



Attenuation of airway hyperreactivity and T helper cell type 2 responses by coumarins from *Peucedanum praeruptorum* Dunn in a murine model of allergic airway inflammation

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ABSTRACT

Ethnopharmacological relevance: The root of *Peucedanum praeruptorum* Dunn (PPD) is a commonly used traditional Chinese medicine for the treatment of asthma. Its major constituents, coumarins, were presumed to be responsible for its efficacy.

Aim of the study: The potential of coumarins from PPD (CPPD) as anti-asthma agent was investigated.

Materials and methods: Female BALB/c mice were sensitized and challenged with ovalbumin (OVA) to induce allergic airway inflammation. CPPD was administered intragastrically before every OVA challenge. Airway reactivity to the intravenous administration of acetylcholine chloride was measured 48 h after final OVA inhalation. Airway inflammation was evaluated by leukocyte counts of bronchoalveolar lavage fluid (BALF) and histopathological analysis of lung lesions. Levels of interleukin (IL)-4, IL-5, IL-10, IL-13 and IFN- γ in BALF and OVA-specific immunoglobulin (Ig) E in serum, and activity of eosinophil peroxidase (EPO) in lung was measured. The percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among CD4⁺ T cells in spleen was analyzed by flow cytometry.

Results: Compared with model group, CPPD significantly reduced airway hyperreactivity and airway eosinophilic inflammation, improved pathologic lesion of the lungs, reduced levels of IL-4, IL-5, IL-13 in BALF and OVA-specific IgE in serum, inhibited the activities of EPO in lung, and up-regulated levels of IL-10 and IFN- γ in BALF as well as the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in spleen.

Conclusion: CPPD can significantly suppress OVA-induced airway inflammation, airway hyperreactivity and Th2 predominant response in mice, showing great therapeutic potential for the treatment of allergic asthma.

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1. Introduction

Allergic asthma is a prevalent and severe disease with ever-increasing worldwide incidence (Anandan et al., 2010), accompanied by recurrent chest tightness and respiratory embarrassment, bringing a great suffering and mortality to patients (Zdanowicz, 2007). Characterized by airway eosinophilia, hyper-responsiveness, hypersecretion and reversible obstruction (Kon and Kay, 1999), allergic asthma is a chronic inflammatory disorder of the airways associated with T-helper 2 (Th2) lymphocyte

predominance induced by allergens, the accumulation of which in peribronchial and perivascular tissues cause inflammatory lesions through the secretion of Th2 cytokines (Brightling et al., 2002).

Corticosteroids are the most powerful and effective anti-asthma drugs up to now (Kilpiö and Hannuksela, 2003). Adverse events were commonly associated with the administration of corticosteroids, especially for long-term use, such as hyperglycemia, hypertension, osteopenia (Barnes, 2001), which cry for highly active anti-asthma drugs with no or lower side effects.

Root of *Peucedanum praeruptorum* Dunn (PPD) is a commonly used traditional Chinese medicine for the treatment of asthma and chronic bronchitis for thousands of years. We have thoroughly investigated coumarins (CPPD) from the roots of PPD, which were categorized into angular dihydropyranocoumarin, linear furocoumarin and simple coumarin types (Hou et al., 2009; Liu et al., 2004). CPPD was found to display potent and diverse pharmacological activities such as relaxing tracheas (Zhao et al., 1999) and arteries (Xu et al., 2010), anti-oxidative effects (Wang et al., 2008), antipyretic-analgesic and anti-inflammatory (Aida et al., 1998). PPD

Abbreviations: PPD, *Peucedanum praeruptorum* Dunn; CPPD, coumarins from *Peucedanum praeruptorum* Dunn; OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; R_L, lung resistance; IL, interleukin; IFN, interferon; Ig, immunoglobulin; EPO, eosinophil peroxidase; HPLC, high performance liquid chromatography; DAD, diode array detection; UV, ultraviolet; ESI-MSⁿ, electrospray ionization tandem mass spectrometry.

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is widely used to treat asthma for many centuries in China, however no systematic mechanism study on it has been performed so far. Its major constituents, coumarins, were presumed to be responsible for its efficacy (Chen et al., 1979; Suzuki et al., 1985; Kong et al., 1996). In the present study, we employed the BALB/c mouse as allergic asthma model to examine the potential of CPPD to block the development of airway inflammation.

2. Materials and methods

2.1. Plant material

The roots of PPD (10 kg) were purchased from Zhejiang Chinese Medical University. Dried roots were thrice extracted with 30 l of light petroleum at a constant temperature of 60 °C, 3 h each. Then the light petroleum solutions were combined and evaporated to get 350 g of yellow oil crude extract by rotary vaporization at 40 °C under reduced pressure. The extract was subjected to chromatographic column over silica gel (100–200 mesh), eluted first with 19 l of petroleum ether:EtOAc (100:3) to remove oils, and then with additional 25 l of petroleum ether:EtOAc (4:1) to get fractions riched in coumarins, which was concentrated under vacuum to get a total of 120 g CPPD, where the content of coumarins over 90% was quantified by ultraviolet spectrophotometer.

2.2. HPLC/UV-DAD/ESI-MSⁿ analysis of CPPD

An Agilent-1100 HPLC (high performance liquid chromatography) system with diode array detector (DAD) (Agilent Corporation, MA, USA) was prepared. The chromatographic separation was performed on a Heder ODS analytical column (5 μm, 250 mm × 4.6 mm I.D., Hanbon, China) with the column temperature set at 25 °C. The mobile phase is consisted of (A) methanol and (B) water (v/v). A gradient elution program of 65–77% A at 0–12 min, 77–80% A at 12–15 min, 80–100% A at 15–25 min was used for the separation of CPPD. The detection wavelength was set at 322 nm and the flow rate at 1.0 ml/min. HPLC/UV-DAD/ESI-MS study was performed on LC/MSD Trap SL (Agilent Corporation, MA,

USA). The MS conditions were as follows: positive ion modes; collision energy (Amplitude): 1.0 V; collision gas: He; drying gas and flow rate: N₂, 9 ml/min; temperature: 350 °C; pressure of nebulizer: 40 psi; high voltage: 3.5 kV. Data acquisition was performed using Agilent Chemstation software (Fig. 1).

2.3. Mice

Forty-eight female BALB/c mice weighing 22–25 g (5–7 weeks) purchased from Vital River Lab Animal Technology Co., Ltd. (Beijing, China) were reared in Animal Center of Nanjing Medical University, adherent to standard guidelines of laboratory animal care (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, 1996). All mice were provided with water and standard chow pellets *ad lib.* for 2 weeks prior to any experimental performances to make them accommodate their new surroundings. All experimental protocols were approved by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.4. Antigen sensitization, challenge, and treatment

Mice were randomly divided equally into six separate groups, each with 8 mice: control, OVA, dexamethasone, and three different doses of CPPD (50, 100 and 200 mg/kg). Except for the control group, all mice were sensitized on days 1 and 14 by intra-peritoneal injection of 10 μg OVA (grade V) (Sigma, St. Louis, MO, USA) dissolved in aluminium hydroxide gel (Sigma–Aldrich, Brøndby, Denmark) in a total volume of 0.2 ml. Two weeks after the sensitization, the mice inhaled 1% OVA in an exposure chamber (55 cm × 30 cm × 30 cm) to challenge their airways with a nebulizer (NE-U11B, Omuron Co., Tokyo, Japan) for 20 min on five successive days. For atomization inhalation, OVA was dissolved in phosphate-buffered saline, nebulized and discharged at 1 ml/min. Mice in groups of CPPD were intragastrically administered, respectively, with 50 mg/kg, 100 mg/kg and 200 mg/kg CPPD dissolved in 0.5% sodium carboxymethyl cellulose solution (CMC-Na) daily before 1 h of OVA challenge. Mice treated with 3 mg/kg dexamethasone (Sigma,

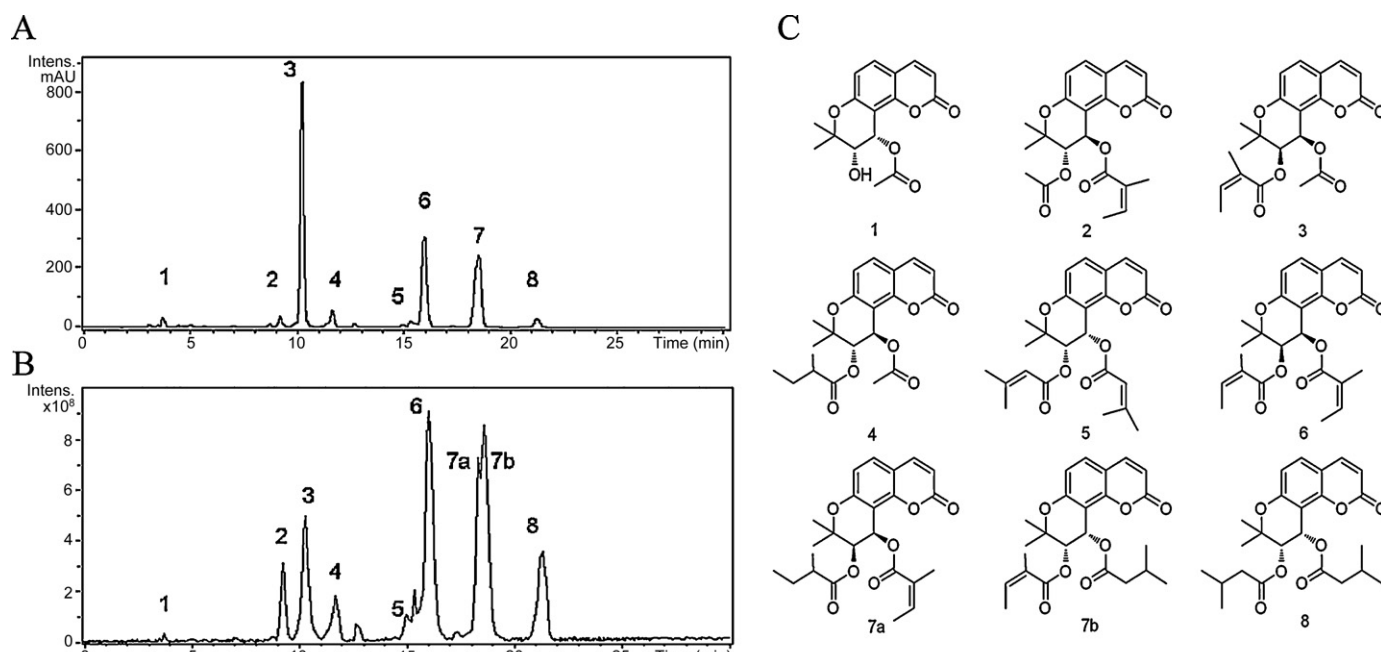


Fig. 1. HPLC/UV-DAD/ESI-MS analyses of coumarins from roots of *Peucedanum praeruptorum* Dunn (CPPD). (A) The representative chromatogram (322 nm). (B) Total ion current chromatogram in the positive ion mode. (C) Structures of coumarins in the chromatograms.

St. Louis, MO) by intra-peritoneal injection 1 h before each airway challenge was used as positive control. The healthy control group inhaled phosphate-buffered saline aerosol and received intragastric feeding of 0.5% CMC-Na.

2.5. Evaluation of airway hyperreactivity

Lung resistance (R_L) induced by acetylcholine chloride, reflecting the development of airway reactivity, was evaluated using AniRes 2005 mouse lung function analysis system (SYNOL High-Tech, Beijing, China) 48 h after the last OVA challenge as described previously (Rui et al., 2006). Mice were anesthetized with pentobarbital sodium (70 mg/kg) by intra-peritoneal injection, intratracheally intubated, placed in a rodent plethysmograph capable and mechanically ventilated at a tidal volume of 6 ml/kg and 90 breaths/min. After reaching to a stable tracing, lung resistance was measured at baseline and following intravenous administration of increasing doses of acetylcholine *via* caudalis vein through a micro-injection pump in progressive doses (10, 30, 90 and 270 μ g/kg) every other 5 min. Data were collected continuously from 5 s to 1 min during each administration to record maximal values of lung resistance that reflects changes in murine airway function. Lung resistance was determined by continuously quantitating the quotient $\Delta P/(\Delta V/\Delta T)$ (where ΔP is the change in tracheal pressure, ΔV is the change in pulmonary volume, and ΔT is the change in time) using a pulmonary mechanics analyzer.

2.6. Measurement levels of the OVA-specific IgE and cytokines

Mice were bled *via* the retro-orbital venous plexus under the anesthesia state. Collected blood samples were centrifuged at $1000 \times g$ for 10 min at 4 °C and then the obtained plasma was stored at –70 °C. Levels of OVA-specific IgE in serum, expressed in ng/ml of serum, were measured by commercially available enzyme-linked immunosorbent assay kits for the OVA-specific IgE, following the manufacturer's instructions (Shibayagi Co., Shibukawa, Japan). The cytokines of IL-4, IL-5, IL-10, IL-13, IFN- γ in BALF, reported in pg/ml of each sample, were measured by enzyme-linked immunosorbent assay kits according to the manufacturer's protocol (Bender MedSystems, Vienna, Austria).

2.7. Leukocyte counts of BALF

The airway was lavaged thrice with 0.4 ml phosphate-buffered saline *via* an intratracheal cannula. The collected lavage fluid was centrifuged ($1000 \times g$ for 10 min at 4 °C). The supernatant was transferred and stored at –70 °C until used to analyze the cytokine production. Cell pellets were resuspended in a phosphate-buffered saline final volume of 0.5 ml. The number of total leukocytes in BALF was counted using a hemocytometer, and differential cell counts were determined using Diff-Quick-stained smears. At least 200 cells were counted on each slide and differentiated according to standard morphologic criteria under light microscopy.

2.8. EPO activity

The EPO activity in lung lobes from BALB/c mice was measured as described previously (Baquero et al., 2010). Briefly, the lung tissues were ground in liquid nitrogen, and centrifuged under 4 °C for 10 min at $1000 \times g$, then supernatants were transferred into tubes and stored at –70 °C before use. The thawed supernatants were plated into 96-well plates at 75 μ l per well, followed by the addition of 1.5 mmol/L *o*-phenylenediamine and 6.6 mmol/L H_2O_2 in 0.05 mol/L Tris-HCl (pH 8.0). After 30 min under room temperature, the reaction was stopped with 75 μ l of 0.2 mol/L citric acid.

Absorbance was then read at 492 nm in a microplate reader (Bio-Rad, USA).

2.9. Flow cytometry for CD4⁺CD25⁺Foxp3⁺ regulatory T cells

To evaluate the effect of CPPD on regulatory T cells in experimental allergic asthma mice, the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among the CD4⁺ T cells was analyzed in spleens of all group mice according to the manufacturer's protocol (BD Biosciences, San Jose, CA). Prepared splenocytes were stained with Anti-Mouse CD4 FITC and Anti-Mouse CD25 APC, then fixed and permeabilized using the Foxp3 Staining Buffer Set and subsequently stained with 0.5 μ g of Anti-Mouse Foxp3 PE. Cells in the lymphocyte gate were used for analysis using a FACSCalibur cytometer equipped with CellQuest software (BD Biosciences).

2.10. Histopathological analyses

After BALF had been collected, the low right lobe of the lungs were collected and fixed in 10% phosphate-buffered formalin, and embedded in paraffin for histological analysis. Tissues were cut into 2- μ m sections and stained with hematoxylin–eosin and Congo red for analysis of inflammatory cells in lung tissue by light microscopy examination. All images were captured using an Olympus DP50 digital camera (Olympus Optical Co., UK) through a BX-51 microscope (Olympus American, Melville, NY, USA) equipped with a reference measurement slide.

2.11. Statistical analysis

Data were given as the means $\pm$ S.E.M. of *n* independent experiments. The SPSS software (SPSS 13.0 for Windows) was used for statistical analysis. Statistical comparisons between groups were evaluated by ANOVA, followed by Bonferroni's test. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effect of CPPD on airway hyperreactivity

Development of increased airway reactivity, a hallmark of typical allergic asthma, can lead to bronchoconstriction and airway over-secretion. To detect the effect of CPPD on airway responsiveness, we assessed lung resistance (R_L) of anesthetized mice in response to increasing concentrations of acetylcholine chloride. The lung resistance induced by acetylcholine chloride at each level of dose ranging from 30 to 270 μ g/kg was significantly increased in the OVA group compared with that in the control group (Fig. 2). Treatment of mice with CPPD before ovalbumin aerosol led to a sharp decrease in lung resistance compared with the OVA group, and such decrease was more obvious in the high-dose of CPPD (Fig. 2). Treatment of mice with dexamethasone showed a similar decrease of lung resistance compared with the high-dose of CPPD (Fig. 2).

3.2. Effect of CPPD on the number of leukocytes in BALF

To investigate the effect of CPPD on the development of airway inflammation induced by OVA challenge, number of total cells and different cells in BALF was counted. The levels of total cells, eosinophils and lymphocytes were markedly increased in mice of the OVA group, much higher than those in mice of the control group (Fig. 3). Treatment of mice with CPPD or dexamethasone substantially inhibited the increase of these leukocytes as compared with the OVA group (Fig. 3).

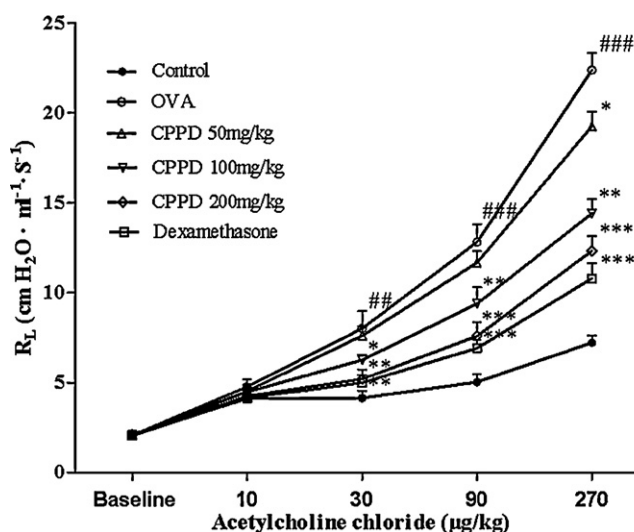


Fig. 2. Effect of CPPD on the lung resistance (R_L) induced by acetylcholine chloride in experimental mice. Values are mean $\pm$ S.E.M. ($n=8$ per group). Significant at: ## $P<0.01$, ### $P<0.001$ vs. control group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. OVA group.

3.3. Effect of CPPD on the OVA-specific IgE, EPO and cytokines

Production of numerous cytokines is an important facet of asthmatic inflammation. The effects of CPPD on the levels of IL-4, IL-5, IL-10, IL-13, IFN- γ in the BALF, EPO in lung and OVA-specific IgE in serum were investigated. Compared with mice of the control group, levels of IL-4, IL-5, IL-13, EPO and OVA-specific IgE were sharply increased and those of IL-10 and IFN- γ greatly were decreased in mice of the OVA group (Fig. 4). However, compared with mice of the OVA group, levels of IL-4, IL-5, IL-13, EPO and OVA-specific IgE were sharply decreased and those of IL-10 and IFN- γ were greatly increased in mice of CPPD groups, which also holds true for mice of dexamethasone group (Fig. 4).

3.4. Effect of CPPD on regulatory T cells

CD4⁺CD25⁺ regulatory T cells played an important role in the suppression of Th2 cell-mediated allergic airway inflammation. We measured the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among CD4⁺ T cells in the spleens of asthmatic animals by flow cytometry (Fig. 5A). The percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells in spleen in mice of OVA group was significantly decreased as compared with those of the control mice (Fig. 5B), which was prevented by the treatment with CPPD or dexamethasone (Fig. 5B).

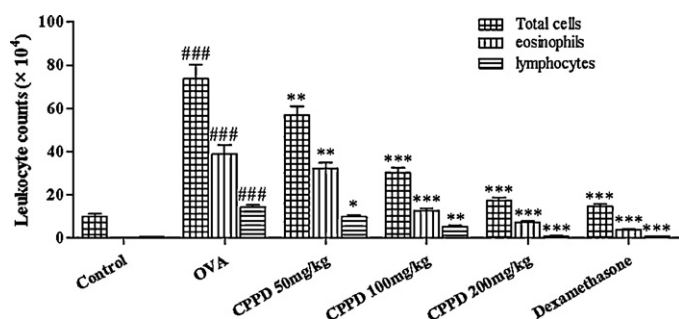


Fig. 3. Effect of CPPD on the number of leukocytes in BALF of experimental mice. Values are mean $\pm$ S.E.M. ($n=8$ per group). Significant at: ### $P<0.001$ vs. control group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. OVA group.

3.5. Effects of CPPD on lung histological changes

Forty-eight hours after the last OVA challenge, lower left part of mice lungs were removed to evaluate the pathologic changes, examined of the lung sections with hematoxylin–eosin and Congo red staining. Mice in the OVA group exhibited a significant inflammatory infiltrate in small airways compared with mice in the control group (Fig. 6A), and most of infiltrating inflammatory cells in inflammatory sites were eosinophils (Fig. 6B). In the CPPD groups, infiltrating inflammatory cells, in particular eosinophils were significantly inhibited as compared with those in the OVA group (Fig. 6A and B). Mice treated with dexamethasone almost eliminated the inflammatory infiltrate (Fig. 6A and B).

4. Discussion

In the present study, it was demonstrated for the first time that coumarins from PPD had potent anti-inflammatory activity in a murine model of allergic airway inflammation. The suppressive effects of CPPD on airway inflammation in mice of the CPPD groups was showed by a marked decrease in airway hyperreactivity and recruitment of inflammatory cells into BALF and lung tissue, in levels of IL-4, IL-5 and IL-13 in BALF, EPO in lung and allergen-specific IgE in serum, and by a marked increase in levels of IL-10 and IFN- γ in BALF, and in the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among the CD4⁺ T cells in spleens compared with those in mice of the OVA group. These results demonstrated the potent anti-inflammatory effect of CPPD in OVA-challenged mice.

The infiltration of lung inflammatory cells (mast cells, lymphocytes and eosinophils), especially those of eosinophils, is representative of inflammatory events of asthma, the severity of which therefore is directly related to the degree of eosinophilia (Sampson, 2000). Oral administration of CPPD markedly inhibited the number of eosinophils in BALF and lung tissues challenged by OVA. EPO is exclusively released by eosinophils and used as a cell specific marker for these cells (Metso et al., 2002). The activity of EPO is often used to evaluate the degree of lung eosinophilia (Collins et al., 1995). It has been shown that patients with allergic rhinitis induced by allergen showed an obvious increase in the number of eosinophil peroxidase-stained nasal cavity mucosal eosinophils (Erjefalt et al., 1999). Our data showed that treatment of mice with CPPD overwhelmingly suppressed the activity of EPO in the lung tissues compared with the OVA group. It demonstrates that CPPD efficiently inhibited the airway eosinophil inflammation in mice challenged by OVA.

Airway hyperreactivity is another striking feature of allergic asthma, leading to episodic bronchoconstriction and obstruction of symptoms (Crimi et al., 1998). The effect of CPPD on lung resistance induced by acetylcholine chloride was used to evaluate the development of airway hyperreactivity in experimental mice. CPPD significantly suppressed airway hyperreactivity in a dose-dependent manner, the effect of its high dosage comparable with that of dexamethasone, which was consistent with previous report of the attenuation of acetylcholine induced bronchoconstriction of rabbit by CPPD *in vitro* (Zhao et al., 1999).

Allergen-induced Th2 response plays a vital role in the mechanism of allergic airway inflammation. The accumulation of Th2 lymphocytes in peribronchial and perivascular lung tissues causes inflammatory lesions through the secretion of Th2 cytokines (Larché et al., 2002). IL-4 and IL-13 are reported to contribute to switch the B lymphocyte to IgE production (Palmqvist et al., 2007) and contribute to airway hyperreactivity and allergic inflammation, as was evident in IL-4- and IL-13-deficient mice (Brusselle et al., 1994; Walter et al., 2001). IL-5 is essential for the proliferation of eosinophils (Yamaguchi et al., 1988), and the subsequent

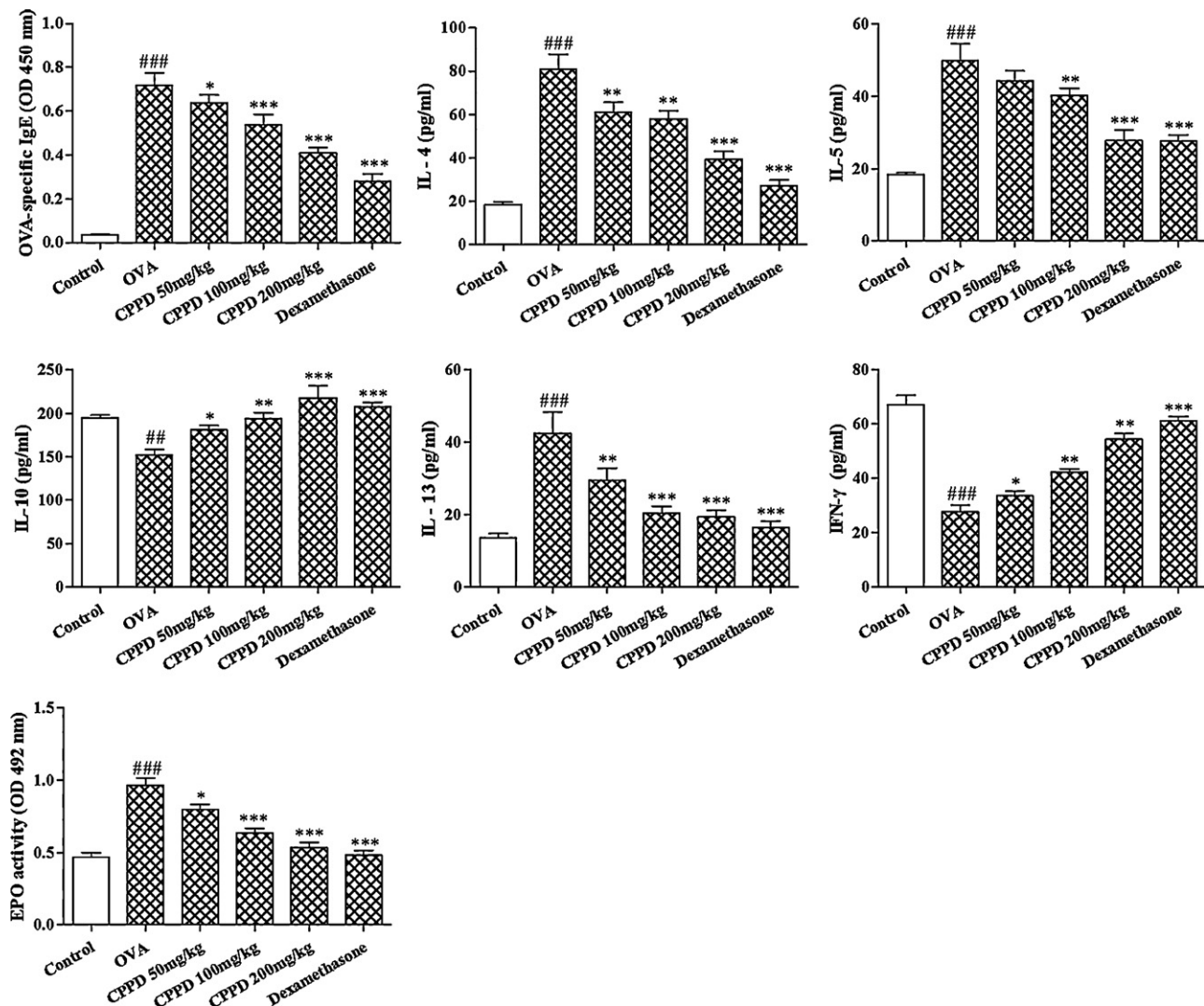


Fig. 4. Effects of CPPD on the levels of IL-4, IL-5, IL-10, IL-13 and IFN- γ in BALF, EPO in lung and OVA-specific IgE in serum of experimental mice. Values are represented as mean $\pm$ S.E.M. ($n=6-8$ per group). Significant at: ## $P<0.01$, ### $P<0.001$ vs. control group; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. OVA group.

onset of airway hyperreactivity and lung damage (Kips, 2001). IL-5 deficiency abolishes eosinophilia, airway hyperreactivity, and lung damage in a mouse asthma model (Foster et al., 1996). CPPD significantly suppressed the predominant Th2 response by preventing

the synthesis of Th2 cytokines (IL-4, IL-5 and IL-13) induced by OVA challenge in mice. IgE-mediated allergic responses are the most common inducers of mast cell activation (Platts-Mills, 2001). CPPD markedly suppressed the OVA-specific IgE levels in serum

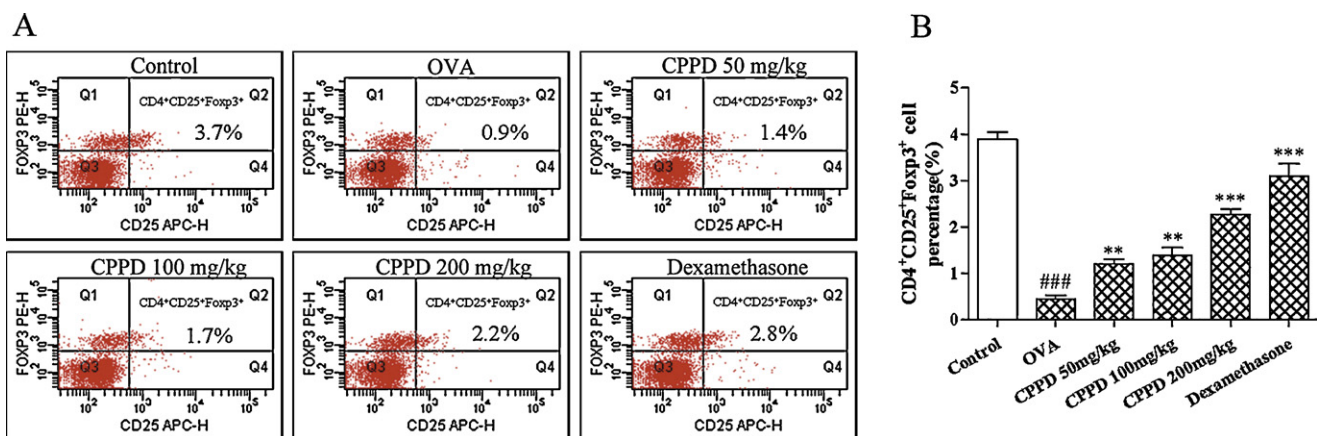


Fig. 5. Effect of CPPD on percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among CD4⁺ T cells in spleens of experimental mice. (A) Flow cytometric analysis of splenocytes stained successively with Anti-Mouse CD4 FITC, Anti-Mouse CD25 APC and Anti-Mouse Foxp3 PE. (B) Bar plot of the average percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells. Values are represented as mean $\pm$ S.E.M. ($n=5-7$ per group). Significant at: ### $P<0.001$ vs. control group; ** $P<0.01$, *** $P<0.001$ vs. OVA group.

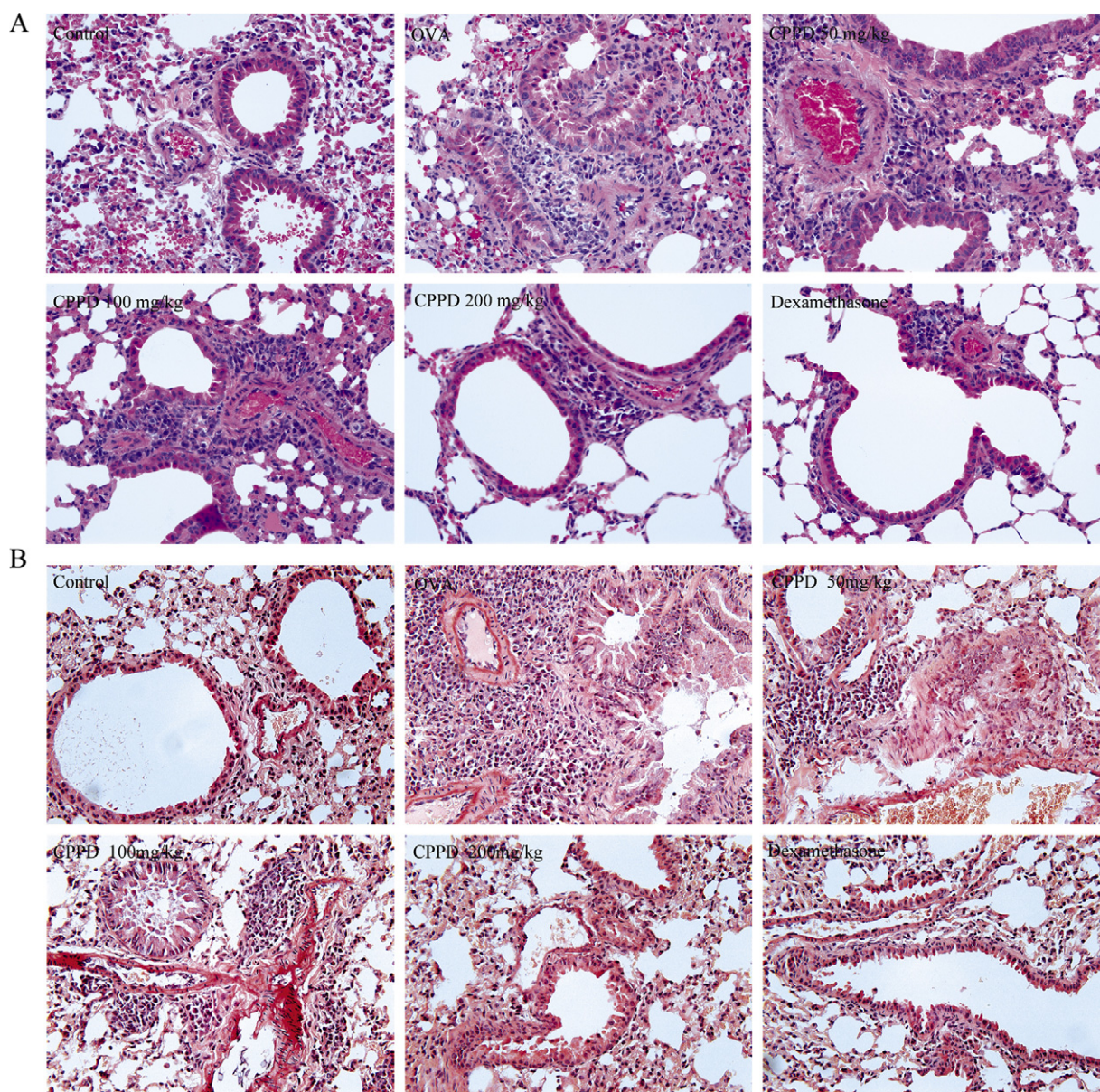


Fig. 6. Effect of CPPD on OVA-induced lung histological changes in experimental mice. (A) Hematoxylin–eosin staining. (B) Congo red staining (eosinophils stained red in cytoplasm and blue in nucleus) ($n = 8$ per group, magnification $\times 400$).

of OVA-challenged mice, which suggested that CPPD might inhibit the release of pro-inflammatory mediators from mast cell in the development of the Th2 predominant allergic reactions.

IL-10 and IFN- γ are known as immuno-modulatory factors in allergic asthma (Lynch et al., 2003). IL-10 plays an important role in proliferative and cytokine responses in T cells (Vieira et al., 1991). Diminished IL-10 production in asthma would result in the increase of pro-inflammatory cytokine synthesis, which contributes to the ingravescence of asthma (Nakagome et al., 2005). Murine studies provided lots of evidences that IL-10 suppresses the development of TH2-type inflammation and airway hyperreactivity (Koya et al., 2007; Grünig et al., 1997). IFN- γ is a potent Th1-type cytokine that can downregulate Th2-predominant immune responses through Th1 pathways (Seder and Paul, 1994; Trinchieri, 1995). Regulation of this effector Th2 asthmatic response by counterbalancing Th1 cells is the goal of new immunotherapy procedures to combat allergic diseases and asthma (Kline et al., 1998; Broide et al., 1998). The decreased levels of IL-10 and IFN- γ were reported both in human and murine allergic asthma (Borish et al., 1996; Wong

et al., 2001; Lee and Kim, 2008). In this study, mice in the OVA group exhibited low levels of IL-10 and IFN- γ , as compared with mice of the control group, which was consistent with previous research (Cho et al., 2008). Treatment of OVA-challenged mice with CPPD significantly prevented the decrease of two cytokines induced by OVA challenge, which suggested that CPPD may inhibit the production of Th2 effector cytokines by regulating the production of Th1 cytokines, and effects the immunomodulatory functions in allergic airway inflammation.

Emerging evidences suggested that regulatory T cells, particularly CD4⁺CD25⁺ regulatory T cells, are the cornerstone component for the generation and maintenance of immunologic tolerance against antigens (O'Garra and Vieira, 2004; Paust and Cantor, 2005). CD4⁺CD25⁺ regulatory T cells were reported to played a key role in modulating Th2-mediated pulmonary inflammation by suppressing the development of a Th2 phenotype that is highly effectively *in vivo* at promoting airway eosinophilia (Akdis et al., 2005; Lewkowich et al., 2005), and prevent pathological findings in animal models of disease (Kearley et al., 2005; Aseffa et al.,

2002; Leech et al., 2007; Kearley et al., 2008). The most specific marker for CD4⁺CD25⁺ regulatory T cells is Foxp3, a recently identified transcription factor that is specifically expressed in these cells (Hori et al., 2003; Fontenot et al., 2005). Our results showed that the percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells among CD4⁺ T cells were substantially reduced in mice of the OVA group as compared with those in mice of the control group, and treatment of OVA-challenged mice with CPPD significantly prevented this decrease, which was consistent with previous report that CD4⁺CD25⁺ regulatory T cells were decreased in the allergic asthma (Lee et al., 2007), and also indicated that the suppression of Th2 cell-mediated allergic inflammation in the airways by CPPD was at least partly due to the effect of CD4⁺CD25⁺ regulatory T cells. The percentage of CD4⁺CD25⁺Foxp3⁺ regulatory T cells was increased in dexamethasone treated mice compared with the OVA group in our experiments, which was consistent with previous researches (Chen et al., 2004; Nguyen and Robinson, 2004; Karagiannidis et al., 2004).

CPPD is composed of about 90% coumarins and some fatty acids and alkynols, such as anchoic acid, lignoceric acid and faltarindiol, which have not been associated with the anti-inflammation or anti-asthma effects of CPPD (Zhang et al., 2006; Miyazawa et al., 1996). Furthermore, some pyranocoumarins isolated from PPD suppress lipopolysaccharide-induced inflammatory response in murine macrophages through inhibition of NF- κ B and STAT3 activation, which play some seminal roles in allergic asthma (Simeone-Penney et al., 2007). All these convincingly support the essential role of coumarins played for the anti-inflammatory effect of CPPD in the OVA-challenged mice.

In summary, our study demonstrated that CPPD effectively suppressed allergic airway inflammation and Th2-predominant responses in BALB/c mice model. The long-term clinic use of PPD in traditional Chinese medicine warranted the safety use of CPPD, which was confirmed by the fact that the median lethal dose (LD50) of CPPD in mice is over 5.0 g/kg in our acute toxicity testing shortly before the present study. Our findings clarified the material foundation of PPD for the treatment of asthma, and favored the further development of CPPD as agents for the treatment of asthma and other allergic diseases.

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