



Aloperine suppresses allergic airway inflammation through NF- κ B, MAPK, and Nrf2/HO-1 signaling pathways in mice

Chongyang Wang^{a,1}, Yun Ho Choi^{b,1}, Zhemin Xian^{c,1}, Mingyu Zheng^d, Hongmei Piao^{c,*}, Guanghai Yan^{a,*}

^a Department of Anatomy, Histology and Embryology, Yanbian University Medical College, Yanji 133002, PR China

^b Department of Anatomy, Medical School, Institute for Medical Sciences, Chonbuk National University, Jeonju 561-180, Jeonbuk, Republic of Korea

^c Department of Respiratory Medicine, Affiliated Hospital of Yanbian University, Yanji 133000, PR China

^d College of Pharmacy, Yanbian University, Yanji 133002, PR China

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ABSTRACT

To explore the effects of aloperine (ALO) on allergic airway inflammation, we investigated whether its mechanism is related with NF- κ B, MAPK, and Nrf2/HO-1 signaling pathways. Histochemical staining and inflammatory cell count were used to observe lung histopathological changes in mice. ELISA was used to detect the content of inflammatory cytokines and IgE in the mouse bronchoalveolar lavage fluid (BALF). Airway hyperresponsiveness (AHR) to inhale methacholine was measured by the plethysmography in conscious mice. Immunohistochemical method was used to detect the expression levels of Nrf2 and HO-1 in lung tissues. The key proteins of MAPK, NF- κ B, and Nrf2/HO-1 in lung tissues were quantitatively analyzed by Western blot. Finally, the in vitro effect of ALO on the production of pro-inflammatory mediators and cytokines by lipopolysaccharide-stimulated RAW 264.7 cells was also evaluated. In the ovalbumin (OVA)-induced asthma mouse model, ALO reduced the exudation and infiltration of inflammatory cells and suppressed goblet cell hyperplasia. ALO-treated asthmatic mice also decreased the protein levels of interleukin (IL)-4, IL-5, IL-13, IFN- γ , and IgE in BALF and attenuated AHR. Furthermore, ALO inhibited the expression of key proteins of MAPK and NF- κ B pathways, and increased the expression of Nrf2/HO-1 in OVA-challenged mice. Additional in vitro study has shown that ALO abrogates the macrophage production of inducible nitric oxide synthase, cyclooxygenase-2, tumor necrosis factor- α , IL-6, and IL-1 β . Taken together, ALO attenuated allergic airway inflammation through regulating NF- κ B, MAPK, and Nrf2/HO-1 signaling pathways. The results suggest the utility of ALO as an anti-inflammatory agent for the treatment of asthma.

1. Introduction

Bronchial asthma represents manifestations of chronic airway inflammation that involves eosinophils, lymphocytes, neutrophils, and mast cells. Its major characteristics are as follows: eosinophilic airway inflammation, increased mucus production, and airway hyperresponsiveness (AHR) [1]. Upregulation of interleukin (IL)-4, IL-5, and IL-13, which are produced by Th2 cells, and downregulation of interferon (IFN)- γ , which is generated by Th1 cells, and the resultant imbalance of Th2/Th1 contribute to asthma pathogenesis, such as the infiltration of eosinophils and the release of immunoglobulin (Ig) E into the lungs [2]. Because as yet, a lot of asthma-related problems remain unclear, it is very urgent to investigate the pathogenic mechanisms of asthma and

find a new therapeutic and preventive target.

A variety of signaling pathways are known to be implicated with the pathophysiology of asthma. Primarily, NF- κ B is a multicellular transcription factor, which plays an important role in modulating immune and inflammatory responses by regulating the production of cytokines [3]. Continuous activation of NF- κ B has been discovered in allergic asthma and inhibition of NF- κ B pathway could alleviate ovalbumin (OVA)-induced asthma [4]. Next, the activation of mitogen-activated protein kinase (MAPK) pathway is also closely related with airway inflammation in an experimental asthma model [5]. The MAPK family is composed of three protein kinases including extracellular signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK), and p38. Furthermore, the nuclear factor-erythroid 2-related factor 2 (Nrf2) and heme

* Corresponding authors.

E-mail addresses: piaohm1969@126.com (H. Piao), ghyan2015@sina.com (G. Yan).

¹ These authors contributed equally to this work and should be considered co-first authors.

oxygenase-1 (HO-1), which are associated with transcriptional activation of antioxidant-response element (ARE) genes, has anti-inflammatory capacity related with cytoprotective responses in asthma. In addition, Nrf2/HO-1 signaling has been reported to be activated by multifarious phytochemicals involved in immune responses [6]. Taken together, NF- κ B, MAPK, and Nrf2/HO-1 pathway has been emerged as the important therapeutic molecular targets in asthma. Accordingly, the suppression of NF- κ B and MAPK family as well as the potentiation of Nrf2/HO-1 pathway might be a welcome method to prevent or treat various inflammatory diseases including asthma.

Previous studies have consistently demonstrated that aloperine, an alkaloid extracted from *sophora alopecuroides*, possesses beneficial properties against tumor growth, infection, inflammation, and immediate-type hypersensitivity [7,8]. Clinically, aloperine has been widely used in oriental countries to treat patients with allergic contact dermatitis, eczema and other skin inflammation. Aloperine has also been reported to ameliorate the inflammatory process in the allergic contact dermatitis model [9]. More interestingly, recent studies further suggest the potential for aloperine to attenuate oxidative stress [10]. These findings prompted us to hypothesize that aloperine could be a good candidate drug for the prevention and treatment of allergic asthma. To address this feasibility, we aim to investigate the protective effect and action mechanism of ALO on airway inflammatory response in a murine model of asthma.

2. Material and methods

2.1. In vivo studies

2.1.1. Animals

The 7-week-old specific pathogen-free (SPF) inbred female BALB/c mice were purchased from House Section of Yanbian University Health Science Center (Yanji, China). The mice were kept under standard laboratory conditions for 7 days prior to experiments and provided with water and standard chow ad libitum. All methods were performed in accordance with the relevant guidelines and regulations. The guidelines of the Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences were followed during the experiments.

2.1.2. Experimental protocols

The asthmatic mouse model was sensitized on days 1, 7, and 14 by intraperitoneal injection of sensitization liquid (200 μ l). The composition of the sensitizing liquid is 10 μ g ovalbumin (OVA, Sigma-Aldrich, China), 1 mg of aluminum hydroxide adjuvant (Imject® Alum; Pierce, USA) and saline. Control group was replaced by an equivalent amount of saline. From day 21 to day 23, the sensitized mice were challenged by exposure to an aerosol of 1% OVA in phosphate-buffered saline (PBS) for 20 min. The bronchoprovocation was carried out in the vented plastic chamber (18 $\times$ 14 $\times$ 8 cm). Aerosol particles of approximately 3–5 μ m in diameter were created from an ultrasonic nebulizer (NE-U12; Omron, Japan), directed into the plastic chamber, and vented to a fume hood. For, experimental grouping, 40 mice were randomly divided into 5 groups (8 mice per group): vehicle group (Control), OVA-administrated model group (OVA), aloperine 100 mg/kg body weight (BW)-treated group (OVA + ALO100), aloperine 200 mg/kg BW-treated group (OVA + ALO 200), and montelukast sodium-treated group (OVA + MON). Control group was exposed to aerosolized PBS and orally administrated 400 μ l of saline. From day 17, aloperine (100 and 200 mg/kg BW, Sigma), dissolved and then diluted further in saline, was administered by oral gavage to each mouse once a day and continuous injection 7 days (the administration time was changed from day 21 to 1 h before challenge). For OVA + MON group, mice were given intraperitoneal administration of montelukast sodium (25 mg/kg) 1 h before OVA stimulation once a day from day 21 to day 23. The mice were sacrificed 24 h after the last challenge.

2.1.3. Histological analysis of lung tissue

The lung tissues of mice were fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. The 4 μ m lung sections were prepared for hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) stains for light microscopic examinations. H&E stain was used to observe the morphologic changes of lung tissue, which reflected the inflammation of bronchus. Goblet cell hyperplasia was evaluated with PAS stain. The percentage of PAS staining-positive cells in the airway epithelium was quantified to estimate the production of mucus. Under 200 $\times$ magnification, each section was randomly selected and photographed for 5 fields.

2.1.4. Bronchoalveolar lavage fluid (BALF)

After anesthesia with 0.75% pentobarbital solution, the skin of the neck was anatomized to expose the trachea on the fixed plate. Then, the catheter was got deeply into the trachea. The lungs were lavaged with 1 ml of cold PBS three times, assuring the recovery of the fluid was > 85%.

2.1.5. Diff-Quik staining

Differential cell counts were performed after cytocentrifugation and staining with Diff-Quik stain (Baxter, Switzerland) in accordance with the manufacturer's protocols.

2.1.6. Enzyme-linked immunosorbent assay (ELISA)

IL-4, IL-5, IL-13, and IFN- γ , in BALF were determined using specific ELISA kits (R&D systems, USA). Quantification of BALF total and OVA-specific IgE was also performed by ELISA kits (BioLegend, USA) according to manufacturer's instructions. Then, the optical density was spectrophotometrically measured at 450 nm using a microplate reader (Biotek Instruments, Inc., USA).

2.1.7. Assessment of AHR

AHR was measured 2 days after the last OVA challenge as described previously [11]. Conscious unrestrained mice were placed in a barometric plethysmographic chamber (All Medicus, Korea) and baseline readings were taken and averaged for 3 min. Aerosolized methacholine (Mch) in increasing concentrations (from 2.5 to 50 mg/ml) was then nebulized through an inlet of the main chamber for 3 min, and readings were taken and averaged for 3 min after each nebulization. The bronchopulmonary resistances are expressed as enhanced pauses (Penh), which were calculated as: [expiratory time (Te)/relaxation time (RT) – 1] $\times$ [peak expiratory flow (PEF)/peak inspiratory flow (PIF)], according to the manufacturer's protocol. The results were expressed as the percentage increase in Penh over the baseline, following challenge performed with each concentration of Mch, where the baseline Penh (after PBS challenge) is expressed as 100%.

2.1.8. Immunohistochemistry staining

Lung sections of 4 μ m were kept in an oven at 60 $^{\circ}$ C for 2 h. The slides were deparaffinized using xylene and dehydrated by graded concentrations of alcohol, then placed in a retrieval solution and incubated in a microwave at high power for 15 min. The slides were then cooled and washed with wash buffer and incubated sequentially with Nrf2 (#12721, 1:200, CST, USA), and HO-1 (#82206, 1:200, CST), and biotin-labeled secondary antibody (#5151, 1:2000, Cell Signaling Technology, USA). Finally, the sections were stained with a diaminobenzidine (DAB) method, counterstained with hematoxylin, cleared in xylene, and fixed. Under 200 $\times$ magnification, 5 different microscopic fields were randomly chosen.

2.1.9. Western blot analysis of the lung

The tissues from experimental mice were lysed in a sample buffer and then denatured. The total, cytoplasmic, and nuclear protein concentrations were determined by the BCA protein estimation kit (Beyotime, China). Equal quantities of proteins samples were separated

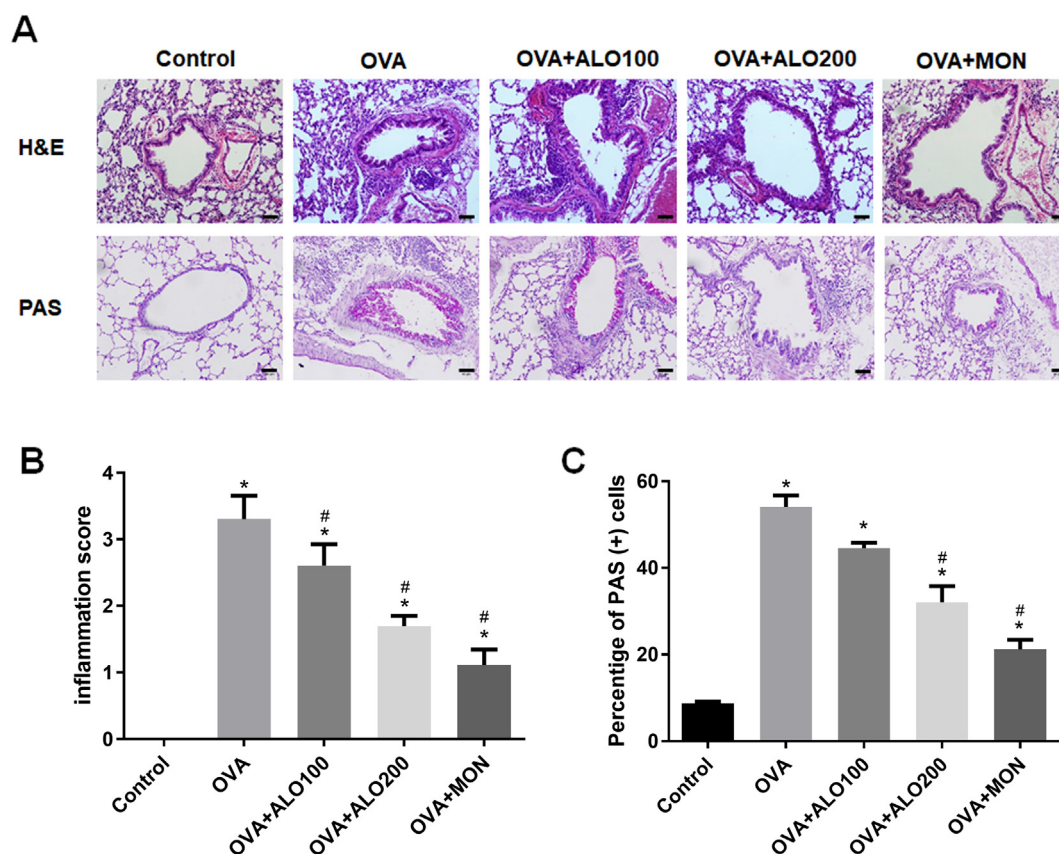


Fig. 1. Effect of aloperine (ALO) on airway inflammation and goblet cell hyperplasia. (A) Lung sections were stained with H&E for measurement of inflammatory cells, PAS for measurement of mucus production around the airways. $\times 200$ magnification, scale bar: 50 μ m. (B, C) The column chart of H&E and PAS were represented the mean $\pm$ S.D. (n = 3). * $p < 0.05$ versus the control group. # $p < 0.05$ versus the OVA group.

by SDS-PAGE. These proteins were then transferred to nitrocellulose membranes. The membranes were blocked with 5% skimmed milk, and incubated with the primary antibodies at 4 °C overnight. The goat anti-rabbit secondary antibody which is conjugated with horseradish peroxidase (#5151, 1:2000, CST) at room temperature (25 °C) for 2 h. Immunodetection was carried out with ECL detection reagent (Beyotime). Photographs were taken and the optical densities of the bands were scanned and quantified with the Chemi-Doc (BioRad, USA). The antibodies of $\text{I}\kappa\text{B}\alpha$ (#4814, 1:1000), phospho (P)- $\text{I}\kappa\text{B}\alpha$ (#2859, 1:1000), NF- κB p65 (#8242, 1:1000), ERK (#4695, 1:1000), P-ERK (#4370, 1:1000), p38 (#8690, 1:1000), P-p38 (#4511, 1:1000), JNK (#9253, 1:1000), P-JNK (#9255, 1:1000), Nrf2 (#12721, 1:1000), HO-1 (#82206, 1:1000), and anti-GAPDH (#2118, 1:1000) were purchased from Cell Signaling Technology.

2.2. In vitro studies

2.2.1. Macrophage cell line culture and viability

The macrophage cell line RAW 264.7 was obtained from the China Cell Line Bank (Beijing, China). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5.5% FBS and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2 incubator. Cells (1×10^6 cells/ml), plated in 96-well plates cells, were treated with variable ALO concentrations (25, 50, 100, and 200 μM) for 24 h time intervals. Afterwards, 10 μl of 3-[4,5-dimethylthazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT, Sigma, USA) were added in each well and incubated for additional 4 h, with a final MTT concentration into each well-plate of 0.5 mg/ml. After removing the supernatant fraction, cells were incubated with 100 μl of DMSO while shaking for 5–10 min. The intensity of colored products formed was quantified by a spectro-photometer at 550 nm. The absorbance of the untreated sample was taken

as 100% viability. Thus, the values of ALO-treated groups were calculated as a percentage compared to the untreated group.

2.2.2. Measurement of NO, PGE_2 , $\text{TNF-}\alpha$, IL-6, and IL-1 β production by ALO-treated macrophages

RAW264.7 cells were stimulated with lipopolysaccharide (LPS) in the presence or absence of 50, 100, or 200 μM ALO for 24 h at 37 °C. NO production was analyzed by adding the Griess reagent (Promega, USA) to the culture supernatant and measuring the nitrite levels using a microplate reader at 540 nm. Secretion of the inflammatory cytokines PGE_2 , $\text{TNF-}\alpha$, IL-6, and IL-1 β was determined using an ELISA kit (R&D systems) according to the manufacturer's instructions, followed by measurements on microplate reader at 450 nm.

2.2.3. Western blot analysis of ALO-treated macrophages

RAW264.7 cells were stimulated with LPS in the presence or absence of 50, 100, or 200 μM ALO for 24 h at 37 °C. Total cell lysates were extracted with RIPA buffer (Cell signaling, USA) and subjected to Western blot analysis to detect β -actin, inducible nitric oxide synthase (iNOS, #13120, Cell Signaling Technology) and cyclooxygenase-2 (COX-2, #12282, Cell Signaling Technology) as described in Section 2.1.9.

2.3. Statistical analysis

The experiments shown are a summary of the data from at least-three experiments and are presented as the mean $\pm$ standard deviation (S.D.). Statistical significance of the data was determined using the ANOVA with Tukey post hoc test; a value of $p < 0.05$ was accepted as statistically significant.

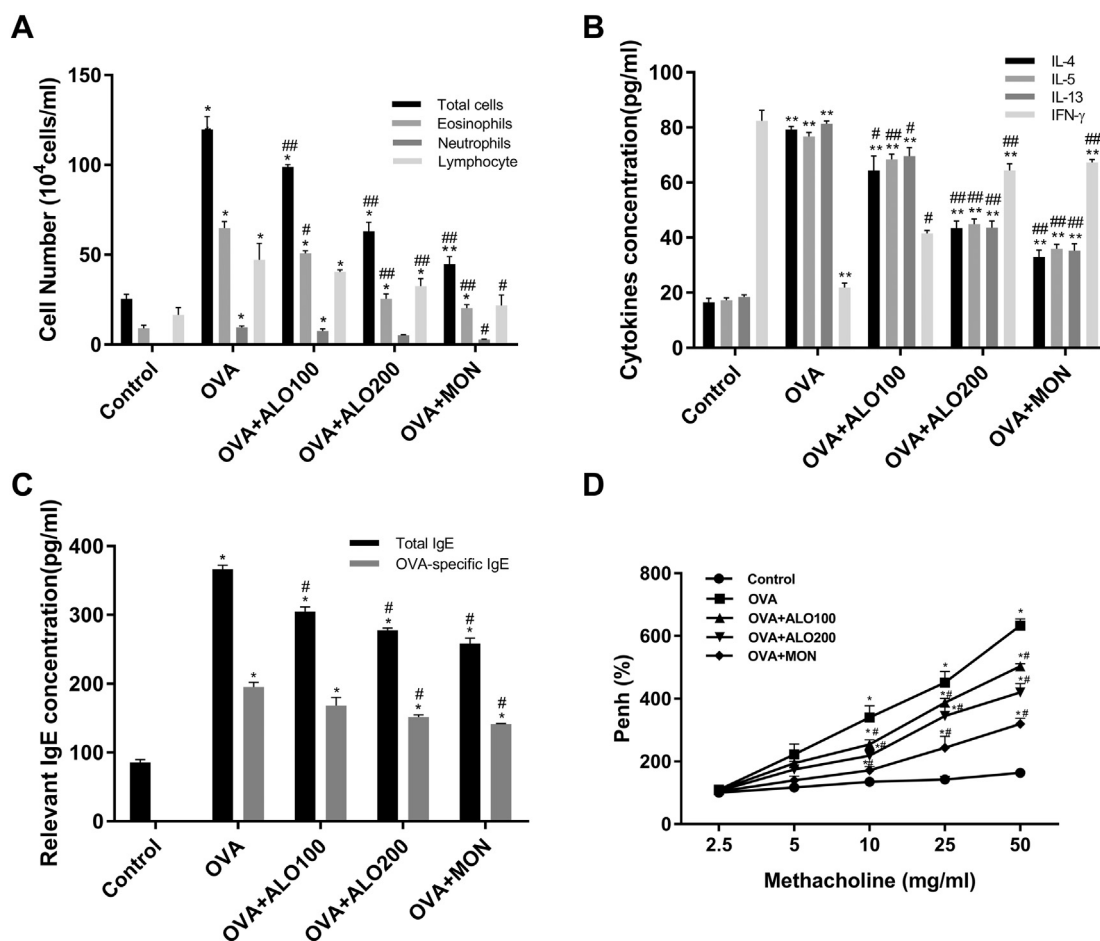


Fig. 2. Effect of aloperine (ALO) on the generation of inflammatory cell infiltration, IL-4, IL-5, IL-13, IFN- γ , and IgE in BALF and AHR. (A) Cell counts in BALF were measured by Diff-Quik staining. (B) The expression levels of IL-4, IL-5, IL-13, and IFN- γ in BALF were measured by ELISA. (C) The concentration of IgE in BALF was measured by ELISA. (D) AHR in OVA-sensitized mice treated with aloperine. Mice were nebulized with various concentrations of methacholine (2.5, 5, 10, 25, and 50 mg/ml) as a bronchoconstrictor. Data are shown as the percentage increase in Penh over the baseline, where the baseline Penh of the saline-treated control group is expressed as 100%. Each value represents the mean $\pm$ S.D. (n = 3). * p < 0.05, ** p < 0.01 versus the control group. # p < 0.05, ## p < 0.01 versus the OVA group.

3. Results

3.1. Effect of ALO on airway inflammation and goblet cell hyperplasia

It is well-known that inflammatory cell infiltration and goblet cell hyperplasia are the crux of the problem in allergic asthma. Hence, we examined whether ALO could alleviate the pathological changes. The number of inflammatory cells was evaluated by H&E stain (Fig. 1A). Compared with the control group, the OVA group showed a great number of infiltrating inflammatory cells around the airway (p < 0.05, Fig. 1B). The OVA + ALO and OVA + MON group clearly showed less inflammatory cell infiltration than the OVA group in a dose-dependent manner (p < 0.05). The production of goblet cells was evaluated by PAS staining. As shown in Fig. 1C, the percentage of PAS-positive cells in the OVA group was significantly higher than the control group (p < 0.05), but the OVA + ALO group could obviously reduce the goblet cells in the airway epithelium and the mucus hypersecretion in a dose-dependent manner (p < 0.05). From these, ALO could remarkably reduce airway inflammation and goblet cell hyperplasia in an asthmatic mouse model.

3.2. Effect of ALO on the generation of inflammatory cell infiltration, IL-4, IL-5, IL-13, IFN- γ , and IgE in BALF and AHR

To understand the effect of ALO on the recruitment of inflammatory

cells into airway, inflammatory cells were counted in BALF. Compared with the control group, the OVA group showed the apparent increase of total inflammatory cells, eosinophils, neutrophils, and lymphocytes in BALF (Fig. 2A). Compared with the OVA group, the OVA + ALO group attenuated total inflammatory cells, eosinophils, neutrophils, and lymphocytes in a dose-dependent manner.

In Fig. 2B, the contents of IL-4, IL-5, and IL-13 in BALF of the OVA group were significantly higher than those in the control group, while the content of IFN- γ of the OVA group was remarkably lower than that in the control group. Compared with the OVA group, the OVA + ALO group could obviously decrease the contents of IL-4, IL-5, and IL-13 and increase the content of IFN- γ in BALF in a dose-dependent manner.

Compared with the control group, the OVA group significantly increased the total IgE and OVA-specific IgE expression in BALF (p < 0.05), while the OVA + ALO group significantly decreased the total IgE and OVA-specific IgE expression in BALF compared to the OVA group (Fig. 2C).

As is shown in Fig. 2D, airway responsiveness was estimated by Penh, and compared with control group, airway responsiveness obviously increased in the OVA group in response to methacholine inhalation. The OVA + ALO group dramatically prevented AHR in response to inhaled methacholine, compared with the OVA group.

Taken together, we suggest that treatment with ALO efficiently inhibits not only the recruitment of inflammatory cells but the production of IL-4, IL-5, IL-13, and IgE and promotes the generation of IFN- γ in

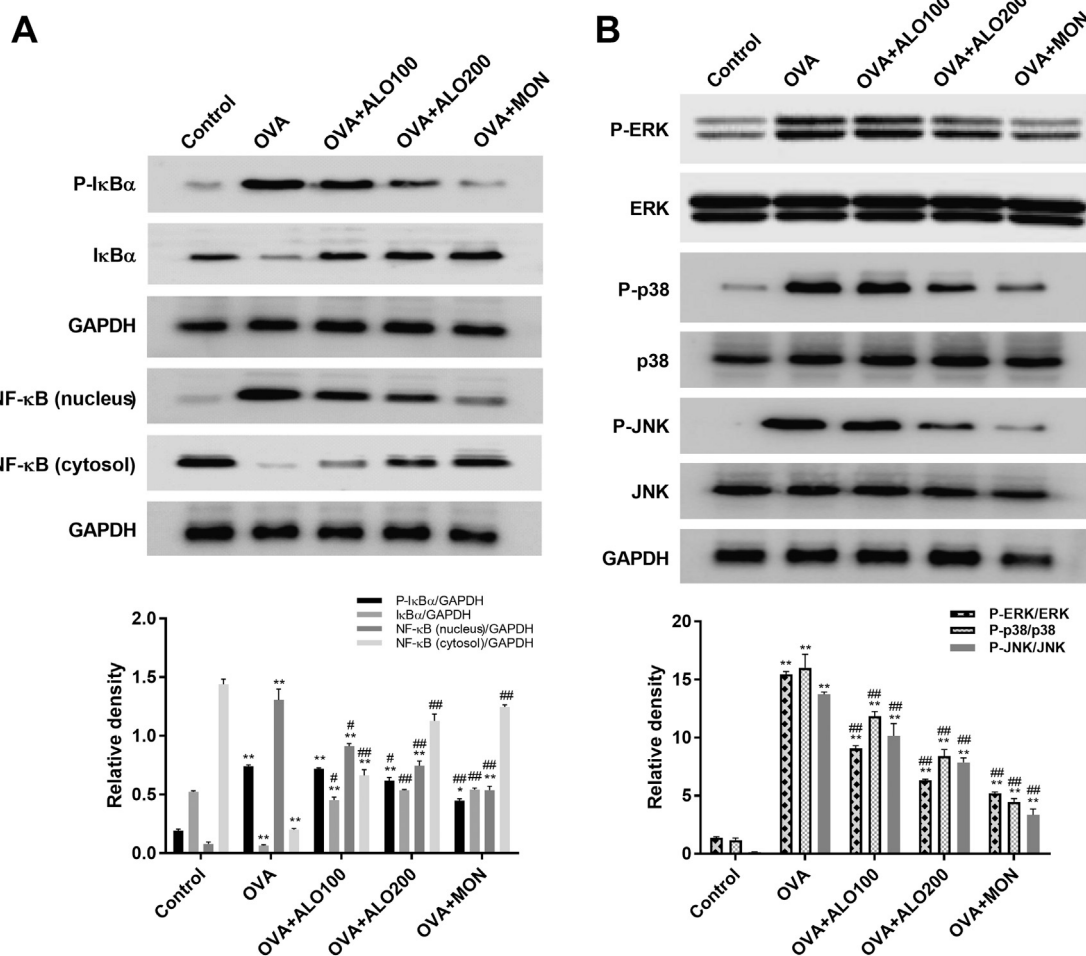


Fig. 3. Effect of aloperine (ALO) on the NF-κB translocation and expression of vital proteins related with the MAPK pathway. The expression of P-IκBα, IκBα, NF-κB (nuclear and cytosol), P-ERK, ERK, P-p38, p38, P-JNK, JNK, and GAPDH were analyzed by Western blot. Each value represents the mean $\pm$ S.D. (n = 3). * p < 0.05, ** p < 0.01 versus the control group. # p < 0.05, ## p < 0.01 versus the OVA group.

BALF, thereby alleviating immune-mediated pathology in asthmatic mice.

3.3. Effect of ALO on the phosphorylation of IκBα and nuclear translocation of NF-κB

As is well-known, NF-κB plays a key role in pathogenesis of allergic inflammatory response via inducing the transcription of numerous pro-inflammatory mediators [4]. To address the issue, the phosphorylation of IκBα and nuclear translocation of NF-κB in asthmatic mice were investigated (Fig. 3A). Compared with the control group, the OVA group increased the expression of phosphorylated (P)-IκBα and NF-κB (nuclear), but the OVA + ALO group had significantly lower levels of NF-κB (nuclear) and P-IκBα than the OVA group in a dose-dependent manner. However, the expression of NF-κB (cytosol) and IκBα were contrary to the expression of P-IκBα and NF-κB (nuclear). The levels of IκBα and NF-κB (cytosol) in OVA group were significantly lower than those in the control group. However, the OVA + ALO group had higher levels of IκBα and NF-κB (cytosol) than the OVA group in a dose-dependent manner. These data indicate that ALO could suppress the nuclear translocation of NF-κB by inhibiting the phosphorylation and subsequent degradation of IκBα. Accordingly, these results represent that ALO might alleviate airway inflammatory reactions by suppressing the NF-κB transcriptional activity.

3.4. Effect of ALO on the expression of vital proteins related with MAPK pathway

Previous study has reported that the generation of various inflammatory cytokines within the asthmatic lung is closely related with MAPK pathways [12]. As shown in Fig. 3B, the phosphorylation of p38, ERK, and JNK was greatly increased in the OVA group, compared with that in the control group (p < 0.01), but these phosphorylated proteins were dose-dependently inhibited by ALO treatment. These findings suggest that ALO plays a crucial role on preventing the development of asthma through limiting the MAPK signaling pathway.

3.5. Effect of ALO on the expression of Nrf2 and HO-1

To assess the antioxidant capacity of ALO, the expression of Nrf2 and HO-1 in allergic mice was investigated. The immunohistochemical staining for Nrf2 and HO-1 in the airways are shown in Fig. 4A. The staining of Nrf2 and HO-1 around the airways was increased in the OVA group, compared with the control group. Treatment with ALO dose-dependently increased the areas of Nrf2 and HO-1 more than the OVA group. This content is in step with the results obtained from Western blot (Fig. 4B). Compared with the control group, the expression of Nrf2 (nuclear) and HO-1 were significantly increased in the OVA group. Furthermore, the expression of Nrf2 (nuclear) and HO-1 in the OVA + ALO group was more increased than that in the OVA group. The expression of Nrf2 (cytosol) was contrary to the expression of Nrf2

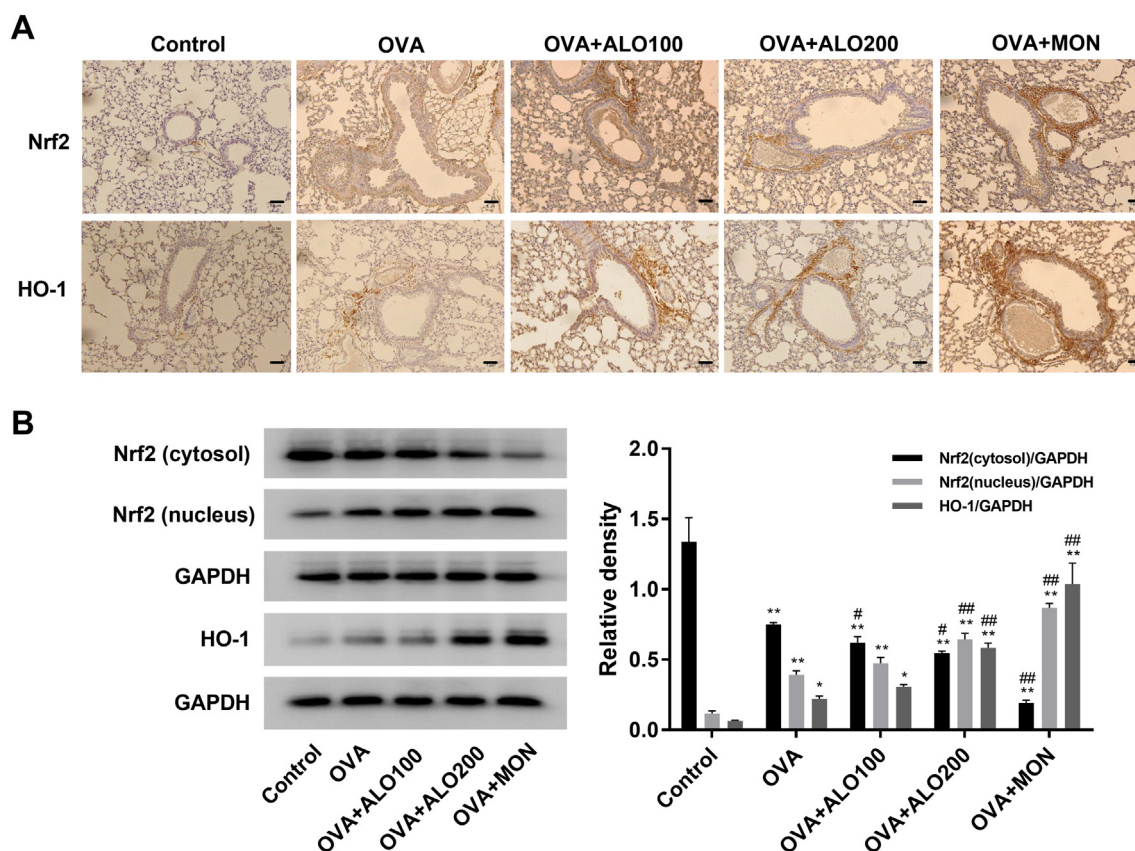


Fig. 4. Effect of aloperine (ALO) on the expression of Nrf2 and HO-1. (A) Immunohistochemical staining was performed to measure the expression of Nrf2 and HO-1. $\times 200$ magnification, scale bar: 5 μ m. (B) The expression of Nrf2 and HO-1 was analyzed by Western blot. Each value represents the mean $\pm$ S.D. (n = 3). * $p < 0.05$, ** $p < 0.01$ versus the control group. # $p < 0.05$, ## $p < 0.01$ versus the OVA group.

(nuclear). These results imply that ALO alleviates allergic airway inflammation by promoting the activation of Nrf2/HO-1 pathway.

3.6. Effect of ALO on the production of inflammatory mediators and cytokines by LPS-stimulated RAW264.7 cells

Previous works have revealed that alveolar macrophages play an essential role in the development of allergic asthma [13]. Therefore, we examined the effect of ALO treatment on macrophage activation by treating RAW 264.7 cells with LPS in the presence of absence of ALO and then measuring their production of the inflammatory mediators NO and PGE₂, their synthases iNOS and COX-2, respectively, and the pro-inflammatory cytokines TNF- α , IL-6, and IL-1 β . ALO treatment significantly inhibited NO and PGE₂ levels (Fig. 5A and B) along with iNOS and COX-2 levels (Fig. 5C). These results indicate that ALO suppressed NO and PGE₂ production by down-regulating iNOS and COX-2 expression. ALO also significantly down-regulated TNF- α , IL-6, and IL-1 β (Fig. 6A, B, and C). Notably, ALO treatment did not have significant cytotoxic effects on RAW 264.7 cells at the maximum concentration used in the in vitro experiments 200 μ M (Fig. 6D).

4. Discussion

To the best of our knowledge, this is the first report showing the inhibitory effect of ALO on bronchial asthma in mice. In the present study, we utilized the murine asthma model to explore the potential effect of ALO on allergic airway inflammation and montelukast sodium was used as the standard reference drug to compare the anti-asthmatic activity of ALO. As mentioned above, the characteristic of chronic airway inflammation is the increasing infiltration of leukocytes, especially eosinophils, which is a main factor in the pathogenesis of asthma

[14]. Our results demonstrate that ALO prominently decreased inflammatory cells infiltration around the airways and goblet cell hyperplasia in OVA-challenged mice. Concretely, ALO prevented the accumulation of eosinophils, neutrophils, and lymphocytes into the airways, as evidenced by an obvious drop in differential cell counts in BALF. Importantly, there was a highly significant reduction in the number of eosinophils in the asthmatic mice treated by ALO. Taken together, it implies that ALO suppresses inflammatory reaction in the asthmatic lung.

Allergic asthma represents that the Th2 cell activity is activated, whereas the activity of Th1 cells is decreased, thereby the imbalance of T1/T2 cells contribute to the pathogenesis of asthma [15]. Th2 cells transfer to the lung and release the Th2 type cytokines, such as IL-4, IL-5, and IL-13, which would promote the development of asthma. However, Th1 cytokines, such as IFN- γ , inhibits the inflammation of asthmatic airways [16]. Our results show that ALO obviously reduced the content of IL-4, IL-5, and IL-13 and enhanced the content of IFN- γ in BALF. Therefore, the data represent that ALO might block airway inflammation in asthmatic mice through inducing a balanced Th1/Th2 immune response.

The production of IgE is related with not only allergic airway inflammation but also bronchial hyperreactivity in a murine asthma model. IgE triggers eosinophils as well as mast cells to secrete cytokines such as IL-4, IL-5, and IL-13 [17]. Our results report that ALO significantly reduced the contents of total IgE and OVA-specific IgE in a dose-dependent manner in allergic mice. From these, it is likely that ALO lowers the production of IgE to inhibit asthmatic airway inflammation.

AHR is a typical clinical symptom of asthma, which is the meaning of morbid change in airflow limitation in response to a provoking stimulus. Even though the mechanism of AHR is unclear, it is convincing

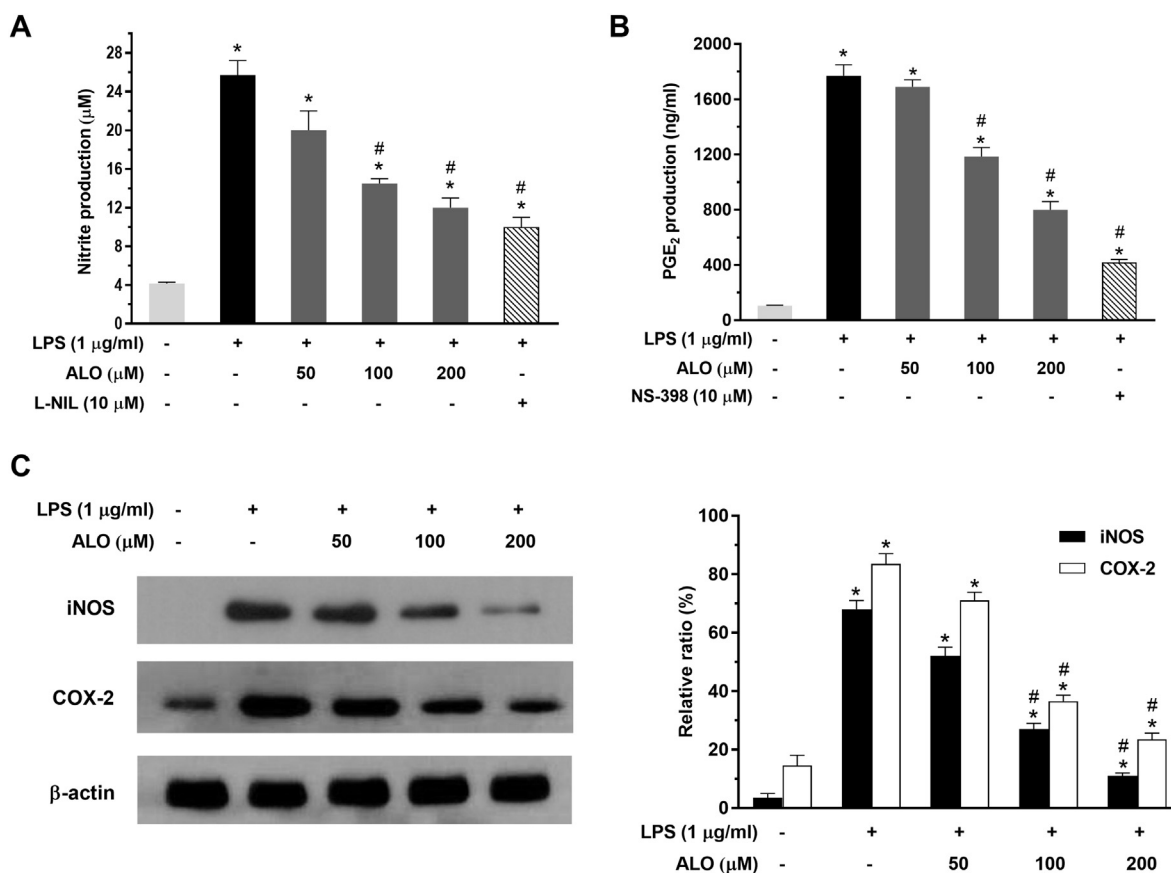


Fig. 5. Effect of aloperine (ALO) on the production of pro-inflammatory mediators and their synthases by lipopolysaccharide-stimulated RAW264.7 cells. The macrophages were treated with lipopolysaccharide (LPS) in the presence or absence of 50, 100, or 200 µM ALO. (A) The nitrite production of the cells was measured using the Griess reagent. (B) The prostaglandin (PG) E₂ production of the cells was measured by ELISA. (C) The expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) was analyzed by Western blot. Each value represents the mean $\pm$ S.D. (n = 3). **p* < 0.05 versus the untreated group. #*p* < 0.05 versus the only LPS-treated group.

that inflammatory factors released during allergic inflammation play an important role in the development of AHR [16]. Our data represent that ALO effectively reduces OVA-induced AHR in response to inhaled methacholine.

Activation of NF- κ B in asthma induces the accumulation of inflammatory cells around the airway and drives most of antigen-induced inflammation, which is an efficient contributor to adaptive immune responses [18]. Concretely, bound I κ B α is quickly phosphorylated by I κ B α kinase when activated by inflammatory cytokines and subsequently phosphorylated I κ B α is degraded by proteasomes. The free dimeric NF- κ B liberated by the dissociation of I κ B α translocates into the nucleus where it induces transcription of various target genes which encode a number of inflammatory cytokines, which are related with the pathogenesis of asthma [19]. The results show that ALO apparently inhibited the translocation of NF- κ B from cytoplasm to nucleus in a dose-dependent fashion in asthmatic mice. Moreover, the phosphorylation and degradation of I κ B α , which is required for NF- κ B activation, was inhibited by ALO in a dose-dependent manner. These findings imply that ALO might attenuate allergic airway inflammation by decreasing the activation of the NF- κ B pathway.

The production of inflammatory cytokines is closely related with MAPK. B-cell receptor primarily activates the ERK pathway which may be involved in allergic conditions, such as asthma, allergy, and anaphylaxis [20]. Moreover, p38 MAPK is essential for phosphorylating the transcription factor GATA-3, which in turn regulates the production of IL-4, IL-5, and IL-13 [21]. Furthermore, JNK and signal transducer and activator of transcription 3 (STAT 3) are closely related with airway inflammation in a murine asthma model [22]. Our results show that

ALO obviously inhibited the phosphorylation of ERK, p38, and JNK. From these, it is suggested that ALO suppresses airway inflammation by limiting MAPK pathway in lungs of OVA-challenged mice (Fig. 4).

Nrf2/HO-1 pathway is one of the most important anti-oxidative stress mechanisms in cellular defense system. Previous studies have shown that HO-1 can effectively alleviate the airway oxidative stress response, inflammation, and excessive mucus secretion in asthma [23]. Under normal physiological conditions, Nrf2, the most important transcription factor for HO-1, is stored in the cytoplasm in combination with the inhibitory protein Keap1. When there is stimulation, Nrf2 is dissociated and transferred to the nucleus, which binds to ARE and activates the transcription of the HO-1 gene, completing the antioxidant effect. In our results, ALO significantly increased the expression of Nrf2 (nuclear) and HO-1 in the lung tissues of OVA-challenged mice. These findings suggest that ALO attenuates airway inflammation by reducing oxidative stress via promoting the activation of Nrf2/HO-1 pathway.

Several studies imply that various herbal constituents can exert anti-inflammatory effects in asthma by suppressing the production of the pro-inflammatory mediators including NO and PGE₂ along with their synthases [24]. These pro-inflammatory mediators and their synthases are considered as immune accelerators that drive macrophage activation in particular. Indeed, these immune facilitators elevate the macrophage production of cytokines (i.e. TNF- α , IL-6, and IL-1 β) and promote the progression of asthma [25–27]. Given these observations, we assessed whether the anti-asthmatic effects of ALO may also be due to its ability to abrogate the macrophage production of pro-inflammatory mediators. Expectedly, LPS-stimulated RAW 264.7 cells pretreated by ALO have shown that ALO inhibits the LPS-induced production of NO

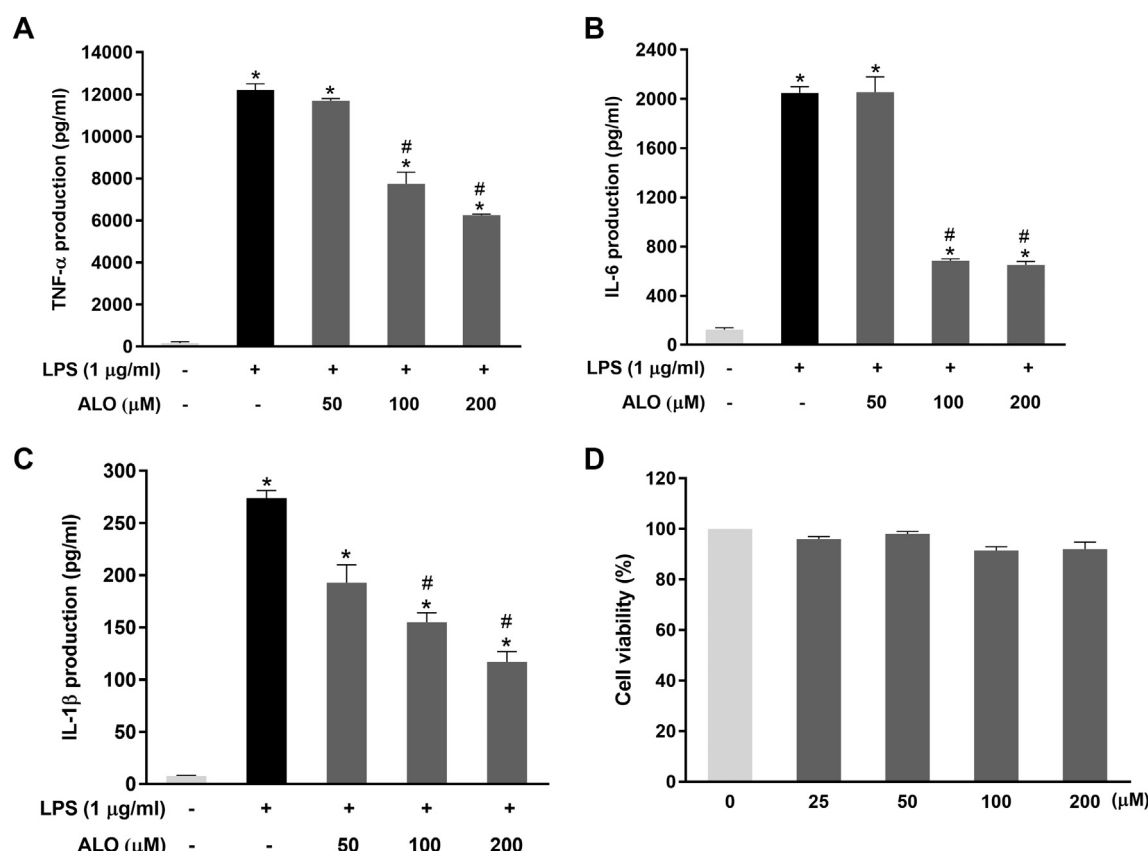


Fig. 6. Effect of aloperine (ALO) on the production of pro-inflammatory cytokines by lipopolysaccharide-stimulated RAW264.7 cells. The macrophages were treated with lipopolysaccharide (LPS) in the presence or absence of 50, 100, or 200 μM ALO. (A–C) The production of TNF-α, IL-6, and IL-1β was measured by ELISA. (D) Cell viability was measured using the MTT assay. Each value represents the mean ± S.D. (n = 3). **p* < 0.05 versus the untreated group. #*p* < 0.05 versus the only LPS-treated group.

and PGE₂ by down-regulating iNOS and COX-2 expression. Moreover, ALO treatment of the macrophage cell line decreased its LPS-induced production of the pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β.

In conclusion, our study demonstrates that ALO ameliorates asthmatic features by inhibiting the generation of IgE as well as regulating the balance of Th1/Th2 through NF-κB, MAPK, and Nrf2/HO-1 signaling pathways in an asthma model. These results explain that ALO might be a potential anti-inflammatory agent in asthma.

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Author contributions

Chongyang Wang, Yun Ho Choi, and Zhemin Xian conceived and designed the experiments; Chongyang Wang and Mingyu Zheng performed the experiments and analyzed the data; Hongmei Piao and Guanghai Yan contributed reagents/materials/analysis tools; Chongyang Wang, Yun Ho Choi, and Zhemin Xian wrote the paper.

Conflicts of interest

The authors declare that there are no financial and commercial conflicts of interest.

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