



Cordycepin alleviates airway hyperreactivity in a murine model of asthma by attenuating the inflammatory process



Xiaofeng Yang^{a,1}, Yanxiang Li^{a,1}, Yanhao He^a, Tingting Li^a, Weirong Wang^b, Jiye Zhang^c, Jingyuan Wei^d, Yanhong Deng^e, Rong Lin^{a,*}

^a Department of Pharmacology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, Xi'an 710061, Shaanxi, PR China

^b Laboratory Animal Center, Xi'an Jiaotong University Health Science Center, Xi'an 710061, Shaanxi, PR China

^c School of Pharmacy, Xi'an Jiaotong University Health Science Center, Xi'an 710061, Shaanxi, PR China

^d Liaoning Province Academy of Analytic Science, Shenyang 110015, Liaoning, PR China

^e College of Veterinary Medicine, Jilin University, Changchun 130062, Jilin, PR China

ARTICLE INFO

Article history:

Received 15 September 2014

Received in revised form 4 April 2015

Accepted 8 April 2015

Available online 22 April 2015

Keywords:

Cordycepin (Cor)

Ovalbumin (Ova)

Allergic inflammation

Hyperresponsiveness

ABSTRACT

Cordycepin (Cor), which is a naturally occurring nucleoside derivative isolated from *Cordyceps militaris*, has been shown to exert excellent antiinflammatory activity in a murine model of acute lung injury. Thus, this study aimed to evaluate the antiasthmatic activity of Cor (10, 20, and 40 mg/kg) and to investigate the possible underlying molecular mechanisms. We found that Cor attenuated airway hyperresponsiveness, mucus hypersecretion, and ovalbumin (Ova)-specific immunoglobulin (Ig) E, and alleviated lung inflammation with decreased eosinophils and macrophages in the bronchoalveolar lavage (BAL) fluid. Notably, Cor reduced the upregulation of eotaxin, intercellular cell adhesion molecule-1 (ICAM-1), IL-4, IL-5, and IL-13 in the BAL fluid. Furthermore, Cor markedly blocked p38-MAPK and nuclear factor-kappaB (NF-κB) signalling pathway activation in the Ova-driven asthmatic mice. In conclusion, this study demonstrated that some of the antiasthmatic benefits of Cor attributable to diets and/or tonics may result from reductions in inflammatory processes and that these antiasthmatic properties involve the inhibition of Th2-type responses through the suppression of the p38-MAPK and NF-κB signalling pathways.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Numerous components of Chinese medicinal herbs exert excellent antiinflammatory effects through the negative regulation of mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) signalling pathways [1–3]. Pharmaceutical and dietary strategies have targeted these signalling cascades to control asthma, and further many natural products with strong pharmacological properties are good candidates for the alleviation or prevention of asthma [4,5]. *Cordyceps militaris* or Dong-Chong-Xia-Cao (winter worm summer grass) in Chinese, which is a caterpillar-grown traditional medicinal mushroom, has been used as a natural invigorant for longevity, endurance, and vitality for thousands of years in China [6]. Cordycepin (Cor, 3'-deoxyadenosine), which is a nucleoside derivative purified from *C. militaris*, is prescribed for various diseases, such as cancer and chronic inflammation. Multiple biological activities of Cor have been recently elucidated, including antiviral, antifungal, antiinflammatory, antihyperglycemic, and antiatherosclerotic activities [7–9]. Kim and

coworkers have previously reported that Cor has antiinflammatory effects in LPS-induced raw 264.7 macrophage cells in association with the suppression of NF-κB activation [10]. Pretreatment with Cor attenuates the inflammatory process, which is an initial step in asthma progression, through antiinflammatory pathways. Nevertheless, no studies have thoroughly explored the possible molecular mechanisms underlying the antiasthmatic activity of Cor in the Ova-driven asthmatic mice.

Cumulative evidence has revealed that the number of individuals with allergic asthma is a rapidly increasing worldwide and that it currently affects approximately 20% of the world's population. It has been described as a global public health problem due to its prevalence, morbidity, and mortality [11,12]. The pathophysiology of allergic asthma is complex and is caused by an aberrant immune response to common allergens, principally coordinated by the Th2-type immune response [13,14]. This allergic reaction is associated with chronic airway inflammation manifested by bronchial hyper-responsiveness, IgE production, mucus hypersecretion, and infiltration of leukocytes, mainly eosinophils, in the airway or lung tissues [13,15]. Several studies have shed light on Th2-dominant eosinophilic inflammation in the airway or lung tissues, aiding in the understanding of the pathogenesis of allergic asthma. Th2 cells participate centrally in all stages of allergic asthma,

* Corresponding author. Tel.: +86 29 82657691; fax: +86 29 82657833.

E-mail address: linrong63@aliyun.com (R. Lin).

¹ Xiaofeng Yang and Yanxiang Li contributed equally to this work.

beginning with their secretion of Th2 cytokines, such as IL-4, IL-5, and IL-13, which are considered to be responsible for many of the features of asthma. Furthermore, these Th2 cell-derived cytokines, representing the hallmarks of Th2 immunity in models of allergic asthma, are involved in the production of immunoglobulin (Ig) E by B cells and also in goblet cell hyperplasia with increased mucus secretion [16–18]. Importantly, during the course of asthma, dendritic cells (DCs), serving as sentinels in the airway because of their roles in antigen presentation, are critical for the development of the Th2 immune response and are also linked with the production of proinflammatory cytokines associated with the toll-like receptor 4 ligand and its downstream signal transduction pathways, typically MAPKs and NF- κ B [19]. Thus, the blockade of MAPKs and NF- κ B activation may be effective in reducing allergic airway inflammation [20,21].

Considering that Cor is a component of the extremely rare medicinal mushroom *C. militaris* and may possess antiallergic functions, as described in the literature. In this study, we investigated whether Cor (10, 20, and 40 mg/kg) ameliorates Ova-induced airway inflammation in addition to its underlying mechanism in a murine model of allergic asthma, attempting to provide evidence of the potential therapeutic values of traditional Chinese medicine for the treatment of asthma.

2. Materials and methods

2.1. Animals and reagents

Male BALB/c mice, 6 weeks old weighing approximately 18 g, were purchased from Shanghai Jingke Industrial Co., Ltd (Certificate: SCXK2003-0003, Shanghai, China). The laboratory temperature was maintained at $24 \pm 1^\circ\text{C}$, and relative humidity was controlled at approximately 60%. Mice were housed in sterilised microisolator cages, with filtered air and autoclaved bedding, food, and water. The animal experimental protocols were approved by the Ethical Committee on Animal Research of Jilin University. Mice were allowed to acclimatise for 1 week before the onset of the experiments with a 12 h light/dark cycle (light 8 am to 8 pm). All experimental procedures were performed in accordance with the guide for the Care and Use of Laboratory Animals established by the US National Institutes of Health. No mice were dead and no apparent signs of exhaustion were observed during the experimental period.

Cordycepin (Cor, purity: >98%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ova (Grade δ), aluminium hydroxide adjuvant, and dexamethasone (Dex, purity: >99.6%) were provided by Sigma-Aldrich Trading Co., Ltd. (St. Louis, MO, USA). Mouse interleukin (IL)-4, IL-5, IL-13, eotaxin, and intercellular cell adhesion molecule 1 (ICAM-1) ELISA kits were obtained from Biolegend (San Diego, CA, USA). The ELISA kit for immunoglobulin (Ig) E was purchased from R&D (Anniston, AL, USA). The primary antibodies including phosphorylated and non-phosphorylated forms of mitogen-activated protein kinases (MAPKs) family and nuclear factor-kappaB (NF- κ B) and the horseradish peroxidase-labelled IgG secondary antibodies were purchased from Cell Signalling Technology Inc (Beverly, MA, USA). Polyclonal rabbit β -actin antibodies were provided by Tianjin Sungene Biotech Co., Ltd (Tianjin, China). Other chemical reagents were obtained from Beijing Dingguo Changsheng Biotech Co., Ltd (Beijing, China). All these reagents were of analytical grade, unless otherwise specified.

2.2. Experimental design

Allergen sensitization/challenge protocol: Male mice were randomly assigned to six groups ($n = 8$) as follows: (1) the Control (Cont) group; (2) the Ova group; (3) the Ova + Cor 10 group; (4) the Ova + Cor 20 group; (5) the Ova + Cor 40 group; and (6) the Ova + Dex group. Hereafter, these group abbreviations are presented to clarify the text. The mice were immunised on days 0, 7, and 14 by

intraperitoneal (i.p.) injection of 20 μg Ova emulsified in 1 mg aluminium hydroxide adjuvant in a total volume of 0.2 mL [22]. On days 23, 24, 25, and 26 after the initial sensitisation, the mice were anaesthetised with an i.p. injection of 0.2 mL of a mixture of ketamine (0.44 mg/mL) and xylazine (6.3 mg/mL) in normal saline. The mice were placed on a board in the supine position. Subsequently, they were intranasally challenged with a 2% (w/v) Ova solution in phosphate-buffered saline (PBS, pH = 7.2), as described previously with minor modifications [4]. The Cont mice received equivalent volumes of PBS without Ova i.p. on days 0, 7, and 14 and then were challenged with PBS without Ova (w/v) each day from days 23 to 26. Airway hyperresponsiveness (AHR) was measured at 24 h after the final Ova challenge on day 27, and then the mice were sacrificed to characterise the protective effects of Cor. The schematic diagram of the treatment schedule is presented in Fig. 1.

Administration of drugs: Mice were administered an intraperitoneal injection of normal saline or Cor (10, 20, and 40 mg/kg, dissolved in normal saline) at 1 h prior to each corresponding Ova challenge on days 23, 24, 25 and 26. Dex, which is a steroid hormone drug of the glucocorticoid class, is a potent inhibitor of airway inflammation and remodelling [23,24]. Thus, in our present study, Dex (2 mg/kg, dissolved in normal saline, i.p.) served as a positive control.

2.3. Collection of blood and bronchoalveolar lavage (BAL) fluid and lung tissue separation

Twenty-four hours after the last challenge, the mice were anaesthetised and bled via the brachial plexus for the collection of blood samples, which were used to estimate IgE production. The collection of BAL fluid was performed three times through a tracheal cannula with 0.5 mL of autoclaved PBS (pH = 7.2) to yield a total volume of 1.3 mL. The recovery rate of the fluid was approximately 87%. The collected BAL fluid samples were centrifuged at 700 $\times g$ for 10 min at 4°C , and the supernatants were frozen at -80°C immediately for further enzyme-linked immunosorbent assays (ELISAs). The cell pellets were then treated and used as samples for inflammatory cell analysis. Lung tissues were harvested simultaneously from mice not subjected to BAL fluid collection and then were treated or stored at -80°C for the other experiments.

2.4. Mouse anti-Ova IgE measurement

The levels of Ova-specific IgE in the serum were measured by an ELISA as described previously [25]. Briefly, microtitre plates were coated with 1% Ova in coating buffer (0.05 M sodium carbonate–bicarbonate, pH = 9.6) overnight at 4°C . After blocking and washing, diluted serum samples (1/20) were added at room temperature for 2 h and

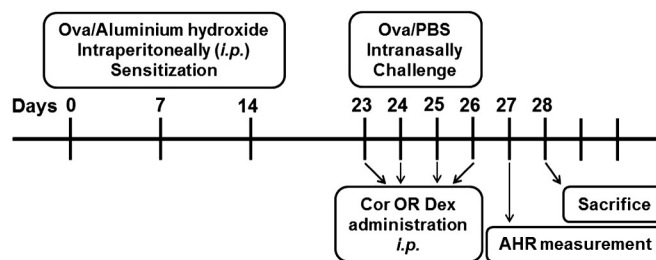


Fig. 1. Experimental protocol for development of allergic asthma and treatment with Cor (10, 20, and 40 mg/kg) or Dex (2 mg/kg) using a murine model. Mice were divided into four groups ($n = 8$) and sensitised via an intraperitoneal injection of 20 μg Ova emulsified in 1 mg aluminium hydroxide in 200 μL PBS on days 0, 7, and 14, respectively. Subsequently, mice were given an intranasal instillation with 2% (w/v) Ova solution in PBS on days 23, 24, 25, and 26 after the initial sensitization. Mice were given an intraperitoneal injection of Cor (10, 20, and 40 mg/kg, dissolved in normal saline) or Dex (2 mg/kg, diluted in normal saline) each day from days 23 to 26 consecutively, 1 h prior to each corresponding Ova challenge. Control mice were sensitised and challenged with equivalent volumes of PBS without drug administration. AHR assay was performed 24 h after the last Ova challenge. Then all mice were sacrificed for further experiments.

then washed and incubated with a biotinylated anti-mouse IgE antibody, followed by multiple washings and incubation for 30 min with extravidin-peroxidase conjugated reagent. For colour development at room temperature, 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate was added, and the reaction was stopped by 2 M sulphuric acid (H_2SO_4). Optical density (OD) values were measured at 450 nm using a Benchmark microplate reader (Bio-Rad, Richmond, CA).

2.5. Bronchoalveolar lavage (BAL) fluid analysis

Differential cell counts of BAL fluid: The cell pellets were again resuspended in 1 mL of red blood cell lysis buffer to remove red cells. Subsequently, leukocytes were repelled by centrifugation at $700 \times g$ for 10 min at $4^\circ C$. Then, these white cell pellets were again resuspended in 1.0 mL of PBS (pH = 7.2) and stained using a KWIK-DIFF™ staining kit (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA). Next, a total of 200 cells from each sample were obtained for differential cell counts, including eosinophils, neutrophils, and macrophages, which were performed by hand with a haemocytometer.

Measurements of cytokines and chemokines: Concentrations were determined in duplicate for each sample. The levels of the cytokines, including IL-4, IL-5, IL-13, eotaxin, and ICAM-1 in the BAL fluid samples, were measured using sandwich enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions.

2.6. Lung histology

For histopathologic assessment, lungs from mice that did not undergo BAL were removed by dissection and placed in 4% paraformaldehyde for 24 h. Lung tissues were sectioned, embedded in paraffin, and cut into $3 \mu m$ sections. Subsequently, tissue sections were stained with Alcian blue-periodic acid Schiff (AB-PAS) for identifying goblet cells in the epithelium and measuring mucus production [26], also, with haematoxylin and eosin (H&E) for infiltration [27]. Photomicrographs were cropped and corrected for brightness and contrast, but not otherwise manipulated. Semi-quantitative analyses of inflammatory cell infiltration and mucus production in lung sections were performed as previously described [28]. Briefly, to determine the severity of inflammatory cell infiltration, peribronchial cell counts were evaluated blind graded on a 5-point scoring system: 0, no cells; 1, a few cells; 2, a ring of cells one cell layer deep; 3, a ring of cells two to four cells deep; 4, a ring of cells of more than four cells deep. To determine the magnitude of mucus production, goblet cell hyperplasia in the airway epithelium was quantified blind using a 5-point grading system: 0, no goblet cells; 1, 25%; 2, 25% to 50%; 3, 50% to 75%; and 4, 75%. Scoring of inflammatory cells and goblet cells was performed in at least three different fields for each lung section.

2.7. Measurement of airway hyperresponsiveness

The airway responsiveness of mice to increasing concentrations of aerosolised methacholine was measured at 24 h after the last Ova challenge. Mice were anaesthetised and tracheotomy was performed, as described previously in detail [29]. The internal jugular vein was cannulated and connected to a microsyringe for intravenous methacholine administration. Baseline airway resistance (RI) and lung compliance (Cdyn), as well as responses to aerosolised saline (0.9% NaCl), were recorded first, followed by responses to increasing doses of aerosolised methacholine (6.25, 12.5, 25, and 50 mg/mL; Sigma, St Louis, MO, USA) in saline using a whole-body plethysmograph system (Buxco, Sharon, CT) [29]. RI represents the main indicator of airflow obstruction. Cdyn indicates lung distensibility, which is defined as the change in volume of the lung driven by a change in pressure across the lung. The results are expressed as a percentage of the respective basal values in response to saline.

2.8. Western blot analysis

Lung tissues were added to Cell Lysis Buffer for Western and IP (Beyotime, Shanghai, China) and homogenised on ice with protease and phosphatase inhibitors (Boehringer, Mannheim, Germany) following the manufacturers' instructions respectively. The homogenate was centrifuged at $14,000 \times g$ for 10 min at $4^\circ C$, the supernatant was aliquoted and then stored at $-80^\circ C$ after removing a small aliquot for protein quantification. Protein concentrations were determined using a BCA protein assay kit (Beyotime, Shanghai, China) according to the manufacturer's protocol. Equivalent amount of 80 μg protein was loaded in each lane of the immunoblot, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride membranes (PVDF). After washing with Tris-buffered saline with 0.1% Tween-20 (TBST), immunoblots were probed with the primary antibodies, including phosphorylated or non-phosphorylated forms of MAPKs family and NF- κB (Cell Signalling Technology, Beverly, MA), and β -actin (Sungene, Tianjin, China). With the use of a peroxidase-conjugated secondary anti-mouse or -rabbit antibodies after another three times washings, bound antibodies were developed using an enhanced chemiluminescence kit (GE Healthcare, Buckinghamshire, UK). Pre-determined molecular weight standards were used as markers (Beyotime, Shanghai, China).

2.9. Statistical analysis

The values were entered into a database and analysed using SPSS software (SPSS for Windows version 13.0, Chicago, IL, USA), and expressed as means $\pm$ SEM. Statistically significant differences between groups were determined by ANOVA followed by Student's *t* test. Survival date was presented by the Kaplan–Meier text and comparisons were made by the log rank test.

3. Results

3.1. Effects of Cor on Ova-specific serum IgE level

To investigate whether Cor regulates Ova-induced serum specific IgE expression, serum samples were obtained and assessed with a commercial ELISA kit. As expected, there was a significant increase in Ova-induced serum-specific IgE secretion in the serum of the Ova-driven mice compared with the control mice, which were sensitised and subsequently challenged without Ova ($^{***}P < 0.01$ vs. the Cont group, Fig. 2A). However, the enhanced provocation of IgE was substantially attenuated by Cor at a dose of 20 and 40 mg/kg ($^*P < 0.05$ or $^{**}P < 0.01$ vs. the Ova group, respectively). Additionally, the protection of Cor at the dose of 40 mg/kg was comparable as that of 2 mg/kg Dex ($P > 0.05$ vs. the Dex + Ova group).

3.2. Effects of Cor on release of eotaxin, ICAM-1, and Th 2-driven cytokines in BAL fluid

Airway inflammation in asthma is considered to be a Th2-predominant immune response. To determine whether Cor suppresses Th2-type inflammation in the lung, we measured the effects of Cor (10, 20, and 40 mg/kg) on Th2-cytokine and chemokine secretion. As shown in Fig. 2B, C, D, E and F, Ova sensitisation and challenge induced marked elevations in eotaxin, ICAM-1, and hallmark Th2 cytokines, including IL-4, IL-5, and IL-13, in the BAL fluid ($^{***}P < 0.01$ vs. the Cont group, respectively). The administration of Cor (20 and 40 mg/kg) noticeably reduced the levels of eotaxin, ICAM-1, IL-4, IL-5, and IL-13 ($^*P < 0.05$ or $^{**}P < 0.01$ vs. the Ova group, respectively). In addition, Dex (2 mg/kg) administration resulted in a comparable reduction in the levels of these Th2-driven cytokines as well as chemokines in the BAL fluid.

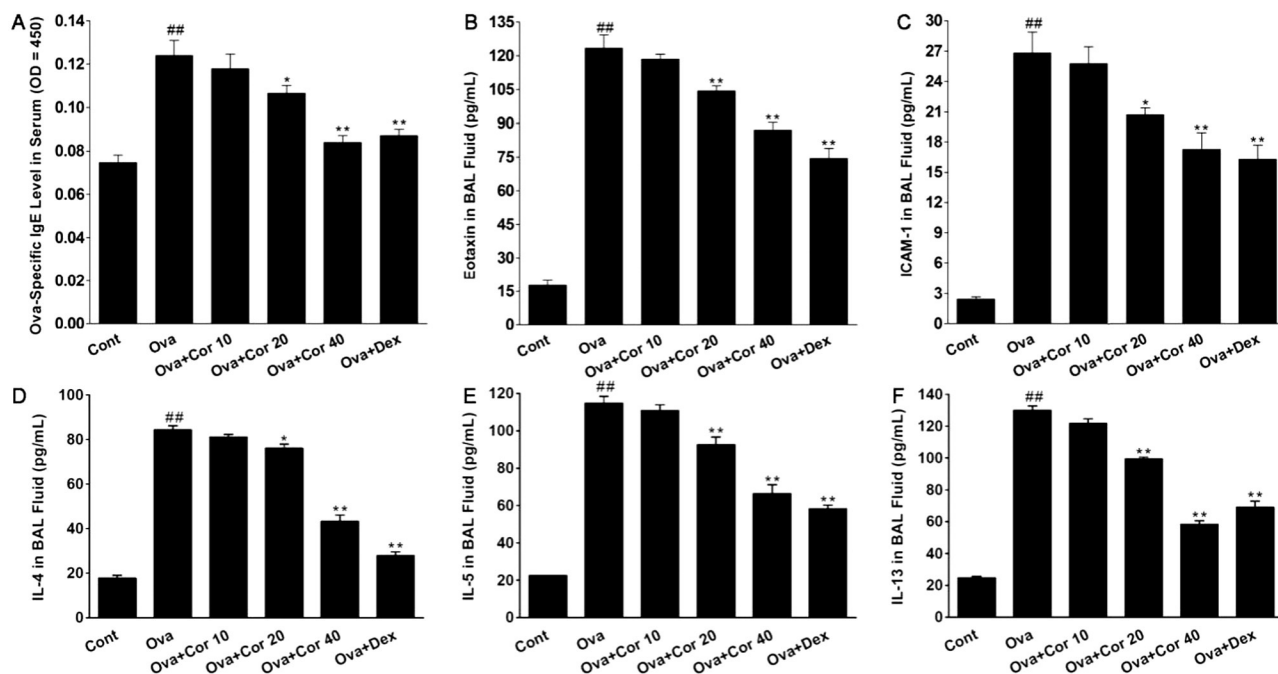


Fig. 2. The effect of Cor (10, 20, and 40 mg/kg) on serum and BAL fluid assessments. Samples of serum and BAL fluid were collected 24 h after the last Ova aerosol challenge. Levels of (A) Ova-specific serum IgE, (B) eotaxin, (C) ICAM-1, and (D, E, and F) Th2 cytokines (IL-4, IL-5, and IL-13) were analysed using specific ELISA kits. The values presented are the means ± SEM (* $P < 0.05$ or ** $P < 0.01$ vs. the Cont group; * $P < 0.05$ or ** $P < 0.01$ vs. the Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; $n = 8$).

3.3. Effects of Cor on inflammatory cell recruitment in BAL fluid

This study determined that Cor treatment may block severe Ova-induced inflammatory cell recruitment to the lungs. Ova challenge significantly increased the total number of cells in the BAL fluid, as well

as the numbers of eosinophils, neutrophils, macrophages, and lymphocytes (** $P < 0.01$ vs. the Cont group, Fig. 3). The administration of Cor (20 and 40 mg/kg) notably prevented the increases in these cells in the BAL fluid (* $P < 0.05$ or ** $P < 0.01$ vs. the Ova group, respectively). In addition, this reduction in Cor at the dose of 40 mg/kg in the

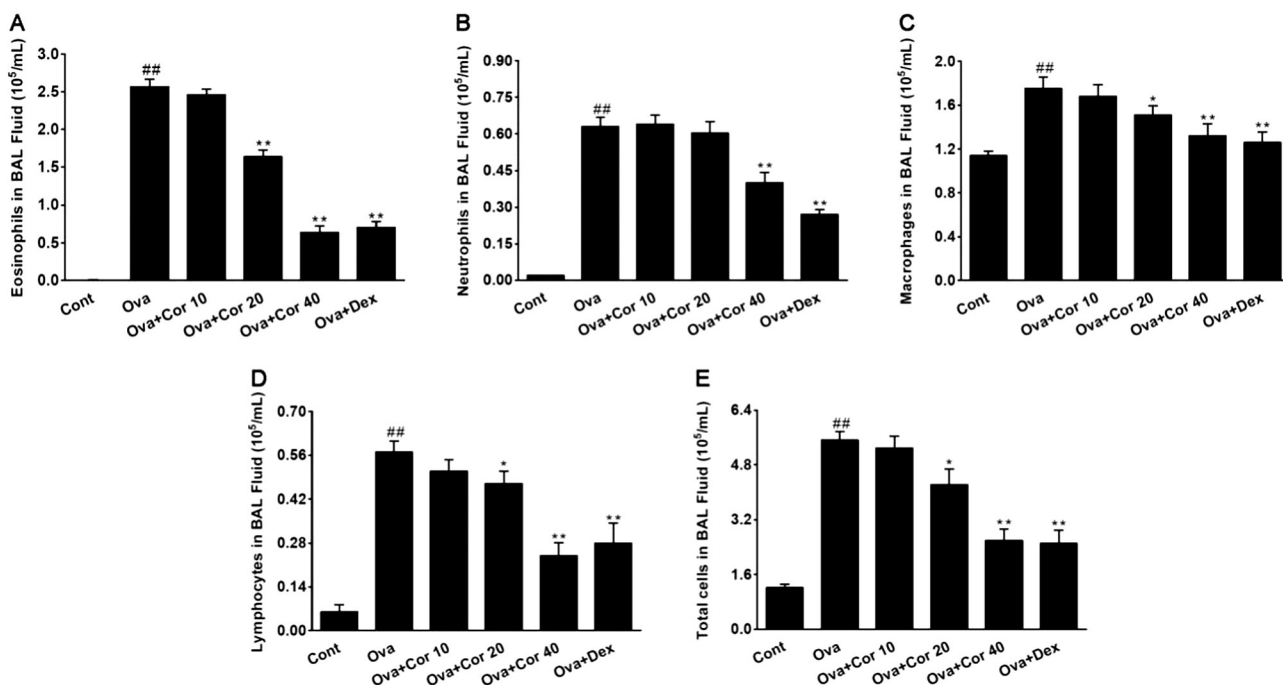


Fig. 3. The effect of Cor (10, 20, and 40 mg/kg) on the recruitment of inflammatory cells in BAL fluid by Kwik-Diff staining. The lavage fluid was centrifuged and red cells were dismissed using its exclusive cell lysis buffer, and then the cell pellets were resuspended in 1 mL of PBS and applied to a slide by cytospinning to obtain differential cell counts, including (A, B, C, D, and E) eosinophils, neutrophils, macrophages, lymphocytes, and total cells. A total of 200 cells were counted per slide. The values presented are the means ± SEM (* $P < 0.05$ or ** $P < 0.01$ vs. the Cont group; * $P < 0.05$ or ** $P < 0.01$ vs. the Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; $n = 8$).

experimental mice was comparable to those of Dex applied at 2 mg/kg ($P > 0.05$ vs. the Dex + Ova group). Importantly, sensitisation and subsequent Ova challenge resulted in accumulation of inflammatory cells around the bronchioles and small vessels, which was consistent with the results of the histological examination of paraffin-embedded lung sections with H&E staining ($^{##}P < 0.01$ vs. the Cont group, Fig. 4A). Compared with the vehicle, treatment with Cor (40 mg/kg) or Dex (2 mg/kg) reduced this accumulation ($^{**}P < 0.01$ vs. the Ova group, respectively).

3.4. Effects of Cor on airway inflammation, hyperplasia and hypertrophy of goblet cells, and mucus overproduction

To elucidate the antiinflammatory properties of Cor, histopathological studies were performed. In accordance with the results of the cell counts, there were abundant inflammatory cells infiltrating into the peribronchial and perivascular areas, as characterised by H&E staining, in the Ova-evoked mice compared with the controls ($^{##}P < 0.01$ vs. the Cont group). The administration of Cor (40 mg/kg) or Dex (2 mg/kg) markedly attenuated the magnitude of inflammatory cells in the lung tissues compared with the Ova group ($^{**}P < 0.01$ vs. the Ova group, respectively, Fig. 4A). To assess the presence of goblet cell hyperplasia, we performed AB-PAS staining on lung section. Both over-expression of mucus and hyperplasia and hypertrophy of goblet cells were detected in the bronchial airways of the Ova-treated mice

(Fig. 4B). In comparison with the Ova group, Cor (40 mg/kg) or Dex (2 mg/kg) administration significantly alleviated mucus secretion and goblet cell hyperplasia ($^{**}P < 0.01$ vs. the Ova group, respectively). Otherwise, no apparent differences were observed between the Cor- and Dex-treated groups ($P > 0.05$ vs. the Dex + Ova group). Semi-quantitative analyses of inflammatory cell infiltration and mucus production in these lung sections were performed as previously described [28], as shown in Fig. 4C and D.

3.5. Effects of Cor on airway hyperresponsiveness to methacholine

To further explore the effect of Cor (40 mg/kg) on airway hyperresponsiveness after exposure to increasing doses of methacholine, we measured airway resistance (RI) and lung compliance (Cdyn) parameters in mechanically ventilated mice. As illustrated in Fig. 5A, Cdyn was markedly decreased in the Ova-challenged mice compared with the control mice ($^{##}P < 0.01$ vs. the Cont group). However, this downregulation in Cdyn was effectively prevented by Cor administration (40 mg/kg, $^{**}P < 0.01$ vs. the Ova group). Additionally, as shown in Fig. 5B, the RI of the Ova-challenged mice was dramatically increased by methacholine challenge compared with that of the control mice ($^{##}P < 0.01$ vs. the Cont group). Accordingly, this upregulation in RI in was also blocked by Cor pretreatment (40 mg/kg, $^{**}P < 0.01$ vs. the Ova group). In addition, the regulatory effects of Cor on the RI and

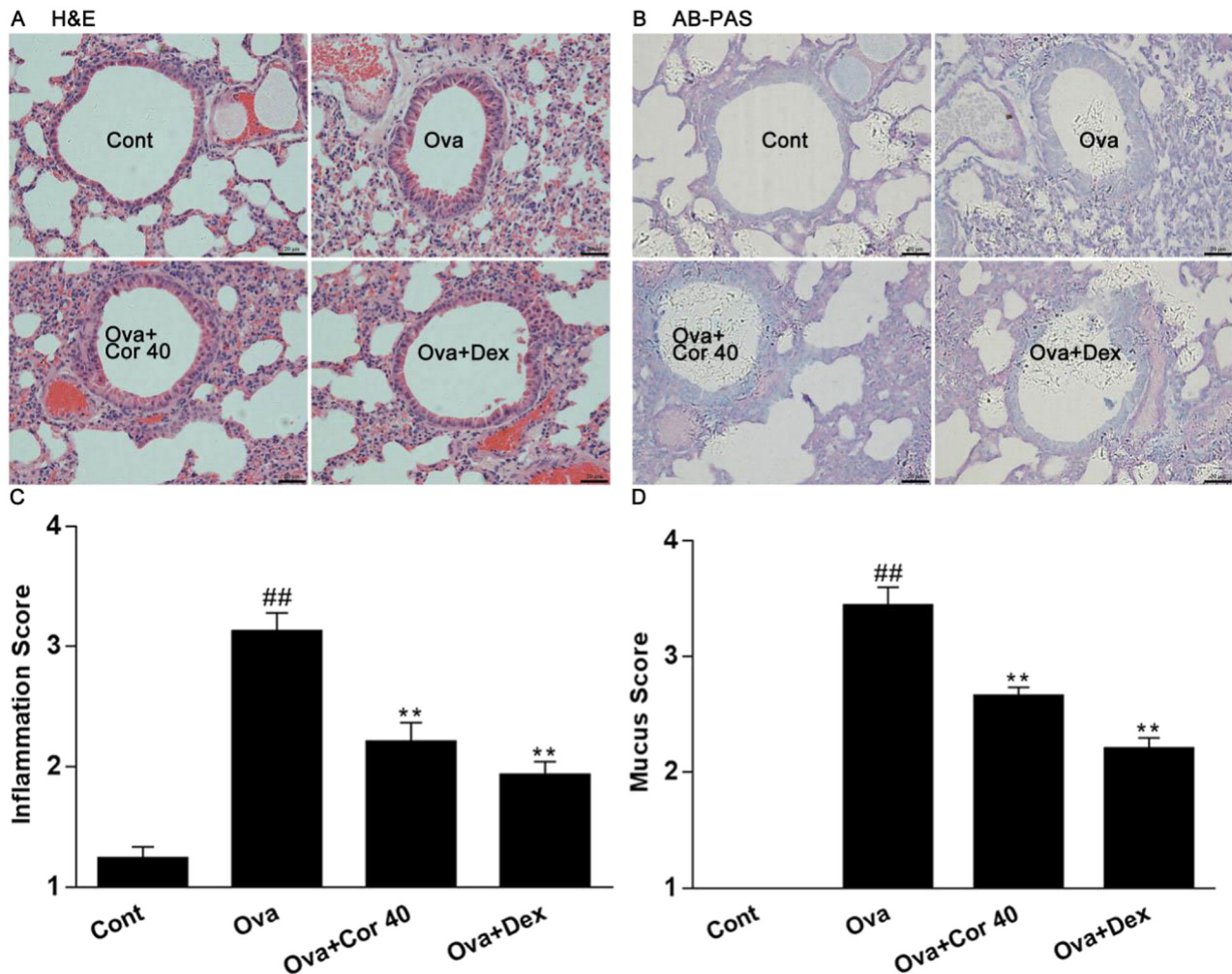


Fig. 4. Effect of Cor (40 mg/kg) on (A) airway inflammation (H&E staining, magnification $\times 400$) and (B) mucus hypersecretion (AB-PAS staining, magnification $\times 400$). Semi-quantitative analyses of (C) inflammatory cell infiltration and (D) mucus production in lung sections were performed as previously described [28]. The scoring data of inflammatory cell infiltration and mucus production were assessed in at least three different fields for each lung section. The values presented are the means $\pm$ SEM ($^{#}P < 0.05$ or $^{##}P < 0.01$ vs. the Cont group; $^{*}P < 0.05$ or $^{**}P < 0.01$ vs. the Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; $n = 8$).

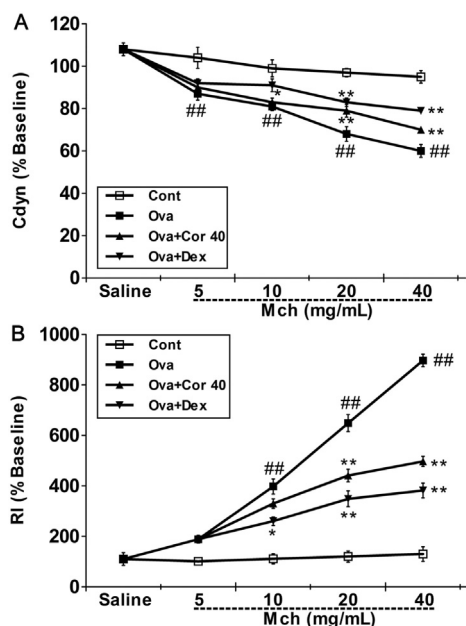


Fig. 5. Effect of Cor (40 mg/kg) on AHR in response to increasing concentrations of aerosolised methacholine (5, 10, 20, and 40 mg/mL) or normal saline was analysed 24 h after the last Ova instillation in mechanically ventilated mice. Airway responsiveness was assessed by percentage change from the baseline level of (A) Cdyn, defined as the change in volume of the lung produced by a change in pressure across the lung and (B) RI, viewed as the pressure driving respiration divided by flow. The values presented are the means $\pm$ SEM (* P < 0.05 or ** P < 0.01 vs. the Cont group; * P < 0.05 or ** P < 0.01 vs. the Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; n = 8).

Cdyn values at the dose of 40 mg/kg were comparable to those of the Dex treatment at 2 mg/kg (P > 0.05 vs. the Dex + Ova group).

3.6. Effects of Cor on MAPKs and NF- κ B signalling pathway activation

To determine whether the therapeutic effect of Cor on Ova-induced asthma occurs through the inactivation of MAPKs and NF- κ B signalling pathways, we examined the protein expression of both phosphorylated and non-phosphorylated forms of the diverse members of these pathways in the mice subjected to the Ova challenge. Within the asthmatic lung, the transcription factor NF- κ B has been implicated in the regulation of airway inflammation, Th2 cell differentiation and recruitment and an elevation in Ova-specific serum IgE secretion [28]. As expected, Ova sensitisation and challenge significantly increased the phosphorylation of NF- κ B and degradation of I κ B in our mouse asthma model, while these alterations were significantly disrupted by the Cor treatment at the dose of 40 mg/kg. We further examined MAPK signalling pathway members, including ERK1/2, p38, and JNK, which may be participated in the process of airway inflammation, and confer protection against certain inflammatory injuries. Interestingly, our results showed that the Cor treatment blocked the activation of Ova-stimulated p38 signalling transduction, but it did not disturb the ERK and JNK signalling pathway. Moreover, no obvious differences were observed between the Ova + Cor 40 and Ova + Dex groups, as illustrated in Fig. 6.

4. Discussion

Asthma, which is a chronic inflammatory disease of the airways, affects many individuals worldwide. The disease can cause severe morbidity and even mortality if it is exacerbated. To date, the treatment strategy for severe asthma consists mainly of the use of bronchodilators (such as β -agonists, anticholinergics, steroids, leukotriene inhibitors, and H1-anti-histamine) [30,31]. However, approximately 5% of patients do not respond to this therapy [31]. Thus, effective therapies that target severe asthma and can alleviate airway inflammation are needed.

Herbal medicines are commonly used to develop traditional formulas to treat asthma in several oriental countries, and growing experimental evidence suggests that numerous components in Chinese medicinal herbs exert excellent anti-inflammatory properties. However, the therapeutic efficacies and accurate mechanisms underlying the activities of such medicines are currently unclear. Kim and coworkers have previously reported that Cor has anti-inflammatory effects in LPS-induced raw 264.7 macrophage cells in association with the suppression of NF- κ B activation [10]. Here, using an Ova-induced mouse model of asthma, we showed that Cor (10, 20, and 40 mg/kg) in a dose-dependent manner delayed the secretion of allergen-specific IgE, eotaxin, and ICAM-1 as well as Th2-associated cytokines, including IL-4, IL-5, and IL-13. Cor (40 mg/kg) also ameliorated Ova-driven goblet cell hyperplasia, mucus hypersecretion, and AHR, which are the cardinal pathophysiological symptoms of allergic airway diseases. Furthermore, the molecular mechanism by which Cor blocks the expression of these inflammatory mediators appeared to involve the disturbances of MAPKs and NF- κ B signalling cascade pathways. These findings indicate that Cor inhibits Th2-dominant inflammation and AHR in Ova-induced asthma, demonstrating its beneficial effects in attenuating asthmatic responses.

Asthma is an allergic inflammatory disease of the airways characterised by airway inflammation, mucus secretion, and elevated IgE levels, resulting in respiratory signs and symptoms (cough, wheezing, and dyspnoea), reversible airflow obstruction, and bronchospasms, which are usually accompanied by persistent AHR [32,33]. Bronchoconstriction attributable to contraction or hypertrophy of airway smooth muscle (ASM) and inflammation within the airway lead to decreased lung function. AHR is a measure of bronchial constriction that is commonly tested in individuals with asthma [30]. We monitored the effects of Cor on AHR by measuring baseline airway resistance (RI) and lung compliance (Cdyn). Reducing airway hyperreactivity has been predicted to alleviate the airway inflammation caused by Th2 cytokines. Allergen inhalation into the airway produces a Th2-dominant response by activating inflammatory cells and increasing levels of IL-4, IL-5, and IL-13 [34]. These experiments demonstrated that Cor inhibited Ova-driven AHR in response to atomised acetylcholine.

The mechanisms of the recruitment of inflammatory cells associated with and presumably causing AHR have been well studied. Lee and colleagues have shown that eosinophil recruitment is induced following exposure to Ova in asthmatic mouse models [35]. Elsner and Kapp have also indicated that migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation [36]. Concerning the rise in the number of eosinophils in the BAL fluid, which is an important hallmark of asthma and allergic disease, we examined whether the beneficial effects of Cor could be potentiated by decreasing the degree of inflammatory cell infiltration. As expected, the present results clearly demonstrated that Cor significantly reduced eosinophil numbers in the BAL fluid. Consistently, Cor also suppressed the Ova-challenged eotaxin level in this fluid. Zhu and coworkers have disclosed that eotaxin-1 accelerates eosinophilia in cooperation with IL-5 and further transmigrates eosinophils into lung tissues [37]. Additionally, it has been reported that leukocyte transmigration into airways is orchestrated by ICAM-1 [38]. Similarly, our results clearly showed that Ova-elevated ICAM-1 expression was reduced by Cor. Thus, we propose that Cor interferes with the recruitment of inflammatory cells, probably by reducing the levels of chemokines and Th2-driven cytokines.

Our present study also attempted to explore the underlying mechanisms of Cor treatment in the amelioration of airway allergic diseases. It is well known that persistent MAPK and NF- κ B activation play essential roles in immune and inflammatory processes, including those of asthma [25,39]. NF- κ B activation has been observed in allergic airway inflammation both in human and animal models of asthma in association with proinflammatory cytokines (predominantly the Th2-type phenotypes) and chemokines (including eotaxin-1 and ICAM-1) [40,41]. Our

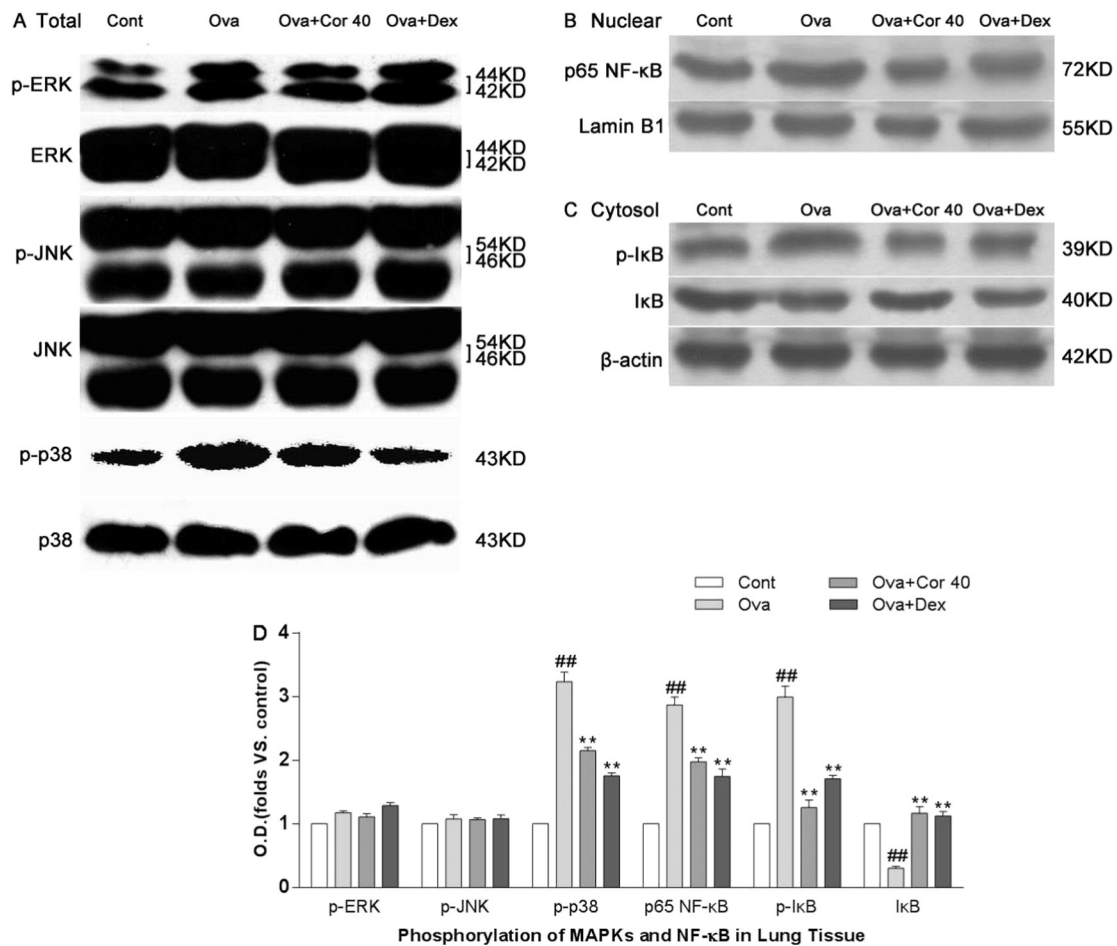


Fig. 6. Effect of Cor (40 mg/kg) on Ova-induced MAPKs and NF- κ B activation. Tissue protein samples were analysed by Western blotting with phosphospecific antibodies. Lamin B1/ β -actin was used as an internal control. Both phosphorylated and non-phosphorylated forms of MAPKs (A, Total) and NF- κ B (B, Nuclear; C, Cytosol) and its inhibitor were subjected to Western blotting. Equivalent amount of 80 μ g protein was loaded in each lane of the immunoblot. Experiments were repeated three times, and similar results were obtained. Means $\pm$ SEM values of p-ERK, p-JNK, p-p38, p65 NF- κ B, p-I κ B, and I κ B quantitative densitometry from different groups. Assay shown is representative of three experiments with similar results. The values presented are the means $\pm$ SEM (* P < 0.05 or ** P < 0.01 vs. the Cont group; * P < 0.05 or ** P < 0.01 vs. the Ova group; ANOVA, comparison for all pairs using Tukey–Kramer HSD; n = 8).

results showed that the elevations in eotaxin, ICAM-1, and Th2-driven cytokines in the BAL fluid were decreased by Cor, suggesting that these reductions might be a consequence of NF- κ B inhibition. Indeed, several previous studies have found that NF- κ B-targeting strategies, such as small-molecule inhibitors and antisense oligonucleotides, are effective in experimental asthma models [28]. Reportedly, the MAPK family is involved in mediating numerous biological functions, including cell proliferation, differentiation, apoptosis, and inflammation [20]. We also investigated the effects of Cor on the MAPK inflammation signalling cascade, and our results showed that Cor treatment abolished Ova-stimulated inflammatory mediators mainly by regulating eosinophilic inflammation-dependent p38 signal transduction but not the ERK or JNK signalling pathway. A reasonable interpretation is likely that Cor targets not only the p38 signalling pathway, but that it also acts on other inflammatory regulatory pathways, such as Akt and other specific chemokine-associated pathways [10,42]. To some extent, our results showed that Cor markedly suppressed the Ova-induced expression of inflammatory mediators in the lungs, which may be due to the inactivation of the p38-MAPK and NF- κ B pathways and the reduced level of Ova-specific IgE. However, additional research would be also worthwhile to address this issue.

In conclusion, our data clearly show that the exposure of mice to Ova results in an asthma-like phenotype. Treatment with Cor before antigen challenge suppressed Th2-type cytokines and elevated IgE levels, attenuated excess airway eosinophilia and mucus hypersecretion, and improved the functional airway response to methacholine. The present

findings suggest that the protective effect of Cor is associated with the modulation of the inflammatory response and AHR, which provides some novel insights into the mechanism underlying asthma. However, further studies should be carried out to explore the therapeutic effect and mechanism underlying the activity of Cor before it is considered a promising candidate for use in clinical practice.

5. Disclosure

All authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 81270347) and the Natural Science Foundation of Shaanxi Province (No. 2012JQ4025).

References

- [1] J.W. Jeong, C.Y. Jin, G.Y. Kim, J.D. Lee, C. Park, G.D. Kim, et al., Anti-inflammatory effects of cordycepin via suppression of inflammatory mediators in BV2 microglial cells, *Int. Immunopharmacol.* 10 (2010) 1580–1586.
- [2] H. Kim, A.S. Naura, Y. Errami, J. Ju, A.H. Boulares, Cordycepin blocks lung injury-associated inflammation and promotes BRCA1-deficient breast cancer cell killing by effectively inhibiting PARP, *Mol. Med.* 17 (2011) 893–900.
- [3] H.Y. Pao, B.S. Pan, S.F. Leu, B.M. Huang, Cordycepin stimulated steroidogenesis in MA-10 mouse Leydig tumor cells through the protein kinase C pathway, *J. Agric. Food Chem.* 60 (2012) 4905–4913.

- [4] S.W. Oh, J.Y. Cha, J.E. Jung, B.C. Chang, H.J. Kwon, B.R. Lee, et al., Curcumin attenuates allergic airway inflammation and hyper-responsiveness in mice through NF-kappaB inhibition, *J. Ethnopharmacol.* 136 (2011) 414–421.
- [5] Y. Deng, M. Guan, X. Xie, X. Yang, H. Xiang, H. Li, et al., Geniposide inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma, *Int. Immunopharmacol.* 17 (2013) 561–567.
- [6] J.Y. Wu, Q.X. Zhang, P.H. Leung, Inhibitory effects of ethyl acetate extract of *Cordyceps sinensis* mycelium on various cancer cells in culture and B16 melanoma in C57BL/6 mice, *Phytomedicine* 14 (2007) 43–49.
- [7] H.S. Tuli, A.K. Sharma, S.S. Sandhu, D. Kashyap, Cordycepin: a bioactive metabolite with therapeutic potential, *Life Sci.* 93 (2013) 863–869.
- [8] Z.G. Chen, D.N. Zhang, L. Cao, Y.B. Han, Highly efficient and regioselective acylation of pharmacologically interesting cordycepin catalyzed by lipase in the eco-friendly solvent 2-methyltetrahydrofuran, *Bioresour. Technol.* 133 (2013) 82–86.
- [9] S.Y. Lee, T. Debnath, S.K. Kim, B.O. Lim, Anti-cancer effect and apoptosis induction of cordycepin through DR3 pathway in the human colonic cancer cell HT-29, *Food Chem. Toxicol.* 60 (2013) 439–447.
- [10] H.G. Kim, B. Shrestha, S.Y. Lim, D.H. Yoon, W.C. Chang, D.J. Shin, et al., Cordycepin inhibits lipopolysaccharide-induced inflammation by the suppression of NF-kappaB through Akt and p38 inhibition in RAW 264.7 macrophage cells, *Eur. J. Pharmacol.* 545 (2006) 192–199.
- [11] G. Jordakieva, J. Wallmann, R. Schmutz, P. Lemell, M. Wegmann, T. Nittke, et al., Peripheral erythrocytes decrease upon specific respiratory challenge with grass pollen allergen in sensitized mice and in human subjects, *PLoS One* 9 (2014) e86701.
- [12] T.A. Chatila, Interleukin-4 receptor signaling pathways in asthma pathogenesis, *Trends Mol. Med.* 10 (2004) 493–499.
- [13] A.C. de Freitas, G.N. Angeli, D.C. Favarin, D.A.E. Lemos, C.J. Lazo, L.H. Faccioli, et al., The effects of proresolution of ellagic acid in an experimental model of allergic airway inflammation, *Mediat. Inflamm.* 2013 (2013) 863198.
- [14] J. Ribeiro-Filho, A.S. Calheiros, A. Vieira-de-Abreu, K.I. de Carvalho, S.M.D. Da, C.B. Melo, et al., Curine inhibits eosinophil activation and airway hyper-responsiveness in a mouse model of allergic asthma, *Toxicol. Appl. Pharmacol.* 273 (2013) 19–26.
- [15] C.B. Mathias, L.A. Guernsey, D. Zammit, C. Brammer, C.A. Wu, R.S. Thrall, et al., Pro-inflammatory role of natural killer cells in the development of allergic airway disease, *Clin. Exp. Allergy* 44 (2014) 589–601.
- [16] C. Malmhall, S. Alawieh, Y. Lu, M. Sjostrand, A. Bossios, M. Eldh, et al., MicroRNA-155 is essential for T2-mediated allergen-induced eosinophilic inflammation in the lung, *J. Allergy Clin. Immunol.* 133 (2014) 1429–1438.
- [17] S.H. Kim, J.H. Hong, Y.C. Lee, Oleanolic acid suppresses ovalbumin-induced airway inflammation and Th2-mediated allergic asthma by modulating the transcription factors T-bet, GATA-3, RORgammat and Foxp3 in asthmatic mice, *Int. Immunopharmacol.* 18 (2014) 311–324.
- [18] S. Ashino, K. Takeda, H. Li, V. Taylor, A. Joetham, P.R. Pine, et al., Janus kinase 1/3 signaling pathways are key initiators of T2 differentiation and lung allergic responses, *J. Allergy Clin. Immunol.* 133 (2014) 1162–1174.
- [19] Z. Hu, R. Chen, Z. Cai, L. Yu, Y. Fei, L. Weng, et al., Salmeterol attenuates the inflammatory response in asthma and decreases the pro-inflammatory cytokine secretion of dendritic cells, *Cell. Mol. Immunol.* 9 (2012) 267–275.
- [20] H. Tang, Y. Sun, Z. Shi, H. Huang, Z. Fang, J. Chen, et al., YKL-40 induces IL-8 expression from bronchial epithelium via MAPK (JNK and ERK) and NF-kappaB pathways, causing bronchial smooth muscle proliferation and migration, *J. Immunol.* 190 (2013) 438–446.
- [21] L.L. Reber, F. Daubeuf, M. Plantinga, L. De Cauwer, S. Gerlo, W. Waelput, et al., A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma, *J. Immunol.* 188 (2012) 3478–3487.
- [22] W.K. Jung, D.Y. Lee, Y.H. Choi, S.S. Yea, I. Choi, S.G. Park, et al., Caffeic acid phenethyl ester attenuates allergic airway inflammation and hyperresponsiveness in murine model of ovalbumin-induced asthma, *Life Sci.* 82 (2008) 797–805.
- [23] X. Ci, X. Chu, C. Chen, X. Li, S. Yan, X. Wang, et al., Oxytetracycline attenuates allergic airway inflammation in mice via inhibition of the NF-kappaB pathway, *J. Clin. Immunol.* 31 (2011) 216–227.
- [24] R.K. Kumar, C. Herbert, P.S. Thomas, L. Wollin, R. Beume, M. Yang, et al., Inhibition of inflammation and remodeling by roflumilast and dexamethasone in murine chronic asthma, *J. Pharmacol. Exp. Ther.* 307 (2003) 349–355.
- [25] C. Xinxin, C. Chi, C. Xiao, X. Xue, Y. Yongjun, C. Junqing, et al., Flufenicol inhibits allergic airway inflammation in mice by p38 MAPK-mediated phosphorylation of GATA 3, *Clin. Immunol.* 138 (2011) 231–238.
- [26] N. Schutze, I. Lehmann, U. Bonisch, J.C. Simon, T. Polte, Exposure to mycotoxins increases the allergic immune response in a murine asthma model, *Am. J. Respir. Crit. Care Med.* 181 (2010) 1188–1199.
- [27] S.P. Guan, L.R. Kong, C. Cheng, J.C. Lim, W.S. Wong, Protective role of 14-deoxy-11,12-didehydroandrographolide, a noncytotoxic analogue of andrographolide, in allergic airway inflammation, *J. Nat. Prod.* 74 (2011) 1484–1490.
- [28] Z. Bao, S. Guan, C. Cheng, S. Wu, S.H. Wong, D.M. Kemeny, et al., A novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor-kappaB pathway, *Am. J. Respir. Crit. Care Med.* 179 (2009) 657–665.
- [29] Z. Bao, S. Lim, W. Liao, Y. Lin, C. Thiernemann, B.P. Leung, et al., Glycogen synthase kinase-3beta inhibition attenuates asthma in mice, *Am. J. Respir. Crit. Care Med.* 176 (2007) 431–438.
- [30] J. Wang, T. Zhang, C. Ma, S. Wang, Puerarin attenuates airway inflammation by regulation of eotaxin-3, *Immunol. Lett.* 163 (2014) 173–178.
- [31] M. Chen, Z. Lv, W. Zhang, L. Huang, X. Lin, J. Shi, et al., Triptolide suppresses airway goblet cell hyperplasia and Muc5ac expression via NF-kappaB in a murine model of asthma, *Mol. Immunol.* 64 (2015) 99–105.
- [32] J. Bousquet, H.J. Schunemann, B. Samolinski, P. Demoly, C.E. Baena-Cagnani, C. Bachert, et al., Allergic Rhinitis and its Impact on Asthma (ARIA): achievements in 10 years and future needs, *J. Allergy Clin. Immunol.* 130 (2012) 1049–1062.
- [33] C.T. McCusker, Use of mouse models of allergic rhinitis to study the upper and lower airway link, *Curr. Opin. Allergy Clin. Immunol.* 4 (2004) 11–16.
- [34] C.J. Liou, P.Y. Cheng, W.C. Huang, C.C. Chan, M.C. Chen, M.L. Kuo, et al., Oral lovastatin attenuates airway inflammation and mucus secretion in ovalbumin-induced murine model of asthma, *Allergy Asthma Immunol. Res.* 6 (2014) 548–557.
- [35] J.H. Lee, J.Y. Lee, T.D. Kim, C.J. Kim, Antiasthmatic action of dibenzylbutyrolactone lignans from fruits of *Forsythia viridissima* on asthmatic responses to ovalbumin challenge in conscious guinea-pigs, *Phytother. Res.* 25 (2011) 387–395.
- [36] J. Elsner, A. Kapp, Regulation and modulation of eosinophil effector functions, *Allergy* 54 (1999) 15–26.
- [37] Y. Zhu, P.J. Bertics, Chemoattractant-induced signaling via the Ras-ERK and PI3K-Akt networks, along with leukotriene C4 release, is dependent on the tyrosine kinase Lyn in IL-5- and IL-3-primed human blood eosinophils, *J. Immunol.* 186 (2011) 516–526.
- [38] D. Proud, R. Leigh, Epithelial cells and airway diseases, *Immunol. Rev.* 242 (2011) 186–204.
- [39] M.Y. Lee, K.S. Ahn, H.S. Lim, J.E. Yuk, O.K. Kwon, K.Y. Lee, et al., Tiarelic acid attenuates airway hyperresponsiveness and inflammation in a murine model of allergic asthma, *Int. Immunopharmacol.* 12 (2012) 117–124.
- [40] J.H. Gong, D. Shin, S.Y. Han, J.L. Kim, Y.H. Kang, Kaempferol suppresses eosinophil infiltration and airway inflammation in airway epithelial cells and in mice with allergic asthma, *J. Nutr.* 142 (2012) 47–56.
- [41] M.Y. Wu, S.K. Hung, S.L. Fu, Immunosuppressive effects of fisetin in ovalbumin-induced asthma through inhibition of NF-kappaB activity, *J. Agric. Food Chem.* 59 (2011) 10496–10504.
- [42] A. Kondrashov, H.A. Meijer, A. Barthet-Barateig, H.N. Parker, A. Khurshid, S. Tessier, et al., Inhibition of polyadenylation reduces inflammatory gene induction, *RNA* 18 (2012) 2236–2250.