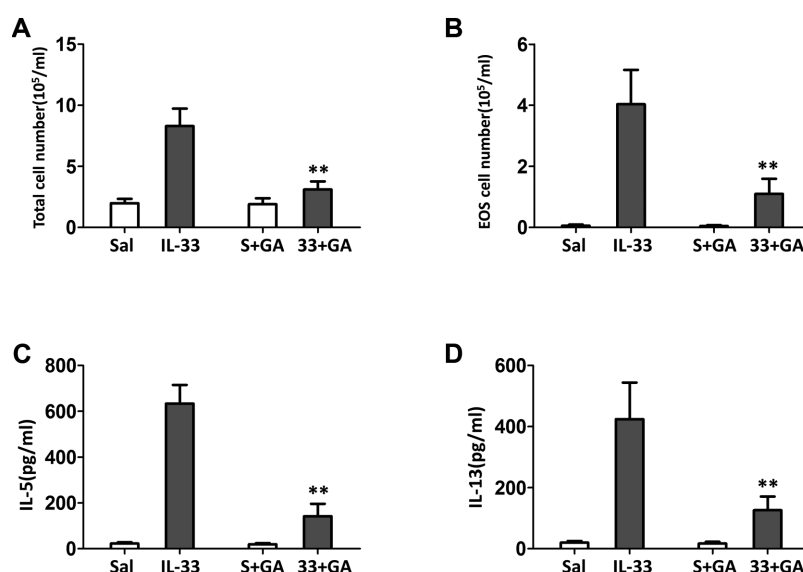


FIGURE 4. Effects of gallic acid on recruitment of inflammatory cells (A, B), and the levels of IL-5 (C) and IL-13 (D) in IL-33-stimulated mice. BALF samples were collected 24 hours after the last challenge. Data are presented as mean $\pm$ standard error of the mean (N = 7). ** p < 0.01 vs OVA group. BALF = bronchoalveolar lavage fluid; GA = gallic acid; IL = interleukin; OVA = ovalbumin; Sal = saline; EOS = eosinophils.

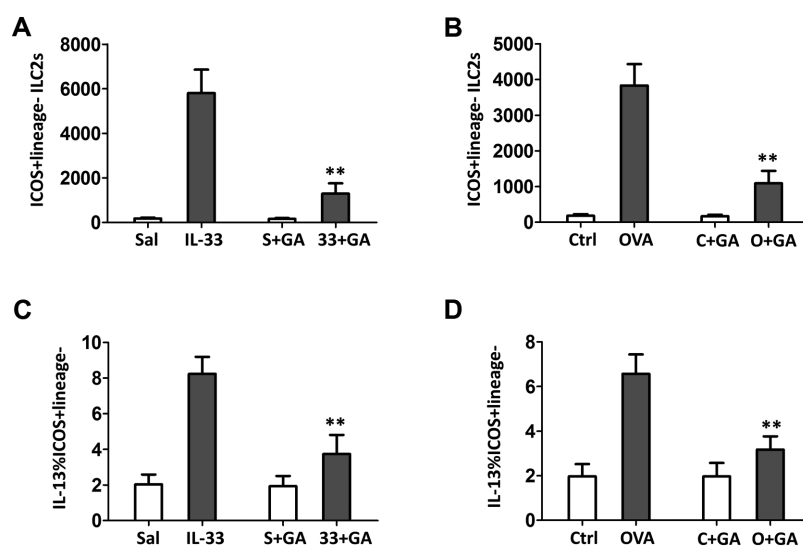


FIGURE 5. Gallic acid suppressed the number of ILC2s and type 2 responsiveness of ILC2s. Twenty-four hours after the last challenge, the lungs were collected and ILC2s (ICOS⁺lineage⁺) were quantified. Data are presented as mean $\pm$ standard error of the mean (N = 7). ** p < 0.01 vs OVA group. Ctrl = control; ILC2s = group 2 innate lymphoid cells; OVA = ovalbumin; Sal = saline.

in this study we tested the efficacy of gallic acid, a potent anti-inflammatory agent, in the treatment of asthma.¹⁷ Using both in vitro and in vivo experiments, we found that gallic acid regulates ILC2s via MyD88-mediated IL-33 expression, reducing IL-5 and IL-13 secretion of ILC2s and, consequently, improving pulmonary inflammation and AHR. Several studies have shown that gallic acid could function as an anti-allergic agent in treating some allergic disorders, including asthma^{13,18}; however, to our knowledge, this is the first report showing that it may have anti-asthma effects by regulating ILC2 activity.

As an effective anti-inflammatory agent, gallic acid, or its synthetic analogs, has been found to inhibit release of histamine and pro-inflammatory cytokines¹³ and also agonize G protein-coupled receptor-35 (a proven target of asthma drugs such as cromolyn and nedocromil).¹⁹ This limited evidence has suggested a possible role for gallic acid in asthma treatment, but few studies have been undertaken to test this possibility.

ILC2s are a novel type of immune cell and have recently been given much attention due to their critical role in local immune reactions in many organs, especially the

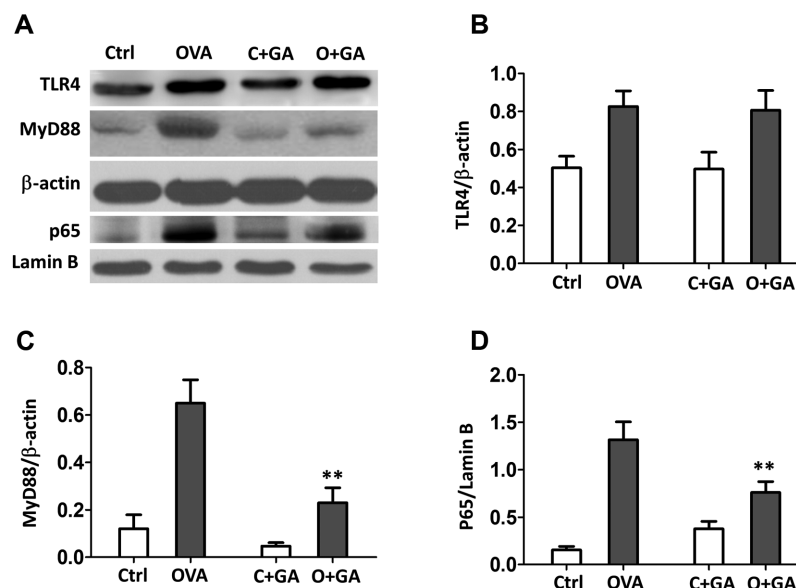


FIGURE 6. Effects of gallic acid on the levels of TLR4, MyD88, and p65 in OVA-induced asthma mouse model. The lung homogenates were collected 24 hours after the last challenge. (A-D) Immunoblotting was used to detect expression of TLR4, MyD88, and p65. Data are presented as mean $\pm$ standard error of the mean (N = 7). ** $p < 0.01$ vs OVA group. Ctrl = control; OVA = ovalbumin; TLR4 = toll-like receptor 4.

lungs.^{20,21} Many pulmonary disorders have been associated with ILC2s, which are also deeply involved in the pathogenesis of asthma. In patients with allergic asthma, epigenetic regulation of ILC2s is associated with asthma, and some of these asthma-associated variants expressed in ILC2s are unique.²² During glucocorticoid treatment, ILC2 frequency and activity are strongly inhibited via MEK/JAK-STAT signaling pathways.²³ Because ILC2s are the main source of IL-5 and IL-13, inactivation of ILC2s is a promising approach in the prevention or reversal of pathogenic progression of asthma.

Several cytokines, including IL-33 and IL-25, provoke ILC2s, with IL-33 being more potent²⁴; therefore, we used IL-33 to activate ILC2s in this study. Both TLR4 and IL-33/ST2 can recruit MyD88 and activate NF- κ B.²⁵ We tested and observed that expression of IL-33, MyD88, and p65 were downregulated by gallic acid, whereas TLR4 expression was not affected, although gallic acid was able to suppress lipopolysaccharide (its receptor is TLR4)-induced p65 activation.²⁶ Our results suggest that, at least in asthma, the IL-33/MyD88/NF- κ B signaling pathway is the target of gallic acid. Intriguingly, lung epithelium is the predominant source of IL-33.²⁷ Thus, we hypothesize that gallic acid may act directly on the lung epithelial cells and reduce their release of IL-33. However, it could still suppress inflammatory cell infiltration and IL-5/IL-13 release in an IL-33-induced mouse model of asthma, suggesting that it may have other mechanisms in mollifying asthma, aside from inhibiting IL-33 expression. Nevertheless, this assumption requires further investigation.

Our study has some limitations. First, only OVA- and IL-33-induced asthma models were tested to show the effects of gallic acid. Because asthma has many etiologies, we cannot simply extrapolate our conclusions to other types of asthma. Second, in this OVA-induced asthma model, we did show treatment efficacy of gallic acid, but we could not determine whether it also has preventive efficacy when used prophylactically. Third, although we focused our analysis on eosinophils only, neutrophils, macrophages, and lymphocytes, as well as other asthma-associated cells like mast cells and smooth muscle cells, were not detected. Furthermore, we did not determine the molecular mechanism by which IL-33 level was attenuated in the lungs by gallic acid. Unfortunately, we did not accurately differentiate the cell type to study the pivotal target cells of gallic acid, but instead we used only the lung homogenates to detect IL-33 expression. In addition, we used AHR and pro-inflammatory cell counts in BALF as substitute outcomes for asthma. Although acceptable, the outcomes may not adequately indicate the biologic and behavioral alterations of asthma. Finally, we focused mainly on the role of ILC2s rather than Th2 cells, which are also important in the pathogenesis of asthma and represent another essential source of Th2 cytokines.

We conclude that gallic acid is able to improve pro-inflammatory cell infiltration and AHR in the OVA-induced mouse model of asthma. The effects of gallic acid may be associated with inactivated ILC2s and suppressed release of IL-5 and IL-13 via the IL-33/MyD88/NF- κ B signaling pathway. Gallic acid or its analogs may be useful in treating asthma.

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