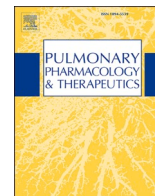




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# Epimedin C modulates the balance between Th9 cells and Treg cells through negative regulation of noncanonical NF- $\kappa$ B pathway and MAPKs activation to inhibit airway inflammation in the ovalbumin-induced murine asthma model

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## ABSTRACT

Allergic asthma is a common airway inflammatory disease and mainly caused by abnormal immune responses to allergens and viruses. The precise mechanisms of airway inflammation and airway hyper-responsiveness (AHR) are still not completely understood. CD4<sup>+</sup> helper T cells (Th cells) serve as critical regulators of allergic immunity. The imbalance between T helper 9 (Th9) cells and forkhead box protein 3 (Foxp3)<sup>+</sup> regulatory T (Treg) cells may contribute to airway inflammation in asthma. Epimedin C, a dominant compound isolated from *Herba Epimedii*, has shown anti-inflammatory effects and the immunoregulatory activity, such as increase of lymphocyte proliferation. However, the protective role of epimedin C in an experimental model of ovalbumin (OVA)-induced allergic airway inflammation and the underlying mechanism remain unknown. Female BALB/c mice were sensitized by intraperitoneal injection (i.p.) of OVA plus aluminum hydroxide (Alum) and subsequently challenged with an aerosol of 3% OVA in saline. Mice were treated with different concentrations of epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) for 4 weeks. Experimental endpoints were evaluated via the analysis of AHR to acetyl- $\beta$ -methacholine (Mch), differential inflammatory cell counts, concentrations of cytokines interleukin-9 (IL-9), IL-4 and IL-10 in bronchoalveolar lavage fluid (BALF), serum OVA-specific IgE level, as well as airway inflammation, mucus secretion and collagen deposition in mice. Mechanistically, we investigated the percentages of Th9 cells and Treg cells, as well as mRNA levels of IL-9 and transcription factor Foxp3 in lungs. Furthermore, the proteins expression of nuclear factor- $\kappa$ B (NF- $\kappa$ B) family members p105/p50, RelA, p100/p52 and RelB, as well as mitogen-activated protein kinase (MAPK) family members extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK was detected. Epimedin C dose-dependently attenuated AHR, airway inflammation, mucus hypersecretion and collagen deposition in OVA-induced murine asthma model. The expression levels of IL-9, IL-4 and OVA-specific IgE were significantly decreased while IL-10 was increased by epimedin C. We further confirmed that epimedin C decreased the percentage of lung Th9 cells with lower mRNA expression of IL-9 and increased the percentage of lung Treg cells with higher mRNA expression of Foxp3. In addition, epimedin C dose-dependently decreased the protein levels of p52, RelB, phosphorylation of ERK1/2 and p38 MAPK which are pivotal to the development of Th9 cells and Treg cells. Collectively, epimedin C could inhibit pathophysiological features of asthma by reconstruction of the balance between Th9 cells and Treg cells through regulation of the noncanonical NF- $\kappa$ B p52/RelB pathway and MAPKs activation. These findings suggest epimedin C as a potential remedy for inflammatory airway diseases.

## 1. Introduction

Asthma is a prevalent immunological disease characterized by airway hyper-responsiveness (AHR), airway inflammation and tissue

remodeling [1,2]. Two forms of asthma have been clinically defined, including the non-allergic (or intrinsic) asthma and allergic (atopic) asthma. Allergic asthma mostly affects children and nearly 90% of all asthmatics [3]. Airway inflammation is the major cause for attacks and development of asthma. When patient's re-exposure to allergens (such

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**Abbreviations**

|                |                                       |
|----------------|---------------------------------------|
| OVA            | ovalbumin                             |
| BALF           | bronchoalveolar lavage fluid          |
| Mch            | acetyl- $\beta$ -methacholine         |
| Alum           | aluminum hydroxide                    |
| AHR            | airway hyper-responsiveness           |
| Th cells       | CD4 <sup>+</sup> helper T cells       |
| Foxp3          | forkhead box protein 3                |
| Th9 cells      | T helper 9 cells                      |
| Treg cells     | Foxp3 <sup>+</sup> regulatory T cells |
| NF- $\kappa$ B | nuclear factor- $\kappa$ B            |
| MAPKs          | mitogen-activated protein kinases     |
| ERK            | extracellular signal-regulated kinase |
| p38 MAPK       | p38 mitogen-activated protein kinase  |
| H&E            | hematoxylin-eosin                     |
| PAS            | periodic acid-Schiff                  |
| qRT-PCR        | quantitative Real-Time PCR            |
| ELISA          | enzyme-linked immunosorbent assay     |

as dust mites, animal dander, dust, mold spores, and pollen) or environmental stimuli, an adaptive immune response can be triggered [4]. The antigens are recognized by dendritic cells (DCs) and other antigen presenting cells (APCs) [5]. APCs process allergens into small peptides and present them to naïve T cells and result in development and mature of subsets of CD4<sup>+</sup> helper T cells (Th cells) [6,7]. Recently T helper 9 (Th9) cells as a major source of the signature cytokine interleukin-9 (IL-9) have shown significance in asthma [8–10]. Th9 cells lead to airway inflammation and allergy [11,12]. Transgenic expression of IL-9 is sufficient in itself to cause bronchial hyper-responsiveness through its effects on the respiratory epithelium and the enhanced release of Th2 cytokines such as IL-4 [13]. Transfer of DO11.10 Th9 cells to recipient mice resulted in allergic airway disease after challenge with ovalbumin (OVA). When these recipient mice were additionally treated with an anti-IL-9 antibody, many of the inflammatory features were blocked [14]. Forkhead box p3 (Foxp3)<sup>+</sup> regulatory T (Treg) cells suppress allergic airway inflammation through inhibiting the function of effector Th cells and production of anti-inflammatory cytokines such as IL-10 and TGF- $\beta$  [6,15]. The balance between Th9 cells and Treg cells could be critical for controlling allergic inflammatory responses in asthma and maintaining airway homeostasis. A recent study demonstrated that the balance between Th9 cells and Treg cells was involved in the control of intestinal inflammation [16].

A new understanding of the pathogenesis of asthma has been made. Although several drugs are available to combat asthma, some patients do not adhere to their therapy, partly because of possible adverse drug reactions and complicated treatment [17,18]. Herbal and natural remedies are the most common kinds of complementary and alternative medicine for asthma and excellent resource for drug discovery [19–21]. Epimedin C is a major flavonoid glycoside derived from *Herba Epimedii* [22]. Our previous studies demonstrated that the combined herbal formulae consisting mainly of *Herba Epimedii* could suppress chronic allergic airway inflammation and AHR through regulating balance between Treg cells and Th17 cells. *Epimedium* flavonoids have also been shown to counteract the suppressive effect of glucocorticoids on the hypothalamic-pituitary-adrenal (HPA) axis function [23,24]. In addition, a study using hydrocortisone acetate-treated murine model showed that epimedin C modulated immune responses of these immunosuppressive mice by increasing proliferation of lymphocyte and production of IL-2 [25]. Although immunomodulatory activity of epimedin C has been reported, it is unclear whether epimedin C might modulate allergic immune responses and attenuate pathophysiological features of asthma through regulating the balance between Th9 cells and Treg cells.

The balance between Treg cells and Th9 cells could be regulated by the development, phenotype shift and function transformation of these cells [26,27]. Extensive literatures showed that the nuclear factor- $\kappa$ B (NF- $\kappa$ B) family of transcription factors played important roles in the development and differentiation of Treg cells and Th9 cells, and consequently regulated the balance between Treg cells and Th9 cells [16,28,29]. Deficiency of the NF- $\kappa$ B family members in CD4<sup>+</sup>T cells could promote differentiation of Treg cells, and inhibit the development of Th9 cells [26]. Besides, mitogen-activated protein kinase (MAPK) family members also play critical roles in allergic inflammatory diseases and differentiation of Treg cells and effector Th cells such as Th9 cells [30–32]. Interestingly, noncanonical NF- $\kappa$ B can induce MAPK activation. MAPK can participate in the activation of NF- $\kappa$ B in the cytoplasm, and in the regulation of its transactivation potential in the nucleus [33]. Importantly, previous studies have demonstrated that anti-inflammatory activities of *Herba Epimedii* and its flavonoids relied on regulating NF- $\kappa$ B and MAPK pathways [34–36]. In the current study, we first showed that the use of epimedin C ameliorated airway inflammation, collagen deposition and AHR in OVA-induced murine asthma model through regulating the balance between Treg cells and Th9 cells. This study also revealed the involvement of noncanonical NF- $\kappa$ B pathway and MAPKs in mediating the therapeutic effects of epimedin C in the murine asthma model.

## 2. Materials and methods

### 2.1. Animals

Five to six-week old female BALB/c mice (18–22 g) were purchased from the Shanghai Jiesijie Laboratory Animal Co., Ltd and raised under specific pathogen-free (SPF) conditions (temperature: 22  $\pm$  2  $^{\circ}$ C; humidity: 40–60%) with a 12 h light/dark cycle. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and were approved by the Ethics Committee on Animal Experiments of School of Pharmacy, Fudan University (permit number: 2019-01-HSYD-DJC-01).

### 2.2. Reagents and antibodies

Epimedin C (C<sub>39</sub>H<sub>50</sub>O<sub>19</sub>,  $\geq$ 98%, Winherb Medical Technology Co., Ltd, Shanghai, China) was suspended in 0.5% sodium carboxy methyl cellulose (CMC-Na) at concentrations of 0.002 g/ml, 0.004 g/ml, 0.008 g/ml and stored at 4  $^{\circ}$ C. Ovalbumin (OVA, grade II and V), aluminum hydroxide (Alum), pentobarbital sodium, acetyl- $\beta$ -methacholine (Mch), collagenase IV, and DNase I were obtained from Sigma-Aldrich (St Louis, MO, USA). Dexamethasone was purchased from Chenxin pharmaceutical Co., LTD (Jining, China). RNAeasy mini kit was acquired from Beyotime Institute of Biotechnology (Shanghai, China). The RevertAid First Strand cDNA synthesis kit was supplied by Thermo Scientific (Carlsbad, CA, USA). Anti-mouse CD4-PE-Cy7, anti-mouse CD25-eF450, anti-mouse IL-9-APC, anti-mouse Foxp3-PE, anti-mouse CD45-eF506, anti-mouse Live/Dead-APC-Cy7, RBC lysis Buffer, and Foxp3 transcription factor staining buffer set were obtained from eBioscience Inc (San Diego, CA, USA). Leukocyte activation cocktail was purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Rabbit anti-mouse polyclonal antibodies recognizing NF- $\kappa$ B1 p105/p50, NF- $\kappa$ B p65 (RelA) were obtained from Abcam (Cambridge, UK). Rabbit anti-mouse polyclonal antibodies recognizing NF- $\kappa$ B2 p100/52, RelB, ERK1/2, phospho-ERK1/2 (p-ERK1/2), p38 MAPK, phospho-p38 MAPK (p-p38 MAPK), mouse anti- $\beta$ -actin monoclonal antibody, and horseradish peroxidase (HRP)-conjugated IgG were all purchased from Cell Signaling Technology (Danvers, MA, USA).

### 2.3. Asthma induction and experimental design

Seventy-two female BALB/c mice were divided into six groups in a

randomized method: (i) Control group, (ii) OVA-induced asthma Model group, (iii) low-dose epimedin C (20 mg/kg/d) treated group, (iv) middle-dose epimedin C (40 mg/kg/d) treated group, (v) high-dose epimedin C (80 mg/kg/d) treated group and (vi) Dexamethasone treated group ( $n = 12$  for each group). Except for the Control group, mice were multi-sensitized with 20  $\mu\text{g}$  of OVA (Grade V) mixed with 2 mg Alum adjuvant in 0.2 ml of saline solution by means of intraperitoneal injection (i.p.) on day 1, 8. Then mice were exposed to 3% OVA (Grade II) solution through an ultrasonic nebulizer for 30 min on day 15, 16 and 17. Starting on day 22, OVA challenge was performed three times per week for 4 consecutive weeks. The mice in the Control group were sensitized and subsequently challenged with saline. From day 22–49, the OVA-induced asthmatic mice in the low-dose, middle-dose, and high-dose epimedin C treated groups were administered intragastrically with epimedin C at doses of 20 mg/kg/d, 40 mg/kg/d and 80 mg/kg/d, respectively. The mice in the Dexamethasone treated group were administered intragastrically with dexamethasone at a dose of 1 mg/kg/d. The mice in the Control group and Model group were administered intragastrically with saline (Fig. 1A). Mice were sacrificed for tissue collection 24 h after the last OVA exposure to collect BALF, blood and lung tissues. Experimental endpoints were assessed, including AHR, OVA-specific IgE in serum, inflammatory cells accumulation and production of cytokines in BALF, as well as pathophysiological features of asthma.

#### 2.4. Assessment of allergen-induced AHR

Twenty-four hours after the last OVA challenge, AHR to Mch was detected by a Buxco's modular and invasive system (Buxco Electronics Inc., NY, USA) as previously described [38]. Briefly, on the 50th day, mice from different groups were anesthetized by i.p. of 50 mg/kg pentobarbital sodium. The tracheas of mice were intubated with an appropriate cannula. Then the mice laid supine inside the body plethysmograph chamber and were induced to breath PBS and increasing concentrations of Mch (3.125, 6.25, 12.5 mg/mL) through a jet nebulizer into the chamber [39]. Airway resistance ( $R_L$ ) and dynamic lung compliance ( $C_{dyn}$ ) in response to Mch were recorded and expressed as a percentage change from the baseline value.

#### 2.5. Inflammatory cell counts, classification and cytokines concentrations in BALF

BALF was collected by flushing 0.3 ml of ice-cold PBS into the lungs three times through a tracheal cannula. BALF was centrifugated at 500g for 10 min at 4 °C. The precipitated cells were suspended in 100  $\mu\text{L}$  PBS for inflammatory cell counts and classification. Differential cell counts were performed as previously described [23]. The concentrations of cytokines IL-9, IL-4 and IL-10 in BALF supernatant were analyzed by ELISA following the manufacturer's recommendations.

#### 2.6. Evaluation of airway inflammation severity and lung histopathology

The lung tissues of mice from each group were perfused and fixed with 4% paraformaldehyde for 24 h, then were embedded in paraffin for histopathological analysis. Three-micrometer sections were mounted on slides and stained with hematoxylin–eosin (H&E), periodic acid-Schiff (PAS) and Masson trichrome. Pathological assessment was conducted blind on randomized sections by light microscopy and Image J software (NIH Bethesda, MD, USA). Inflammatory changes were scored for a semi-quantitative scale of 0–4 for cellular infiltration around the airways, 0, no infiltration, 1, few inflammatory cells, 2, a ring of inflammatory cells 1 cell layer deep, 3, a ring of inflammatory cells 2 to 4 cells deep, and 4, a ring of inflammatory cells greater than 4 cells deep [40]. PAS staining evaluated the level of airway mucus secretion. The result was quantified as the airway epithelium staining positive areas for mucus/the perimeter of the basement membrane of bronchiole [41].

Airway collagen deposition was scored for a semi-quantitative scale of 0–3 for collagen in peribronchial space, 0, no collagen in peribronchial space, 1, a thin layer of peribronchial collagen deposits, 2, a cluster of peribronchial collagen deposits, and 3, a thick layer of peribronchial collagen deposits [42].

#### 2.7. Measurement of OVA-specific IgE in serum

The blood samples were collected from the retro-orbital venous plexus. Serum sample was separated by centrifugation of the blood at 1500g for 10 min at 4 °C. The serum OVA-specific IgE level was measured by ELISA according to the manufacturer's instruction.

#### 2.8. qRT-PCR based analysis of gene expression

Isolation of total RNA from the lung tissues was performed using RNAeasy mini kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Quality of total RNA was assured based on A260/A280 absorbance ratio determined using a spectrophotometer (NanoDrop Products, Wilmington, DE). The A260/A280 ratio of 1.9–2.2 was used as a purity criterion for all samples. DNA was synthesized from 500 ng of RNA by using the RevertAid First Strand cDNA synthesis transcription kit (Thermo Fisher Scientific, Carlsbad, CA, USA). 2  $\mu\text{L}$  cDNA was amplified in each PCR reaction mixture. GAPDH gene was used as the internal control and the threshold cycles ( $C_t$ ) of all genes were used for calculation of parameter gene expression by  $2^{-\Delta\Delta C_t}$  method. The primers were designed using Primer-BLAST and the sequences were: IL-9, forward 5'-cagcaccacatggggcatcag-3', reverse 5'-tgggacggagagacacaagcag-3'; Foxp3, forward 5'-aagaatgccatccgccacaacc-3', reverse 5'-tacgtgccactgtctctcttc-3'; GAPDH, forward 5'-caaggtctgtgggcaaggtcatc-3', reverse 5'-tctccaggcggcagctgac-3'.

#### 2.9. Flow cytometry analysis of Th9 cells and Treg cells in the lungs of mice

For Th9 cells and Treg cells analysis, the lung tissues of mice from each group were digested for 45 min at 37 °C in RPMI medium with 1 mg/mL collagenase IV, 200  $\mu\text{g}/\text{mL}$  DNase I (Sigma-Aldrich, St. Louis, MO, USA) and were filtered through 40  $\mu\text{m}$  cell strainers. Red cells were lysed with RBC lysis Buffer (eBioscience Inc., San Diego, CA, USA). The mononuclear cell suspension was centrifuged, first stained for fluorescent conjugated primary monoclonal antibodies (mAbs) including Live/Dead mAb, APC-Cyanine 7, CD45 mAb, eFlour 506, CD4 mAb, PE-Cyanine 7, CD25 mAb, eFluor 450 (eBioscience, Inc. San Diego, CA, USA). Intracellular staining of IL-9 was performed after restimulation of cells with leukocyte activation cocktail (BD Biosciences, Franklin Lakes, NJ, USA) for 6 h. Then cells were fixed, permeabilized with Foxp3/transcription factor staining buffer set and stained with IL-9 mAb, APC, Foxp3 mAb, PE (eBioscience, Inc. San Diego, CA, USA) according to the manufacturer's instructions. After that, labeled cells were examined using an Attune NxT (Thermo Fisher Scientific) and data were analyzed using FlowJo software from Tree Star.

#### 2.10. Western blotting analysis

The total protein was extracted from lung tissues by using RIPA protein extraction reagent, and the concentration of each protein sample was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto the polyvinylidene difluoride (PVDF) membranes. The membranes were then blocked with 5% of non-fat milk at room temperature for 1 h, and followed by incubation with different primary antibodies including p105/p50, RelA, p100/p52, RelB, ERK1/2, p-ERK1/2, p38 MAPK, p-p38 MAPK and  $\beta$ -actin at 4 °C overnight. On the next day, the HRP-conjugated secondary antibodies were utilized to

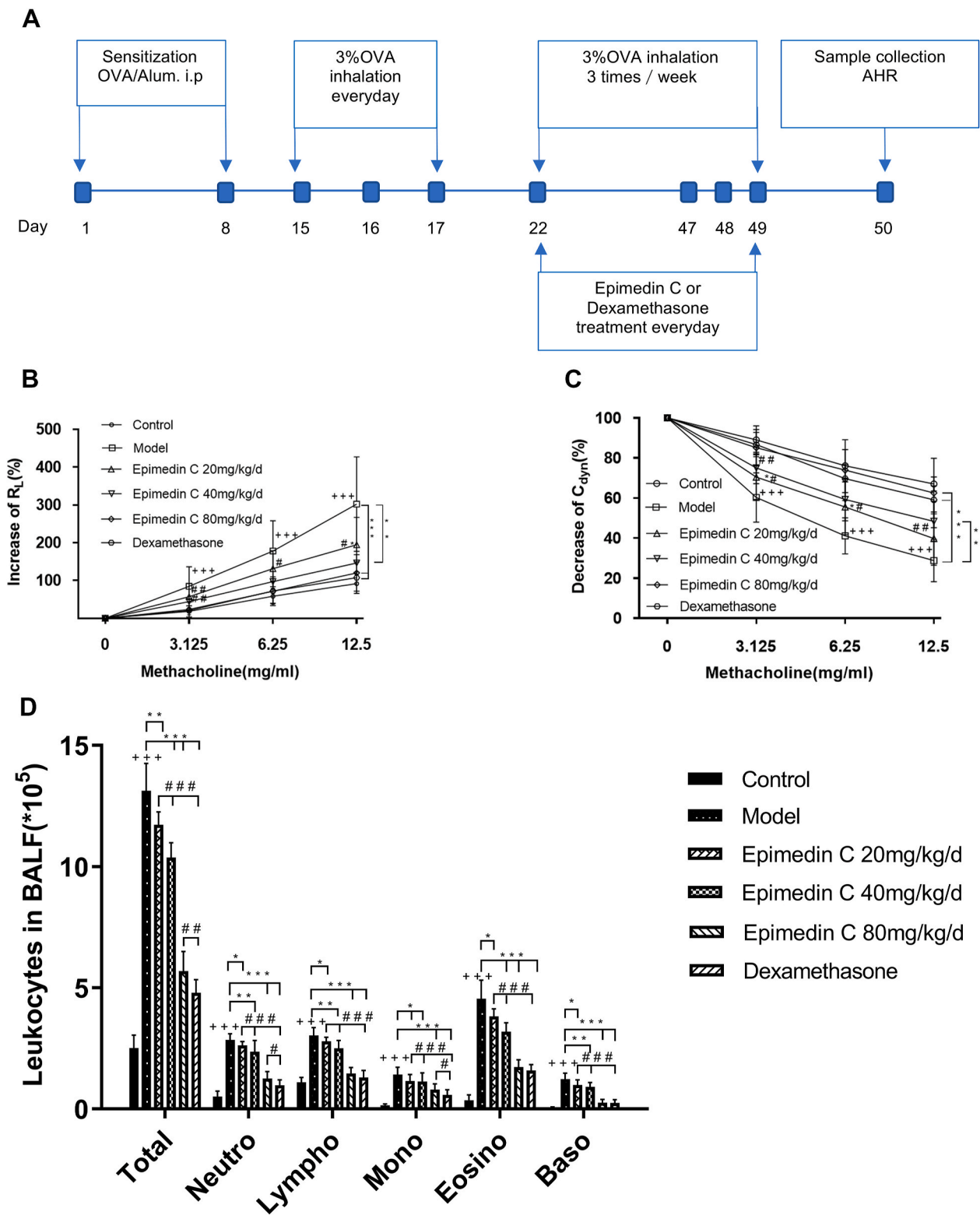


**Fig. 1.** The effects of epimedin C on AHR and BALF inflammatory cell counts in OVA-induced asthmatic mice. (A) Mice were sensitized and challenged with OVA in a 50-day asthma induction protocol; (B, C) following the last OVA challenge, mice were anesthetized and induced to breath increasing concentrations of Mch, changes in AHR including airway resistance ( $R_L$ ) and dynamic lung compliance ( $C_{dyn}$ ) were detected. Epimedin C treatment improved the AHR in asthmatic mice; (D) the BALF was collected and inflammatory cells in BALF were classified and counted; Epimedin C treatment decreased total and differential leukocyte counts in asthmatic mice. The values indicate the mean  $\pm$  SEM.  $n = 8$  to 12 mice/group. Significance is represented by  $+++ P < 0.001$  compared to Control group,  $*** P < 0.001$ ,  $** P < 0.01$  and  $* P < 0.05$  compared to Model group,  $### P < 0.001$ ,  $## P < 0.01$  and  $\# P < 0.05$  compared to Dexamethasone treated group.

incubate membranes for 1 h. The expressions of target molecules were detected by chemiluminescence method. To explain possible difference in the protein load, the intensity of each band was normalized to  $\beta$ -actin.

### 2.11. Statistical analysis

The experimental data were analyzed by using SPSS software package (version 25.0, SPSS Inc., Chicago, IL, USA). Data are expressed as the mean  $\pm$  standard error of the mean (SEM) and representative of three independent experiments. Non-parametric two-sample *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test or post hoc Dunnett's test was used for comparisons between groups. Statistically differences were considered to be significant when *P*-values < 0.05 (two-tailed).

## 3. Results

### 3.1. Epimedin C effectively suppressed AHR in OVA-induced murine asthma model

In OVA-induced asthmatic mice following 28-day intragastric administration of epimedin C at different concentrations, the effective modulation of AHR including  $R_L$  and  $C_{dyn}$  was established. Mice in the Model group had a significant enhancement of AHR, with a remarkable increase in  $R_L$  and decrease in  $C_{dyn}$ , as compared with the Control group ( $P < 0.001$ ). Administration of a middle-dose and a high-dose of epimedin C (40 mg/kg/d and 80 mg/kg/d) and 1 mg/kg/d dexamethasone resulted in a marked decrease in  $R_L$ , with a concomitant increase in  $C_{dyn}$  compared with the Model group ( $P < 0.01$  or  $P < 0.001$ ). Treatment of a low-dose of epimedin C (20 mg/kg/d) also caused notable regulatory effects on  $R_L$  to Mch at 12.5 mg/ml, and on  $C_{dyn}$  to Mch at 3.125 mg/ml and 6.25 mg/ml compared with the Model group ( $P < 0.05$ ). Except for treatment of a low-dose of epimedin C (20 mg/kg/d) on  $R_L$  and on  $C_{dyn}$  to Mch at 3.125 mg/ml, 6.25 mg/ml, 12.5 mg/ml, and a middle-dose of epimedin C (40 mg/kg/d) on  $R_L$  and  $C_{dyn}$  to Mch at 3.125 mg/ml, there was no significant difference among epimedin C and dexamethasone treatment in regulating AHR ( $P > 0.05$ ). The results implied that the suppressive effect of epimedin C on AHR was similar to that of dexamethasone and the high-dose of epimedin C (80 mg/kg/d) was shown to be more effective than the middle-dose and low-dose of epimedin C (Fig. 1B–C).

### 3.2. Epimedin C inhibited airway inflammatory cells accumulation in OVA-induced murine asthma model

The accumulation of inflammatory cells including total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils and basophils were significantly elevated in BALF of OVA-induced murine asthma model, as compared with the Control group ( $P < 0.001$ ). Treatment of epimedin C and dexamethasone strikingly reduced the counts of inflammatory cells in BALF when compared to the Model group ( $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$ ). Except for the efficacy of epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) in reducing total and differential leukocytes, treatment of a high-dose of epimedin C (80 mg/kg/d) caused an obvious decrease in counts of lymphocyte, eosinophil and basophil ( $P < 0.001$ ), which was similar to that of dexamethasone (1 mg/kg/d) ( $P > 0.05$ ). It seemed that the efficacy of a high-dose of epimedin C on decrease of inflammatory cell counts was better in comparison with other doses of epimedin C. Dexamethasone treatment had a slightly stronger effect on decreasing total leukocyte, neutrophil and monocyte counts in BALF of the asthmatic mice than that of high-dose of epimedin C treatment ( $P < 0.05$  or  $P < 0.01$ ) (Fig. 1D).

### 3.3. Epimedin C attenuated airway inflammation in OVA-induced murine asthma model

The H&E staining revealed the dense infiltration of inflammatory cells into peribronchiolar and perivascular connective tissues of the mice in the Model group, as compared with the Control group ( $P < 0.001$ ). Treatment with a high-dose of epimedin C (80 mg/kg/d) and dexamethasone (1 mg/kg/d) significantly inhibited the increased infiltration of inflammatory cells around the airways ( $P < 0.01$  or  $P < 0.001$ ). The inhibitory effects of the low-dose and middle-dose of epimedin C (20 mg/kg/d, 40 mg/kg/d) on airway inflammation were weaker than that of dexamethasone ( $P < 0.05$ ). There was no significant difference between the high-dose of epimedin C (80 mg/kg/d) and dexamethasone treatment ( $P > 0.05$ ) (Fig. 2A–B).

### 3.4. Epimedin C attenuated airway mucus secretion and collagen deposition in the lungs of OVA-induced murine asthma model

The PAS staining revealed that the level of airway mucus secretion of mice in the Model group was significantly higher than that in the Control group ( $P < 0.001$ ). In the epimedin C treated groups, the severity of mucus secretion decreased as the concentration of epimedin C increased, indicating that epimedin C dose-dependently ameliorated mucus hypersecretion. The suppressive effects of the high-dose and the middle-dose of epimedin C (80 mg/kg/d, 40 mg/kg/d) on mucus secretion were more obvious ( $P < 0.001$  or  $P < 0.01$ ) and similar to that of dexamethasone ( $P > 0.05$ ) (Fig. 3A–B). The collagen deposition in the lungs of mice in the Model group demonstrated significant increase by the use of Masson trichrome staining ( $P < 0.001$ ). Treatment with epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) and dexamethasone reduced collagen deposition around the bronchioles and blood vessels of OVA-induced asthmatic mice, as compared with untreated asthmatic mice ( $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$ ). The effect of the high-dose of epimedin C (80 mg/kg/d) on reducing collagen deposition was better than that of dexamethasone ( $P < 0.05$ ) (Fig. 4A–B).

### 3.5. Epimedin C reduced concentrations of OVA-specific IgE, cytokines IL-9, IL-4 and increased concentration of IL-10 in serum or BALF

Only a tiny amount of OVA-specific IgE was detected in mice in the Control group. Immunizing and challenging with OVA markedly increased serum OVA-specific IgE level in mice ( $P < 0.001$ ). A significant reduction of serum OVA-specific IgE level was detected in epimedin C treated groups and dexamethasone treated group, as compared to Model group ( $P < 0.001$ ). The concentrations of proinflammatory cytokines IL-9 and IL-4 in BALF were significantly elevated in the Model group ( $P < 0.001$ ), while the IL-10 concentration in BALF was markedly decreased ( $P < 0.01$ ), as compared to the Control group. Treatment with three different doses of epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) and dexamethasone (1 mg/kg/d) significantly reduced the levels of IL-9, IL-4 and increased IL-10 level in asthmatic mice, as compared to untreated asthmatic mice ( $P < 0.01$  or  $P < 0.001$ ). The inhibitory effects of the low-dose and middle-dose of epimedin C (20 mg/kg/d, 40 mg/kg/d) on OVA-specific IgE and proinflammatory cytokines were weaker than that of dexamethasone ( $P < 0.001$  or  $P < 0.05$ ). There was no significant difference in levels of IL-9, IL-4 and OVA-specific IgE between the high-dose of epimedin C (80 mg/kg/d) and dexamethasone treatment ( $P > 0.05$ ). Importantly, the effect of a high-dose of epimedin C (80 mg/kg/d) on up-regulation of IL-10 concentration was stronger than that of dexamethasone ( $P < 0.001$ ) (Fig. 5A–D).

### 3.6. Epimedin C regulated gene expression of IL-9 and Foxp3 in the lungs of OVA-induced murine asthma model

The pulmonary expression of a key transcription factor gene of Treg cells (*Foxp3*) and a specific Th9 cells inflammatory gene (*IL9*) was

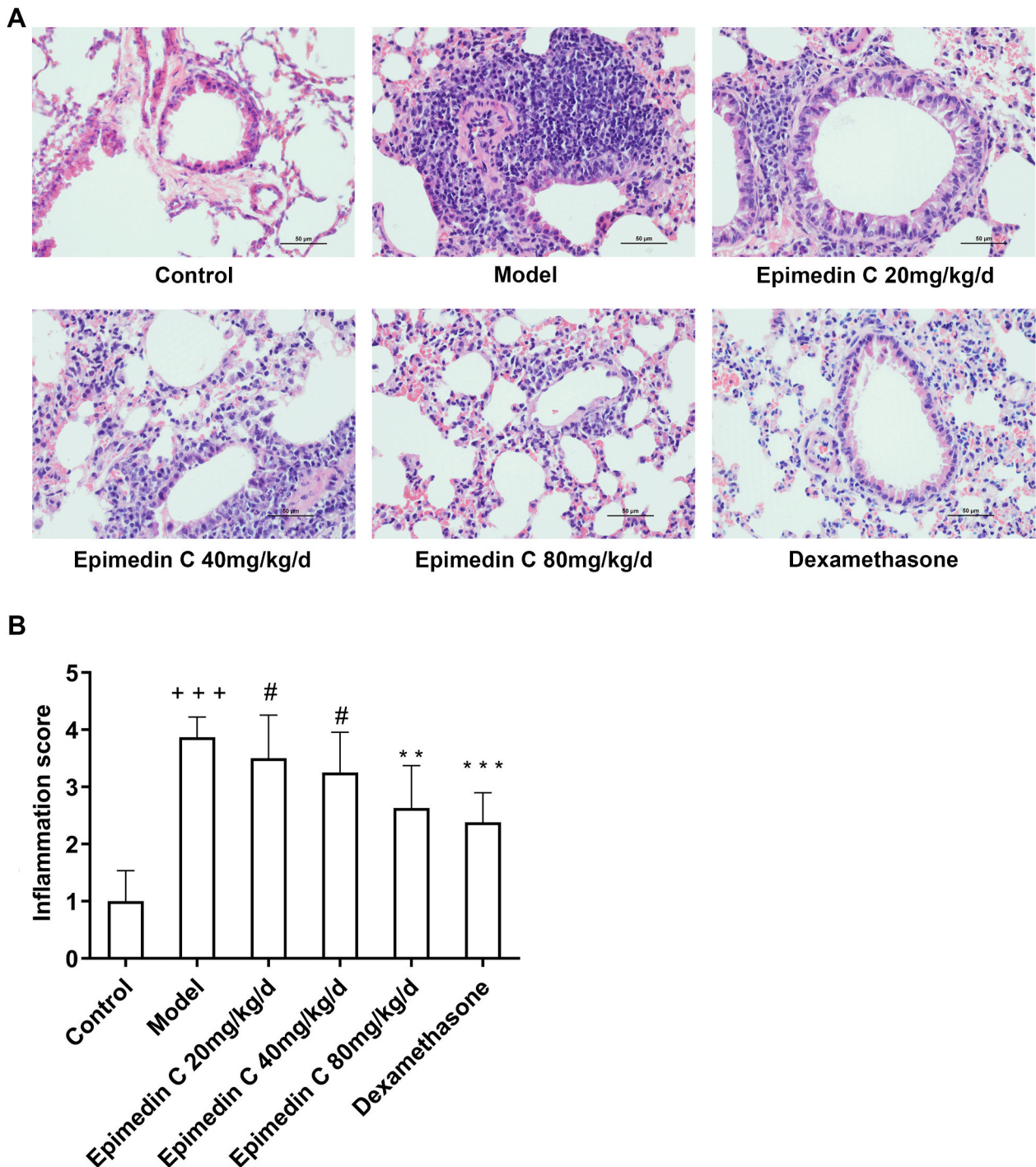


**Fig. 2.** The effect of epimedin C on airway inflammation in OVA-induced asthmatic mice. (A) lung sections were stained with H&E to assess the airway inflammation of mice from each group; scale bar 50  $\mu$ m; (B) inflammation scores were assessed and analyzed; The high-dose of epimedin C (80 mg/kg/d) significantly inhibited the infiltration of inflammatory cells into lung tissues in asthmatic mice. The values indicate the mean  $\pm$  SEM.  $n = 8$  mice/group. Significance is represented by +++  $P < 0.001$  compared to Control group, \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  and \*  $P < 0.05$  compared to Model group, ###  $P < 0.001$ , ##  $P < 0.01$  and #  $P < 0.05$  compared to Dexamethasone treated group.

evaluated by qRT-PCR. OVA challenge led to a significant increase in IL-9 mRNA expression in lungs of mice in the Model group, as compared to the Control group ( $P < 0.001$ ). The Foxp3 mRNA expression was slightly decreased in the Model group compared to Control group ( $P > 0.05$ ). The middle-dose and high-dose of epimedin C (40 mg/kg/d, 80 mg/kg/d) treatment dose-dependently up-regulated Foxp3 mRNA expression as well as down-regulated IL-9 mRNA expression in lungs of asthmatic mice ( $P < 0.01$  or  $P < 0.001$ ). The low-dose of epimedin C (20 mg/kg/d)

also down-regulated IL-9 mRNA expression in lungs of asthmatic mice ( $P < 0.05$ ). The IL-9 mRNA expression was also affected by dexamethasone treatment ( $P < 0.001$ ). There was no significant difference in IL-9 mRNA expression among the three different concentrations of epimedin C and dexamethasone treatment ( $P > 0.05$ ). The effect of a high-dose of epimedin C (80 mg/kg/d) on up-regulation of Foxp3 mRNA expression was stronger than that of dexamethasone ( $P < 0.001$ ) (Fig. 6A–B).

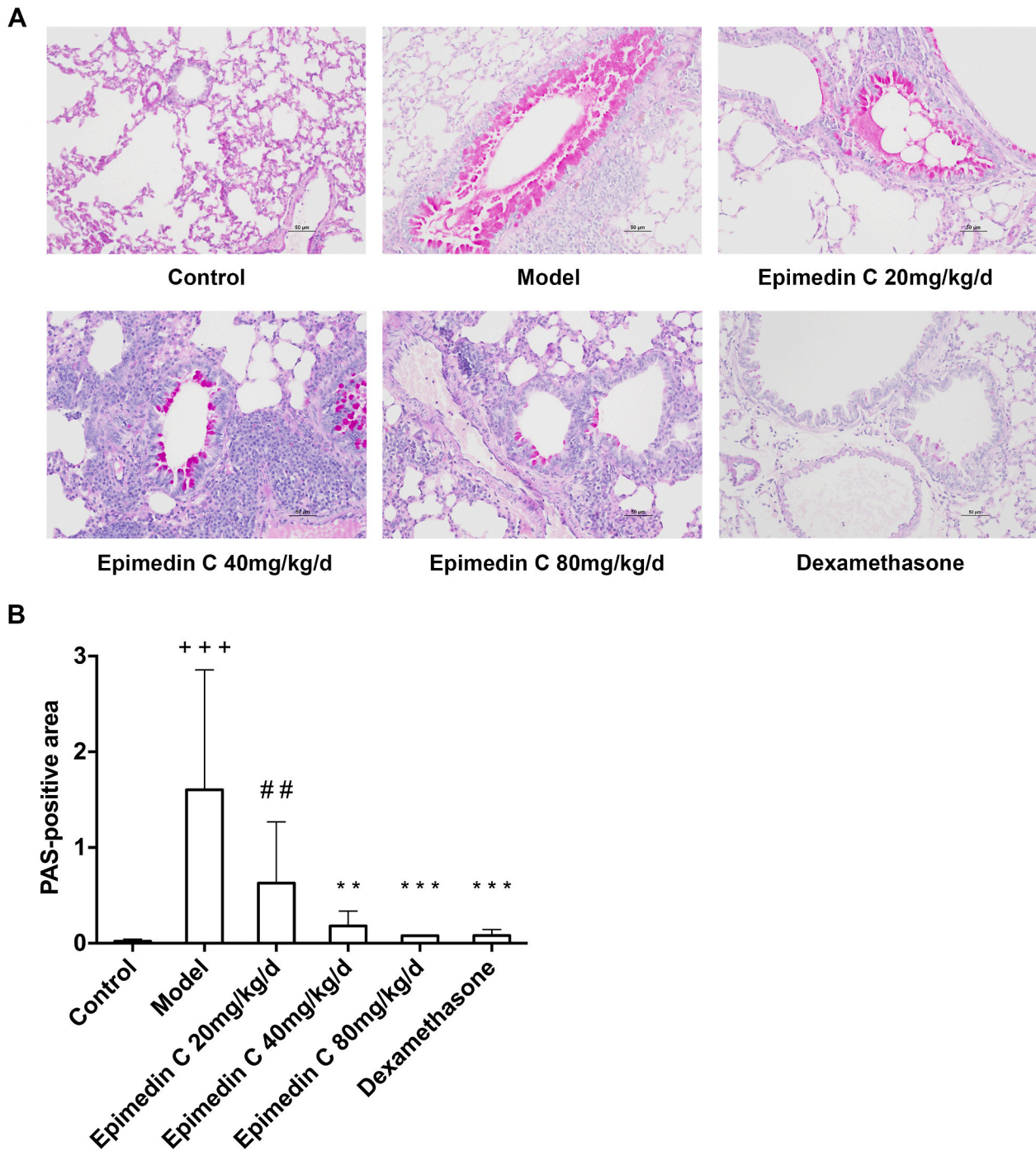


**Fig. 3.** The effect of epimedin C on airway mucus hypersecretion in OVA-induced asthmatic mice. (A) PAS-stained sections of the lungs were used to evaluate airway mucus production; scale bar 50 µm; (B) PAS-positive area were assessed and analyzed; The high-dose (80 mg/kg/d) and middle-dose (40 mg/kg/d) of epimedin C treatment significantly ameliorated mucus hypersecretion in the lungs of asthmatic mice. The values indicate the mean ± SEM. n = 8 mice/group. Significance is represented by +++ P < 0.001 compared to Control group, \*\*\* P < 0.001, \*\* P < 0.01 and \* P < 0.05 compared to Model group, ### P < 0.001, ## P < 0.01 and # P < 0.05 compared to Dexamethasone treated group.

### 3.7. Epimedin C regulated the balance between Th9 cells and Treg cells in OVA-induced murine asthma model

To recognize whether epimedin C regulated the balance between Th9 cells and Treg cells, the flow cytometry detection of lung CD4<sup>+</sup> IL9<sup>+</sup> (Th9) cells and CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> (Treg) cells of mice were counted. Th9 cells and Treg cells were analyzed in the CD45<sup>+</sup> gated cells which represent all leukocytes in single cell suspensions from the lung tissues. CD45 is a receptor-linked protein tyrosine phosphatase that is amongst

the most abundant proteins within the T cell plasma membrane and is required for T cell development, differentiation and activation via the T cell receptor (TCR) [43,44]. The percentage of Treg cells in lungs of mice in the Model group was slightly decreased, as compared to the Control group (P > 0.05). In contrast, the percentage of Th9 cells in lungs of mice in the Model group was greatly increased when compared to the Control group (P < 0.001). Treatment with different concentrations of epimedin C led to a significant reduction in the percentage of Th9 cells and an increase in the percentage of Treg cells compared to the Model group (P

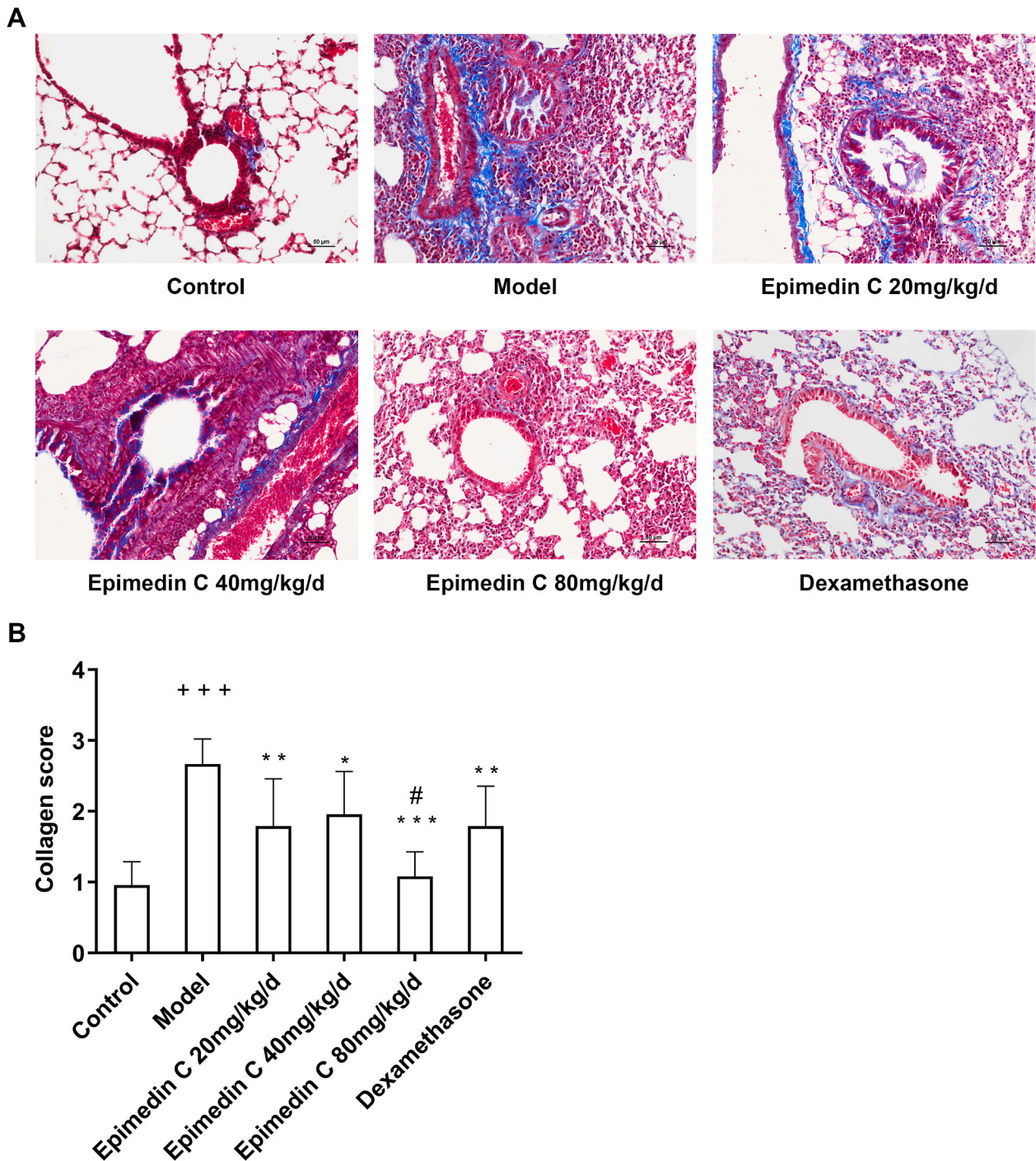


**Fig. 4.** The effect of epimedin C on airway collagen deposition in OVA-induced asthmatic mice. (A) Masson trichrome-stained sections of the lungs were used to evaluate airway collagen deposition; scale bar 50  $\mu$ m; (B) semi-quantitative scoring was analyzed; Epimedin C treatment significantly reduced collagen deposition around both bronchioles and blood vessels in asthmatic mice. The values indicate the mean  $\pm$  SEM.  $n = 8$  mice/group. Significance is represented by +++  $P < 0.001$  compared to Control group, \*\*  $P < 0.01$ , \*  $P < 0.05$  compared to Model group, ###  $P < 0.001$ , ##  $P < 0.01$  and #  $P < 0.05$  compared to Dexamethasone treated group.

$< 0.01$  or  $P < 0.001$ ). It is noteworthy that three doses of epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) displayed more obvious potential in increasing percentage of Treg cells than that of dexamethasone in asthmatic mice ( $P < 0.01$  or  $P < 0.001$ ). There was no obvious statistical difference among middle-dose and high-dose of epimedin C (40 mg/kg/d, 80 mg/kg/d) and dexamethasone (1 mg/kg/d) treatment in decreasing Th9 cells ( $P > 0.05$ ). These data suggested that epimedin C restored the balance between Th9 cells and Treg cells in OVA-induced murine asthma model (Fig. 7A–E).

### 3.8. Epimedin C inhibited activation of the noncanonical NF- $\kappa$ B pathway

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) family of transcription factors, including NF- $\kappa$ B1 (p105), RelA, NF- $\kappa$ B2 (p100), RelB, and c-Rel have key roles in the development and differentiation of different helper T cell lineages [28]. Recent studies suggested that the noncanonical NF- $\kappa$ B pathway mediated activation of p52/RelB complex to regulate the development and differentiation of Th9 cells and Treg cells [12,16,26,45]. To address the question of whether the two NF- $\kappa$ B signaling

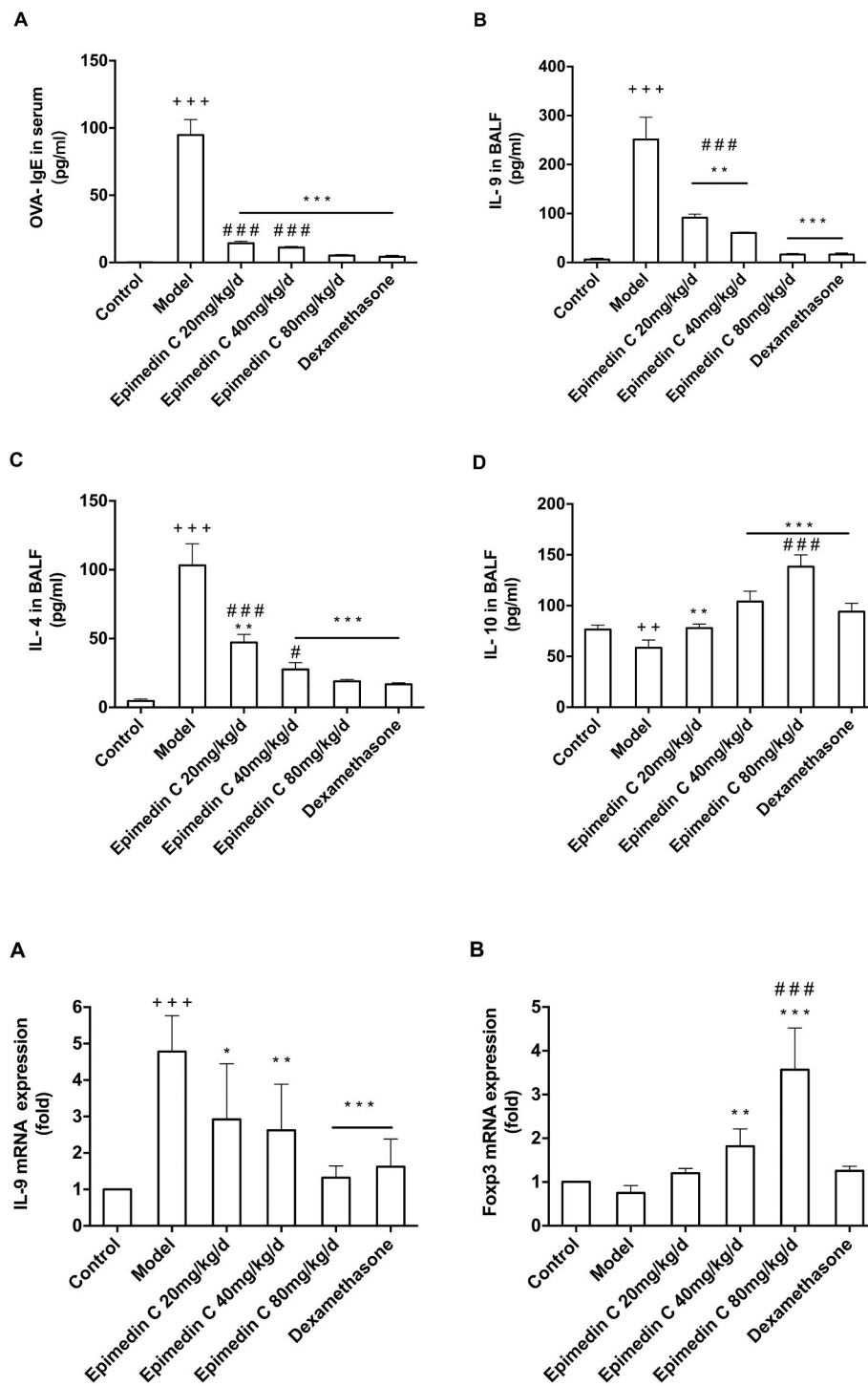


**Fig. 5.** The effects of epimedin C on the OVA-specific IgE level and concentrations of IL-9, IL-4 and IL-10 in serum or BALF of OVA-induced asthmatic mice. The levels of (A) OVA-specific IgE, (B) IL-9, (C) IL-4 and (D) IL-10 were analyzed by ELISA. Epimedin C treatment dose-dependently reduced concentrations of OVA-specific IgE, IL-9, IL-4 and increased concentration of IL-10 in serum or BALF of asthmatic mice. The values indicate the mean  $\pm$  SEM.  $n = 6$  mice/group. Significance is represented by  $+++ P < 0.001$ ,  $++ P < 0.01$  compared to Control group,  $*** P < 0.001$ ,  $** P < 0.01$  and  $* P < 0.05$  compared to Model group,  $### P < 0.001$ ,  $## P < 0.01$  and  $# P < 0.05$  compared to Dexamethasone treated group.

**Fig. 6.** The effects of epimedin C on gene expression of IL-9 and Foxp3 in lungs of OVA-induced asthmatic mice. Total RNA isolated from lung tissues of mice were analyzed by qRT-PCR. (A) The IL-9 mRNA expression and (B) the Foxp3 mRNA expression were analyzed. Epimedin C treatment dose-dependently down-regulated IL-9 mRNA expression and up-regulated Foxp3 mRNA expression in lungs of asthmatic mice. The values indicate the mean  $\pm$  SEM.  $n = 6$  mice/group. Significance is represented by  $+++ P < 0.001$  compared to Control group,  $*** P < 0.001$ ,  $** P < 0.01$  and  $* P < 0.05$  compared to Model group,  $### P < 0.001$ ,  $## P < 0.01$  and  $# P < 0.05$  compared to Dexamethasone treated group.

pathways were involved in epimedin C-mediated the balance between Th9 cells and Treg cells, the expression of p105, p50, RelA, p100, p52, RelB in mice lungs was detected by Western blotting. The p52, RelB protein expression levels were significantly increased in the Model group compared to the Control group ( $P < 0.001$  or  $P < 0.01$ ). Treatment with different concentrations of epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) and dexamethasone suppressed expression of p52 and RelB in lungs of asthmatic mice, as compared to untreated asthmatic mice ( $P < 0.05$ ,  $P < 0.01$  or  $P < 0.001$ ). Furthermore, the activation of non-canonical NF- $\kappa$ B pathway (p52 and RelB) seemed to be more strongly inhibited by a high-dose of epimedin C (80 mg/kg/d) than low-dose and middle-dose of epimedin C (20 mg/kg/d, 40 mg/kg/d) (Fig. 8A and C).

However, there seemed to be no obvious effect of epimedin C on the regulation of canonical NF- $\kappa$ B pathway (p50, RelA) ( $P > 0.05$ ) (Fig. 8B and D). Therefore, it was suggested that epimedin C regulated the balance between Th9 cells and Treg cells through inhibiting activation of noncanonical NF- $\kappa$ B pathway.

### 3.9. Epimedin C inhibited activation of the ERK1/2 and p38MAPK

In addition to evaluating NF- $\kappa$ B, we also evaluated the components of MAPKs activation that have been associated with allergic airway inflammation and involved in development and differentiation of Th cells [46,47]. The pulmonary protein expression of phosphorylation of

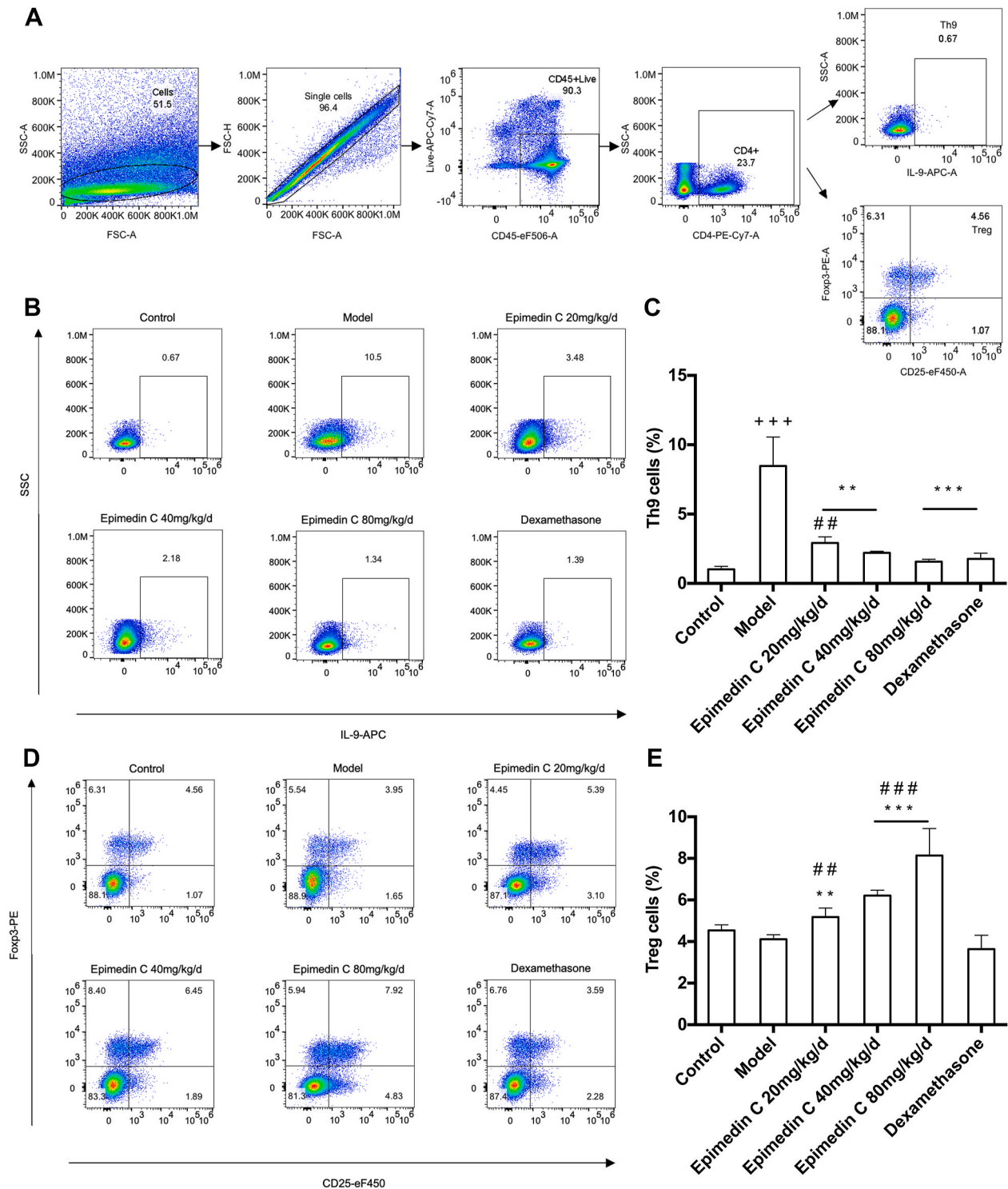


**Fig. 7.** The effects of epimedin C on the percentage of Th9 cells and the percentage of Treg cells in OVA-induced asthmatic mice. Th9 cells and Treg cells from lungs of mice from each group were analyzed by flow cytometry. (A) Gating strategy for lung Th9 cells and Treg cells (B) Color FACS plots depicting Th9 cells and (D) Treg cells. (C) the percentage of Th9 cells and (E) the percentage of Treg cells were analyzed; Epimedin C treatment significantly decreased the percentage of Th9 cells and increased the percentage of Treg cells in lungs of asthmatic mice. The values indicate the mean  $\pm$  SEM.  $n = 8$  mice/group. Significance is represented by +++  $P < 0.001$  compared to Control group, \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  and \*  $P < 0.05$  compared to Model group, ###  $P < 0.001$ , ##  $P < 0.01$  and #  $P < 0.05$  compared to Dexamethasone treated group.

ERK1/2 (p-ERK1/2) and phosphorylation of p38 MAPK (p-p38 MAPK) was greatly increased in the Model group when compared to the Control group ( $P < 0.001$ ). Treatment with three different doses of epimedin C and dexamethasone significantly decreased the protein levels of p-ERK1/2 and p-p38 MAPK in asthmatic mice, as compared to untreated

asthmatic mice ( $P < 0.01$  or  $P < 0.001$ ). The high-dose of epimedin C (80 mg/kg/d) displayed more obvious potential in decreasing the protein level of p-ERK1/2 than that of dexamethasone in asthmatic mice ( $P < 0.01$ ) (Fig. 9A and C). However, the inhibitory effects of three doses of epimedin C (20 mg/kg/d, 40 mg/kg/d, 80 mg/kg/d) on p-p38 MAPK

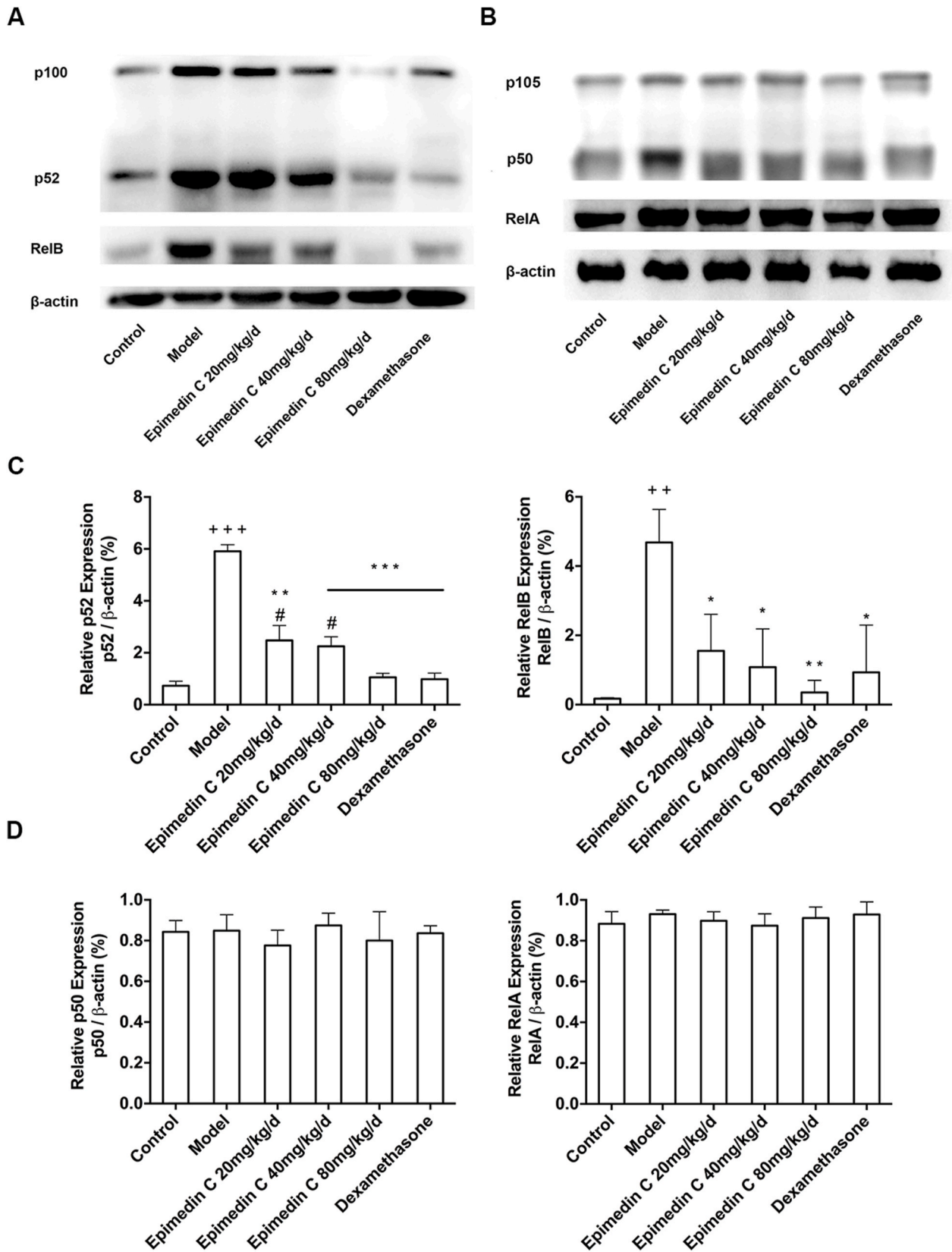


**Fig. 8.** The effects of epimedin C on the proteins expression of p105, p50, RelA, p100, p52, RelB in lungs of asthmatic mice. (A, C) The total levels of p100, p52 and RelB in mice from each group; Epimedin C dose-dependently inhibited the expression levels of p52 and RelB beyond the basal level; (B, D) the total levels of p105, p50, RelA in mice from each group; There was no considerable effect of epimedin C on expression levels of p50, RelA. The values indicate the mean  $\pm$  SEM.  $n = 3$  mice/group. Significance is represented by <sup>+++</sup>  $P < 0.001$ , <sup>++</sup>  $P < 0.01$  compared to Control group, <sup>\*\*\*</sup>  $P < 0.001$ , <sup>\*\*</sup>  $P < 0.01$  and <sup>\*</sup>  $P < 0.05$  compared to Model group, <sup>###</sup>  $P < 0.001$ , <sup>##</sup>  $P < 0.01$  and <sup>#</sup>  $P < 0.05$  compared to Dexamethasone treated group.

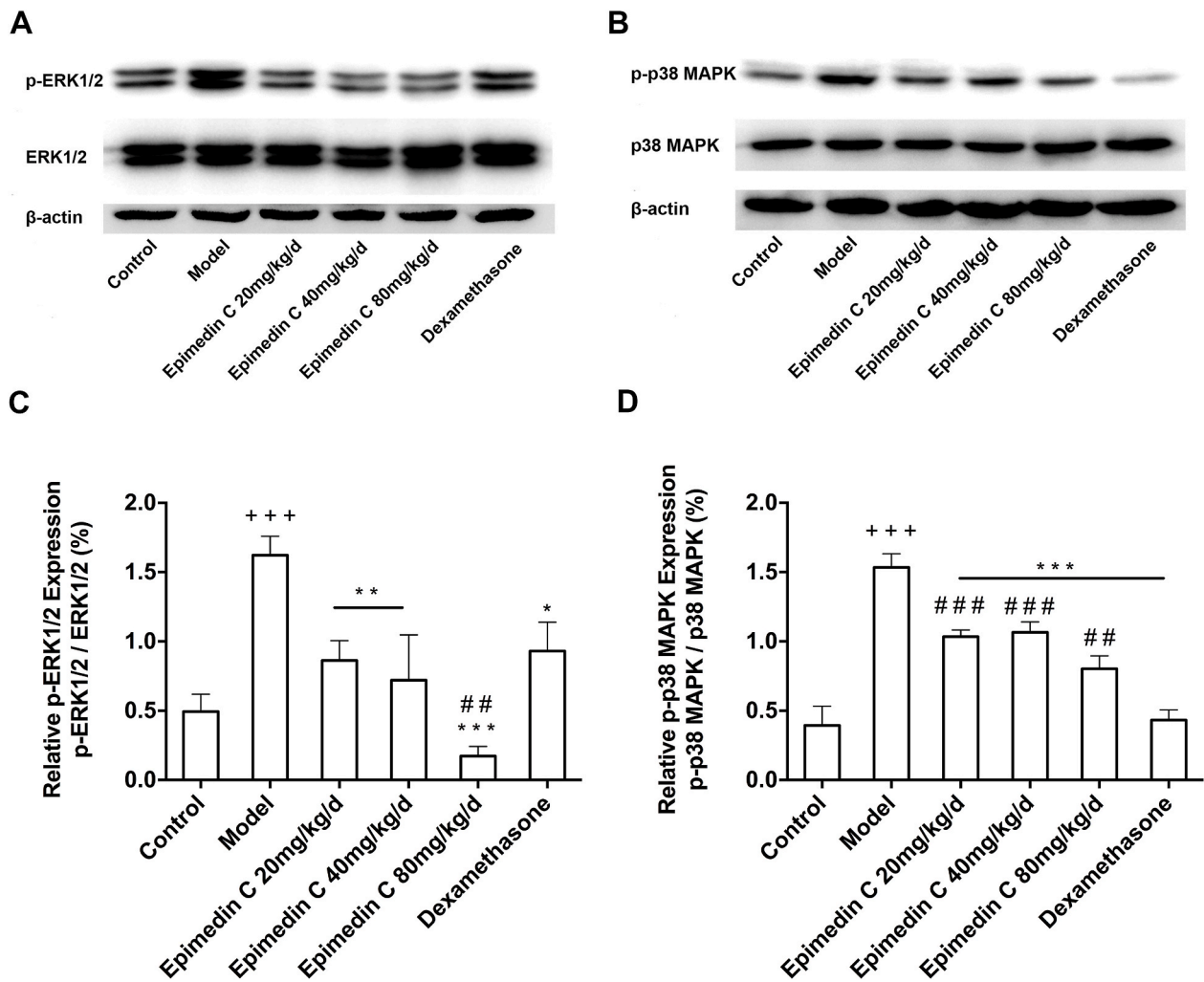


**Fig. 9.** The effects of epimedin C on the protein expression of p-ERK1/2, ERK1/2, p-p38 MAPK, p38 MAPK in lungs of asthmatic mice. (A, C) The protein levels of p-ERK1/2 and ERK1/2 in mice from each group; Epimedin C dose-dependently inhibited the expression level of p-ERK1/2 beyond the ERK1/2 level; (B, D) the protein levels of p-p38 MAPK and p38 MAPK in mice from each group; Epimedin C inhibited the expression level of p-p38 MAPK beyond the p38 MAPK level. The values indicate the mean  $\pm$  SEM.  $n = 3$  mice/group. Significance is represented by +++  $P < 0.001$  compared to Control group, \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$  and \*  $P < 0.05$  compared to Model group, ###  $P < 0.001$ , ##  $P < 0.01$  and #  $P < 0.05$  compared to Dexamethasone treated group.

expression were weaker than that of dexamethasone ( $P < 0.01$  or  $P < 0.001$ ) (Fig. 9B and D). These data suggested that the activation of ERK and p38 MAPK in OVA-induced asthmatic mice was significantly inhibited by the treatment with epimedin C.

#### 4. Discussion

The original herb *Epimedium* and *Epimedium*-containing formula have been suggested as potential therapeutic agents for asthma [48–50]. As an active ingredient of herb *Epimedium*, epimedin C has been extensively studied for its anti-inflammatory properties [51–53]. However, the potential role of epimedin C in ameliorating pathophysiology of asthma has not been reported until now. AHR and airway inflammation are the most critical pathophysiological features of asthma. AHR is associated with increased decline in lung function, increased risk for the development of asthma [54] and increased likelihood of the persistence of wheeze from childhood to adulthood [55]. Meanwhile, treatment strategies targeting inhibition of AHR in conjunction with improvement of symptoms and lung function lead to reduction in the risk of mild exacerbations while alleviating chronic airways inflammation [56,57]. The inflammatory cells or cytokines likely contribute to AHR. AHR and airway inflammation may be in a harmful circle of positive feedback. In

asthmatic subjects, there is a positive correlation between the severity of AHR and the number of eosinophils and metachromatic cells in sputum [58,59], as well as the number of mast cells in the airways [60]. In the present study, we found that epimedin C dose-dependently suppressed AHR in OVA-induced asthmatic mice, and showed similar efficacy with dexamethasone treatment. Epimedin C also showed strong inhibition of airway inflammation including the decrease in accumulation of the total leukocytes, lymphocytes, eosinophils and basophils. Furthermore, the airway mucus hypersecretion and collagen deposition in lungs of asthmatic mice were reduced by epimedin C treatment. Based on our results, we think that epimedin C may be the main active ingredient representing the original herb *Epimedium* and *Epimedium*-containing formula in asthma treatment [22,23]. Considering that epimedin C has a wide range of improvements in inflammation, we also deduce that the inhibitory effect of epimedin C on airway inflammation may contribute to improvement of AHR.

Chronic airway inflammation in allergic asthma was mainly driven by various immune cells-mediated immune processes [61].  $CD4^+$  helper T cell subsets including Th2 cells, Th9 cells, Th17 cells have been demonstrated to augment inflammatory responses of asthma by secretion of different types of cytokines such as IL-4, IL-9 and IL-17 [7]. In contrast, Treg cells protect against allergic inflammation by suppressing

T effector cells and related cytokines such as IL-4, as well as by secretion of the anti-inflammatory and immunosuppressive cytokine IL-10 [15, 62]. The transcription factor Foxp3 is essential for differentiation and suppressive function of Treg cells in asthma [63]. More recently, studies using various murine asthma models and peripheral blood mononuclear cells from asthmatic patients suggested that Th9 cells played a critical role in the pathogenesis of allergic airway inflammation by secretion of cytokine IL-9 [11–13,64]. Accordingly, the balance between Th9 cells and Treg cells may be a critical determinant for controlling the allergic inflammatory responses in asthma and maintaining airway homeostasis. Here, OVA exposure triggered Th9 cell-mediated immune responses and concomitant up-regulated gene expression and secretion of proinflammatory cytokine IL-9 in lungs or BALF of mice. On the contrary, lung Treg cell-mediated suppression of inflammatory responses was impaired by decrease of percentage of Treg cells, as well as down-regulation of both Foxp3 gene expression and IL-10 secretion. Epimedin C treatment significantly increased the percentage of Treg cells and decreased the percentage of Th9 cells in lungs of asthmatic mice. Meanwhile, epimedin C decreased the IL-9 mRNA expression and IL-9 secretion, as well as increased transcription factor Foxp3 mRNA expression and IL-10 secretion. These results support the hypothesis that epimedin C suppresses allergic airway inflammation through regulation of the balance between Th9 cells and Treg cells.

The balance between Th9 cells and Tregs cells can be regulated by the development and differentiation of these cells [26]. The NF- $\kappa$ B family of transcription factors consists of five subunits: RelA, NF- $\kappa$ B 1 (p105), RelB, NF- $\kappa$ B 2 (p100) and c-Rel, which play central roles in the development and functional divergence of Th9 cells and Treg cells [16, 28]. Generally, the activation of NF- $\kappa$ B occurs through both canonical pathway and noncanonical pathway [65]. Upon activation of canonical NF- $\kappa$ B pathway, the NF- $\kappa$ B dimer which consists of p50, RelA, or c-Rel is released from inhibition of I $\kappa$ B and translocate into the nucleus to activate target genes. Once noncanonical NF- $\kappa$ B pathway is activated, IKK $\alpha$  phosphorylates p100 resulting in its ubiquitination and partial process into p52 which forms a heterodimer with RelB to translocate into the nucleus to bind to specific sites for expression of target genes. In activated CD4<sup>+</sup> T cells, p50 can bind and 'close' the Foxp3 locus through mediating site-specific histone deacetylation, and consequently, inhibit induction of Treg cells. Furthermore, p50 can also activate STAT6 which allow IL9 locus to be accessible through mediating site-specific histone hyperacetylation, and consequently, promote induction of Th9 cells [43]. On the other hand, p52 and RelB complexes could directly bind to IL-9 promoter region to induce transcription of IL-9. The noncanonical NF- $\kappa$ B p52-RelB pathway most likely coordinate with other factors downstream of TGF- $\beta$  and IL-4 signaling to induce differentiation of Th9 cells [12]. Furthermore, the noncanonical NF- $\kappa$ B pathway restricts differentiation of Treg cells. A current study has shown that Foxp3<sup>+</sup> Treg cells from RelB-deficient mice were functional and capable of suppressing T effector cells. Activation of RelB also correlated with the dysfunction of Treg cells in vivo [66]. Previous studies found that epimedin C could suppress the NF- $\kappa$ B pathway and inhibit secretion of proinflammatory cytokines TNF- $\alpha$  which was activated through NF- $\kappa$ B pathway [51,53,67]. Importantly, noncanonical NF- $\kappa$ B pathway can induce MAPK activation [33]. Activation of MAPK family members and noncanonical NF- $\kappa$ B pathway can be mediated by NF- $\kappa$ B-inducing kinase (NIK) [47]. A previous study demonstrated that NLRP12-mediated inhibition of noncanonical NF- $\kappa$ B pathway was accompanied by down-regulation of ERK activation with inhibitory effect on inflammation in colitis [69]. MAPK family members ERK and p38 MAPK are also involved in regulating Th cells development and function [47]. ERK activation can induce phosphorylation of STAT6 as well as enhance IL-4 expression and IL-4R signaling pathway, which are required for Th9 cell differentiation [70,71]. Blocked activation of ERK could upregulate development of Treg cells [72]. Moreover, p38 MAPK activation can also promote IL-4 expression and suppress Treg cells differentiation in OVA-induced asthmatic mice [30]. Thus, we deduced that inhibition of

NF- $\kappa$ B pathway as well as ERK and p38 MAPK activation may, at least partially, interpret the reconstruction of balance between Th9 and Treg cells by epimedin C treatment. In this study, epimedin C at concentrations of 20 mg/kg/d, 40 mg/kg/d and 80 mg/kg/d significantly inhibited expression of p100/p52 and RelB, as well as phosphorylation of ERK1/2 and p38 MAPK. However, we observed subtle differences of p105/p50 and RelA in the untreated asthmatic mice and epimedin C-treated asthmatic mice, which did not achieve statistical significance. Thus, this result give rise to speculation of that epimedin C regulates the balance between Th9 cells and Treg cells by inhibiting noncanonical NF- $\kappa$ B pathway and activation of ERK and p38 MAPK.

Overall, the airway inflammation, collagen deposition and AHR in OVA-induced murine asthma model were significantly inhibited by oral administration of epimedin C. Epimedin C exerted its effect by modulating balance between Th9 cells and Treg cells through negative regulation of noncanonical NF- $\kappa$ B pathway and ERK, p38 MAPK activation. These findings explore the mechanism involving epimedin C treatment of asthma and provide a novel potential strategy for treating inflammatory disorders with traditional Chinese medicine.

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## CRediT authorship contribution statement

**Muhua Huang:** contributed to designing the work, performed the experiments. **Ying Wei:** performed the experiments. **Jingcheng Dong:** contributed to designing the work.

## Declaration of competing interest

The authors declare no conflict of interest.

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