Curcumin attenuates ovalbumin-induced asthma via regulating Th9 cell production

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Abstract: Curcumin is a polyphenol compound with anti-inflammatory effect. It can reduce the oxidative stress response of asthmatic patients by inhibiting the production of NO, thus alleviating the occurrence of airway inflammation. In this study, we further studied the molecular mechanism of curcumin in ovalbumin (OVA)-sensitized mice. Compared with OVA group, injection of curcumin reduced the number of inflammatory cells in bronchoalveolar lavage fluid (BALF) and attenuated airway sensitivity in mice. ELISA tests showed that curcumin reduced levels of tumor growth factor (TGF)-β, interleukin (IL)-4, IL-9, and IL-10 in BALF; IL-9 and IL-10 were also reduced in lung tissues. Western blot showed that curcumin inhibited collagen deposition and α-smooth muscle actin (α-SMA) secretion in lung tissues; meanwhile TGF-β and phosphorylated Smad3 was down-regulated. Flow cytometry analysis showed that the proportion of T helper (Th) 9 cells in peripheral blood mononuclearcells decreased after curcumin injection. These results suggested that curcumin could attenuate OVA-induced asthma in mice via regulating Th9 differentiation. Inhibition of TGF-β/Smad signaling pathway was probably involved.

Key words: curcumin, asthma, interleukin 9, T helper cell 9, tumor growth factor-β

Introduction

Asthma is a complex airway disease characterized by chronic inflammation and airway hyper-responsiveness. According to surveillance data of the Global Initiative for Asthma (GINA), there are estimated to be 300 million asthmatic patients worldwide (1). At present, there are about 30 million asthmatic patients in China and asthma has become an important public health problem (2).

Curcumin is a kind of fat-soluble polyphenol pigment extracted from *Curcuma longa*, which has good anti-inflammatory, anti-oxidation, anti-microbial and other immune regulatory effects (3). It has many clinical applications in cardiovascular system diseases, digestive system diseases and tumors (4). Previous study showed that dietary supplementation of curcumin could improve airway inflammation in allergic asthmatic mice, reduce the number of inflammatory cells around the bronchus, and reduce the levels of interleukin (IL)-4 and tumor growth factor (TGF)-β in bronchoalveolar lavage fluid (BALF) (5). Naïve $CD4^+$T cells differentiates into T helper (Th) 0 cells after stimulation of antigens, and then continues to differentiate into Th9 cells in the presence of TGF-β and IL-4, in which STAT and Smad are the main signaling pathways involved (6,7). Th9 cells preferentially secrete high level of IL-9. The involvement and therapeutic value of IL-9 in asthma has been elucidated in a large number of studies. IL-9 has been identified as an essential factor in mucosal immunity and airway remodeling (8,9). Therefore, we hypothesized that curcumin might play a role in regulating IL-9 secretion and alleviating asthma susceptibility by affecting the differentiation of Th9 cells.

Materials and methods

Animals and ovalbumin (OVA) sensitization

Female BALB/c mice (8-10 weeks of age) at specific pathogen free grade were used in this study. The animal work protocol was reviewed and approved by the Institutional Animal Work Committee of the First Affiliated Hospital of Chengdu Medical College. Twenty-four mice were randomly divided into three groups: control

group, ovalbumin (OVA) sensitization group, and curcumin treatment group. The mice in OVA group and curcumin group were injected with 0.2 ml of sensitization solution (containing 400 μg of aluminium hydroxide and 100 μg of OVA) intraperitoneally on day 1, 7, and 14, respectively. From day 21-day 24, the mice were challenged with 1% OVA solution (once a day for 30 min) to induce asthma using an ultrasonic nebulizer. OVA powder was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Curcumin administration

From day 21-day 24, curcumin was dissolved in 0.9% saline and intraperitoneally injected (20 mg/kg body weight) 30 min before OVA challenge. Curcumin was purchased from Sigma-Aldrich.

Measurement of airway responsiveness

After 24 h of the last challenge, the mice were subjected to intraperitoneal injection of pentobarbital sodium (90 mg/kg body weight), tracheal intubation, and connection to animal ventilator. The respiratory rate was adjusted to 90 times/min and tidal volume was 200 ml. Different doses of acetylcholine chloride (10, 30, 90, 270 μg/kg body weight) were administered via caudal vein. The maximum inspiratory airway resistance (RL) was recorded as reported (10).

Leukocyte count in BALF

After measurement of airway responsiveness, 2 ml of PBS was injected into the tracheal intubation and left lung lavage was performed three times. BALF was centrifuged at 4℃ and 1500 r/min for 10 min. The precipitation was suspended with 0.5 ml of PBS. Total cell numbers of 0.1 ml suspension were counted with a hemocytometer. Cell smears were prepared with 0.1 ml suspension and stained with eosin and methylene blue for differential cell counting. Two independent investigators counted the cells under an optical microscope and approximately 200 cells were counted.

Measurement of cytokines in BALF

The supernatant of BALF after centrifugation was collected for detection of

cytokine of tumor growth factor-β, IL-4, IL-9, and IL-10. Levels of these cytokines were quantified by ELISA according to the manufacturer's instruction (Cusabio Technology, Wuhan, China).

Histological examination

Left lung tissues of mice were fixed with 4% (v/v) paraformaldehyde for 24 h. After paraffin embedding, the lung tissues were sectioned continuously into 3 μm thickness. The slices were dehydrated with gradient alcohol, dewaxed with xylene, rehydrated with gradient alcohol, and then stained with hematoxylin and eosin (HE). The stained sections were observed under optical microscope.

Western blot

Total protein was extracted from the right lung tissue of mice. The protein concentration was determined according the instruction of BCA kit (Cusabio Technology). Twenty μg of total protein was electrophoresed at 80 V voltage in 10% SDS-PAGE. After electrophoresis, the protein bands were transblotted onto PVDF membrane at 300 mA and 4℃. After blocking with 5% skimmed milk at room temperature for 1 h, the membrane was incubated with primary rabbit antibodies against IL-9 (ab133675), IL-10 (ab9969), α-smooth muscle actin (SMA, ab32575), matrix metalloproteinases (MMP) 9 (ab228402), TGF-β1 (ab92486), Smad (ab40854), Smad3 (phospho S423+S425, ab52903), and GAPDH (ab181602) at 4℃ for overnight. After routine washing with TBST, the second antibody (goat anti-rabbit IgG, ab97051) was added and incubated at room temperature for 2 h. The antibodies were purchased from Abcam (Cambridge, UK). The protein bands were then visualized with ECL reagent and analyzed with Image J software.

Flow cytometry sorting of Th9 cells

After collection of BALF, peripheral blood mononuclear cells (PBMC) were isolated from venous blood by using lymphocyte separation solution (Tianjin Hao Yang Biological Manufacture Co. Ltd., Tianjin, China). Cells were suspended in RPMI 1640 medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and the cell density was adjusted to 5×10^6/ml. The cells were then inoculated in

24-well plates and incubated in 37℃ and 5% CO_2 incubator. Cells were stimulated with PMA/ionomycin for 4 h and followed by BFA/monensin incubation for 2 h. Cells were collected and stained with antibody against PE-CD4 at room temperature for 30 min. Cells were then fixed and permeabilized. Antibody against FITC-IL-9 was added and incubated for 30 min. All the antibodies and relative reagent were purchased from eBioscience (San Diago, CA, USA). The cells were analyzed by flow cytometry (FACS Aria by BD Bioscience, San Jose, CA, USA) to calculate the proportion of IL-9^+ cell subsets (Th9 cells) in total $CD4^+$ T lymphocytes.

Statistics

SPSS version 17.0 was used in data analysis. Continuous variables were expressed as mean ± standard deviation (SD). One-way ANOVA was used for comparison among multiple groups when continuous data accorded with normal distribution. A *P* value < 0.05 was considered as statistical significance.

Results

Curcumin attenuates airway hyper-response and infiltration of inflammatory cells in OVA-sensitized mice

Acetycholine chloride was used to stimulate airway resistance in mice. There was no significant difference in airway resistance between groups at low dose (10 μg/kg and 30 μg/kg); at medium and high dose (90 μg/kg and 270 μg/kg), airway resistance in asthma group was significantly higher than that in other groups (P<0.05). Curcumin groups could reduce airway resistance in mice and restore it to the level of control group, as shown in Figure 1A. Pathological pictures of typical lung tissue stained with HE are shown in Figure 1B. Under the light microscope, the alveolar wall of normal mice was intact with no exudates in the alveolar cavity. Alveolar epithelial cells arranged orderly and alveolar wall thickness was normal. Pathological pictures of asthmatic mice showed impaired alveolar wall structure, interstitial congestion, edema, alveolar wall thickening, and infiltration of inflammatory cells such as eosinophils and lymphocytes around the bronchus. The changes of alveolar

structure and the infiltration of inflammatory cells in curcumin treated mice were less severe than those in asthmatic mice, but there were still pathological changes such as alveolar septum thickening. In the control group, the white blood cells in BALF were mainly monocytes, with a few lymphocytes and very few eosinophils and neutrophils. The proportion of eosinophils, lymphocytes, and neutrophils in BALF of asthmatic mice increased significantly compared with the control group (all $P<0.01$); the proportion of above-mentioned white blood cells in curcumin group was significantly lower than that in the asthma group, but still higher than that in the control group (Figure 1C).

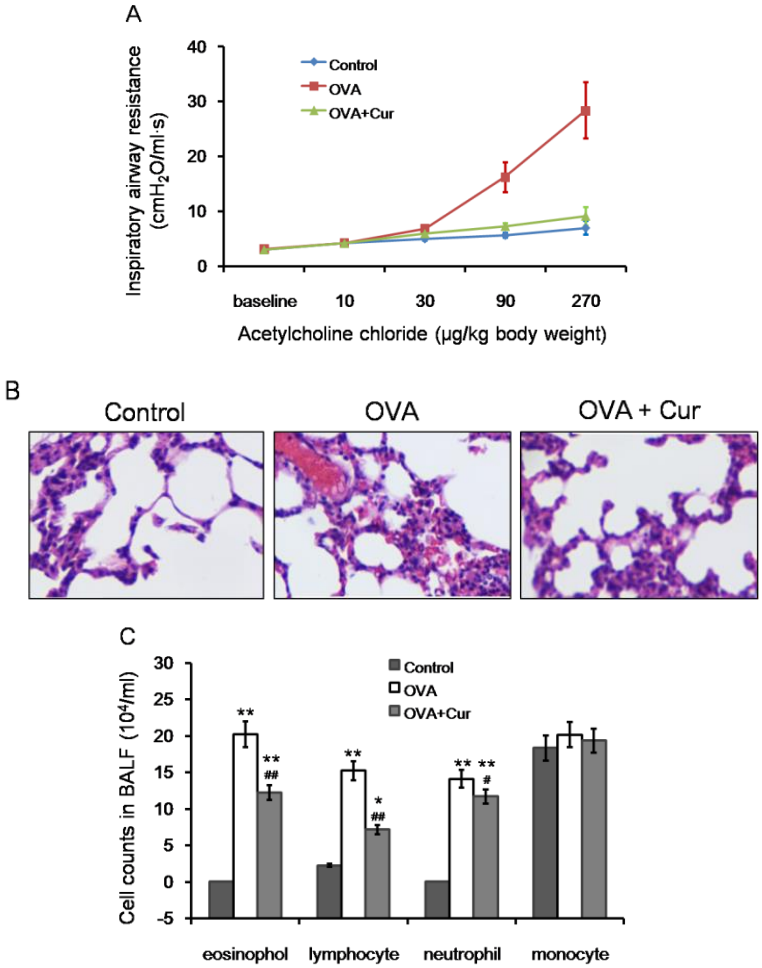


Figure 1. Curcumin attenuates airway hyper-response and infiltration of inflammatory cells in OVA-sensitized mice

A: Typical histological sections of different groups (×200 magnification). B: Effect of curcumin on the development of airway hyper-responsiveness. C. Effect of curcumin on the leukocyte counts in BALF.

* $P< 0.05$ vs. control; ** $P< 0.01$ vs. control; $^{\#}P < 0.05$ vs. OVA; $^{\#\#}P< 0.01$ vs. OVA.

Curcumin decreases production of inflammatory cytokines and collagen deposition via suppressing TGF-β/Smad signaling

According to the pathological results of HE, we further tested the exudation of inflammatory factors in BALF (Figure 2A). The contents of TGF-β and IL-4 in BALF of OVA-sensitized mice were significantly higher than those of control mice. Because TGF-β and IL-4 can promote the naïve T cells to differentiate into Th9 cells, we also detected exudation of IL-9 and IL-10, the main effector cytokines of Th9 cells. IL-9 and IL-10 also increased in BALF of OVA-sensitized mice. The content of TGF-β, IL-9, and IL-10in BALF of curcumin-treated mice was significantly decreased, but still higher than that of control mice. Although the decrease of IL-10 was not obvious after curcumin treatment, it was reduced to the same level as the control group. Western blot also showed that the expression of IL-9 and IL-10 in lung tissues of OVA-sensitized mice increased, and decreased after curcumin treatment (Figure 2B-2C). Western blot showed that the contents of α-SMA and MMP9 in lungs of OVA-sensitized mice were higher than those of control group, while curcumin could reduce the secretion and accumulation of these extracellular matrix (ECM) components. Smad pathway is a classical downstream pathway of TGF signal. Western blot showed that TGF/Smad pathway could be activated by OVA, while curcumin could inhibit the expression of TGF-β and phosphorylated Smad3 (S423+S425). The results suggested that curcumin could treat asthma through the TGF/Smad pathway.

Curcumin reduces Th9 differentiation in PBMC

The purified mouse PBMC-derived $CD4^+$ T cells were analyzed by flow cytometry to detect Th9 cells. The results showed that the proportion of Th9 cells in PBMC of OVA-sensitized asthmatic mice increased significantly, while curcumin decreased the proportion of Th9 cells (Figure 3). The results suggested that the mechanism of curcumin reducing IL-9 content in BALF and lung tissue might be related to the inhibition of Th9 cell differentiation.

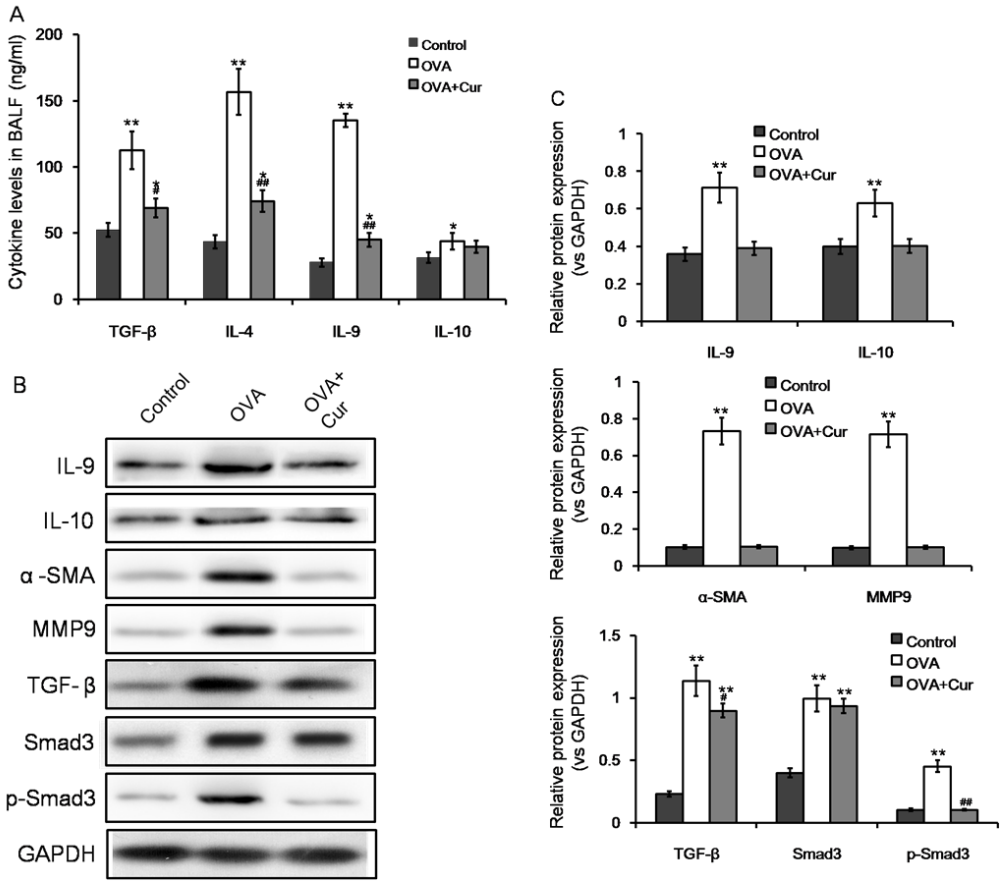
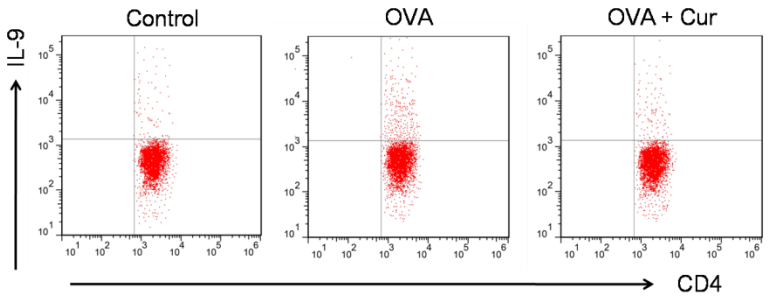


Figure 2. Curcumin decreases production of inflammatory cytokines and collagen deposition via suppressing TGF-β/Smad signaling

A: Levels of TGF-β, IL-4, IL-9, and IL-10 in BLAF. B-C: Protein levels of IL-9, IL-10, α-SMA, MMP9, TGF-β, Smad3, and p-Smad3 in lung tissues. * P< 0.05 vs. control; ** P< 0.01 vs. control; $^{\#}P$ < 0.05 vs. OVA; $^{\#\#}P$< 0.01 vs. OVA.



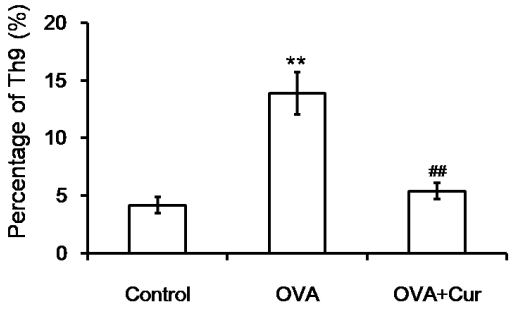


Figure 3. Curcumin inhibits Th9 differentiation in peripheral blood mononuclear cells

** P< 0.01 vs. control; $^{\#\#}P$< 0.01 vs. OVA.

Discussion

Asthmatic patients have high airway responsiveness to various provocative factors, which can cause airway stenosis, manifested as recurrent wheezing, dyspnea, chest tightness or cough, often occurring and aggravating at night and morning. Our research studied the effect of curcumin on airway inflammation and airway hyper-responsiveness in acute asthma. The results showed that curcumin could reduce the recruitment of airway inflammatory cells, the expression of inflammatory factors, and alleviate airway hyper-responsiveness. Our results also suggested that curcumin could reduce the production of IL-9 and IL-10 by inhibiting Th9 cell differentiation.

Eosinophils generally account for less than 1% of white blood cells. In the course of bronchial asthma, eosinophils increase and infiltrate airway tissue is a characteristic of airway inflammation (11). Eosinophils can release a variety of cytokines and cytotoxic protein particles, causing airway epithelial damage, airway epithelial stripping, and airway hyper-responsiveness (12). In this study, we also found that lung tissue sections of asthmatic mice showed a large number of inflammatory cell infiltration, mucosal epithelial exfoliation, narrow lumen, and a large increase in eosinophil content in BALF, presenting typical asthmatic symptoms. It is found that eosinophils can secrete eosinophil cation protein (ECP), which induces fibroblasts to secrete TGF-β1 (13); ECP can also make fibroblasts transform into myofibroblasts and secrete a large number of ECM components, causing thickening of airway wall,

eventually leading to airway remodeling (14). Consistent results were found in this study. The content of TGF-β increased in BALF and lung tissue of asthmatic mice; ECM components, α-SMA and MMP9, also increased in lung tissues. Curcumin treatment significantly inhibited eosinophil production and reduced content of TGF-β, α-SMA and MMP9. Similar results were reported before. Curcumin plays an anti-fibrotic role in bleomycin-induced pulmonary fibrosis animals, and the mechanism could be explained by inhibiting transformation of lung fibroblasts into myoblasts (15,16).

Asthma is usually considered to be an inflammatory reaction of Th2 type (17). The high secretion of Th2 cytokine IL-4 and the high secretion of TGF-β by fibroblasts constitute the conditions for inducing Th9 differentiation. Although IL-9 can also be secreted by Th2, it is mainly an effector of Th9 cells, so the differentiation of Th9 should be changed when asthma occurs. Our study showed that the proportion of Th9 cells increased significantly in OVA-sensitized mice, and the content of IL-9 changed accordingly. IL-10, another effector of Th9, also increased after OVA sensitization and decreased after curcumin treatment. These results suggest that Th9 is indeed involved in the occurrence of asthma, and curcumin can target Th9 cells to alleviate asthma symptoms. In addition to inhibiting the Th9 differentiation, previous study has confirmed that curcumin’s antioxidant effect is also a mechanism for alleviating asthma (5). Nevertheless, little is known about the therapeutic value and molecular mechanism of curcumin in asthma, and further research is needed.

References

1. Masoli M, Fabian D, Holt S, et al: The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy 59:469-478, 2004.
2. ZH C, PL W, HH S: Asthma research in China: A five-year review. Respirology Suppl 3:10-19, 2013.
3. Goel A, Kunnumakkara AB, Aggarwal BB: Curcumin as "Curecumin": From kitchen to clinic. Biochem Pharmacol 75:787-809, 2008.
4. Hatcher H, Planalp RP, Cho J, et al: Curcumin: from ancient medicine to current clinical

trials. Cell Mol Life Sci 65:1631-1652, 2008.

5. Moon D, Kim M, Lee H, et al: Curcumin attenuates ovalbumin-induced airway inflammation by regulating nitric oxide. Biochem Biophys Res Commun 375:275-279, 2008.
6. Elyaman W, Bassil R, Bradshaw EM, et al: Notch receptors and Smad3 signaling cooperate in the induction of interleukin-9-producing T cells. Immunity 36:623-634, 2012.
7. Kaplan MH, Hufford MM, Olson MR: The development and in vivo function of T helper 9 cells. Nat Rev Immunol 15:295-307, 2015.
8. Zhou Y, Mclane MP, Levitt RC: Th2 cytokines and asthma-Interleukin-9 as a therapeutic target for asthma. Respir Res 2:80-84, 2001.
9. Oh CK, Raible D, Geba GP, et al: Biology of the interleukin-9 pathway and its therapeutic potential for the treatment of asthma. Inflamm Allergy Drug Targets 10:180-186, 2011.
10. Du Q, Chen Z, Zhou L, et al: Inhibitory effects of astragaloside IV on ovalbumin-induced chronic experimental asthma. Can J Physiol Pharmacol 86:449-457, 2008.
11. Walford HH, Doherty TA: Diagnosis and management of eosinophilic asthma: a US perspective. J Asthma Allergy 7:53-65, 2014.
12. Matucci A, Vultaggio A, Maggi E, et al: Is IgE or eosinophils the key player in allergic asthma pathogenesis? Are we asking the right question? Respir Res 19:113, 2018.
13. Possa SS, Leick EA, Prado CM, et al: Eosinophilic inflammation in allergic asthma. Front Pharmacol 4:46-46, 2013.
14. Bystrom J, Amin K, Bishopbailey D: Analysing the eosinophil cationic protein - a clue to the function of the eosinophil granulocyte. Respiratory Research 12:10-10, 2011.
15. Liu D, Gong L, Zhu H, et al: Curcumin inhibits transforming growth factor β induced differentiation of mouse lung fibroblasts to myofibroblasts. Front Pharmacol 7:419, 2016.
16. Punithavathi D, Venkatesan N, Babu M: Curcumin inhibition of bleomycin-induced pulmonary fibrosis in rats. Br J Pharmacol 131:169-172, 2000.
17. Steinke JW, Borish L: Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. Respir Res 2:66-70, 2001.