



# Curcumin attenuates allergic airway inflammation by regulation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs)/Th17 balance in ovalbumin-sensitized mice

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## ABSTRACT

The present study aimed to determine the protective effects and the underlying mechanisms of curcumin on ovalbumin (OVA)-induced allergic inflammation in a mouse model of allergic asthma. Asthma mice model was established by ovalbumin. A total of 60 mice were randomly assigned to six experimental groups: control, model, dexamethasone (2 mg/kg), and curcumin (50 mg/kg, 100 mg/kg, 200 mg/kg). Airway resistance (Raw) was measured by the forced oscillation technique, differential cell count in BAL fluid (BALF) was measured by Wright–Giemsa staining, histological assessment was measured by hematoxylin and eosin (HE) staining, BALF levels of Treg/Th17 cytokines were measured by enzyme-linked immunosorbent assay, Treg cells and Th17 cells were evaluated by flow cytometry (FCM). Our study demonstrated that curcumin inhibited OVA-induced increases in eosinophil count; interleukin (IL)-17A level were recovered in bronchoalveolar lavage fluid increased IL-10 level in bronchoalveolar lavage fluid. Histological studies demonstrated that curcumin substantially inhibited OVA-induced eosinophilia in lung tissue. Flow cytometry (FCM) studies demonstrated that curcumin remarkably inhibited Th17 cells and significantly increased Treg cells. The results in vivo show ovalbumin-induced significantly broke Treg/Th17 balance; curcumin treatments markedly attenuated the inflammatory in asthma model by regulating Treg/Th17 balance. Our findings support the possible use of curcumin as a therapeutic drug for patients with allergic asthma.

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## 1. Introduction

Asthma is a clinical diagnosis based on episodic symptoms and variable airway obstruction [1]. It has been generally recognized that the disease is also characterized by variable degrees of chronic inflammation and structural alterations in the airways [2]. Allergen-specific CD4<sup>+</sup>T cells are believed to play a pivotal role in the development of asthma [3]. Traditionally, it is 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 production of allergen-specific immunoglobulin (Ig)E and the release of mediators from mast cells [4]. Th1 cells suppress Th2 immune responses by secreting interferon IFN- $\gamma$ . IL-4 induces class switching in IgG1 and IgE, where as IFN- $\gamma$  is involved in IgG2a class switching. Accordingly, estimation of IgE production and Th1/Th2 cytokine balance is an important tool in the evaluation of asthma [5]. Recently, regulatory T cells (Treg cells) and T-helper17 (Th17) cells have been described as two distinct subsets from Th1 and Th2 cells that play opposite roles in vivo [6]. Treg cells expressing the forkhead/winged helix transcription factor (Foxp3) have an anti-inflammatory role and maintain tolerance to self-components through direct contact with cells or by releasing anti-inflammatory cytokines such as IL-10 [7]. Th17

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cells expressing retinoic acid receptor-related orphan receptor  $\gamma$ t (ROR $\gamma$ t) play crucial roles in the induction of autoimmune tissue injuries and inflammation by producing cytokines such as IL-17, tumor necrosis factor (TNF)- $\alpha$ , and IL-6. The balance between Th17 and Treg cell action may be important in the development/prevention of inflammatory and autoimmune diseases such as asthma [8].

Curcumin is an illustrious dietary ingredient of the Indian subcontinent [9]. Curcumin, in the form of the herbal powder, turmeric (given the name curry spice by the British), has been used for centuries as an anti-inflammatory remedy in Asian [10]. Turmeric has been studied extensively for its biological activities, predominantly as follows: antiinflammatory and antiarthritic [11], antioxidant [12], antimicrobial [13], anti-leishmanial [14], hepatoprotective [15], anticancer [16], vasodilator [17], hypolipidaemic [18], antiplatelet [19], hypoglycaemic [20], choleric [21], antiulcer [22], antiscabies [23], wound healing [24], immunomodulatory [25], neuroprotective [26], antidepressant [27] and effective in Alzheimer's disease [28]. Curcumin has been used for the treatment of various inflammatory diseases in certain countries [29]. Curcumin has been reported to exert an anti-inflammatory effect, through desensitizing the nociceptor ion channel TRPA1 (As TRPA1 is specific to certain nociceptors, it is an attractive target for various therapeutic conditions such as pain, asthma, and cough) [30] and curcumin has been reported to exert an anti-asthma effect, through NF- $\kappa$ B inhibition [31]. The purposes of this study were to determine whether curcumin exerts an anti-inflammatory effect via regulation of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (Tregs)/Th17 balance in ovalbumin-sensitized mice.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Curcumin (purity > 95%) was purchased from National Institutes for Food and Drug Control (Beijing, China). Dexamethasone was purchased from Xiansheng drug store (Nanjing, China). Ovalbumin (OVA), methacholine (Sigma Chemical Co., St Louis, MO), aluminum hydroxide (Pierce Biotechnology, Rockford, USA), and Wright–Giemsa staining (Nanjing Jiancheng Bioengineering institute, Nanjing, China). ELISA kits (R&D, Minneapolis, MN, USA).

### 2.2. Animals

Animal experiment was carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University China Pharmaceutical University. A total of 60 female BALB/c mice were obtained from the Experimental Animal Center of China Pharmaceutical University (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 experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China.

### 2.3. Sensitization and challenge

All animals were allowed to acclimatize for 5 days prior to treatment. After that they were separated randomly into 6 groups ( $n = 10/\text{group}$ ) as follows: control group, model group, dexamethasone group (2 mg/kg), and curcumin treatments (50 mg/kg, 100 mg/kg, 200 mg/kg). Sensitization and challenge treatment protocols for the different groups in this study are summarized in (Fig. 1). Mice (except control group) were sensitized by intraperitoneal (i.p.) injection of 10  $\mu$ g ovalbumin and 1 mg aluminum hydroxide. Mice were sensitized on two occasions, seven days apart. One week after the second sensitization, mice (except control group) were challenged with 2.5% ovalbumin-PBS, from day 14 to day 21, during the day 22 to day 28, mice (except control group) were orally gavaged dexamethasone group (2 mg/kg), curcumin treatments (50 mg/kg, 100 mg/kg, 200 mg/kg), and then challenged with 2.5% ovalbumin-PBS.

### 2.4. Measurement of airway resistance (Raw)

The forced oscillation technique was used to measure respiratory mechanics. Regular ventilation was interrupted and a computer generated volume signal that consisted of waveforms of mutually primed frequencies was delivered to the airway opening. Piston displacement and cylinder pressure were measured. Impedance values were obtained before and after the delivery of increasing concentrations of methacholine aerosols. Prior to the start of the methacholine concentration response curve, two total lung capacity breaths were delivered. Methacholine dissolved in saline was given via an ultrasonic nebulizer (Hudson RCI, Teleflex Medical) in increasing concentrations (0.125 mg ml<sup>-1</sup>, 0.25 mg ml<sup>-1</sup>, 0.5 mg ml<sup>-1</sup>, and 1 mg ml<sup>-1</sup>). After delivery of each aerosol, forced oscillations were delivered every 15 s, over a 5-min duration. In between impedance measurements, regular ventilation was resumed. The peak response for each variable was determined.

### 2.5. Collection of bronchoalveolar lavage (BAL) fluid

Mice were sacrificed using an overdose of 50 mg/kg of pentobarbital 24 h after the last challenge, and tracheotomy was performed. After instilling ice-cold PBS (0.5 mL) into a lung, BAL fluid was obtained by three successive aspirations (total volume 1.5 mL) via tracheal cannulation [32]. BAL fluid was centrifuged (250 g, 4 °C, for 5 min) and each supernatant was collected and stored at -70 °C. BAL fluid (100  $\mu$ L) was placed on a slide and centrifuged (200 g, 10 min, at 4 °C) to fix the cells, using a cytospin machine (Hanil Science Industrial, Seoul, Korea). After the slides were dried, cells were fixed and stained using Wright–Giemsa stain according to the manufacturer's instructions. The slides were quantified for differential cell count by counting a total of 200 cells/slide at 40 magnification. The absolute leukocyte count was calculated by multiplying each leukocyte percentage by the total cell count.

### 2.6. Histological assessment

Lung tissues were detached from the mice and fixed with Carnoy's solution overnight at 4 °C. The fixed tissues were



Fig. 1. Sensitization and challenge treatment protocols for the different groups in this study.

embedded in paraffin and cut into 4- $\mu$ m sections with microtome (Leica, Nussloch, Germany). The sections were placed on slide glasses, deparaffinized, and stained with hematoxylin and eosin (Sigma, Korea) in order to examine the cells that had infiltrated into the peribronchial connective tissues. A peribronchial cell count based on a five point scoring system was performed as previously described to estimate the severity of leukocyte infiltration [33]. The scoring 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.

## 2.7. Enzyme-linked immunosorbent assay detection of plasma cytokines IL-10 and IL-17A

The concentrations of IL-10 and IL-17A in the BAL fluid were measured by ELISA according to the manufacturer's instructions (R&D, Minneapolis, MN, USA). The lower detection limits of these assays were 3.9 pg/ml (IL-10) and 2 pg/ml (IL-17A). All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

## 2.8. Flow cytometric analysis of Tregs/Th17 cells

The spleen was removed from each sacrificed mouse and placed in a tube containing RPMI1640 media (Gibco). A single cell suspension was prepared by disrupting the spleen using Cell disrupter (Beckman), cells were equally distributed into tubes and washed once in phosphate-buffered saline (PBS). For the Th17 analysis, cells were incubated with allophycocyanin (APC) anti-mouse CD4 (BD Biosciences, East

Rutherford, NJ, USA) at 4 °C for 20 min. For Treg analysis, cells were incubated with fluorescein isothiocyanate (FITC) anti-mouse CD4 and phycoerythrin (PE) anti-mouse CD25 at 4 °C for 30 min. Following surface staining, cells were fixed and permeabilized according to the manufacturer's instructions, and then stained with phycoerythrin (PE) anti-mouse IL-17A (eBioscience, San Diego, CA, USA) for Th17 detection or with APC anti-mouse Foxp3 (eBioscience) for Treg detection. Isotype controls (eBioscience) were treated to enable correct compensation and confirm antibody specificity. FACS analysis was performed using FACS Calibur and CellQuest software (BDBiosciences).

The fluorescence strength was represented by value of mean (mean fluorescence intensity, MFI) and the data then analyzed using Flowjo software version 7.6.1.

## 2.9. Statistical analysis

All values were expressed as the mean  $\pm$  SDs, and analyzed by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using SPSS version 13.0 software; a p-value of less than 0.05 was considered significant and  $p < 0.01$  was considered to be statistically very significant.

## 3. Results

### 3.1. Effect of curcumin on airway resistance (Raw)

Measurements of airway resistance (Raw), during methacholine-induced constriction are shown in (Fig. 2). The Raw value of the OVA-challenged group was significantly higher than that of the PBS control group ( $P < 0.05$ ) when

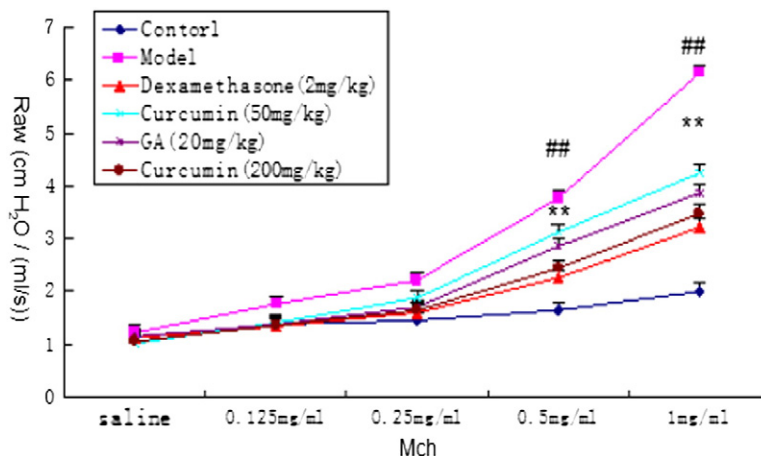


Fig. 2. Effect of curcumin on airway resistance (Raw). Values are expressed as means  $\pm$  SDs. Compared with control: #  $P < 0.05$ , ##  $P < 0.01$ ; compared with model: \*  $P < 0.05$ , \*\*  $P < 0.01$ .

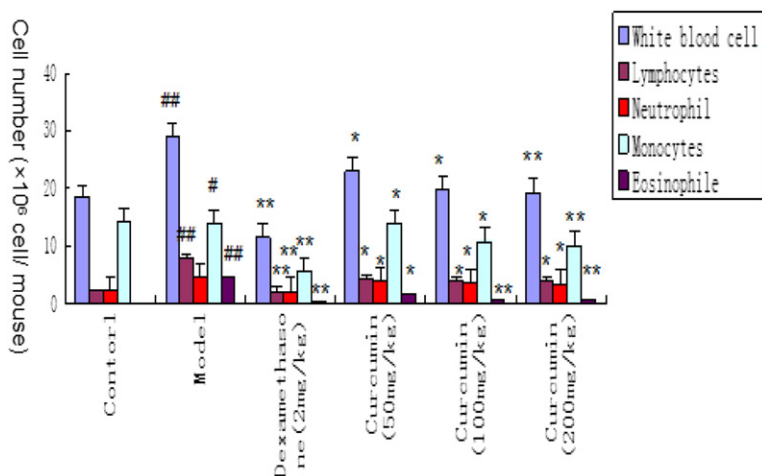


**Fig. 3.** Effect of curcumin on OVA-induced eosinophilia and BAL fluid composition. Values are expressed as means  $\pm$  SDs. Compared with control: # $P < 0.05$ , ## $P < 0.01$ ; compared with model: \* $P < 0.05$ , \*\* $P < 0.01$ .

concentrations of  $0.125\text{--}1.000\text{ mg ml}^{-1}$  methacholine. OVA-sensitized and-challenged mice treated with curcumin, displayed a reduced mean Raw upon exposure to  $0.5\text{--}1.0\text{ mg ml}^{-1}$  compared with the response of the OVA-sensitized and -challenged group. Positive control mice given with dexamethasone showed a Raw decrease similar to that achieved using curcumin.

### 3.2. Effect of curcumin on OVA-induced eosinophilia and BAL fluid composition

We examined changes in total cell levels in the BAL fluid to determine the effects of curcumin on asthma. Suppression of eosinophilia by curcumin in OVA-challenged mice was estimated by counting of cells recruited to the BAL fluid 24 h after the last challenge. As shown in (Fig. 3), OVA induced a

marked influx of leukocytes into BAL fluid in the PBS control group. Eosinophil levels markedly increased in the BAL fluid of OVA-challenged mice. OVA-sensitized and-challenged mice treated with curcumin displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction.

### 3.3. Effect of curcumin on OVA-induced eosinophilia in lung tissue

Because curcumin inhibits inflammatory cell recruitment into the BAL fluid, we next examined the anti-asthmatic effects of the drug. Lung tissue was collected 24 h after the final OVA challenge. In OVA-induced asthmatic lung tissue, we observed a marked infiltration of inflammatory cells into perivascular and peribronchial connective tissues,

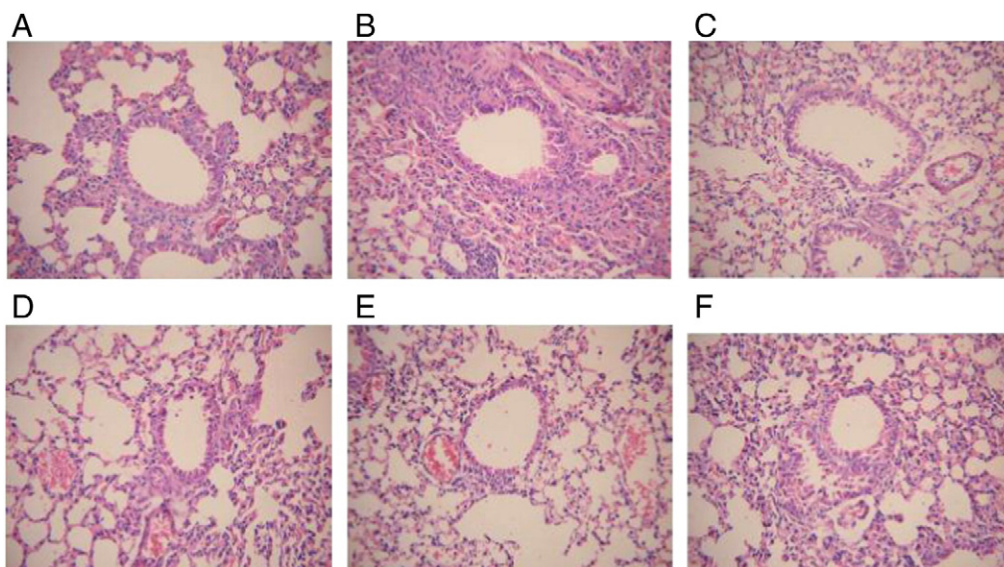


**Fig. 4.** Pathological changes of lung tissues observed by HE staining (Light microscopy,  $\times 200$ ): A (control); B (model); C (dexamethasone); D [curcumin treatment ( $50\text{ mg/kg}$ )]; E [curcumin treatment ( $100\text{ mg/kg}$ )]; and F [curcumin treatment ( $200\text{ mg/kg}$ )].

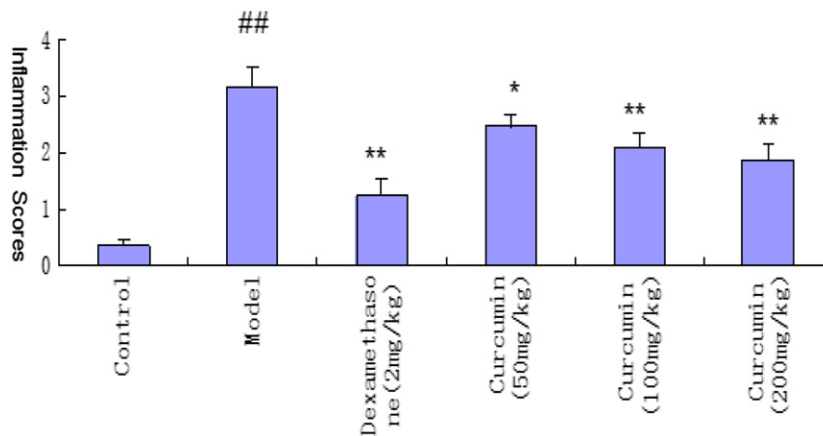


**Fig. 5.** Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections. Values are expressed as means  $\pm$  SDs. Compared with control: # $P < 0.05$ , ## $P < 0.01$ ; compared with model: \* $P < 0.05$ , \*\* $P < 0.01$ .

compared with the normal tissue. Moreover, most leukocytes were eosinophils. The control group displayed no change in the extent of inflammatory cell infiltration, similar to what was seen in dexamethasone-treated positive control mice. Eosinophilia in OVA-sensitized and challenged mice treated with curcumin was significantly attenuated compared with the level seen in OVA-challenged mice as shown in (Fig. 4) and (Fig. 5).

### 3.4. Effect of curcumin on cytokine levels in BAL fluid

To determine whether curcumin influenced cytokine secretion into BAL fluid, IL-10 and IL-17A in the BAL fluid were measured by ELISA 24 h after the final challenge. As shown in (Fig. 6), as for IL-17A, OVA challenge triggered significant increases in cytokine levels in the BAL fluid, compared with control fluid. In OVA-sensitized and -challenged mice treated with curcumin, the rises in cytokine levels were significantly suppressed, compared with the concentrations seen in the

OVA-challenged group; as for IL-10, OVA challenge triggered significant decreases in cytokine levels in the BAL fluid, compared with control fluid. In OVA-sensitized and -challenged mice treated with curcumin, the rises in cytokine levels were significantly strengthened, compared with the concentrations seen in the OVA-challenged group.

### 3.5. Effect of curcumin on Tregs and Th17 cells

As shown in (Fig. 7), (Fig. 8) and (Fig. 9), the frequencies of Treg cells in model group ( $1.10 \pm 0.34\%$ ) were lower than those in the normal group ( $0.53 \pm 0.15\%$ ), while in dexamethasone group and treatment groups were significantly higher than those in model group ( $P < 0.05$ ); the frequencies of Th17 cells in model group ( $0.97 \pm 0.22\%$ ) were higher than those in the normal group ( $9.62 \pm 1.57\%$ ), while in dexamethasone group and treatment groups were significantly lower than those in model group ( $P < 0.05$ ).

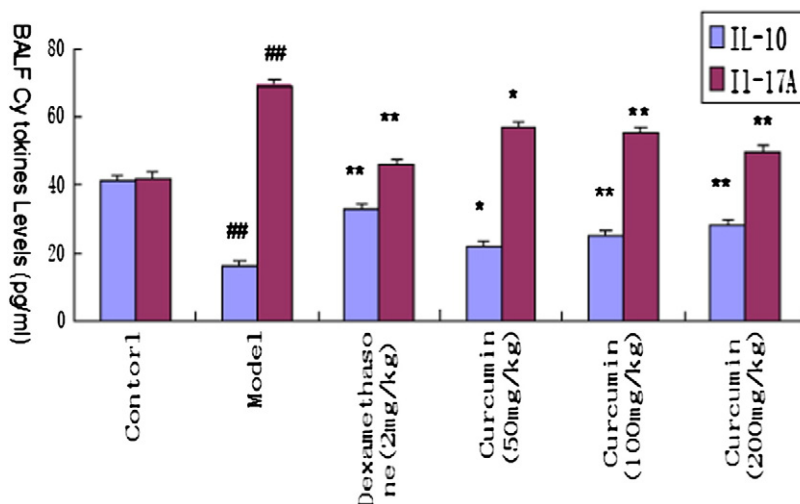


**Fig. 6.** Effect of curcumin on cytokine levels in BAL fluid. Values are expressed as means  $\pm$  SDs. Compared with control: # $P < 0.05$ , ## $P < 0.01$ ; compared with model: \* $P < 0.05$ , \*\* $P < 0.01$ .

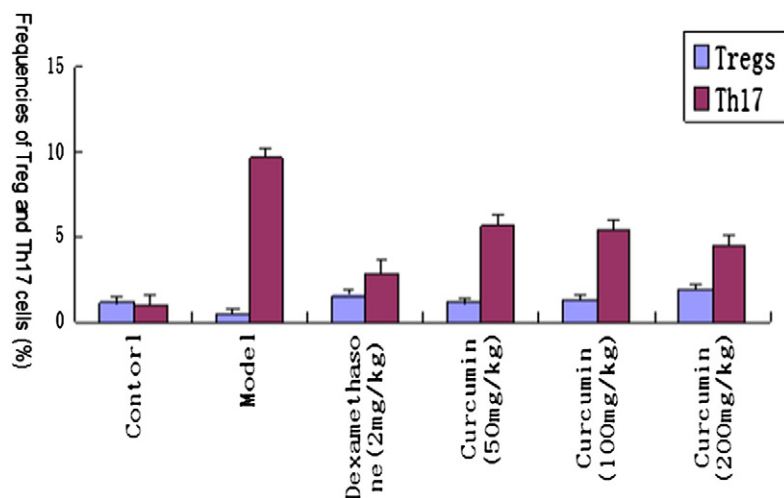


**Fig. 7.** Frequencies of Treg and Th17 cells. Values are expressed as means  $\pm$  SDs. Compared with control: #P < 0.05, ##P < 0.01; compared with model: \*P < 0.05, \*\*P < 0.01.

#### 4. Discussion

Asthma, a chronic inflammatory disease of the airways, affects many individuals worldwide. The disease may cause

severe morbidity, and even mortality if exacerbated. To date, a variety of drugs have been developed to treat asthma; these include steroids, leukotriene inhibitors, mast cell stabilizers, and  $\beta_2$  adrenergic agonists. Many nature products were

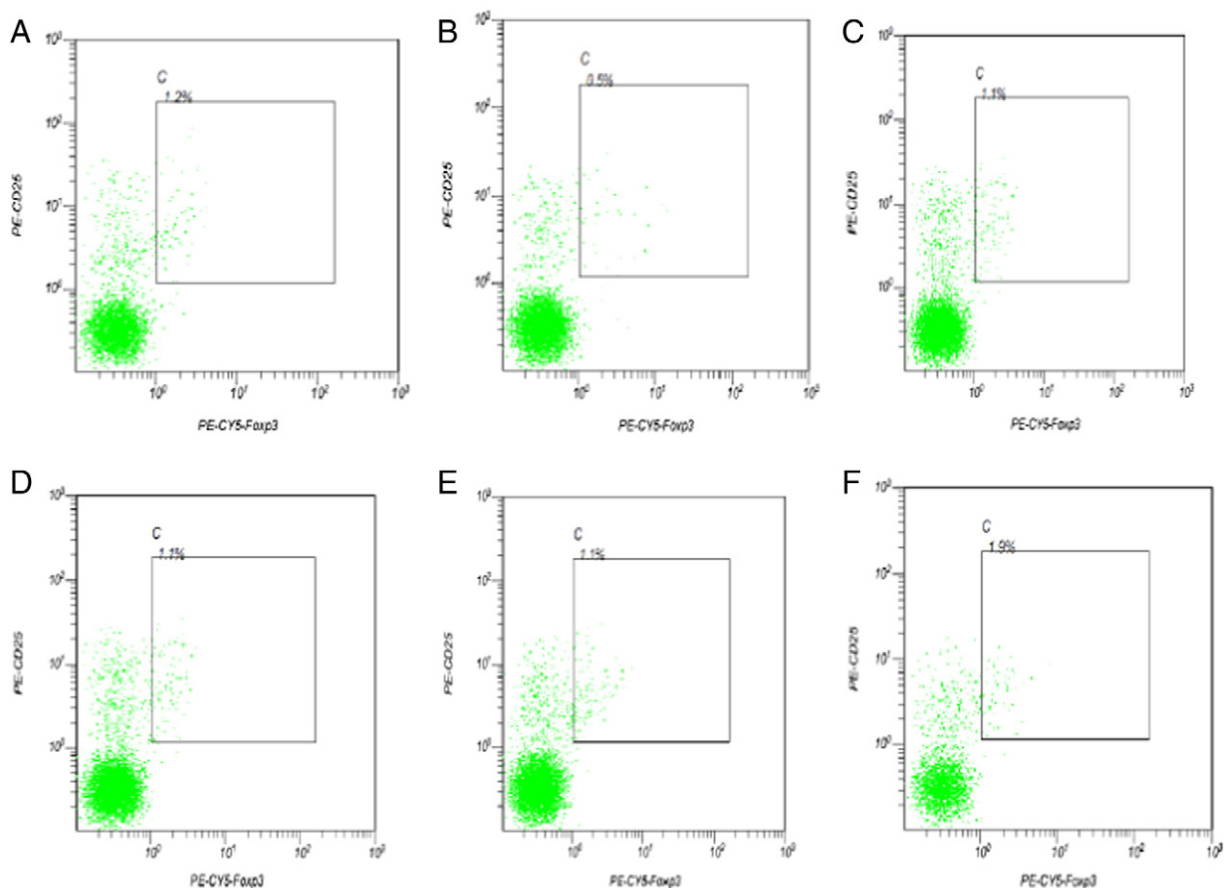


**Fig. 8.** Effect of curcumin on Tregs: A (control); B (model); C (dexamethasone); D [curcumin treatment (50 mg/kg)]; E [curcumin treatment (100 mg/kg)]; and F [curcumin treatment (200 mg/kg)].

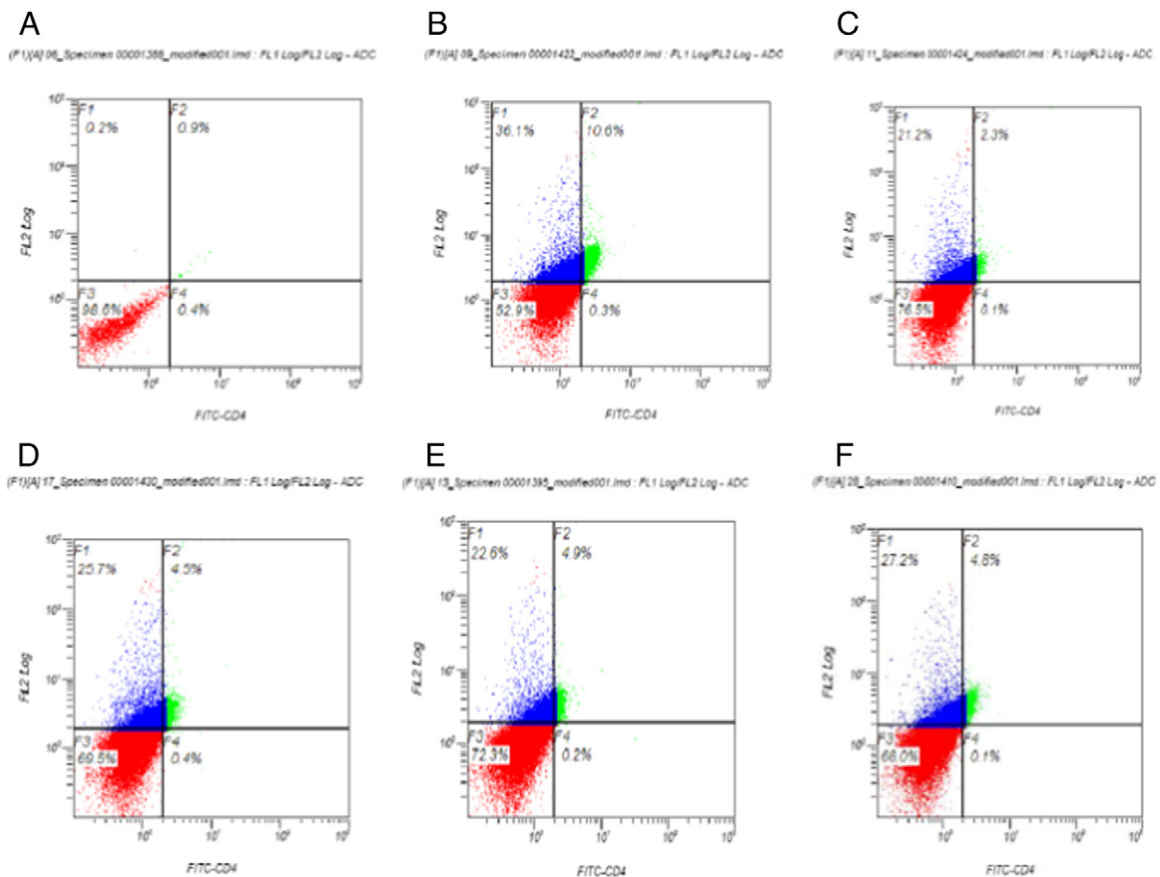


**Fig. 9.** Effect of curcumin on Th17: A (control); B (model); C (dexamethasone); D [curcumin treatment (50 mg/kg)]; E [curcumin treatment (100 mg/kg)]; and F [curcumin treatment (200 mg/kg)].

commonly used to treat asthma, but the therapeutic efficacies and modes of action of such medicines are currently unclear. Here, using an OVA-induced mouse model of asthma, we show for the first time that curcumin can be effective as a therapeutic drug for the treatment of allergic asthma.

Asthma is defined as a variable level of airway obstruction usually accompanied by AHR. Bronchoconstriction attributable to contraction or hypertrophy of airway smooth muscle (ASM), and inflammation within the airway, leads to decreased lung function [34]. AHR is a measure of the bronchial constriction commonly observed in individuals with asthma. We monitored the effects of curcumin on AHR by measuring Raw. These experiments disclosed that curcumin inhibited the OVA-induced AHR in response to inhaled methacholine.

The mechanisms of recruitment of inflammatory cells associated with and presumably causing AHR have been well-studied. Migration of inflammatory cells, specifically eosinophils and lymphocytes, into the lung is a major contributor to the development of allergic airway inflammation [35]. A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that curcumin significantly reduces eosinophil numbers in the BAL fluid and lung tissue.

Previous studies documented that the underlying process that drives and maintains the asthmatic inflammatory response appears to be an imbalance of the equilibrium between T helper

cell type 1 (Th1) and T helper cell type 2 (Th2) immune response, with a bias towards an increased Th2 response [36]. Recently, regulatory T cells (Treg cells) and T-helper17 (Th17) cells have been described as two distinct subsets from Th1 and Th2 cells that play opposite roles in vivo [6]. The Treg plays an important role in regulating the immune response. Tregs can reduce inflammation and allergic reactions through their inhibitory functions. Using a murine model of asthma Dongaci et al. [37] provided evidence that the diminished levels of Tregs resulted in increased airway hyperreactivity, thus linking Tregs to airway physiology [38]. Given the capacity of Tregs to suppress disease-promoting immune responses, enhancing Treg function in patients represents an attractive therapeutic strategy for treating allergic diseases, including asthma. Data suggest that IL-17 produced by Th17 cells plays an important role in the pathogenesis of different chronic inflammatory processes, such as rheumatoid arthritis, autoimmune blistering skin diseases, psoriasis, inflammatory bowel diseases, and multiple sclerosis [39–41]. Recent studies using mouse asthma models have shown that Th17 cells and their cytokines, such as IL-17A and IL-17F, are involved in antigen-induced neutrophil recruitment as well as the enhancement of Th2 cell-mediated eosinophil recruitment into the airways [42]. Previous studies with asthmatic patients have shown the increased levels of Th17 cytokines in lung tissue, sputum, bronchoalveolar lavage fluid, and correlation of IL-17 with airway hypersensitivity as

well as asthma severity [43–45]. In this study, our data show that curcumin significantly suppressed IL-17A cytokine level; and significantly increased IL-10 cytokine level. Moreover, curcumin significantly suppressed Th17 cells and increased Tregs.

In summary, curcumin effectively suppressed IL-17A, improved IL-10 and inhibited both the recruitment of eosinophils and mucus overproduction, in mice with OVA-induced asthma. Airway inflammation and bronchial hyperresponsiveness were attenuated in the presence of curcumin. Moreover, curcumin effectively suppressed Th17 cells and increased Tregs. Our findings support the possible use of curcumin as a therapeutic drug for patients with allergic asthma.

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