

## Original Article

# Cornuside ameliorates airway inflammation via Toll-like receptor 4 and Notch1 in asthmatic mice induced by lipopolysaccharide and ovalbumin

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**Abstract:** To determine the potential role of cornuside in the regulation of allergic airway inflammation in lipopolysaccharide (LPS) and ovalbumin (OVA)-induced asthma. BALB/c mouse model of LPS and OVA-induced asthma was used to evaluate the effect of cornuside on Toll-like receptor 4 (TLR4) and Nocth1 signaling pathway. Hematoxylin and eosin staining was performed for histological studies of murine lung tissues. Enzyme-linked immunosorbent assay was employed to measure protein levels in serum, while Western blotting was carried out for the determination of protein expression in tissues. Airway hyperresponsiveness was measured 2 days after the last OVA challenge. After induction by LPS and OVA, the mice had increased numbers of inflammatory cells, increased levels of IL-4, IL-5 and IL-13 in bronchoalveolar lavage fluids (BAL) and lung tissues, increased total and OVA-specific IgE levels in serum and increased expression of TLR4 and Nocth1 and phosphorylation of STAT3 in lung tissues. Administration of cornuside markedly reduced airway inflammatory cell recruitment and peribronchiolar inflammation, decreased the production of various cytokines in BAL fluids and lung tissues, and lowered the levels of total and OVA-specific IgE in serum. In addition, the increased expression of TLR4 and Nocth1 and phosphorylation of STAT3 after LPS and OVA inhalation was inhibited by the administration of cornuside. The present study demonstrates that the signaling pathway between TLR4 and Notch1 may be able to coordinate with STAT3 and cornuside regulates TLR4 and Nocth1 signaling pathway to attenuate allergic airway inflammation in LPS and OVA-induced asthma. These findings provide a crucial molecular mechanism for the potential use of cornuside to prevent and/or treat asthma and other airway inflammatory disorders.

**Keywords:** Cornuside, asthma, TLR4, Notch1, STAT3, airway inflammation

## Introduction

Asthma remains a major cause of morbidity in developed nations and is a leading cause of hospitalization [1]. The characteristic features of asthma are airway inflammation, epithelial damage, reversible airflow obstruction, airway hyperreactivity, and airway remodeling [2]. It is well recognized that respiratory infection mediates allergic airway inflammation [3]. However, genetic and environmental factors that contribute to progression of asthma remain poorly understood. Pattern recognition receptors (PRRs) is an umbrella term for several receptor families that have specific abilities to recognize various microbes, and to initiate

innate host defensive reactions [4, 5]. Toll-like receptors (TLRs) are the best characterized PRR family comprising 10 members (13 in mice) [4]. TLR4 is shown to be a receptor for lipopolysaccharide (LPS) [6, 7]. Exposure to LPS increases the severity of asthma, and activates TLR4 signaling in the regulation of T helper 2 (Th2) cells-driven lung inflammations [8]. LPS induces lung inflammation of asthmatic mice and airway hyperresponsiveness (AHR) via TLR4 pathway [8-11]. Collectively, these studies have shown that contaminating LPS and TLR4 signaling are necessary for priming pro-inflammatory T helper cell responses to inhaled ovalbumin in asthma.

Several studies have shown that TLR4 activation by LPS promotes inflammatory mechanisms including nuclear factor (NF)- $\kappa$ B and Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT) pathways [10]. STAT3 is a well-known transcription factor that regulates a variety of cellular processes, including cell proliferation and survival, oncogenesis and cancer metastasis. STAT3 also has a close interaction with Notch receptors in various physiological and pathological conditions, including proliferation, differentiation, and apoptosis [12]. Upon ligand-dependent activation, Notch is cleaved and releases the Notch intracellular domain, which participates in a transcriptional complex in the nucleus to regulate Notch-dependent gene expression [13]. Blockade of Notch has been reported to inhibit T lymphocyte activation and airway inflammation in asthma [14, 15]. TLR4 and Notch signaling pathways are all involved in asthma, but these cell signaling pathways are not completely independent of each other. For example, cross-talks take place between different signaling pathways. We hypothesize that signaling pathways between TLR4 and Notch coordinate with each other in regulating airway inflammation in asthma.

Cornuside is a kind of secoiridoid glucoside isolated from the fruit of *Cornus officinalis* Sieb. et Zucc., which is a traditional oriental medicine for treating inflammatory diseases and invigorating blood circulation. A crude extract from the fruits of *Cornus officinalis* has been found to have pharmacological actions such as anti-neoplastic, anti-inflammatory, hepatoprotective, and antidiabetic effects [16-18]. Recent reports have demonstrated that cornuside could suppress the expression of cytokine-induced pro-inflammatory and adhesion molecules in human endothelial cells, and could protect cultured rat cortical cells against damages induced by oxygen-glucose deprivation [19, 20]. Furthermore, cornuside has been demonstrated to inhibit LPS-induced nitric oxide (NO) production in cultured mouse macrophages [21]. Our previous studies have revealed that cornuside inhibits acute hepatic injury induced by carbon tetrachloride, and suppresses LPS-induced inflammatory mediators by inhibiting NF- $\kappa$ B activation in RAW 264.7 macrophages [22, 23]. However, a direct relationship between

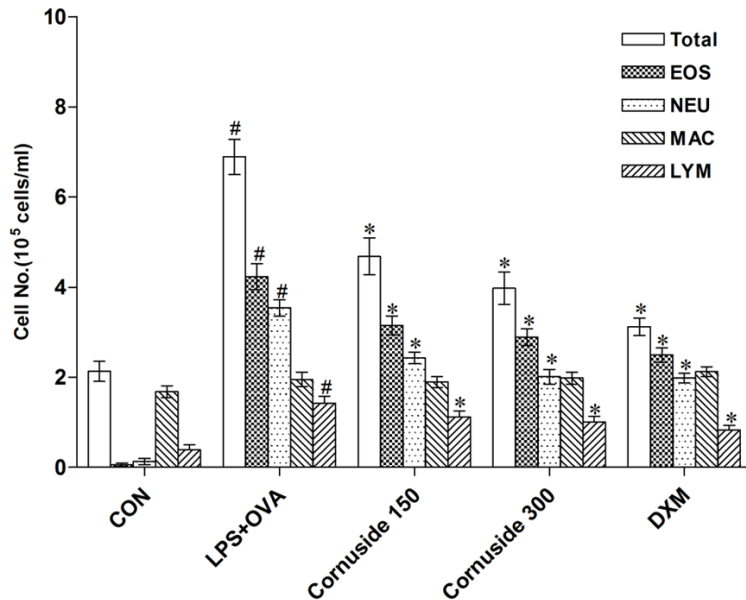
cornuside and airway inflammation has not yet been understood in murine model of asthma. In the present study, we determine the effect of cornuside on airway inflammation in animal model of asthma. In addition, we examine whether TLR4 and Notch signaling pathways are involved in the pathophysiological process of asthma.

### Materials and methods

#### Animals

Specific pathogen-free (SPF) inbred female BALB/c mice (7 weeks) were purchased from House Section of Yanbian University Health Science Center (Yanji, China). The mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided with water and standard chow *ad libitum*. The experiments were performed in compliance with the guidelines approved by Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences. The mice were immunized intraperitoneally with 10  $\mu$ g of ovalbumin (OVA; Grade V, chicken egg albumin; Sigma-Aldrich, St. Louis, MO, USA) and 1.0 mg of aluminum hydroxide adjuvant (Inject Alum; Pierce, Rockford, IL, USA). A booster injection of 10  $\mu$ g of OVA and 1.0 mg aluminum hydroxide adjuvant was given 10 days later. From day 17 to day 19, the immunized mice were challenged by exposure to aerosol of 1% OVA in phosphate-buffered saline (PBS) for 20 min. The broncho-provocation was carried out in vented plastic chamber (18  $\times$  14  $\times$  8 cm) adapted for mice. Aerosol particles of approximately 3-5  $\mu$ m in diameter were created from an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan), directed into the plastic chamber, and vented to a fume hood. Each group consisted of seven animals. The saline-treated mice used as controls were exposed to aerosolized saline. Cornuside (purity > 99.0%, CAS No. 13118957-6) with the molecular formula  $C_{24}H_{30}O_{14}$  (molecular weight: 542.49; Shandong Engineering Research Center for Nature Drug, Yantai, China) was dissolved in sterile saline [150, or 300 mg/kg body weight (BW)] or dexamethasone (DXM; 1.0 mg/kg body weight), and administered by oral gavage to each animal at 24 h intervals on days 13-19.

## Treatment of asthma with cornuside



**Figure 1.** Effect of cornuside on total and differential cellular components of BAL in LPS and OVA-induced asthmatic mice. Numbers of counted cells of each type in BAL. LPS and OVA-induced asthmatic mice were orally given cornuside or DXM (positive control) daily on days 13-19 for seven consecutive days after the first sensitization. The effects of cornuside and DXM on LPS and OVA-induced changes in cell counts in BAL fluid were analyzed 24 h after the last OVA challenge. Results of five independent experiments (7 mice/group) were expressed as means  $\pm$  standard errors of the mean. #,  $P < 0.05$  compared with control group; \*,  $P < 0.05$  compared with LPS and OVA-induced group.

### LPS exposure

In brief, the mice were exposed to 0.1  $\mu$ g LPS (from *Escherichia coli* O111:B4; Sigma-Aldrich, St. Louis, MO, USA) on day 2 of sensitization an hour before antigen administration and on each day of OVA-aerosol challenge in 100  $\mu$ L sterile saline [24]. Mice in control group were given LPS-free saline.

### Harvest of bronchoalveolar lavage (BAL) fluid and cytospin preparations

Immediately following assessment of airway responsiveness, the mice were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight; Wuhan Boster Biological Technology, Ltd., Wuhan, China) and the tracheas were cannulated while gently massaging the thorax. The lungs were lavaged with 0.7 ml of PBS. BAL fluid samples were collected and the number of total cells in a 0.05 mL aliquot was counted using a hemocytometer. The remaining samples were centrifuged at 200 g for 10 min at 4°C (model 5424R, Eppendorf-

Netheler, Hamburg, Germany) and the supernatants were stored at -70°C for the assay of IL-4, IL-5 and IL-13 levels. The cell pellets were resuspended in PBS and cytospin preparations (Cytospin 3, Shandon Life Sciences, Astmor, UK) of BAL cells were stained with Diff-Quik solution (International Reagents, Kobe, Japan). Two independent, blinded investigators counted the cells using a microscope (CX31 microscope; Olympus Corporation, Tokyo, Japan). Approximately 400 cells were counted in each of four different random locations. Inter-investigator variation was  $< 5\%$ . The mean number from the two investigators was used to estimate cell differentials.

### Hematoxylin-eosin (HE) staining

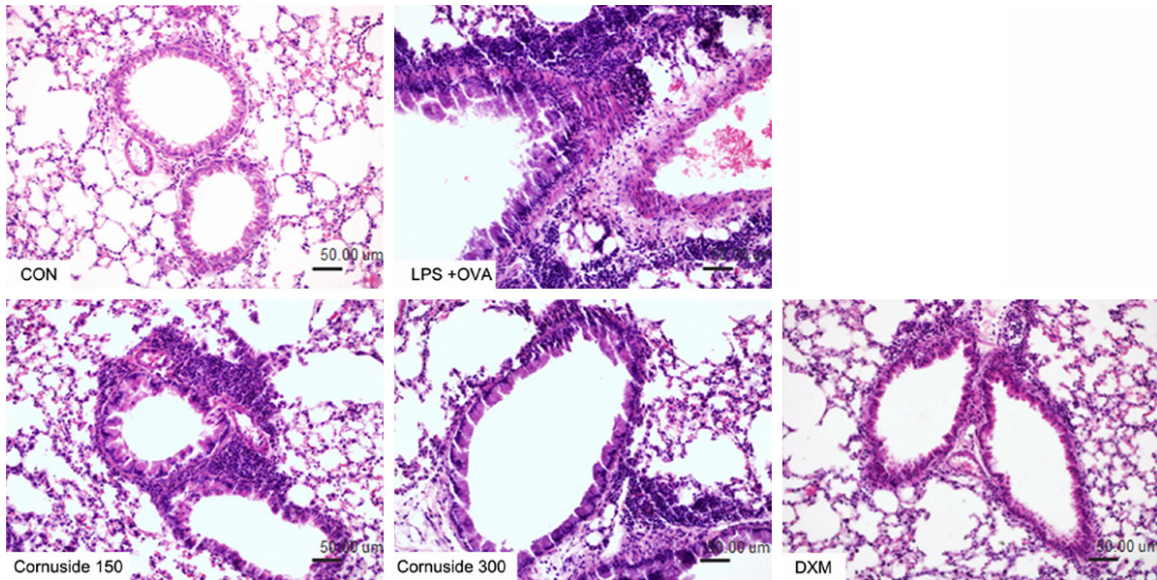
After BAL preparation, murine lung was resected, fixed with 4% paraformaldehyde, and embedded in paraffin. Specimens were cut into 4  $\mu$ m sections by Leica model 2165 rotary microtome (Leica, Nussloch, Germany). The microsections were stained with hematoxylin and eosin (Richard-Allan Scientific, Kalamazoo, MI, USA) and examined under the microscope (CX31 microscope; Olympus Corporation, Tokyo, Japan) with a magnification of  $\times 100$ .

### Enzyme-linked immunosorbent assay (ELISA)

IL-4, IL-5, and IL-13 levels in BAL and IgE in serum were determined using mouse ELISA kits for IL-4, IL-5, IL-13 and IgE (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

To measure total IgE and OVA-specific IgE, 96-well microtiter plates were coated overnight with isotype-specific coating (total IgE) and 10 mg/mL OVA in PBS-Tween 20 (OVA-specific IgE). After washing and blocking of plate, samples were incubated for 2 hours. Subsequently, 96-well plates were washed, and HRP-con-

## Treatment of asthma with cornuside



**Figure 2.** Effect of cornuside on pathological changes in lung tissues in LPS and OVA-induced asthmatic mice. Hematoxylin and eosin stained sections of lung tissues (magnification,  $\times 200$ ). Paraffin-embedded lung sections were prepared 24 h after the last OVA challenge and stained with hematoxylin and eosin. Images were representative of five independent experiments. EOS, eosinophil; NEU, neutrophil; MAC, macrophage; LYM, lymphocyte; BAL, bronchoalveolar lavage fluid; OVA, ovalbumin; DXM, dexamethasone. CON, saline-treated mice; LPS+OVA, LPS and OVA-induced asthmatic mice; Cornuside 150, LPS and OVA-induced asthmatic mice treated with 150 mg/kg cornuside; Cornuside 300, LPS and OVA-induced asthmatic mice treated with 300 mg/kg cornuside; DXM, LPS and OVA-induced asthmatic mice treated with 1 mg/kg DXM.

jugated goat anti-mouse total IgE and OVA specific IgE were added. After washing four times, 200  $\mu$ L of o-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The plate was incubated for 10 min in the dark and then the absorbance was determined at 450 nm using a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA, USA). Total IgE and OVA-specific IgE concentrations were calculated from a standard curve generated using 250 ng/mL recombinant IgE.

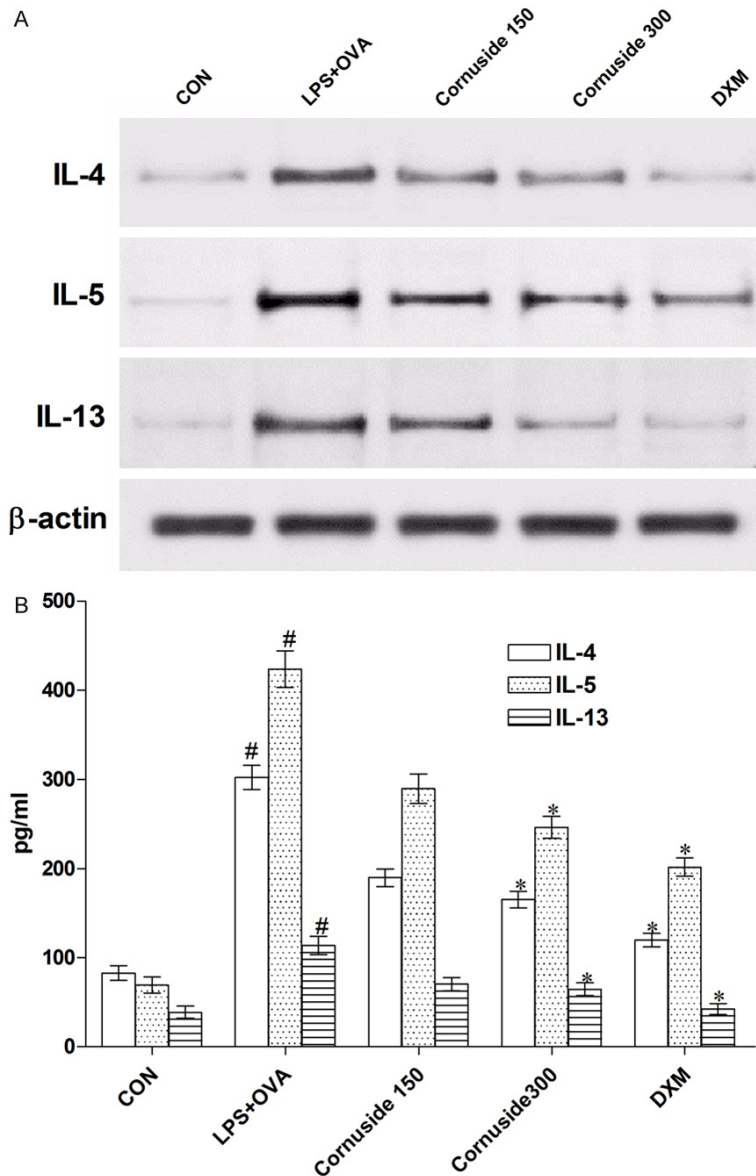
### Western blotting

Lung tissues were homogenized in the presence of protease inhibitors and protein concentrations were determined using the Bradford reagent (Bio-Rad, Hercules, CA, USA). Protein sample (30  $\mu$ g) from the lung homogenates was loaded per lane on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel. Electrophoresis was then performed. The proteins were then transferred to nitrocellulose membranes. Western blotting analysis was performed using polyclonal antibodies against IL-5, TLR4, STAT3, p-STAT3, notch1,  $\beta$ -actin (Santa Cruz, Santa Cruz, CA, USA), IL-13 (R&D

Systems, Minneapolis, MN, USA) and IL-4 (Serotec, Oxford, UK). The binding of all the antibodies was detected using an electrochemiluminescence detection system (Amersham, Arlington Heights, IL, USA) according to the manufacturer's instructions.

### Assessment of AHR

Airway responsiveness was measured 2 days after the last OVA challenge according to the method described by Choi et al. [25]. Conscious unrestrained mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, 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. Bronchopulmonary resistances were expressed as enhanced pauses (Penh), which were calculated as:  $[\text{expiratory time (Te)}/\text{relaxation time (RT)}-1] \times [\text{peak expiratory flow (PEF)}/\text{peak inspiratory flow (PIF)}]$ , according to the manufacturer's protocol. The results were expressed as the percentage of increase in



**Figure 3.** Effect of cornuside on Th2 cytokines in BAL fluids and lung tissues of LPS and OVA-induced asthmatic mice. A. Protein expressions of IL-4, IL-5 and IL-13 in lung tissues after 24 h measured by Western blotting. B. The levels of IL-4, IL-5 and IL-13 in BAL fluids quantified by ELISA. BAL fluids were collected 24 h after the last challenge. Results from five independent experiments with 7 mice/group were given as means  $\pm$  SEM. #,  $P < 0.05$  compared with control; \*,  $P < 0.05$  compared with LPS and OVA -induced group. CON, saline-treated mice; LPS+OVA, LPS and OVA-induced asthmatic mice; Cornuside 150, LPS and OVA-induced asthmatic mice treated with 150 mg/kg cornuside; Cornuside 300, LPS and OVA-induced asthmatic mice treated with 300 mg/kg cornuside; DXM, LPS and OVA-induced asthmatic mice treated with 1 mg/kg DXM.

Penh over the baseline, following challenges performed with each concentration of Mch, where the baseline Penh (after PBS challenge) was expressed as 100%.

To examine the effect of cornuside on pathological features of asthma, HE staining was used. Histological analyses revealed that mice with asthma induced by LPS and OVA had more widespread perivascular and peribronchiolar inflam-

### Statistical analysis

Data were expressed as means  $\pm$  SEM. Statistical evaluation of the data was performed using ANOVA, followed by Dunnett's *post-hoc* test by SPSS 18.0 statistical software (IBM, Armonk, NY, USA). Results with  $P < 0.05$  were considered statistically significant.

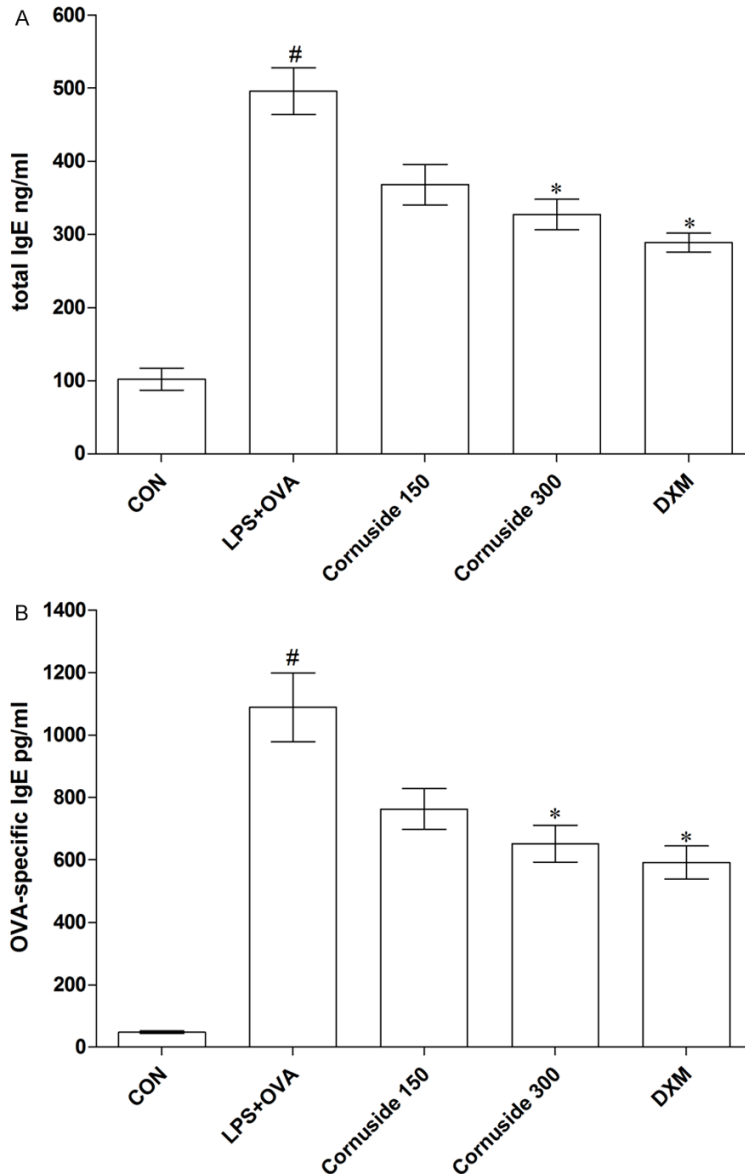
### Results

#### *Cornuside attenuates cellular changes in BAL fluids in asthmatic mice induced by LPS plus OVA*

To study the effect of cornuside on cells in BAL fluids in asthmatic mice induced by LPS and OVA, samples were collected and the number of total cells was counted using a hemocytometer. Total numbers of eosinophils, lymphocytes, and neutrophils in BAL fluids were significantly increased at 24 h after LPS and OVA inhalation compared with those after saline inhalation. By contrast, the numbers of these cells were significantly reduced by the administration of cornuside or reference drug DXM (**Figure 1**). The result suggests that cornuside attenuates cellular changes in BAL fluids in asthmatic mice induced by LPS and OVA.

#### *Treatment with cornuside inhibits the infiltration of inflammatory cells and attenuates airway inflammation induced by LPS and OVA*

## Treatment of asthma with cornuside



**Figure 4.** Effect of cornuside on (A) total IgE and (B) OVA-specific IgE levels in serum of OVA-induced asthma. Serum was collected 24 h after the last challenge. The level of IgE was quantified by ELISA. Results from five independent experiments with 7 mice/group were given as means  $\pm$  SEM. #,  $P < 0.05$  compared with control; \*,  $P < 0.05$  compared with LPS and OVA-induced group. CON, saline-treated mice; LPS+OVA, LPS and OVA-induced asthmatic mice; Cornuside 150, LPS and OVA-induced asthmatic mice treated with 150 mg/kg cornuside; Cornuside 300, LPS and OVA-induced asthmatic mice treated with 300 mg/kg cornuside; DXM, LPS and OVA-induced asthmatic mice treated with 1 mg/kg DXM.

matory cell infiltration compared with control. However, mice treated with cornuside or DXM showed significantly reduced degrees of inflammatory cell infiltration in peribronchiolar and perivascular regions (**Figure 2**). These results indicate that treatment with cornuside inhibits

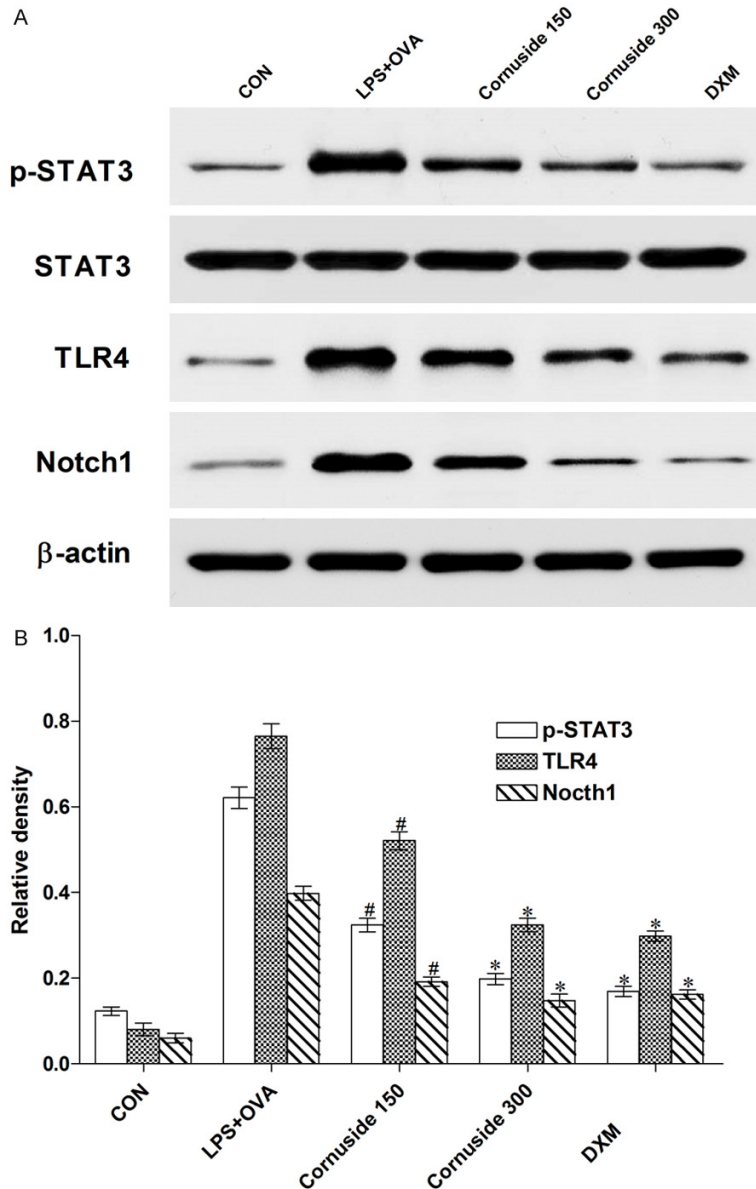
the infiltration of inflammatory cells and attenuates airway inflammation induced by LPS and OVA.

*Cornuside reduces the increased in IL-4, IL-5 and IL-13 protein levels in lung tissues and BAL fluids of mice with asthma induced by LPS and OVA*

To measure IL-4, IL-5 and IL-13 protein levels in lung tissues and BAL fluids, Western blotting and ELISA were carried out, respectively. Western blotting analysis revealed that IL-4, IL-5 and IL-13 protein levels in lung tissues were increased significantly at 24 h after LPS and OVA inhalation compared with those after saline inhalation. Of note, the increased IL-4, IL-5 and IL-13 levels were significantly reduced by the administration of cornuside or DXM (**Figure 3A**). Consistently, ELISA revealed that the levels of IL-4, IL-5 and IL-13 in BAL fluids were increased significantly at 24 h after LPS and OVA inhalation compared with those after saline inhalation. Moreover, the increased IL-4, IL-5 and IL-13 levels were significantly reduced by the administration of cornuside or DXM (**Figure 3B**). These results suggest that cornuside reduces the increased in IL-4, IL-5 and IL-13 protein levels in lung tissues and BAL fluids of mice with asthma induced by LPS and OVA.

*Cornuside lowers the increased total IgE and OVA-specific IgE levels in the serum of mice with asthma induced by LPS and OVA*

To determine the levels of total IgE and OVA-specific IgE, ELISA was performed at 24 h after



**Figure 5.** Effect of cornuside on the expression of TLR4 and Nocth1 and the phosphorylation of STAT3 in lung tissues of LPS and OVA-induced asthmatic mice. LPS and OVA-induced asthmatic mice were orally given cornuside or DXM daily on days 13-19 for seven consecutive days after the first sensitization. Lung homogenates were prepared 24 h after the last OVA challenge. A. The expression of TLR4 and Nocth1 and the phosphorylation of STAT3 assessed by Western blotting. B. Quantification of Western blots by densitometric analysis relative to β-actin or total STAT3. Results from five independent experiments with 7 mice/group were given as means ± SEM. #, P < 0.05 compared with control; \*, P < 0.05 compared with LPS and OVA-induced group. CON, saline-treated mice; LPS+OVA, LPS and OVA-induced asthmatic mice; Cornuside 150, LPS and OVA-induced asthmatic mice treated with 150 mg/kg cornuside; Cornuside 300, LPS and OVA-induced asthmatic mice treated with 300 mg/kg cornuside; DXM, LPS and OVA-induced asthmatic mice treated with 1 mg/kg DXM.

nificantly after LPS and OVA inhalation compared with those after saline inhalation (Figure 4). By contrast, the increased total IgE and OVA-specific IgE levels in serum were significantly reduced by the administration of cornuside or DXM (Figure 4). The result indicates that cornuside lowers the increased total IgE and OVA-specific IgE levels in the serum of mice with asthma induced by LPS and OVA.

*Cornuside inhibits the expression of TLR4 and Nocth1 and the phosphorylation of STAT3 in lung tissues of asthmatic mice induced by LPS and OVA*

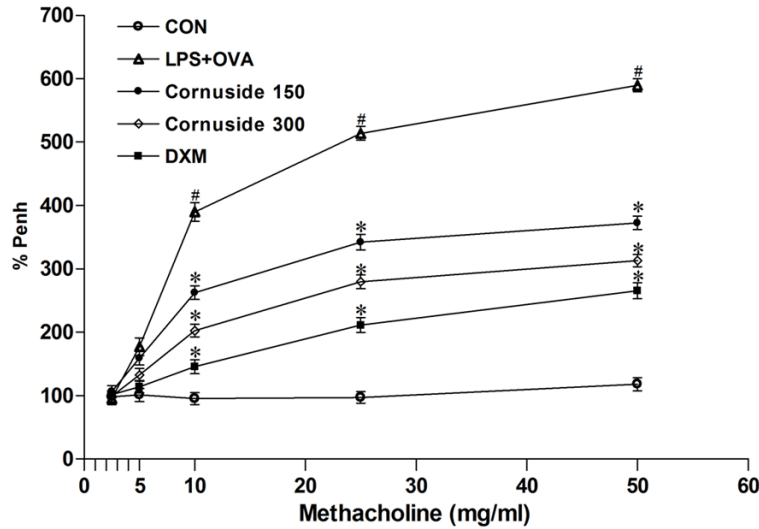
To test the expression of TLR4 and Nocth1 and the phosphorylation of STAT3 in lung tissues, we used Western blotting. Western blots revealed that the levels of TLR4 and Nocth1 and the phosphorylation of STAT3 in lung tissues were increased at 24 h after LPS and OVA inhalation compared with those in control. Furthermore, the increased TLR4 and Nocth1 levels and STAT3 phosphorylation at 24 h after LPS and OVA inhalation were decreased by the administration of cornuside or DXM (Figure 5). The result suggests that cornuside inhibits the expression of TLR4 and Nocth1 and the phosphorylation of STAT3 in lung tissues of asthmatic mice induced by LPS and OVA.

*Cornuside reduces Mch-induced AHR in mice with asthma induced by LPS and OVA*

the final OVA challenge. Total IgE and OVA-specific IgE levels in serum were increased sig-

To investigate the effect of cornuside on the development of AHR in mice, we determined

## Treatment of asthma with cornuside



**Figure 6.** Effect of cornuside on LPS and OVA-induced AHR. All animals were nebulized with various concentrations of methacholine (2.5, 5, 10, 25, and 50 mg/mL) as a bronchoconstrictor. Data were expressed as the percentage increase in Penh over the baseline, where the baseline Penh of the saline-treated control group was expressed as 100%. Results from five independent experiments with 7 mice/group were given as means  $\pm$  SEM. #,  $P < 0.05$  compared with control; \*,  $P < 0.05$  compared with LPS and OVA-induced group. CON, saline-treated mice; LPS+OVA, LPS and OVA-induced asthmatic mice; Cornuside 150, LPS and OVA-induced asthmatic mice treated with 150 mg/kg cornuside; Cornuside 300, LPS and OVA-induced asthmatic mice treated with 300 mg/kg cornuside; DXM, LPS and OVA-induced asthmatic mice treated with 1 mg/kg DXM.

airway responsiveness using Penh. The data showed that Penh was significantly increased in LPS and OVA-induced asthmatic mice in response to Mch inhalation compared with control mice. Cornuside or DXM dramatically prevented AHR to inhaled Mch (**Figure 6**). The result indicates that cornuside reduces Mch-induced AHR in mice with asthma induced by LPS and OVA.

### Discussion

Inflammatory and allergic asthma is characterized by the infiltration of eosinophils, mast cells, and T lymphocytes into airway epithelia [26, 27]. The interactions between these cells and airway epithelial cells play important roles in the pathogenesis of asthma [28]. Specific cytokines such as IL-4, IL-5, and IL-13 cause several key features of allergic asthma [29]. LPS is a major component of the outer membrane of gram-negative bacteria and its exposure is a major risk factor for asthma [30]. There is apparent controversy about the role of LPS in asthma and allergic inflammation as evidenced by epidemiological and experimental

studies, which show that LPS exhibits exacerbating role on asthma [8, 31]. In the present study, we have used LPS and commercial OVA to induce inflammatory responses and investigated the effects of cornuside on airway inflammation in a murine model of allergic asthma. Our results have revealed that LPS and commercial OVA induce classical pathophysiology of asthma and that cornuside reverses or prevents these effects in asthma model mice. Counts of total cells, eosinophils, lymphocytes and neutrophils in BAL fluid were much lower in LPS and OVA-induced mice treated with cornuside. Expression of Th2 cytokines such as IL-4, IL-5 and IL-13 is increased after the induction of asthma. Cornuside significantly reduced IL-4, IL-5 and IL-13 expression that is enhanced by LPS and OVA induction. Consistent

with these biochemical findings, HE staining has shown that cornuside treatments ameliorate airway inflammation.

Airway inflammation is connected by complex signaling networks. Therefore, the molecular mechanisms of this disorder are poorly understood. There is abundant evidence that demonstrates that activation of TLR pathway conditions subsequent responses to sensitization with soluble antigens. In the absence of adjuvant, stimulation of TLR4 on airway structural cells has been shown to be necessary for priming innate immune responses and for the development of airway disease in response to inhaled house dust mite allergen [32]. Recent reports have also shown that TLR4 expression on stromal cells promotes Th2-biased allergic sensitization to OVA via the airways and the development of subsequent airway disease [33], and exposure to LPS increases the severity of asthma, which activates TLR4 signaling in the regulation of Th2-driven airway disease [34]. In the present study, LPS and OVA have stimulated TLR4 expression associated with allergic inflammation in OVA-in-

duce asthmatic model mice. Cornuside has suppressed the increase of TLR4 enhanced by LPS and OVA. Activated TLR4 leads to the promotion of inflammatory mechanisms including several downstream pathways of mitogen-activated protein kinase, NF- $\kappa$ B, and JAK/STAT [35]. STAT proteins, cytokine-inducible transcription factors, are crucial for cytokine signaling and acute phase responses [36]. However, their role in mediating allergic responses in asthma is not well defined. One study has shown that airway epithelial STAT3 is responsible for allergic inflammation by modulating Th2 cell recruitment and effector function in a murine model of chronic asthma [37]. Likewise, inhibition of STAT3 ameliorates experimental asthma by modulating lung CD11c (+) dendritic cell phenotype and function [38]. Therefore, targeting of STAT3 may provide the basis for a novel therapy for asthmatic inflammation. The present study indicates that STAT3 activation may be linked to TLR4 signaling and inhibited in LPS and OVA-induced mice that are treated with cornuside.

Notch signaling pathway is involved in the control of cell identity, proliferation, differentiation and apoptosis in various animals [39]. Mammalian Notch receptors (Notch1-4), a family of transmembrane proteins, have traditionally been thought to play an important role in the regulation of cellular development, differentiation, and apoptosis [12, 40]. However, recent studies have demonstrated that inhibition of Notch activation dramatically decreases T cell proliferation in both CD4 and CD8 cells [41], and jagged is an allophycocyanin-derived signal for IL-4-independent Th2 differentiation, with Delta being an APC-derived Th1 differentiation signal [42]. However, after Notch1 is blocked by Notch1-specific small interfering RNA, IL-4 is decreased and IFN- $\gamma$  is increased in activated lung T cells, suggesting that Notch1 signal plays a role in Th1/Th2 differentiation of asthma [43]. Notch pathway crosstalk with STAT3 is also implicated and activation of Notch signaling can be promoted by enhancing STAT3 phosphorylation [44, 45]. We hypothesize that Notch pathway may also be involved in LPS and OVA-induced asthma. Notably, our study has shown that TLR4 and Notch1, including STAT3, have high expression in LPS and OVA-induced asthmatic mice, which is decreased by cornuside. These results sug-

gest that TLR4 and Notch signaling pathways are all involved in asthma, and crosstalk occurs between the two cell signaling pathways. Furthermore, the signaling pathway between TLR4 and Notch may be able to coordinate with STAT3.

In summary, we have examined the effects of cornuside on airway inflammation in LPS and OVA-induced asthmatic mice. At the same time, signaling pathway crosstalk between TLR4 and Notch in this process is elucidated. After treatment with cornuside, expression of TLR4 and Notch1 and phosphorylation of STAT3 in LPS and OVA-induced asthmatic mice are reduced. In addition, the level of Th2 cytokine, airway inflammation, and airway AHR are also attenuated. It is suggested that the blocking effect of cornuside on LPS and OVA-induced airway inflammation is mediated in part by regulating TLR4 and Notch pathways. The present study also provides a crucial molecular basis for the preventive and/or therapeutic capability of cornuside, for allergic airway diseases.

### Acknowledgements

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### Disclosure of conflict of interest

None.

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