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RESEARCH ARTICLE



The effects of chrysophanol on ovalbumin (OVA)-induced chronic lung toxicology by inhibiting Th17 response

Chunyan Zhang^a, Yafan Song^a, Congxia Wang^a, Ling Zhao^a, Huafeng Kang^b, Xiaobin Ma^b, Jing Wang^c, Tianzhu Zhang^c, Wang Shumin^c and Chunhua Ma^c

^aDepartment of Cardiology, the Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an, P.R. China; ^bDepartment of Oncology, the Second Affiliated Hospital, Medical School of Xi'an Jiaotong University, Xi'an, P.R. China; ^cChangchun University of Chinese Medicine, Changchun, P.R. China

ABSTRACT

Chrysophanol (CH), extracted from plants of *Rheum* genus, possesses various pharmacological effects including anti-inflammatory activity. The purpose of the present study was to evaluate the protective effects and the underlying mechanisms of CH on ovalbumin (OVA)-induced asthma in mice. Fifty mice were randomly assigned to five experimental groups: control group, model group, dexamethasone (2 mg/kg) group and CH (5 and 10 mg/kg) groups. The number of eosinophil cells and the production of interleukin-6 (IL-6), IL-1 β , IL-17A and tumor necrosis factor- α in bronchoalveolar lavage fluid (BALF) were measured. In addition, pulmonary histopathology, airway resistance (Raw), T-helper17 (Th17) cells frequency and ROR γ t expression were evaluated. Our study demonstrated that CH effectively decreased eosinophil count and inflammatory cytokines production in BALF. In addition, treatment with CH significantly inhibited the Raw, Th17 percentage and ROR γ t expression in OVA-induced animals compared with those in model group. Histological studies also demonstrated that CH significantly suppressed OVA-induced eosinophilia in lung tissue compared with model group. Our findings supported that CH can prevent allergic asthma in the mouse model.

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KEYWORDS

Chrysophanol; lung toxicology; ROR γ t; Th17 cell

Introduction

Asthma is a clinical disease based on airway eosinophilic inflammation, overproduction of cytokines and variable air-flow obstruction (Gupta et al., 2014). It has been generally recognized that asthma is also characterized by variable degrees of chronic inflammation and structural alterations in the airways (Ogurlu et al., 2014). Allergen-specific CD4⁺ T cells are believed to be high related to the development of asthma (Nakashima et al., 2014). Traditionally, it is widely thought that Th2-mediated chronic inflammation triggers and perpetuates a vicious circle of tissue injury–tissue repair culminating in remodeling. Th2 cells synthesize high levels of interleukin IL-4, IL-5 and IL-13, which lead to the overproduction of allergen-specific immunoglobulin (Ig)E and the release of mediators from mast cells (Lee et al., 2012). T-helper17 (Th17) cell, another T lymphocyte subset, has been recently discovered and may be involved in the pathogenesis of several immune-mediated disorders (Hayashi et al., 2013). Th17 cells are characterized by the production of various cytokines, including IL-17, IL-6, tumor necrosis factor (TNF)- α and IL-22. IL-17 is associated with tissue pathology in autoimmune disorders. Furthermore, anti-IL-17 reduces eosinophils infiltration in experimental murine asthma model (Sergejeva et al., 2005). IL-17 induces the recruitment and increases the survival rate of airway macrophages (Yuan et al., 2014). These

facts suggest that IL-17 and Th17 cell play regulatory role in autoimmune disorders.

Chrysophanol (CH), a member of the anthraquinone family, was originally extracted from plants of *Rheum* genus. CH has been shown to have multiple pharmacological effects including the anti-inflammatory activity (Kim et al., 2010), for which the underlying mechanisms remain to be elucidated. However, few studies focused on its mechanism of asthma modulation, thus, the present study was undertaken to determine the potential mechanisms of CH on allergic asthma.

Materials and methods

Chemicals and reagents

CH (purity > 90%) was purchased from National Institutes for Food and Drug Control (Beijing, China) and Dexamethasone (Dex) was provided by Xiansheng Drug Store (Nanjing, China). Both CH and Dex were dispersed in 0.5% sodium carboxymethyl cellulose (CMC-Na solution). Ovalbumin (OVA) and methacholine were supplied by Sigma Chemical Co. (St Louis, MO). Aluminum hydroxide was purchased from Pierce Biotechnology (Rockford, IL). Wright–Giemsa staining kit was provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). ELISA kits were purchased from (R&D,

Minneapolis, MN). Anti-ROR γ t and anti-GAPDH were obtained from ABCAM.

Animals

Specific pathogen-free female BALB/c mice (2014556), aged 6–8 weeks, which routinely screened serologically for relevant respiratory pathogens, were purchased from Jiangning Qinglongshan Animal Cultivation Farm (Nanjing, China). Mice were maintained in an animal facility under standard laboratory conditions for 1 week prior to experiments, and provided with water and standard chow *ad libitum*. All the experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Sensitization, airway challenge and treatment

Mice were randomly assigned to five groups (each of 10 animals): (1) normal control, (2) asthma-model control, (3) asthma treated with Dex (2 mg/kg, administered by gavage) group, (4) asthma treated with CH (5 mg/kg, administered by gavage) group, (5) asthma treated with CH (10 mg/kg, administered by gavage) group. Allergic asthma was induced by OVA using the method described by Ma et al. (2013b). Mice were immunized via intraperitoneal (i.p.) administration with 10 μ g chicken OVA and 2 mg aluminum hydroxide in 200 μ l phosphate buffered saline (PBS) (pH 7.4) on days 0 and 14. Mice were exposed to a 1% (w/v) OVA solution in PBS for 20 min using an ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) and treated with all drugs and vehicles (0.5% CMC-Na) through oral route at a volume of 0.1 ml/g of body weight on days 28, 29, and 30 after initial sensitization. Animals were sacrificed 48 h after the last challenge (thus on day 32) to evaluate the suppressive effects of CH. A schematic diagram of the treatment schedule is illustrated in Figure 1.

Measurement of airway resistance (Raw)

The forced oscillation technique was employed to detect respiratory mechanics (Wang et al., 2014b). Regular ventilation was interrupted and the generated volume signal which

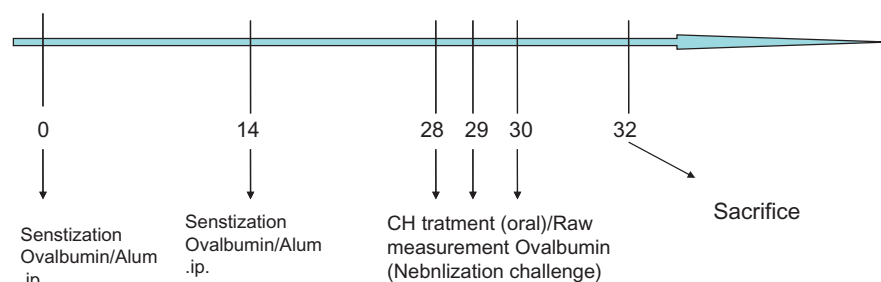
composed of waveforms of mutually primed frequencies was delivered to the airway opening. Piston displacement and cylinder pressure were examined. Impedance values were recorded before and after the delivery of increasing concentrations of acetylcholine aerosols. Prior to the start of the acetylcholine concentration response curve, two total lung capacity breaths were delivered. Acetylcholine (Sigma) dispersed in saline was given through an ultrasonic nebulizer (Hudson RCI, Teleflex Medical) in increasing concentration manners (0.125, 0.25, 0.5 and 1 mg/ml). After delivery of each aerosol, forced oscillations were delivered every 15 s, over a 5-min duration. Regular ventilation was resumed in between impedance measurements. The peak response for each variable was measured.

Collection of BALF

Mice were sacrificed with an overdose of 50 mg/kg of pento-barbital 48 h after the last challenge. The collection of BALF was carried out three times via atracheal cannula with autoclaved PBS. A total volume of 1.3 ml BALF samples were instilled up and centrifuged at 1500 rpm for 10 min at 4 °C, the supernatants were stored in –80 °C for analysis of cytokine concentrations and the pellet was resuspended in 100 μ l of saline, centrifuged onto slides and stained with Wright–Giemsa staining. The slides were quantified for differential cell count.

Pulmonary histopathology

In order to examine the cells that infiltrated into the peribronchial connective tissues and airway tissues, lung tissues and airway tissues were collected from the mice and fixed with normal 10% neutral-buffered formalin at 4 °C for 48 h. The fixed tissues were embedded in paraffin and cut into 4- μ m sections with amicrotome (Leica, Nussloch, Germany). Then, the sections were placed on glasses slide, deparaffinized and stained with hematoxylin and eosin according to the standard protocol. Peribronchial and airway cells count based on a five-point scoring system was performed to evaluate the severity of leukocyte infiltration. The scoring



Mice were immunized with 10 μ g chicken OVA and 2 mg aluminum hydroxide in 200 μ l phosphate buffered saline (PBS) (pH 7.4), on days 0 and 14. Mice were given CH 1 h before then were exposed to a 1% (w/v) OVA solution in PBS, for 20 min, using an ultrasonic nebulizer on days 28, 29, and 30 after initial sensitization. Animals were sacrificed 48 h after the last challenge (thus on day 32) to characterize the suppressive effects of CH.

Figure 1. Sensitization, challenge treatment protocols for the different groups in this study.

system was: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cell layers deep and 4, a ring of cells more than 4 cell layers deep.

Cytokine assays

The levels of IL-6, IL-1 β , IL-17A and TNF- α in BALF were measured by ELISA according to the manufacturer's instructions (R&D). Briefly, the BALF samples were added in triplicate to 96-well plates with 100 μ l per well. After incubated with appropriate biotin conjugated antibodies for 2 h, the samples were then aspirated and washed five times. The substrate solutions were added to each well for another incubation of 30 min at room temperature in the dark. The optical density of each well was read at 450 nm using a microplate reader.

Flow cytometric analysis of Th17 cells

The spleen was removed from the sacrificed mice and placed in a tube containing RPMI1640 media (Gibco). After that the single cell suspension was prepared by disrupting the spleen with cell disrupter (Beckman). Cell suspension was distributed into tubes and washed with PBS. Then, the cells were incubated with FITC antihuman CD4 at 4°C for 30 min. After surface staining, the samples were fixed and permeabilized according to the manufacturer's instructions and stained with PE anti-mice IL-17A (eBioscience) for Th17 analysis.

Western blotting

The lung tissues were homogenized, washed with PBS and lysed in a RI PA buffer to obtain extracts of lung proteins. The samples were loaded to 10% SDS-PAGE gels and were electrotransferred to nitrocellulose. After blocked with 5% non-fat milk, the blots were incubated with appropriate concentration of specific antibody at 4°C overnight, then the blots were incubated with horseradish peroxidase-conjugated second antibody. Two hours later, the proteins were visualized using an ECL Key-GEN system (KeyGEN Biotechnology, Nanjing, China) and detected with a ClinX ChemiScope chemiluminescence imaging system (Gel Catcher 2850, China).

Statistical analysis

All values were expressed as the mean $\pm$ SD and analyzed by one-way ANOVA with Tukey multiple comparison test. A p value of less than 0.05 was considered significant.

Results

Effect of CH on Raw

The analysis of Raw during acetylcholine-induced constriction are shown in Figure 2. The Raw value of the OVA challenged group was remarkably higher than that of the control group ($p < 0.05$). By contrast, CH (5 and 10 mg/kg) groups showed a significant decrease compared with those in model group.

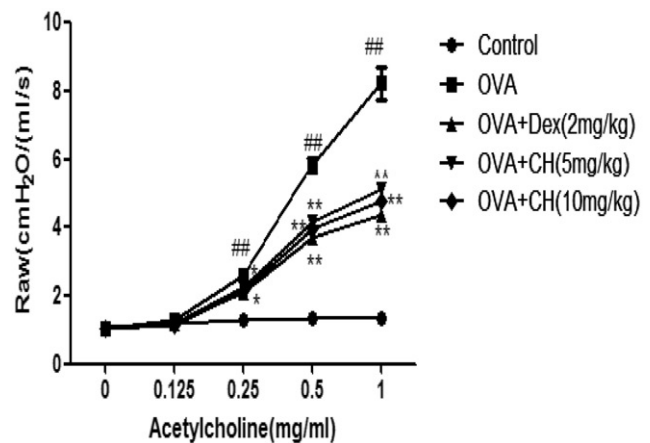


Figure 2. Effect of CH on Raw. Values are expressed as means $\pm$ SDs. Compared with control: # $p < 0.05$, ## $p < 0.01$. Compared with OVA: * $p < 0.05$, ** $p < 0.01$.

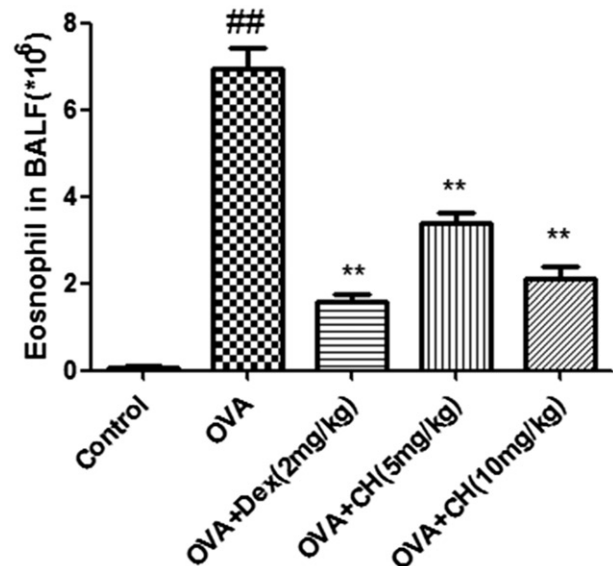


Figure 3. Effect of CH on OVA-induced eosinophilia. Values are expressed as means $\pm$ SDs. Compared with control: # $p < 0.05$, ## $p < 0.01$. Compared with OVA: * $p < 0.05$, ** $p < 0.01$.

Effect of CH on OVA-induced eosinophilia in BALF

The inhibition of eosinophilia caused by CH in OVA-challenged mice was estimated by counting of cells in BALF 48 h after the last challenge. As shown in Figure 3, eosinophil levels dramatically increased in BALF of OVA-challenged mice. On the contrary, treatment with CH displayed significant reduction of eosinophilia.

Effect of CH on cytokine levels in BALF

The productions of IL-6, IL-1 β , IL-17A and TNF- α in BALF were examined with commercial ELISA kits. As revealed in Figure 4, compared with control group, the levels of inflammatory cytokines in model group were markedly increased ($p < 0.05$). The CH treatment groups could markedly enhance levels of IL-6, IL-1 β , IL-17A and TNF- α compared with those in model group.

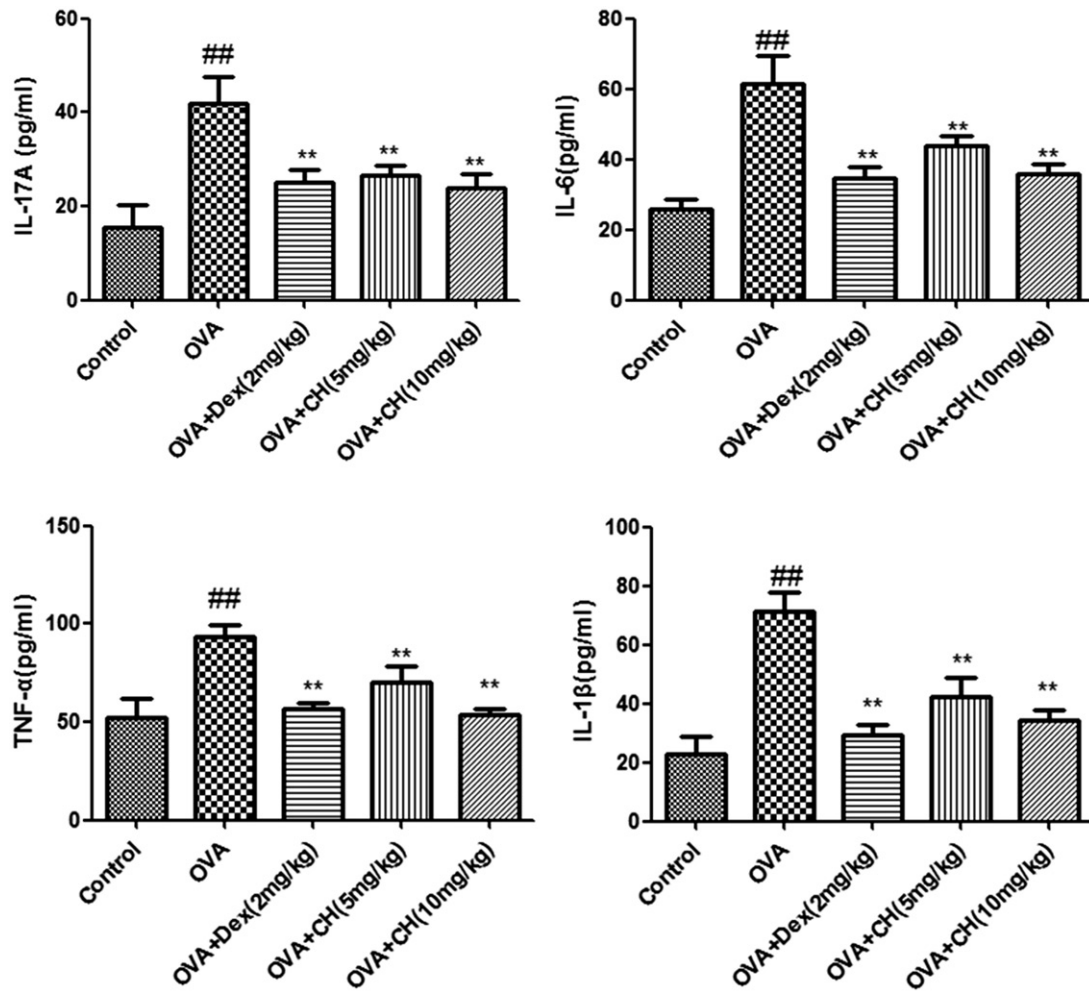


Figure 4. Effect of CH on cytokine levels in BALF. Values are expressed as means $\pm$ SDs. Compared with control: $\#p < 0.05$, $\#\#p < 0.01$. Compared with OVA: $*p < 0.05$, $**p < 0.01$.

Effect of CH on airway tissue

Airway tissue was collected 48 h after the final OVA stimulation. In OVA-induced asthmatic airway tissue, we observed obvious infiltration of inflammatory cells into airway tissue compared with those in normal tissue. The control group displayed no change in the extent of inflammatory cell infiltration, similar to that in Dex-treated positive control mice. Eosinophilia of CH treatment group was significantly attenuated compared with those in OVA-sensitized and challenged mice (Figure 5).

Effect of CH on Th17 cells

Flow cytometric analysis was performed to evaluate the immunosuppressive properties of CH. As shown in Figure 6, the frequencies of Th17 cells in model group were higher than that in control group, while in treatment groups and Dex group, they were significantly lower than that in model group.

Effect of CH on ROR γ t

Transcription factor ROR γ t is essential for the development and function of Th17 cell. As shown in Figure 7, ROR γ t level

in model group was higher than that in control group, while the ROR γ t expressions were significantly inhibited in CH treatment groups and Dex group than that of model group.

Discussion

Asthma, an inflammatory airway disease with severe morbidity or even mortality, affects about 300 million individuals worldwide (Chong et al., 2014; Jang et al., 2014). To date, a variety of medicines have been developed to treat asthma including leukotriene inhibitors, mast cell stabilizers, steroids and β 2 adrenergic agonists (Ma et al., 2013a). Herbal medicines are commonly applied to treat asthma in the Orient. The previous studies demonstrated the protective effects of CH on cerebral ischemia/reperfusion (Zhang et al., 2014). Rim et al. also elicited the anti-inflammatory activity of CH in RAW 264.7 and 3T3-L1 cells (Rim et al., 2013). However, the therapeutic efficacies and the underlying mechanism of CH on OVA-induced asthma are still unclear. Herein, we performed the present study to evaluate the therapeutic efficiency of CH on OVA-induced asthma in mice and investigate its potential mechanism.

Asthma is characterized by a variable level of airway obstruction accompanied by airway hyperresponsiveness

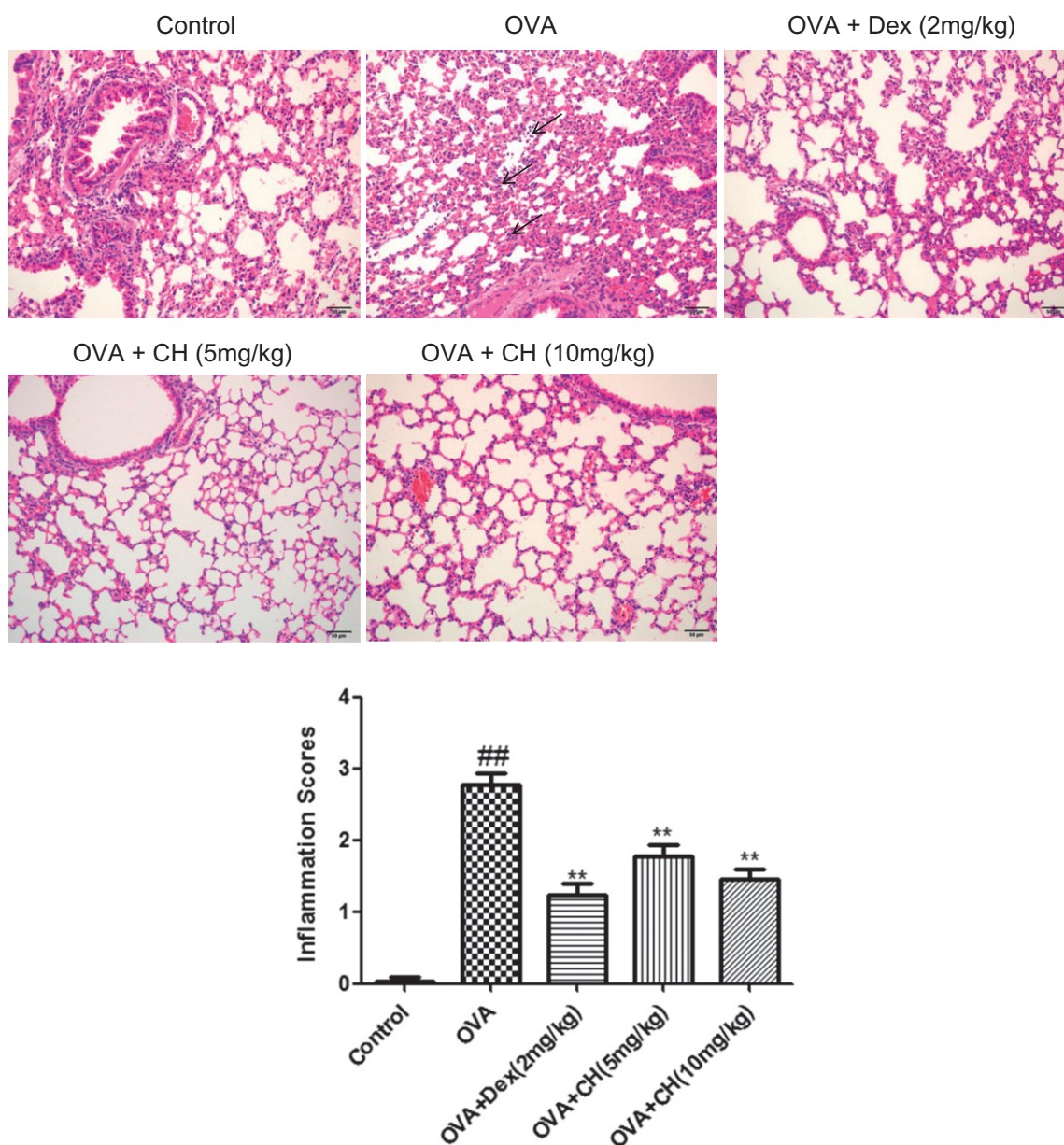


Figure 5. Pathological changes of lung tissues observed by HE staining. (Light microscopy, $\times 200$): A (control); B (model); C (Dex, 2 mg/kg); D [CH (5 mg/kg)]; E [CH (10 mg/kg)]. Black arrow represents inflammation cells. Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections. Values are expressed as means $\pm$ SDs. Compared with control: $\#p < 0.05$, $\#\#p < 0.01$. Compared with OVA: $*p < 0.05$, $**p < 0.01$.

(AHR) (Tang et al., 2014). The contraction or hypertrophy of airway smooth muscle and inflammation within the airway lead to bronchoconstriction which consequently conduce to the pulmonary dysfunction (Chauhan et al., 2014; Subhashini et al., 2013). AHR is a critical index of the bronchial constriction commonly observed in individuals with asthma (Wang et al., 2014a). The Raw was measured to monitor the effects of CH on AHR. Our experimental data displayed that CH inhibited the OVA-induced AHR in response to inhaled acetylcholine.

The recruitment of inflammatory cells led to AHR has been well-studied. Migration of inflammatory cells, specifically eosinophils, into the lung is an important contributor to the pathogenesis of allergic airway inflammation (Li et al., 2014b). The increased number of eosinophils in the BALF is the key feature of asthma. Our data clearly demonstrated

that CH significantly reduced eosinophil numbers in BALF and airway tissue. Meanwhile, CH also attenuated the histopathologic change in lung tissue. Taken together, the results suggested that the CH effectively ameliorated the condition in OVA-induced asthma in mice.

Th17 cells are the major immune cells in the development/prevention of autoimmune diseases (Liu et al., 2014). Recently, Th17 cells have been described as two distinct subsets from Th1 and Th2 cells which play opposite roles in asthma (Mohammadzadeh et al., 2014). Accumulating evidence reported that Th17 was responsible for the regulation of autoimmunity reaction (Lu et al., 2013). Notably, Th17 enhances the eosinophilic airway inflammation in a mouse model of asthma (Wakashin et al., 2008). Th17 cells expressing retinoic acid receptor-related orphan receptor γ t (ROR γ t) play a crucial role in induction of autoimmune tissue injuries

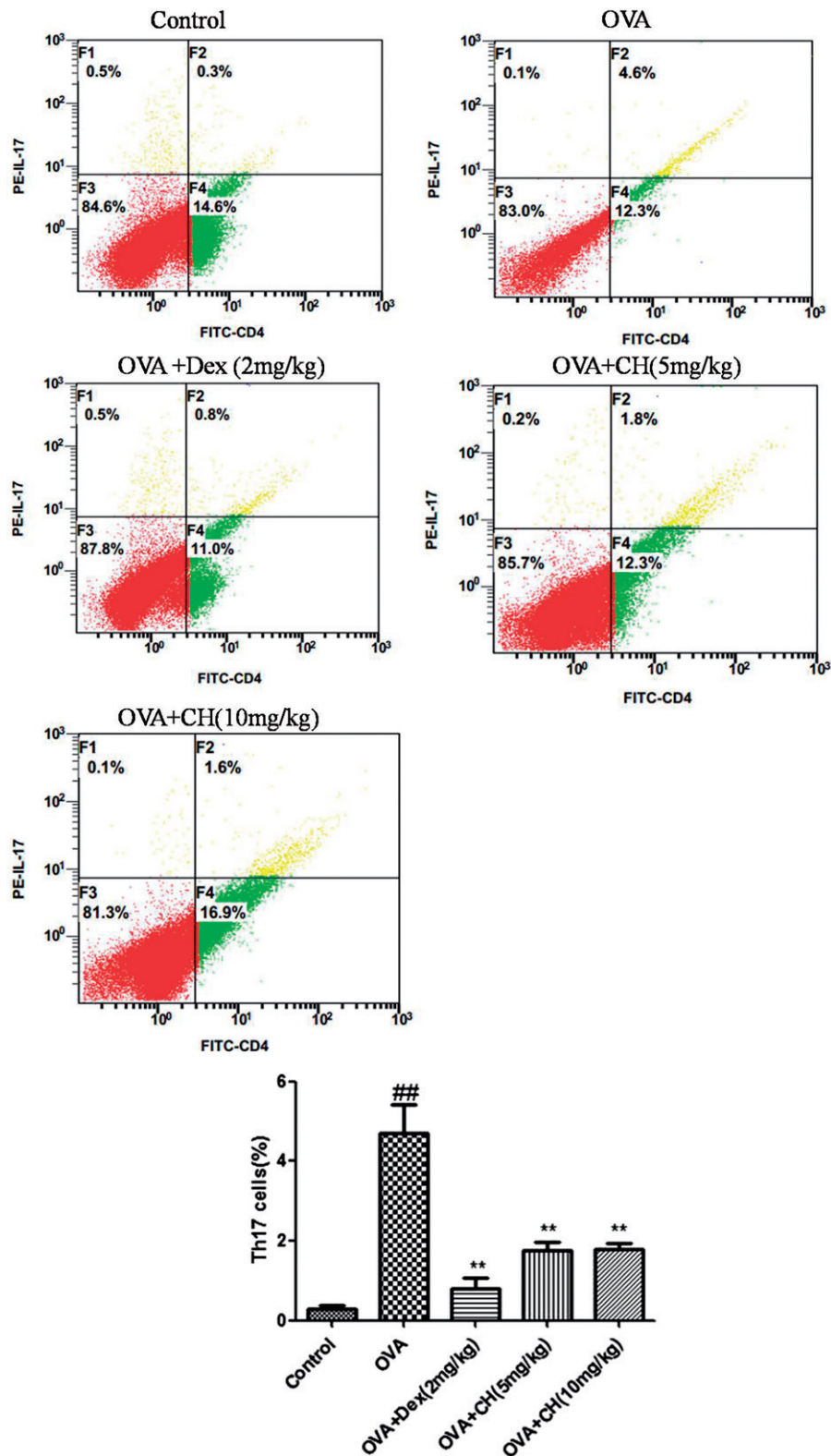


Figure 6. Effect of CH on Th17 cells and the percentage of Th17 cells in different groups. Values are expressed as means $\pm$ SDs. Compared with control: # $p < 0.05$, ## $p < 0.01$. Compared with OVA: * $p < 0.05$, ** $p < 0.01$.

by producing cytokines such as IL-17A, IL-1 β , TNF- α and IL-6 (Li et al., 2014a). The elevated level of IL-17A is correlated with the severity of mucus secretion and airway hypersensitivity (Ji et al., 2014). IL-1 β is closely associated with the alveolar epithelial repairment and the production of other cytokines (Geiser et al., 2001). IL-6 contributes to the acute-

phase response of inflammation and participates in the initiation of inflammatory cascade (Lou et al., 2015). TNF- α is a pivotal cytokines during the development of pulmonary disorder and is widely acknowledged to play an essential role in the motivation of innate immune response (Chen et al., 2015). The specific cytokine IL-17A could synergize with TNF-

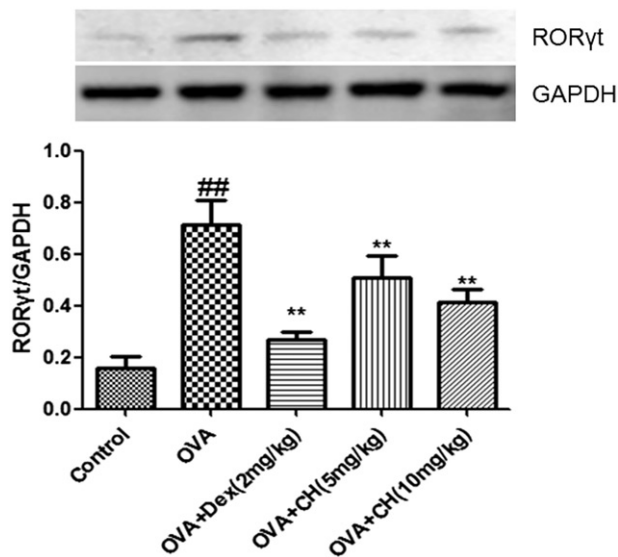


Figure 7. Effect of CH on ROR γ t. Values are expressed as means $\pm$ SDs. Compared with control: # $p < 0.05$, $p < 0.01$. Compared with OVA: * $p < 0.05$, ** $p < 0.01$.

α which stimulates the proliferation of T cells and promote the generation of IL-6 (Xiong et al., 2014). Our data showed that CH significantly suppressed the inflammatory cytokines production, Th17 cells population and ROR γ t expression. These findings indicated that CH exhibited the protective activity against asthma through the inhibition of Th17 response.

In this study, CH effectively suppressed IL-6, IL-1 β , IL-17A and TNF- α and inhibited both the recruitment of eosinophils and bronchial hyperresponsiveness in mice with OVA-induced asthma. Moreover, CH effectively inhibited Th17 cell frequency and ROR γ t expression. Our findings supported that CH can prevent symptoms of allergic asthma in the mouse model and the potential mechanism of CH application for asthma patients should be studied in the future.

Disclosure statement

The authors declare no conflicts of interest.

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