

RESEARCH COMMUNICATION



α -Asarone alleviates allergic asthma by stabilizing mast cells through inhibition of ERK/JAK2-STAT3 pathway

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Abstract

Asthma is a heterogeneous disease related to numerous inflammatory cells, among which mast cells play an important role in the early stages of asthma. Therefore, treatment of asthma targeting mast cells is of great research value. α -Asarone is an important anti-inflammatory component of the traditional Chinese medicine *Acorus calamus* L, which has a variety of medicinal values. To investigate whether α -asarone can alleviate asthma symptoms and its mechanism. In this study, we investigated the effect of α -asarone on mast cell activation in vivo and in vitro. The release of chemokines or cytokines, AHR (airway hyperresponsiveness), and mast cell activation were examined in a mast cell-dependent asthma model. Western blot was performed to determine the underlying pathway. α -Asarone inhibited the degranulation of LAD2 (laboratory allergic disease 2) cells and decreased IL-8, MCP-1, histamine, and TNF- α in vitro. α -Asarone reduced paw swelling and leakage of Evans blue, as well as serum histamine, CCL2, and TNF- α in vivo. In the asthma model, α -asarone showed an inhibitory effect on AHR, inflammation, mast cells activation, infiltration of inflammatory cells, and the release of IL-5 and IL-13 in lung tissue. α -Asarone decreased the levels of phosphorylated JAK2, phosphorylated ERK, and phosphorylated STAT3 induced by C48/80. Our findings suggest that α -asarone alleviates allergic asthma by inhibiting mast cell activation through the ERK/JAK2-STAT3 pathway.

KEYWORDS

asthma, ERK/JAK2-STAT3, mast cell, α -Asarone

Abbreviations: ACh, acetylcholine chloride; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; CCL, C-C motif chemokine ligand; Cst, Static lung compliance; DEX, dexamethasone; DMSO, dimethyl sulfoxide; G, tissue damping; IL, interleukin; LAD2, laboratory allergic disease 2; MCP-1, Monocyte chemoattractant protein-1; MRGPRX2, MAS-related G-protein coupled receptor-X2; OVA, ovalbumin; PAS, periodic acid-Schiff; Rn, Newtonian resistance; Rrs, respiratory resistance; TNF, tumor necrosis factor.

1 | INTRODUCTION

Asthma is a heterogeneous disease characterized by chronic airway inflammation, airway hyperresponsiveness to a variety of stimuli, variable reversible airflow limitation, and a series of airway structural changes with the course of the disease, namely airway remodeling.^{1,2} Worldwide, asthma affects more than 300 million people,

and the prevalence of adults diagnosed is approximately 4.3%. there are significant differences between countries, the incidence rate of diagnosis in China is only 0.2%, however, with the Westernization of lifestyle and the development of society, this figure in China is rapidly increasing.^{3–6}

Airway hyperresponsiveness is related to a variety of lymphocytes, such as eosinophils, basophils, neutrophils, macrophages, and mast cells, in which mast cells play an important role in lung inflammation and airway remodeling.⁷ In the process of allergic asthma, resident tissue-based mast cells migrate to the inflammatory site and

stimulate the contraction of the bronchial smooth muscle bundle by releasing a variety of inflammatory factors, leading to various pathophysiological changes in asthma.⁸ Inhaled allergens activate mast cells in two ways. One way is to activate lymphoid B cells, which leads to the secretion of a specific IgE and the participation of airway epithelial cells, antigen-presenting cells, interleukins (IL-4, IL-5, IL-13), and other Th2 cytokines; the other is through direct activation by binding to the MRGPRX2 (MAS-related G-protein coupled receptor-X2) on the cytoplasmic membrane of mast cells.⁹ IgE binds to FcεR1 (high-affinity IgE receptor) on the cytoplasmic

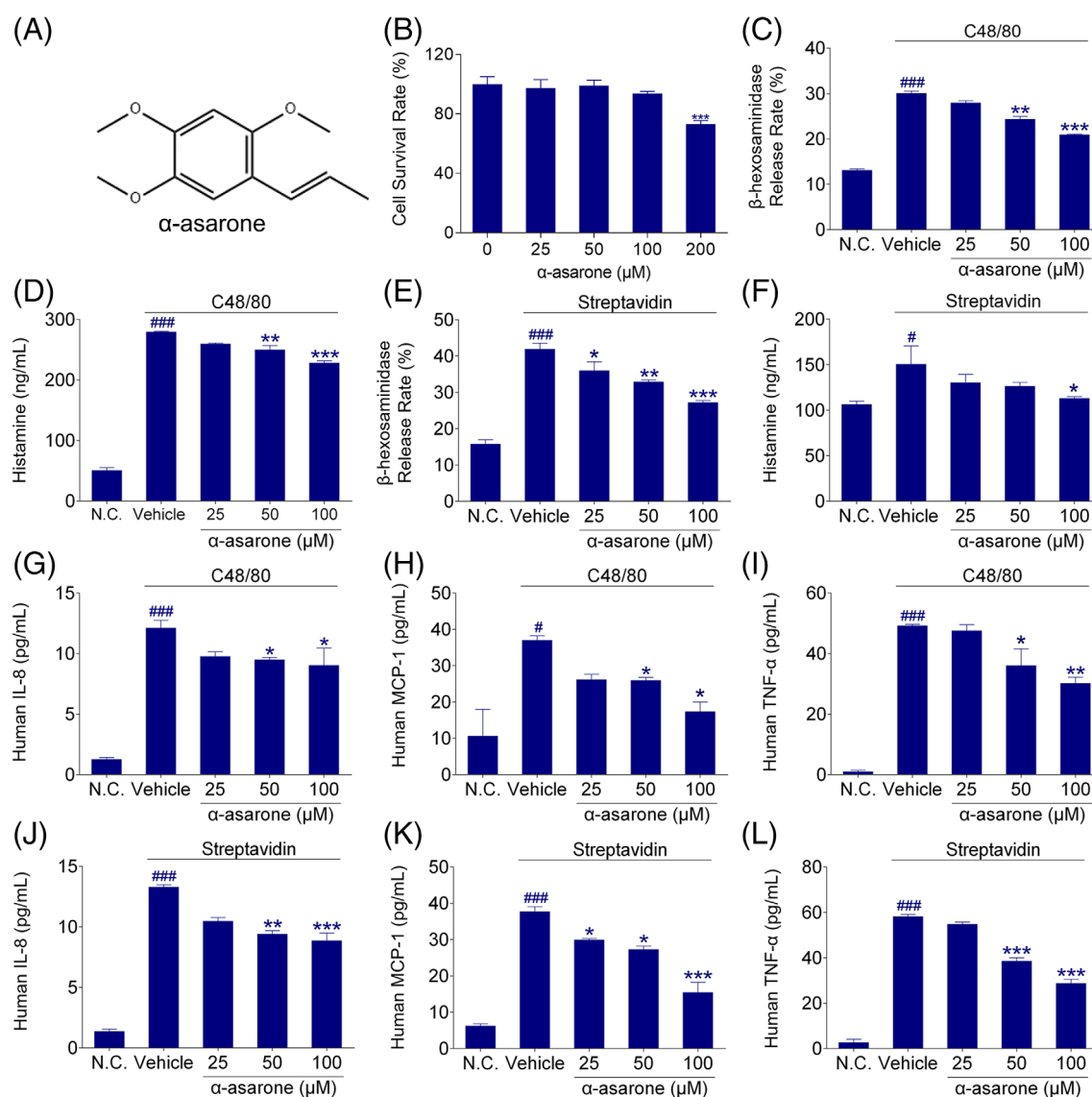


FIGURE 1 α-Asarone inhibits degranulation and cytokine release in LAD2 cells. Tyrode's solution was used as the negative control (N.C.), and the vehicle group was challenged with an equal volume of 30 μg/ml C48/80 or 100 ng/ml streptavidin. (A) Structure of α-asarone. (B) Toxicity of α-asarone after 24 h of treatment of LAD2 cells at the appropriate concentrations. (C–D) the β-hexosaminidase and histamine release of LAD2 cells induced by C48/80 after α-asarone treatment. (E–F) the β-aminohexose and histamine release of LAD2 cells induced by streptavidin after α-asarone treatment. (G–I) cytokine release in LAD2 cells induced by C48/80. (J–L) cytokine release in LAD2 cells induced by streptavidin. Experiments were repeated three times, and data are presented as mean ± SD and analyzed using a two-tailed unpaired Student's t-test. Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$, versus vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group); $n = 3$

membrane, leading to mast cell activation.^{10–12} Activated mast cells can produce a variety of inflammatory factors, such as histamine, interleukin, and prostaglandin, which can cause smooth muscle contraction, mucosal secretion, allergic inflammation, and inflammatory cell infiltration.¹³ Furthermore, mast cells contribute to the recruitment and activation of other immune cells, particularly eosinophils, which are the hallmarks of chronic asthmatic response and airway remodeling.¹⁴

At present, the commonly used drugs for the treatment of allergic asthma are as follows: β -2 receptor agonists, anti-inflammatory drugs including hormones, and leukotriene modulators, which have side effects when used long-term.^{15,16} Monoclonal antibody drugs such as reslizumab, benralizumab, and omalizumab present a huge economic burden to patients, and biological treatment is not successful in all patients.^{17,18} Therefore, it is necessary to develop drugs for treating allergic asthma. Traditional Chinese medicine is an important source of new bioactive small molecules. *Acorus calamus* L., which has been used as a medicinal plant in China for over 2000 years, has a variety of biological activities and is used to treat many ailments such as inflammation, chest pain, rheumatism, bronchitis, cough, fever, cramps, depression, and tumors.^{19,20} To date, many studies have reported the anti-inflammatory and antioxidant effects of α -asarone, as shown in Figure 1A, which is one of the active ingredients of *Acorus calamus* L..^{20–23} However, whether α -asarone could reduce allergic reaction as well as alleviate allergic asthma and its mechanism is still unclear.

2 | MATERIALS AND METHODS

2.1 | Chemicals

α -Asarone (purity >98%, HPLC) was purchased from Herbest (Baoji, Shannxi, China), Evans blue, *p*-nitrophenyl *N*-acetyl- β -D-glucosamide (β -hexosaminidase), biotinylated human IgE were purchased from Sigma-Aldrich (St. Louis, MO). DEX (Dexamethasone) (purity >98%), OVA (Ovalbumin) (grade V, purity $\geq$ 98%), Streptavidin, ACh (acetylcholine chloride), and histamine were purchased from Sigma-Aldrich (St. Louis). StemPro™-34 medium, L-glutamine, human stem cell factor, and penicillin–streptomycin were provided by Cell Signaling Technology (MA), as well as Abbkine-Cell Counting Kit was from Abbkine Inc (California, USA). All ELISA kits were purchased from Sino Biological (Beijing, China). Triton X-100 was obtained from Qingdao Meigao Chemical Co., Ltd. (Qingdao, China).

Antibodies: JAK2, phosphorylated JAK2 (Tyr1007/1008), STAT3, and phosphorylated STAT3 (Tyr727) were purchased from proteintech (Chicago, USA). ERK1/2 and phosphorylated ERK1/2 (Thr202/Tyr204), were purchased from Cell Signaling (Boston, MA). GAPDH was purchased from Sino Biological (Beijing, China).

2.2 | Animals

Female C57BL/6 mice (8 weeks old) were purchased from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, China). The animals were kept in separate cages in a large colony room, with free water access and standard dry feed twice a day. The breeding environment is 20–25°C, relative humidity is 40%, and the light/dark cycle is 12 hours. All experiments, involving equal treatments in animals, were conducted by investigators blinded to the conditions.

2.3 | Ethical considerations

This study was conducted in strict accordance with the recommendations stated in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The experimental protocols for the animal experiments were approved by the Animal Ethics Committee in Xi'an Jiaotong University, Xi'an, China (Permit Number: XJTU 2019–713).

2.4 | Cell lines

The Laboratory of Allergic Disease 2 (LAD2) human mast cells (suspension cell) were kindly provided by Daniel Lee (NIH, USA). Cells are maintained in StemPro medium containing StemPro nutrient supplement (10 ml/L), 1:100 penicillin–streptomycin, 2 mM glutamine, 100 ng/ml human SCF in 5% carbon dioxide 37°C. The medium was replaced for half a week, and the cells are maintained at 2×10^6 cells/ml.

2.5 | Cytotoxicity assay

1×10^6 LAD2 cells per well were seeded in a 96-well plate and treated with different concentrations of α -asarone (0, 25, 50, 100, and 200 μ M) for 24 hours, five parallel controls were set for each concentration. An abbkine-Cell Counting Kit assay (California, USA) was used to determine the Cytotoxicity assay Cell viability.

2.6 | Measurement of β -hexosaminidase

Biotinylated human IgE (100 ng/ml) was used to sensitize LAD2 cells that were used to observe IgE-mediated allergic reactions overnight. The cells used to observe anaphylactoid reactions did not undergo any pretreatment. All the cells were incubated with α -asarone (0, 25, 50, and 100 μ M) which was dissolved in Tyrode's solution (TM buffer, 6.954 g/L sodium chloride, 0.353 g/L potassium chloride, 0.282 g/L calcium chloride, 0.143 g/L MgSO_4 , 0.162 g/L KH_2PO_4 , 2.383 g/L informed, 0.991 g/L glucose, and 1 g/L bovine serum albumin [BSA], pH 7.4) for 30 minutes, after that, 100 ng/ml streptavidin or 30 μ g/ml C48/80 were used to stimulate LAD2 cells for 30 minutes respectively. Cells were lysed with 0.1% (v/v) Triton x-100 for 5 minutes to get intracellular β -hexosaminidase. All the supernatants were collected and incubated with 1 mM *p*-Nitrophenyl *N*-acetyl- β -D-glucosamide in 0.1 M citric acid/sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The hydrolysis reaction was terminated with 150 μ l stop buffer (0.1 M $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 11), and Absorbance 405 nm was measured with FlexStation 3. The percentage of β -hexosaminidase release was calculated as follows: the extracellular supernatant absorbance $\times$ 100%/the sum of the extracellular supernatant absorbance and the intracellular supernatant absorbance.

2.7 | Paw swell in C48/80 & OVA-induced mice

A total of 40 mice were divided into two parts. 20 mice were stimulated by intraperitoneal injection of 0.5 mg/kg bw OVA (resolved in saline) twice before the experiment, with an interval of 1 day, to evaluate the allergic reaction mediated by IgE. The remaining 20 untreated mice were used to evaluate C48/80 induced pseudo-allergic reactions. Both two parts of mice were randomly divided into four groups with five mice in each group. 0, 5, 10 and 20 mg/kg bw α -asarone (resolved in 0.5% CMC-Na, 0.2 ml) were administered once by gavage respectively in both parts. After intragastric administration for 30 minutes, mice were anesthetized by intraperitoneal injection of 0.8 mg/kg bw pentobarbital sodium (resolved in saline, 0.2 ml). The thickness of the left and right hind paws of mice were recorded. Evans blue normal saline solution (0.2 ml, 4 mg/ml) was injected into tail vein. 5 μ l Irritants (50 μ g/kg bw OVA used to evaluate the allergic reaction mediated by IgE, 30 μ g/kg bw C48/80 used to evaluate the pseudo-allergic reactions.) were injected into the left paw of mice with a microinjector, and the same volume of normal saline was injected into the right hind paw of mice as a

negative control. After 15 minutes, the mice were sacrificed and the thickness of the left and right paws was recorded. The photos of paws were taken and paw tissues were collected into 1.5 ml eppendorf tubes, dried for 24 hours at 50°C, and weighed. Evans blue was extracted by adding the 450 μ l acetone-saline (7:3) mixture into eppendorf tubes and cutting paw tissues into pieces. The supernatant was collected into a 96-wells plate (200 μ l/well) and the absorbance value was measured at 620 nm with a spectrophotometer (Bio-rad, CA).

2.8 | Chemokine release assay

Biotinylated human IgE (100 ng/ml) was used to sensitize LAD2 cells (1×10^6 /well in a 96-well plate) overnight, which was used to observe IgE-mediated allergic reactions. The cells used to observe anaphylactoid reactions did not undergo any pretreatment. After the culture medium was removed, cells were incubated with different concentrations of α -asarone (0, 25, 50, 100 μ M) and streptavidin or C48/80 for 6 hours at 37°C respectively. Human TNF- α , IL-8, and MCP-1 concentrations in the supernatant were detected with ELISA kits according to provided instructions.

All the mice were divided into two parts. One part was stimulated by intraperitoneal injection of 0.5 mg/kg bw OVA twice before the experiment, with an interval of 1 day, which was used to evaluate IgE-mediated allergic reactions; The others without pretreatment were used to evaluate C48/80 induced anaphylactic reactions. One hour before the challenge by injection of 50 μ g/kg bw OVA or 30 μ g/kg bw C48/80 to mice tail vein respectively, different doses of α -asarone (0, 5, 10, 20 mg/kg bw) were randomly divided into different groups. The blood of mice was collected 7 hours after OVA or C48/80 stimulation, Mouse TNF- α and CCL2 concentrations in the supernatant were detected with ELISA kits according to provided instructions.

2.9 | Histamine release assay

Biotinylated human IgE (100 ng/ml) was used to sensitize LAD2 cells (1×10^6 /well in a 96-well plate) overnight, which was used to observe IgE-mediated allergic reactions. The cells used to observe anaphylactoid reactions did not undergo any pretreatment. After the culture medium was removed, cells were incubated with different concentrations of α -asarone (0, 25, 50, 100 μ M) and streptavidin or C48/80 for 1 hour at 37°C respectively. The supernatants and cell lysates were collected. Histamine release assay was carried out using a LC-ESI-MS/MS method which is reported previously.²⁴

2.10 | IgE release assay

ELISA kits were used to measure serum IgE of OVA-induced type I allergic mouse model as well as IgE level in BALF of OVA-induced allergic asthmatic mice according to the manufacturer's instructions.

2.11 | OVA-induced lung allergic inflammation in mice

Briefly, adult female mice were randomly divided into the following groups (5 mice per group): N.C. (0.5% CMC-Na, 0.2 ml), vehicle (0.5% CMC-Na, 0.2 ml), experimental (5, 10, 20 mg/kg bw α -asarone resolved in 0.5% CMC-Na, 0.2 ml), and Dex group (5 mg/kg bw resolved in 0.5% CMC-Na, 0.2 ml). The mice in each group were intraperitoneally sensitized with 0.5 mg/kg bw OVA (resolved in physiological saline, 0.2 ml) on Days 1, 3, 5, and 7, while the N.C. group was sensitized with physiological saline. On days 21, 23, 25, 27, and 29, mice in each group were given corresponding dose of α -asarone orally administrated via gavage, 30 minutes later, mice were placed in sealed containers and given filtered 1% OVA (resolved in physiological saline, 4 ml) inhalation for 30 minutes each time to induce asthma attacks. The vehicle group was atomized and inhaled with same volume of physiological Saline. After the last challenge, mice were anesthetized and evaluated for AHR severity with flexiVent apparatus (SCIREQ). Mice were sacrificed, and lung tissues were subjected to staining.

2.12 | Measurement of airway reactivity in mice

Mice were anesthetized by intraperitoneal injection of pentobarbital sodium salt (1%) and ventilated. Baseline lung function was measured using a flexiVent apparatus (SCIREQ), as described previously. Mice were exposed to acetylcholine, and Rn (Newtonian resistance), Rrs (respiratory resistance), G (tissue damping), and Cst (Static lung compliance) were determined using flexiVent.

2.13 | BALF collection

The BALF collection assay was the same as the protocol of the previous study.²⁵

2.14 | Lung histology

The lung histology assay was the same as the protocol of the previous study.²⁵

2.15 | Western blot analysis

LAD2 cells were seeded into a 6-well plate (1×10^7 cells/well), cells were then incubated with α -asarone (0, 25, 50, and 100 μ M) for 30 minutes followed with 30 μ g/ml streptomycin stimulation for 30 minutes, the details was the same as the protocol of the previous study.²⁶

2.16 | Statistical analyses

All results were repeated at least three times and calculated as the mean $\pm$ SD. Independent sample variance analysis was used to determine significance in statistical comparisons using SPSS 18.0 (IBM, USA). Differences were considered significant at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$, versus Vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group).

3 | RESULTS

3.1 | α -Asarone inhibited degranulation and cytokine release in LAD2 cells

To investigate the inhibitory effect of α -asarone (Figure 1A) on LAD2 cell activation in vitro, the cytotoxic effect of α -asarone on LAD2 cells was first evaluated to exclude the inhibitory effect of drugs that interfered with cell proliferation. The survival rate of LAD2 cells exceeded 90% after treatment with 100 μ M α -asarone, indicating that α -asarone showed no obvious proliferative toxicity to LAD2 cells at this particular concentration. However, higher cytotoxicity was observed at a concentration of 200 μ M (Figure 1B). Therefore, we determined 100 μ M α -asarone as the maximum concentration for cell experiments. Next, we determined the inhibitory effect of different concentrations of α -asarone on C48/80 induced LAD2 cells by measuring the release of β -hexosaminidase and histamine. α -Asarone showed a dose-dependent inhibitory effect on C48/80 induced degranulation of LAD2 cells. The maximum inhibitory effect was observed at a maximum of 100 μ M. α -Asarone inhibited half of the β -aminohexose release at the concentration of 100 μ M when compared with the vehicle group. The release of histamine decreased from 279.80 ± 0.73 ng/ml to 228.40 ± 3.34 ng/ml (Figure 1C-D). ELISA was used to detect MCP-1, IL-8, and TNF- α in the supernatant of the C48/80-induced cell model. α -Asarone inhibited IL-8, MCP-1, and TNF- α levels in a dose-dependent manner. Specifically, with the pretreatment of α -asarone, MCP-1 decreased from 37.03 ± 1.66 pg/ml to 17.40 ± 3.73 pg/ml, IL-8 decreased from 12.12 ± 0.64 pg/ml to

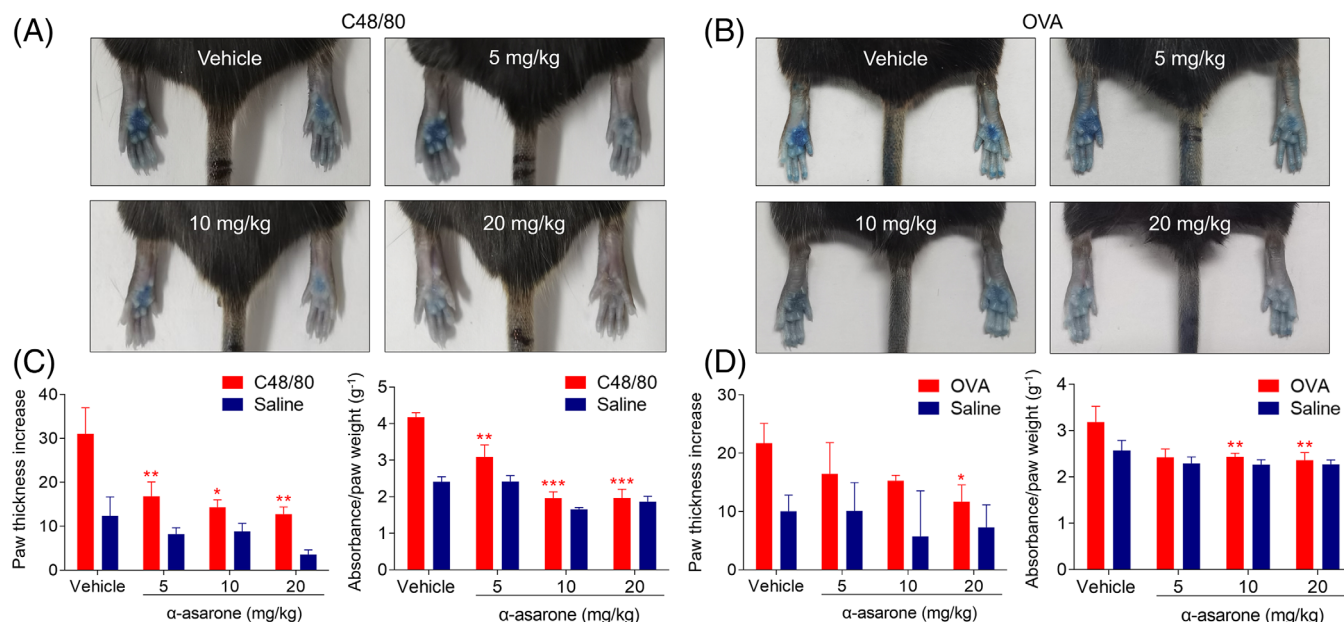


FIGURE 2 α -Asarone inhibits local inflammatory reactions. Mice were stimulated with 30 μ g/ml C48/80 or 50 μ g/ml OVA in the left paws. The right paws were stimulated with physiological saline as a negative control (saline). (A) Pictures of paw swelling in mice stimulated with C48/80 after α -asarone administration. (B) Pictures of paw swelling in mice stimulated with OVA after α -asarone administration. (C) Paw swelling rate and Evans blue exudation in mice stimulated with C48/80 from different groups. (D) Paw swelling rate and Evans blue exudation in mice stimulated with OVA from different groups. Experiments were repeated three times. Data are presented as mean $\pm$ SD. Two-tailed unpaired Student's *t*-tests were used for the analysis. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$, versus vehicle group; $n = 5$

9.05 $\pm$ 1.41 pg/ml, and TNF- α decreased from 49.27 $\pm$ 0.40 pg/ml to 30.19 $\pm$ 1.98 pg/ml (Figure 1G–I). We also verified the inhibitory effect of α -asarone on the streptavidin-induced activation of LAD2 cells. α -Asarone inhibited the release of β -hexosaminidase and histamine in a dose-dependent manner; it inhibited half the release of β -hexosaminidase and histamine at a concentration of 100 μ M (Figure 1E–F). Additionally, MCP-1 levels decreased from 37.76 $\pm$ 1.87 pg/ml to 15.49 $\pm$ 3.94 pg/ml, IL-8 decreased from 13.30 $\pm$ 0.26 pg/ml to 8.87 $\pm$ 0.89 pg/ml, and TNF- α decreased from 58.25 $\pm$ 1.19 pg/ml to 28.79 $\pm$ 2.38 pg/ml following pretreatment with α -asarone (Figure 1J–L).

3.2 | α -Asarone inhibited local inflammatory reactions

To further verify the anti-allergic activity of α -asarone in vivo, a mouse paw swelling and extravasation model was established. The rate of increase in paw thickness and leakage rate of Evans blue were recorded to evaluate the severity of the allergic reaction. The left paws, which were injected with C48/80 (30 μ g/ml) or OVA (50 μ g/ml), were assigned to the treated group, right paws injected with Saline were assigned to the saline group.

Photographs of each mouse were taken (Figure 2A–B). α -Asarone reduced paw swelling and Evans blue leakage in a dose-dependent manner. When the administration concentration reached 20 mg/kg bw, α -asarone inhibited nearly half of the paw swelling and Evans blue leakage (Figure 2C–D).

3.3 | α -Asarone inhibits LAD2 mediated systemic anaphylaxis

Activated mast cells can produce many cytokines, including CCL2 and TNF- α , which can reflect the severity of inflammation and allergic reactions, and were chosen as the targets of our investigation.²⁷ After 30 minutes of α -asarone intragastric administration, C48/80 or OVA was injected through the tail vein to trigger systemic anaphylaxis. Histamine, as a pre-stored mediator, can be detected in the serum after 1 hour of injecting C48/80 or OVA, while cytokines were detected 8 hour after injection. Specifically, α -asarone significantly reduced serum TNF- α ; it almost completely inhibited the release of TNF- α caused by C48/80 and decreased the level of CCL2 and histamine in serum in a dose-dependent manner in the C48/80 induced mouse allergic model (Figure 3A). Additionally, it is obvious that α -asarone inhibited

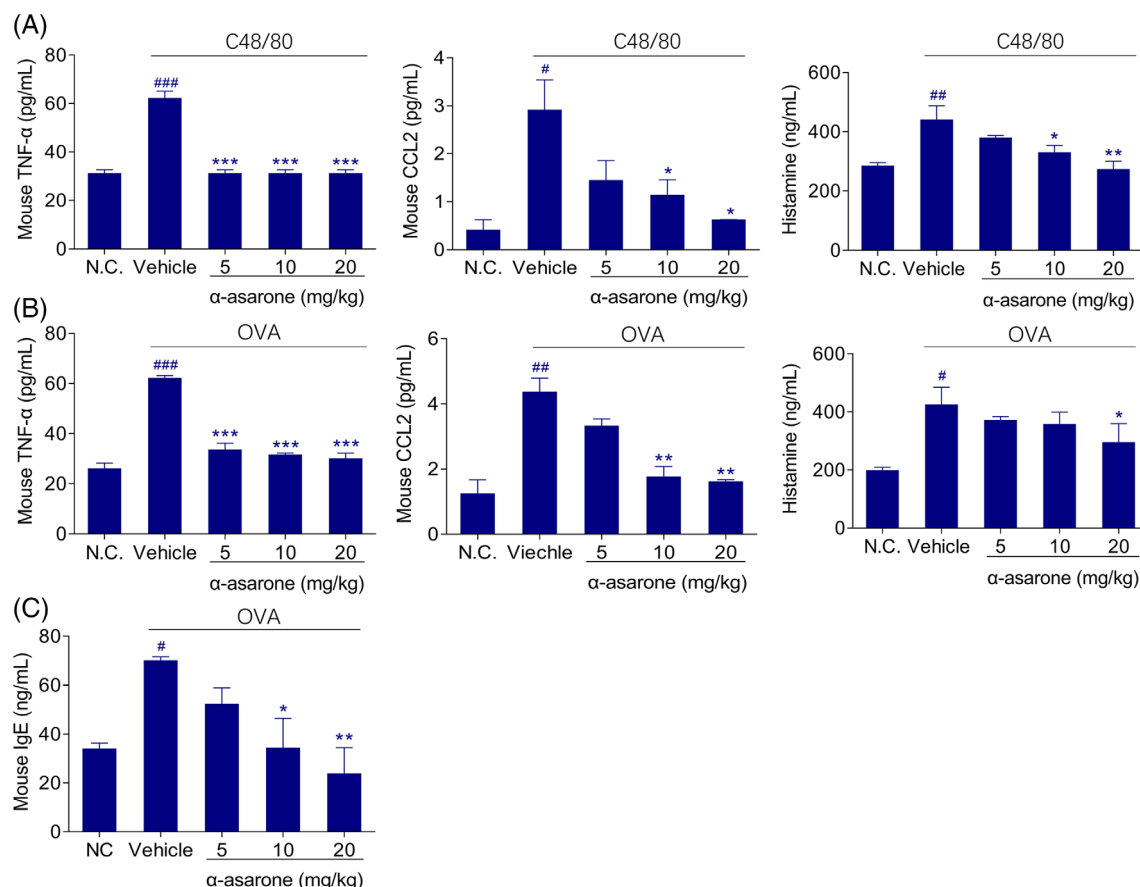


FIGURE 3 α -Asarone reduces systemic anaphylaxis. After oral administration of α -asarone at different concentrations (5, 10 and 20 mg/kg bw), 0.3 mg/kg bw C48/80 was injected into the tail vein, and an equal volume of saline was injected into the negative control group (N.C.). (A) the levels of TNF- α , CCL2, and histamine in serum induced by C48/80 in mice. (B) the levels of TNF- α , CCL2, and histamine in serum induced by OVA in mice. Data are presented as mean $\pm$ SD. Two-tailed unpaired Student's t-tests were used for the analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, versus vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group); $n = 5$

inflammatory factors release in the OVA-induced mouse allergic model. α -Asarone reduced TNF- α in serum by about 90% at the concentration of 5 mg/kg bw; The CCL-2 in serum was almost completely inhibited when the administration concentration reached 10 mg/kg bw; Histamine release in serum was decreased by α -asarone in a dose-dependent manner (Figure 3B). The level of serum IgE was regarded as an important indicator of the severity of individual type I anaphylaxis.²⁸ Therefore, we investigated the level of serum IgE in each group (Figure 3C). The serum IgE concentration of mice in N.C. group was 34.17 ± 2.13 ng/ml. After OVA stimulation, the IgE concentration of pre-sensitized mice was increased significantly (70.15 ± 1.42 ng/ml, $p < 0.05$ vs. N.C.). α -Asarone inhibited the increase of IgE level of pre-sensitized mice induced by OVA in a dose-dependent manner. The serum IgE levels were decreased to 24.31 ± 15.11 ng/ml when administrated concentration reached 20 mg/kg.

3.4 | α -Asarone alleviated OVA-induced asthma-like symptoms in mice

A murine model of OVA-induced asthma was used to determine whether α -asarone could relieve AHR in mice. Measurements of Rn, G, Rrs, and Cst during ACh-induced AHR illustrated that AHR to ACh was significantly increased in the OVA group compared with that in the N.C. group. Oral administration of α -asarone (5, 10, and 20 mg/kg bw) effectively reduced AHR in a dose-dependent manner. α -Asarone showed the same inhibitory effect as DEX (5 mg/kg bw) when the dosage reached 10 mg/kg bw (Figure 4B). The density of inflammatory cells reflects the severity of asthma. Therefore, we measured the changes in total cell levels and various inflammatory cell levels in BALF to determine the effect of α -asarone on the OVA-induced mouse asthma model. The OVA challenge significantly induced the infiltration of inflammatory cells into the BALF

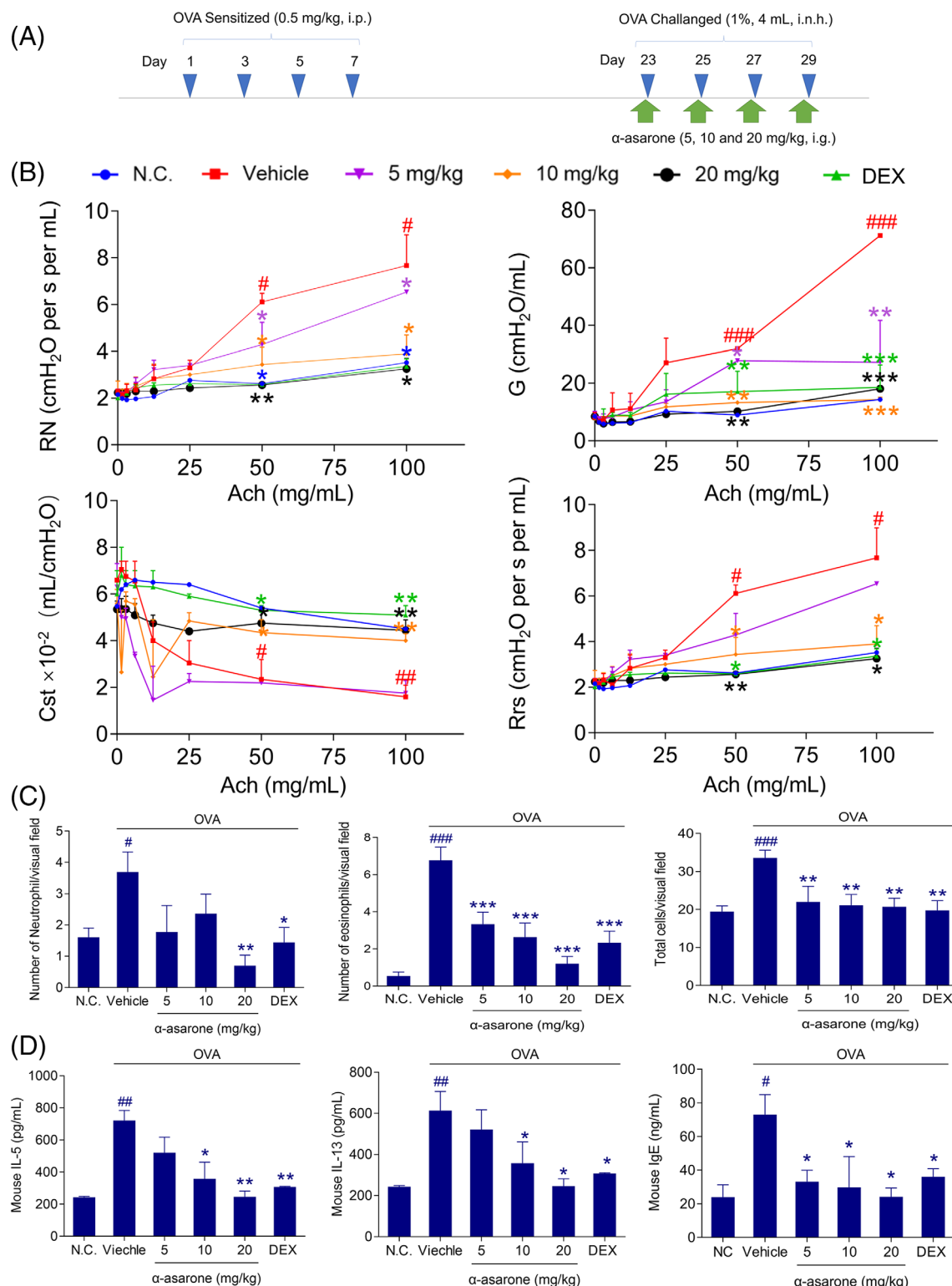


FIGURE 4 α -Asarone alleviated OVA-induced asthma-like symptoms in mice. (A) Protocol for sensitization and challenge in mice with induced asthma. (B) Airway responses to methacholine measured 24 h after the last OVA or phosphate buffered saline (PBS) challenge on day 27. (C) 24 h after the last challenge, BALF was collected, and the numbers of eosinophils, neutrophils, and total cells were determined. (D) IL-5 and IL-13 were detected in BALF on day 29. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, versus vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group); $n = 5$

compared with the N.C. group. Mice treated with α -asarone displayed fewer eosinophils, neutrophils, and Total cells than those in the vehicle group (Figure 4C).

Next, we measured the effect of different concentrations of α -asarone on the levels of relevant chemokines and cytokines in BALF. The levels of cytokines, such as IL-5

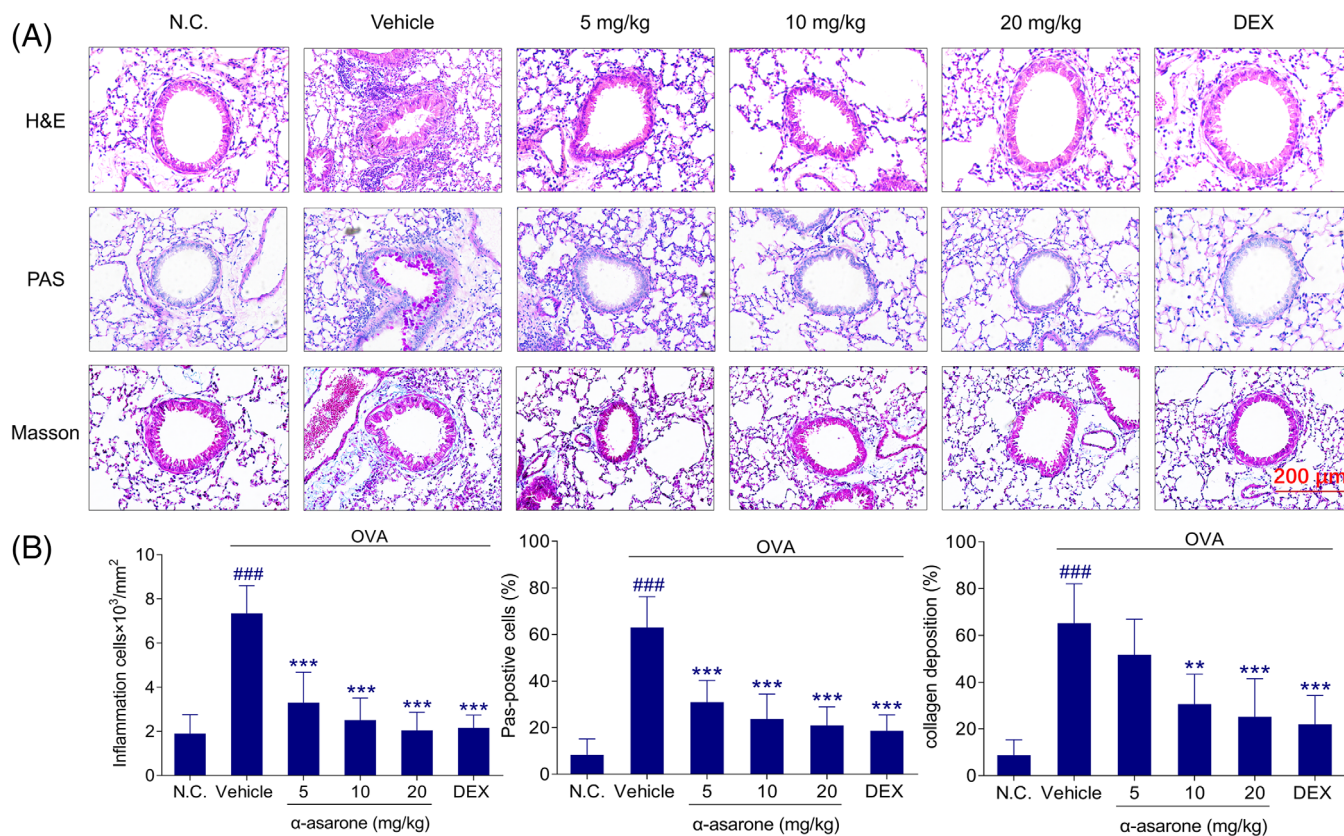


FIGURE 5 α-Asarone inhibited mucus production and glycogen and collagen deposition in the lungs of OVA-induced asthma. (A) on day 29, lung sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson. (B) Quantification of inflammatory cells, PAS-positive bronchial epithelial cells, and Masson-positive bronchial epithelial cells were scored as described in the materials and methods section. Data are presented as mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, versus vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group); $n = 5$

and IL-13, were significantly increased in the BALF of the vehicle group compared to those in the N.C. group. α-Asarone significantly reduced IL-5 and IL-13 levels in a dose-dependent manner. In the mast cell-dependent allergic asthma model of murine, the IgE concentration in BALF reached 73.04 ± 11.93 ng/ml, which is much higher than that of the N.C. group (22.19 ± 9.37 ng/ml), α-asarone inhibited the increase of IgE in BALF in asthma model murine in dose dependent manner, the IgE declined to 24.13 ± 4.79 ng/ml at the administrated concentration of 20 mg/kg. (Figure 4D).

Hematoxylin and Eosin (H&E) staining illustrated that there were more extensive inflammatory infiltrates in the peribronchial areas in the lung of the vehicle group than in the N.C. group. However, in mice treated with α-asarone, there was a decrease in inflammatory infiltrates, and the severity of inflammatory infiltration decreased with an increase in drug concentration. Pulmonary inflammation and asthma symptoms can lead to severe airway remodeling, which mainly manifests as mucus secretion and collagen deposition.²⁹ Periodic acid-

Schiff (PAS) and Masson staining showed that compared with the vehicle group, the α-asarone administration groups showed a significantly reduced deposition of glycogen and collagen in the lung tissue of asthmatic mice (Figure 5A–B).

3.5 | α-Asarone inhibited mast cell activation in the lung of OVA-induced asthma

Mast cell activation is an important inducer of asthma symptoms.³⁰ Avidin staining showed that the density and degranulation rate of mast cells in the vehicle group were higher than those in the N.C. group. With the increase in α-asarone concentration, the density and release rate of pulmonary mast cells tended to decrease and were similar to those of the N.C. group. These results demonstrated that α-asarone alleviated lung inflammation by inhibiting the activation and degranulation of mast cells in the lungs (Figure 6A–B).

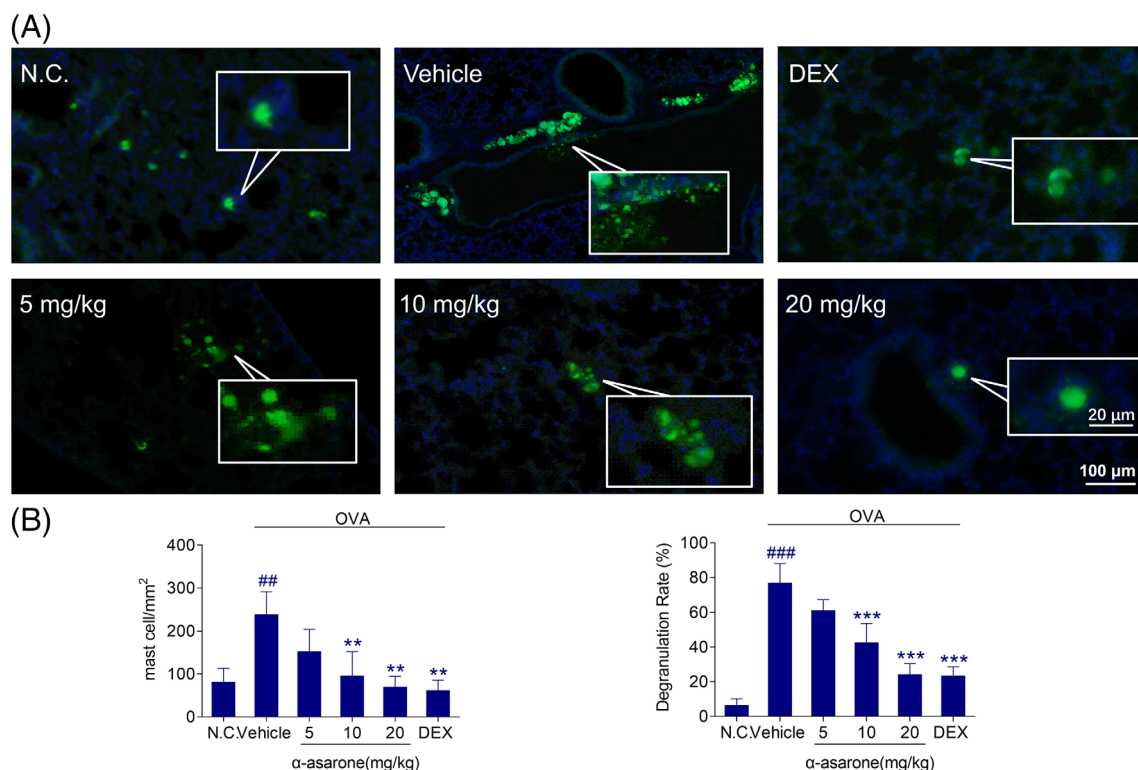


FIGURE 6 α -Asarone inhibited the activation of mast cells in the lungs of OVA-induced asthma. (A) Lung sections were stained with FITC-avidin on day 29. (B) the number and degranulation rate of mast cells in lung tissue. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, versus vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group); $n = 5$

3.6 | α -Asarone inhibits mast cell activation through ERK / JAK2-STAT3 pathway

STAT3 is an important nuclear transcription factor that plays an important role in cell proliferation, apoptosis, inflammation, immunity, and cancer. The JAK/STAT3 pathway is considered a classical inflammatory pathway.³¹ ERK is a protein kinase that contains serine/threonine residues and its role in cell cycle regulation, cell proliferation, differentiation, and apoptosis are widely known.³² To investigate whether these proteins were involved in mast cell activation and to verify that α -asarone inhibits mast cell activation, we measured the expression levels of JAK2, ERK1/2, and STAT3, as well as their phosphorylation levels; GAPDH was used as the internal parameter for protein phosphorylation analysis. Samples from different groups (N.C., vehicle, α -asarone at 25, 50, and 100 μ M) were analyzed. The results showed that pSTAT3, pERK, and pJAK2 were significantly increased in the vehicle group. However, α -asarone inhibited the phosphorylation of these proteins in a dose-dependent manner. Specifically, when the concentration reached 50 μ M, the phosphorylation of STAT3 and JAK2 was almost completely inhibited, while the

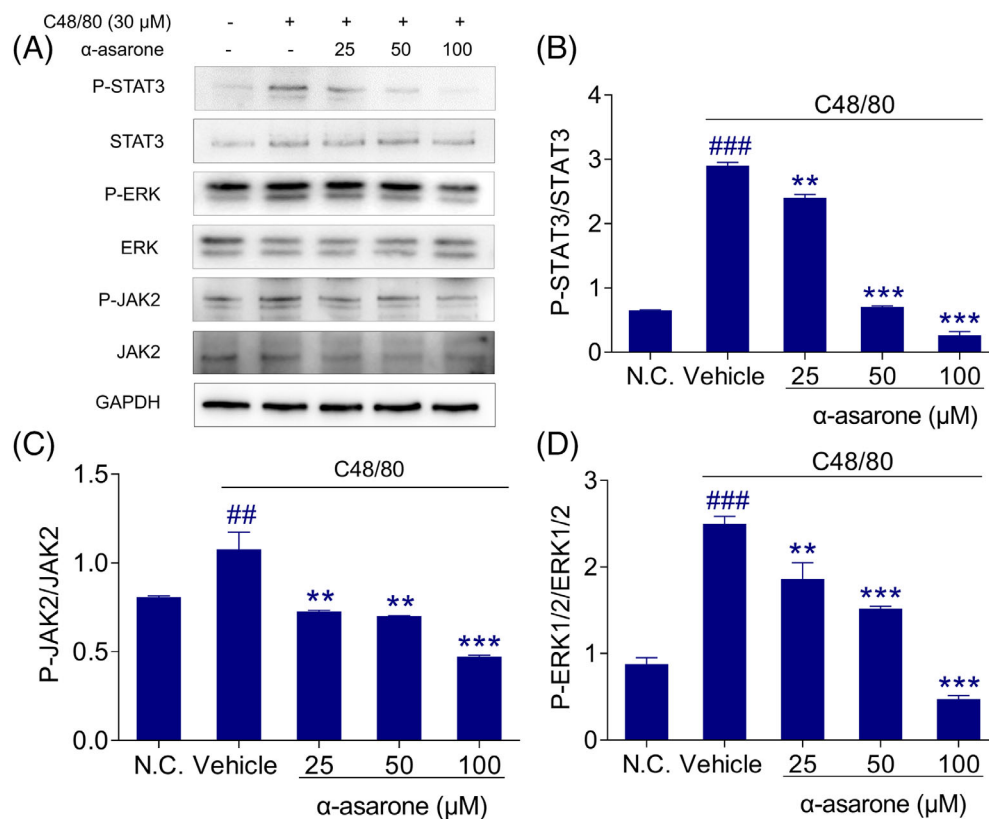
phosphorylation of ERK1/2 was almost completely inhibited at a concentration of 100 μ M (Figure 7A–D).

4 | DISCUSSION

As one of the main components of *Acorus calamus* L., which has been used for 2000 years in China, α -asarone has been widely reported for its anti-inflammatory, anti-rheumatic, and therapeutic effects on digestive and nervous system diseases.²³ In this study, we first verified the inhibitory effect of α -asarone on mast cell-mediated pseudo-allergic reactions and type I anaphylactic reactions in vitro and in vivo. Second, a murine model of OVA-mediated asthma was established to evaluate the effect of α -asarone on asthma and related symptoms. Finally, we observed the activation and degranulation of mast cells in lung tissue by immunofluorescence staining. Additionally, the ERK/JAK2-STAT3 pathway was observed to determine the mechanism of α -asarone-stabilizing mast cells.

Mast cells exist in almost all vascularized tissues and are an important source of bioactive secretory mediators, including diverse cytokines and growth factors.³³ There is strong evidence that mast cells play an important role in

FIGURE 7 α -Asarone inhibits the ERK/JAK2-STAT3 pathway in LAD2 cells. (A–D) C48/80 increased the phosphorylation of these proteins. At different concentrations of α -asarone (25, 50, and 100 μ M), this increase in phosphorylation was inhibited. Experiments were repeated three times. Data are presented as mean $\pm$ SD. Two-tailed unpaired Student's *t*-tests were used for the analysis. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, versus vehicle group; # $p < 0.05$, ## $p < 0.01$, and ### $p < 0.005$, versus N.C. (negative control group); $n = 3$



many types of innate or adaptive immune responses, including their contribution to direct and chronic IgE-related allergic diseases. Mediated by MRGPRX2, mast cells can be directly activated by exogenous antigens or substance P, which is a neuropeptide widely distributed in fine nerve fibers, leading to the release of a variety of secretions, including TGF- β 1, CCL2, CXCL2, CXCL8, VEGF, TNF, and granule-associated cytokines.²⁷ IgE-mediated chronic allergic reactions can also cause mast cells to release many specific bioactive secretions, including chemokines, regulatory cytokines, mitogens, growth factors, and inflammatory cytokines.²⁷ Both MRGPRX2 and IgE-mediated mast cell activation release TNF- α and CCL2; therefore, these two cytokines were selected as indicators to observe the degree of mast cell activation or individual inflammatory response.

A mouse model of OVA as well as C48/80 induced skin inflammation were established to determine the anti-anaphylactic effect of α -asarone. However, the right paws treated with saline showed a certain extent of toe swelling and Evans blue leakage. We inferred that although the experiment focused on local allergic reactions, within 30 minutes, it is possible that some allergic mediators such as histamine may enter the systemic circulatory system, resulting in slight exudation and swelling of the right paws. Similar phenomena have also been reported in some studies.^{34–36}

Allergic asthma is a heterogeneous disease characterized by chronic airway inflammation and airway hyperresponsiveness.³⁷ Antigens, microorganisms, dust mites, and other stimuli lead to the maturation and differentiation of plasma cells within the airway of epithelial cells and DC cells, resulting in the production of specific IgE, which is crucially involved in mast cell-mediated type I allergic reactions.^{11,37} Recent studies have shown that mast cells may also be activated by foreign antigens directly, in addition to the above processes.³⁸ Activated mast cells play an important role in the pathophysiology of asthma.¹² Secretions derived from mast cells such as histamine, prostaglandin D2, and LTC4 are capable of inducing mucus secretion and mucosal edema.³⁹ The mouse asthma model used to evaluate the effect of α -asarone in vivo is strictly mast cell-dependent.⁴⁰ MC-dependent airway inflammation and remodeling have been demonstrated in mast cell-deficient mice that have a C57BL/6 background.⁴¹ Such models mimic many human features of asthma, including inflammatory responses in the lung, increases in MCs, and eosinophil and mucus production. Using this asthma model, we observed that treatment with α -asarone in OVA-challenged mice reduced AHR, airway inflammation, mucin content, and collagen levels (Figure 4B, 5A). Furthermore, the effect of α -asarone (20 mg/kg bw) was comparable to that of DEX (5 mg/kg bw). The number of

degranulating mast cells increased in the lungs of OVA-challenged mice, which was suppressed by treatment with α -asarone (Figure 6A–B). This suppressive action of α -asarone is consistent with the in vitro and in vivo results, where α -asarone inhibited mast cells.

Although asthma relieving effect has been demonstrated in our study, the toxic effects of α -asarone can not be ignored. It is reported that α -asarone showed acute toxicity, subchronic toxicity and chronic toxicity when administered at high doses, α -asarone may have mutagenicity, teratogenicity, and genotoxicity as well.^{42,43} Furthermore, the hepatotoxicity of α -asarone has aroused widespread concern. It is reported that α -asarone is cytotoxic to human and rat hepatocyte cell.^{44,45} There are also research reports that B6C3F1 male mice developed liver cancer when the i.p. dosage of α -asarone is higher than 50 mg/kg bw.⁴³ α -Asarone is mainly metabolized by interacting with cytochrome P450 (CYP450) enzymes in hepatocytes and liver microsomes in vivo, the metabolites are active epoxides that can be adducted with DNA. The metabolic mode of α -asarone and its metabolites may cause hepatotoxicity and genotoxicity.⁴⁵ Although the dosage was used as low as possible in this study, which were much lower than the reported toxic dose, based on the above report on the toxicological effects of α -asarone, it is extremely urgent to reduce the toxicity of α -asarone by multiple ways, such as changing the dosage form, compound modification or structural modification and others.

In conclusion, α -asarone could alleviate allergic asthma by stabilizing mast cells through the inhibition of the ERK/JAK2-STAT3 pathway.

AUTHOR CONTRIBUTIONS

Nan Wang, and Haoyun Bai designed research; Haoyun Bai, Zhenqi Zhou, Wen Zhang, Chaohua Feng, and Zhuoyin Xue performed research; Nan Wang contributed new reagents or analytic tools; Haoyun Bai, Shiling Hu, and Yongjing Zhang analyzed data; Haoyun Bai wrote the paper.

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CONFLICT OF INTEREST

All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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