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Epigallocatechin gallate ameliorates airway inflammation by regulating Treg/Th17 imbalance in an asthmatic mouse model☆

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ABSTRACT

Epigallocatechin gallate (EGCG) is a polyphenol that is found in green tea that has been shown to ameliorate airway inflammation in an ovalbumin-sensitized asthmatic mouse model. The purpose of this study was to investigate whether the immunomodulatory and anti-inflammatory effects of EGCG by regulating the regulatory T cell (Treg)/Th 17 cells balance in this model. Female BALB/c mice were sensitized and challenged with ovalbumin by intraperitoneal injection. EGCG was administered to asthmatic mice intraperitoneally 1 h before each OVA challenge. Airway hyperresponsiveness (AHR) was measured, and lung inflammatory infiltrations were assessed by hematoxylin and eosin (HE) staining. Serum OVA-specific IgE levels, Interleukin-10 (IL-10) levels and Interleukin-17A (IL-17A) levels in the bronchoalveolar lavage fluid (BALF), serum, and splenocyte culture supernatants were measured by ELISA. Flow cytometry was used to assess the effects of EGCG on the frequency of $CD4^+CD25^+Foxp3^+$Treg cells in the splenocytes and real-time PCR method was used to measure the expression of Forkhead box P3 (Foxp3) mRNA and retinoid-related orphan receptor gammat (RORγt) mRNA in the lung tissue. The results showed that administration of EGCG significantly decreased AHR and OVA specific IgE in the serum, increased IL-10 levels in the BALF, serum, and splenocyte culture supernatant, and the frequency of $CD4^+CD25^+Foxp3^+$Treg cells in the splenocytes in asthmatic mice. Administration of EGCG also ameliorated airway inflammation and eosinophil infiltrations in asthmatic mice. These results suggested that EGCG likely ameliorated OVA-induced airway inflammation by increasing the production of IL-10, the number of $CD4^+CD25^+Foxp3^+$Treg cells and expression of Foxp3 mRNA in the lung tissue, and it could be an effective agent for treating asthma.

1. Introduction

Bronchial asthma is a heterogeneous disease that is typically characterized by airway inflammation, airway hyperresponsiveness (AHR) and airway remodeling [1,2]. Its pathogenesis is complex and has not yet been fully understood. Related studies have confirmed that T cells played an important role in the development of asthma [3]. Previous studies have shown that the typical symptoms of asthma such as recurrent wheezing and shortness of breath were a part of an inflammatory reaction that was caused by Th2 cells and other inflammatory cells, such as eosinophils, macrophages and neutrophils, as well as inflammatory cytokines and chemokines [4]. Th2 superior response is the mechanism for the formation and development of asthma. The cytokines such as Interleukin (IL)-4 and IL-5 which were secreted by Th2 cells have been shown to play a vital role in allergic airway inflammation [4,5]. IL-4 is pivotal for the initiation of Th2 inflammatory responses [6]; IL-5 is known to play an important role in eosinophil maturation, differentiation, recruitment and survival [7]. However, the treatment of cytokines secreted by Th2 cannot completely effectively reduce airway inflammation of asthma. Therefore, the pathogenesis of asthma cannot be simply summarized as Thl/Th2 imbalance. Scholars have confirmed that Treg and Thl7 cells and their representative cytokines IL10 and IL-17 were significantly related to the onset of asthma. And the immune imbalance of Treg/Thl7 is also one of the important mechanisms for asthma [8–12]. The equilibrium between Treg and Th17 cells is extensively deliberated in the studies of allergic diseases including asthma.

The prevalence of asthma is increasing in both developed and developing countries [13]. Although great progress has been made in the diagnosis and treatment of asthma, it remains a severe public health

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issue, particularly in children [14]. At present, inhaled glucocorticoids (ICS) are the first choice for the treatment of asthma. Although inhaled glucocorticoids are usually effective, long-term use of glucocorticoids may lead to many side effects, and also may cause drug dependence or drug resistance. Therefore, it is still urgent to find a safe and effective drug that can replace glucocorticoids for asthma treatment.

Epigallocatechin-3-gallate (EGCG) is the major polyphenol extracted from green tea. It has many biological effects, and obvious preventive and therapeutic effects on anti-inflammatory, antivirus, anti-tumor, anti-oxidant and immunomodulatory activities [15–18]. Previous studies have shown that EGCG can enhance the activity of Treg cells in autoimmune disease. However, the immunomodulatory effect of EGCG in asthma has still not been clarified. Therefore, we hypothesized that EGCG may play a role in ameliorating airway inflammation in an ovalbumin-sensitized asthmatic mouse model via maintaining the balance between Treg cells and Th17 cells. In the present study, we sought to determine the immunomodulatory effect of EGCG on airway inflammation and identified its underlying mechanisms in ovalbumin (OVA) sensitized asthmatic mice model, so as to provide theoretical basis for clinical prevention and treatment of asthma.

2. Materials and methods

2.1. Drugs and reagents

EGCG, OVA and aluminum hydroxide were purchased from Sigma (Sigma-Aldrich Beijing, China). The purity of EGCG was determined to be above 99% by HPLC analysis.

2.2. Animals and experimental protocol

Fifty healthy female BALB/c mice (specific-pathogen-free, 18–22 g, 4–6 weeks of age) were purchased from the experimental animal center of Shengjing Hospital of China Medical University. All experimental protocols involving animals were approved by the China Medical University Animal Care Committee and complied with the guidelines of the China Council on Animal Care. All the mice were housed in an environment with temperature of 22 ± 2 °C, relative humidity of 50 ± 1%.

Fifty mice were randomly divided into five groups (n = 10/group): (1) the negative control group that was sensitized and challenged with phosphate-buffered saline (PBS); (2) the asthma group that was sensitized and challenged with OVA (Grade V, Sigma-Aldrich Beijing, China); (3) OVA + EGCG (5 mg/kg) treatment group that received 5 mg/kg EGCG by intraperitoneal (i.p.) injection 1 h before each OVA challenge; (4) OVA + EGCG (50 mg/kg) treatment group that received 50 mg/kg EGCG by i.p. injection 1 h before each OVA challenge; and (5) OVA + dexamethasone (DXM) treatment group that received 2 mg/kg DXM by i.p. injection 1 h before each OVA challenge. This group was used as a positive control.

The mice, except the negative control group, were sensitized on days 1, 8 and 15 by i.p. injection of 20 μg OVA emulsified in 1 mg aluminum hydroxide in 200 μL of PBS. After the initial sensitization, from the day 22, the mice were challenged by an aerosolized of 1% OVA for 30 min/d in PBS using an ultrasonic nebulizer for 7 days. In the negative control group, OVA was replaced by PBS during sensitization and challenge. All of the drugs (EGCG and DXM) were dissolved in PBS in the same volume for administration (0.2 mL/mouse). EGCG (5 or 50 mg/kg per day) was administered by i.p. injection for 7 days 1 h before each challenge. DXM (2 mg/kg) was used as a negative control for the drug and was administered in the same manner. On day 29, enhanced pause (Penh) was assessed and on day 30, the mice were sacrificed. Serum, bronchoalveolar lavage fluid (BALF), lung tissue and splenocytes were collected for future analyses (Fig. 1).

2.3. Measurement of AHR to methacholine

Airway responsiveness was indirectly assessed 24 h after the last challenge using a single whole body plethysmography chamber. Each conscious mouse was challenged with methacholine aerosols in increasing concentrations (3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL in saline) for 3 min. After each methacholine challenge, Penh values were calculated for 3 min.

2.4. Collection of BALF and cell counts

Bronchoalveolar lavage (BAL) was performed 48 h after the last challenge. First, the mice were anesthetized and their tracheas were isolated by blunt dissection. A catheter was inserted into the airway. Three same 0.5 mL of PBS was dispensed into the lungs and gently aspirated three times. Each BALF sample was centrifuged (1000 rpm, 10 min at 4 °C) and the cell pellet was resuspended in 1 mL saline. To perform the differential leukocyte cell count, 0.1 mL of the cell suspension was drop on a glass slide and stained with Wright-Giemsa staining. Two hundred (200) nucleated cells were then examined under a microscope and classified as macrophages, lymphocytes, eosinophils and neutrophils.

2.5. Measurement of OVA-specific IgE level in serum

48 h after the last challenge, mice were anesthetized with 5% chloral hydrate, and blood was collected from the left side of the heart. Serum OVA-specific IgE measurement was performed using mouse OVA-IgE enzyme-linked immunosorbent assay (R&D ELISA, DoBio Biotech, Shanghai, China).

2.6. Histological examination of lung tissue by hematoxylin and eosin (HE) stain

Lungs were removed from the mice after sacrifice and then fixed with 4% paraformaldehyde. Routine histological staining methods were used. Sections of the left lung (4 μm thick) were stained with hematoxylin and eosin (H&E) staining to evaluate eosinophil infiltration.

2.7. Measurement of IL-10 and IL-17A levels in the serum, BALF and splenocyte culture supernatant

Spleens were removed 48 h after the last challenge and single-cell suspensions were prepared by gently pressing the spleen through a sterile 200-μm nylon cell screener. Then, splenocytes were cultured in RPMI 1640 culture media supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. 1×10^6 cells/mL from each group were seeded in 24-well flat-bottom plates at 37 °C in a 5% CO_2 incubator. The cells were stimulated with OVA (100 mg/L) for 48 h. The supernatant was collected and stored at −80 °C for further use.

IL-10 and IL-17A levels were measured in the serum, and the culture supernatants from BALF and splenocytes using commercial ELISA kits (R&D ELISA KIT, DoBio Biotech, Shanghai, China).

2.8. Flow cytometry analysis (FCA)

Splenocytes were analyzed for $CD4^+CD25^+Foxp3^+$ expression using a Treg cell staining reagent according to the manufacturer's instructions. Briefly, cells (1×10^5) were washed in flow cytometry staining buffer. Then, the cells were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD25 antibodies in staining buffer for 30 min at 4 °C. Next, the cells were fixed and permeabilized in a fixation/permeabilization solution for 30 min and stained with 0.5 μg of APC-labeled anti-mouse Foxp3. Finally, the cells were resuspended in flow cytometry staining buffer and analyzed by flow cytometry using a FACS

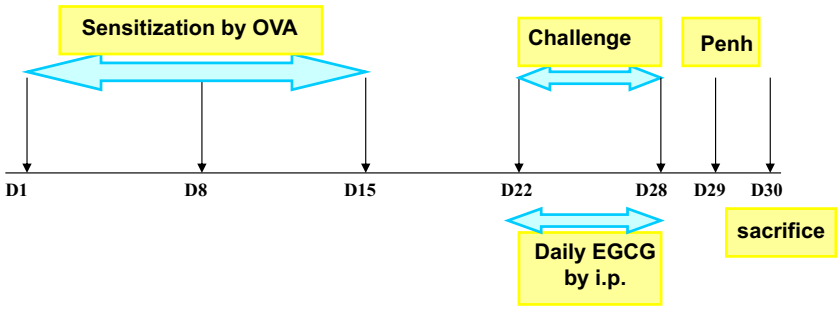

Fig. 1. Schematic illustration of the experimental design for establishing an asthmatic mouse model using OVA sensitization and challenge.

Calibur instrument with Cell Quest software (Beckman Coulter, USA).

2.9. Analysis of Foxp3 mRNA and RORγt mRNA expression in the lung tissue by quantitative real-time PCR

Quantitative real-time PCR was performed to determine the levels of Foxp3 mRNA and RORγt mRNA expression in the different mouse treatment groups. After the lung tissue was collected from each group of mice, the total RNA was extracted from the lung tissues using RNA iso™ Plus reagent (Takara, Dalian, China) and quantified using a spectrophotometer. Following quantification, 4 μg of mRNA was reverse transcribed to cDNA. The quantitative real-time PCR assays were conducted using an ABI PRISM 7500 real-time PCR System (Applied Biosystems, Foster City, CA, USA). PCR amplification was performed using the SYBR Prime Script™ RT-PCR kit reagent (Takara, Dalian, China). The PCR conditions were 40 cycles of denaturation at 95 °C for 5 s and annealing and extension at 60 °C for 30 s. Target mRNA levels were normalized to the housekeeping gene β-actin. The primers used were: Foxp3: forward 5′-ACTGGGCTTCTGGGTATGTC-3′, reverse 5′-TTTAGCTTGCGGCTCCTAAT-3′, RORγt: forward 5′-GACCCCACCTC ACA-3′, reverse 5′-AGTAGGCCACATTACACTGCT-3′, β-actin: forward 5′-CTGTGCCCATCTACGAGGGCTAT-3′, reverse 5′-TTTGATGTCACGCA CGATTTCC-3′. The gene expression data was analyzed using the $2^{-\Delta\Delta CT}$ method.

2.10. Statistical analysis

SPSS version 19.0 software was used for statistical analysis. All data were presented as the mean ± standard deviation (SD). Comparisons were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test. Calculations were performed with Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA, USA). P values of < 0.05 were considered statistically significant difference.

3. Results

3.1. Effect of EGCG on alleviating AHR in vivo

To assess the effects of EGCG on airway responsiveness, we measured as the Penh values induced by increasing doses of methacholine (Mch) (3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, 25 mg/mL and 50 mg/mL). As shown in Fig. 2, the AHR increased with the concentration of Mch inhaled in each group. The AHR of mice in the asthmatic group was significantly higher in comparison to the normal control group after induction by Mch ($P < 0.05$). However, treatment with EGCG and DXM caused an obvious decrease in the Penh values ($P < 0.05$).

3.2. Serum OVA-IgE measurement

As shown in Fig. 3A, serum OVA-IgE levels were significantly higher in the OVA-sensitized group than in the normal control group ($P < 0.05$). Serum OVA-IgE levels in the EGCG-treated groups were significantly decreased compared to the untreated asthmatic mice, but still higher than that in the normal control group ($P < 0.05$).

3.3. Effect of EGCG on alleviating airway inflammation and eosinophils infiltration in asthmatic mice

H&E staining of the lung tissue indicated that the asthmatic group had large quantities of inflammatory cell infiltrates in the peribronchial regions including eosinophils, neutrophils, and lymphocytes (Fig. 4), particularly eosinophils. The inflammatory cell infiltration was markedly reduced by treatment with EGCG. The number of inflammatory cells in BALF was also measured in different groups. Compared with the normal control group, the number of eosinophils and total white blood cells in BALF was significantly elevated in the asthmatic group ($P < 0.05$). By contrast, the number of eosinophils in the BALF was significantly lower in the EGCG treated group (Fig. 3B and C), and there was dose dependent effects of EGCG. These results supported that EGCG inhibited airway inflammation and inflammatory cells infiltration in the lung tissue of OVA-induced asthmatic mice.

3.4. Effects of EGCG on the IL-10 and IL-17A levels in the serum, splenocyte culture supernatants and BALF

To understand the effects of EGCG on the balance of Treg and Th17, we measured Treg and Th17 cytokines: IL-10 and IL-17A levels in the serum, BALF, and splenocyte culture supernatants by ELISA. The levels of IL-17 in the OVA-induced asthmatic mice was significantly reduced by EGCG, whereas the levels of IL-10 were upregulated by EGCG and DXM treatment in the serum, splenocyte culture supernatants and BALF of mice (Fig. 5, $P < 0.05$).

3.5. Effects of EGCG on the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen

Imbalances between Treg and Th17 cells involve in contributing to the pathogenesis of asthma. $CD4^+CD25^+Foxp3^+$ Treg cells play an important role in the immunoregulation of asthma, and the induction of allergen-specific Tregs has become an appealing strategy for asthma therapy. Thus, the relative proportions of Treg cells in the splenocytes were determined after treatment with different doses of EGCG.

Flow cytometric analysis was used to assess the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen. As shown in Fig. 6, the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen of OVA-induced asthmatic mice were markedly reduced compared with the normal control group (6.21% vs 10.4%, $P < 0.05$). Treatment with high dose of EGCG (50 mg/kg) significantly increased the frequency of Treg cells compared to untreated asthmatic mice (8.34% vs 6.21%, $P < 0.05$). However, treatment with low dose of EGCG (5 mg/kg) had no efficacy on increasing the frequency of Treg cells (7.11% vs 6.21%, $P > 0.05$). Treatment with high dose of EGCG had remarkable potential in the augmenting $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen of asthmatic mice, and the efficacy was as obvious as DXM treatment

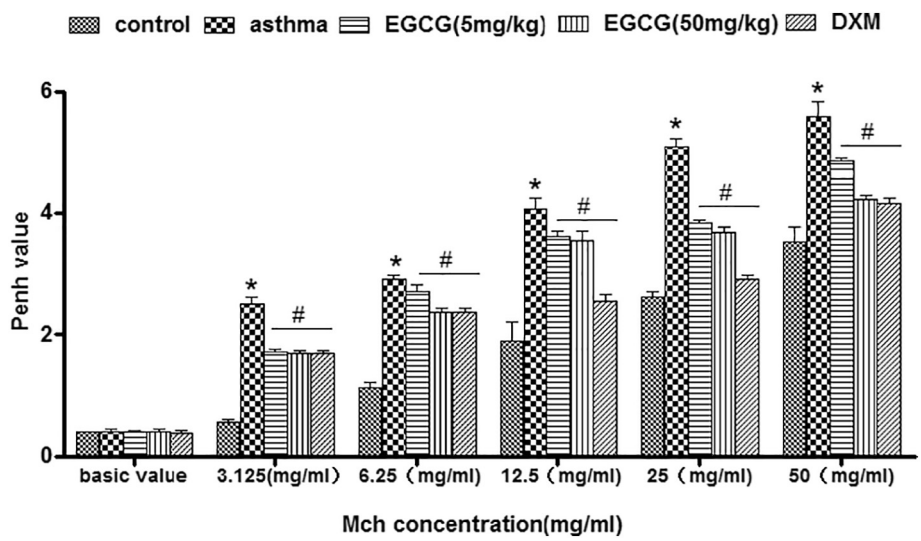

Fig. 2. Effects of EGCG on airway hyperresponsiveness. Penh values represent the airway responsiveness. Data are presented as means ± SD, $*P < 0.05$, compared with control group, $\#P < 0.05$, compared with asthma group.

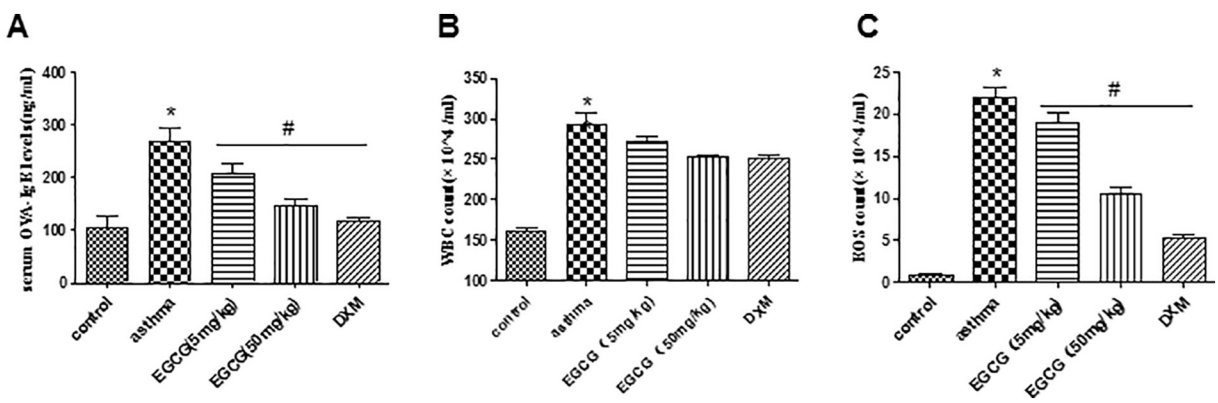

Fig. 3. Effects of EGCG on airway inflammation (A) OVA-IgE levels in the serum, (B) total white blood cells in the BALF, (C) the number of eosinophils in the BALF. Data are presented as means ± SD, $*P < 0.05$, compared with control group. $\#P < 0.05$, compared with asthma group.

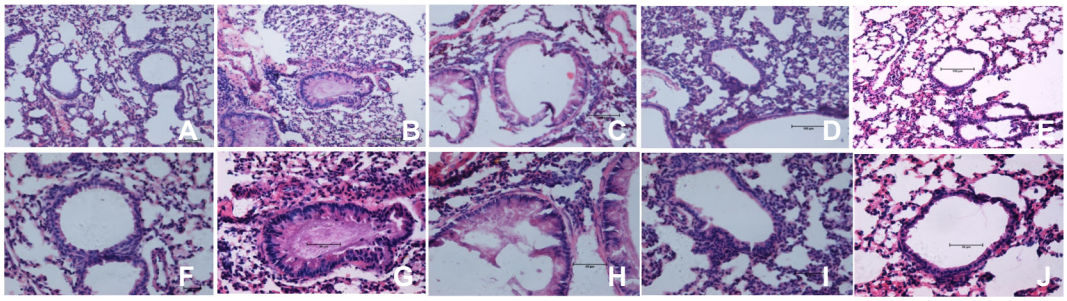

Fig. 4. Effects of EGCG on airway inflammation. Hematoxylin and eosin (HE) staining of lung tissue at 200× magnification from the: (A) control group, (B) asthma group, (C) EGCG (5 mg/kg) group, (D) EGCG(50 mg/kg) group, and (E) dexamethasone (DXM) group. Representative HE staining of the lung tissue at 400× magnification from the: (F) control group, (G) asthma group, (H) EGCG (5 mg/kg) group, (I) EGCG (50 mg/kg) group, and (J) DXM group.

group ($P > 0.05$).

3.6. EGCG improved the expression of Foxp3 mRNA and inhibited the expression of RORγt mRNA in the lung tissues of asthmatic mice

Foxp3 mRNA was expressed in the lung tissues of the normal control group, but was significantly lower in the asthma group ($P < 0.01$). Administration of EGCG at different doses significantly increased the expression of Foxp3 mRNA compared to asthmatic mice ($P < 0.01$), however the levels were, still lower than that in the normal control group. RORγt mRNA expression was remarkably higher after OVA challenge, EGCG and DXM treatment induced a remarkable reduction in RORγt level in the lung tissue of asthmatic mice (Fig. 7; $P < 0.01$).

4. Discussion

Bronchial asthma is a chronic inflammatory disorder of the airways that is characterized by chronic airway inflammation, airway of the airways that is characterized by chronic airway inflammation, AHR and irreversible airway remodeling. The pathogenesis of asthma is mediated

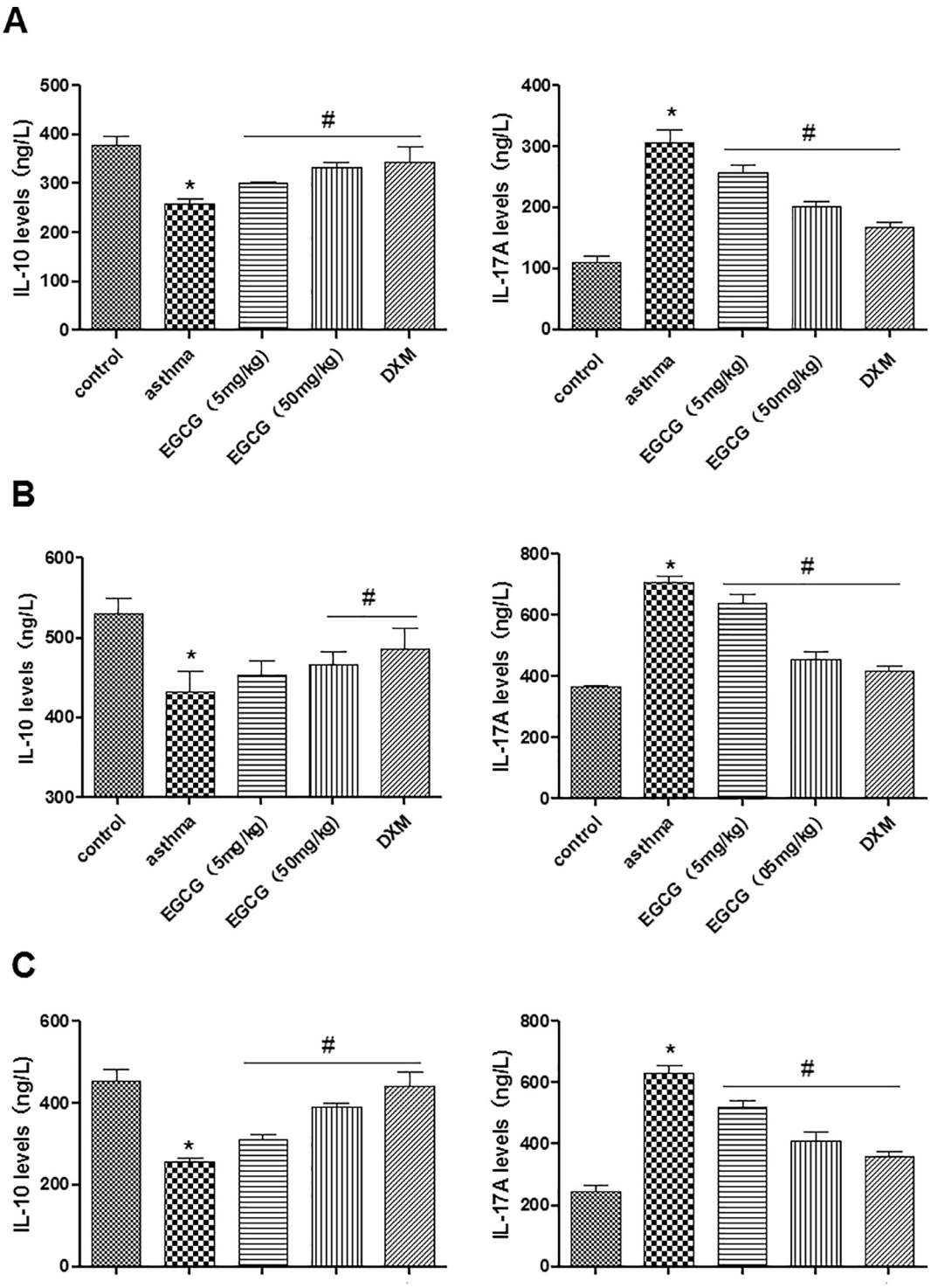

Fig. 5. Effects of EGCG on the production of IL-10 and IL-17A levels in the serum, bronchoalveolar lavage fluid (BALF), and splenocytes. Data are presented as means ± SD, $*P < 0.05$, compared with control group, $\#P < 0.05$, compared with asthma group.

by immune cells such as eosinophils, T lymphocytes, mast cells and neutrophils and other cellular elements. During the past three decades, the incidence of childhood asthma has increased sharply each year, particularly in developing countries, and poses a serious threat to the physical and mental health of children. To improve the quality of life for children with asthma, it is critical to understand how to effectively treat and control the disease. Currently, inhaled corticosteroids are the first-line clinical medication for asthma treatment. Previous studies have found that even on long-term medication, airway remodeling cannot be completely prevented. Given this challenge, developing a novel safe and effective drug to inhibit the airway inflammation and airway remodeling is still an urgent issue.

Recently natural extracts such as curcumin, mangosteen extract, and Lunasin have been shown to have anti-inflammatory effects, which may be able to ameliorate airway inflammation through their immunomodulatory effects, and may be a new mean of research for the treatment of asthma [15–18]. Imbalances in the T lymphocyte subsets contribute to the pathogenesis of airway inflammation in asthma,

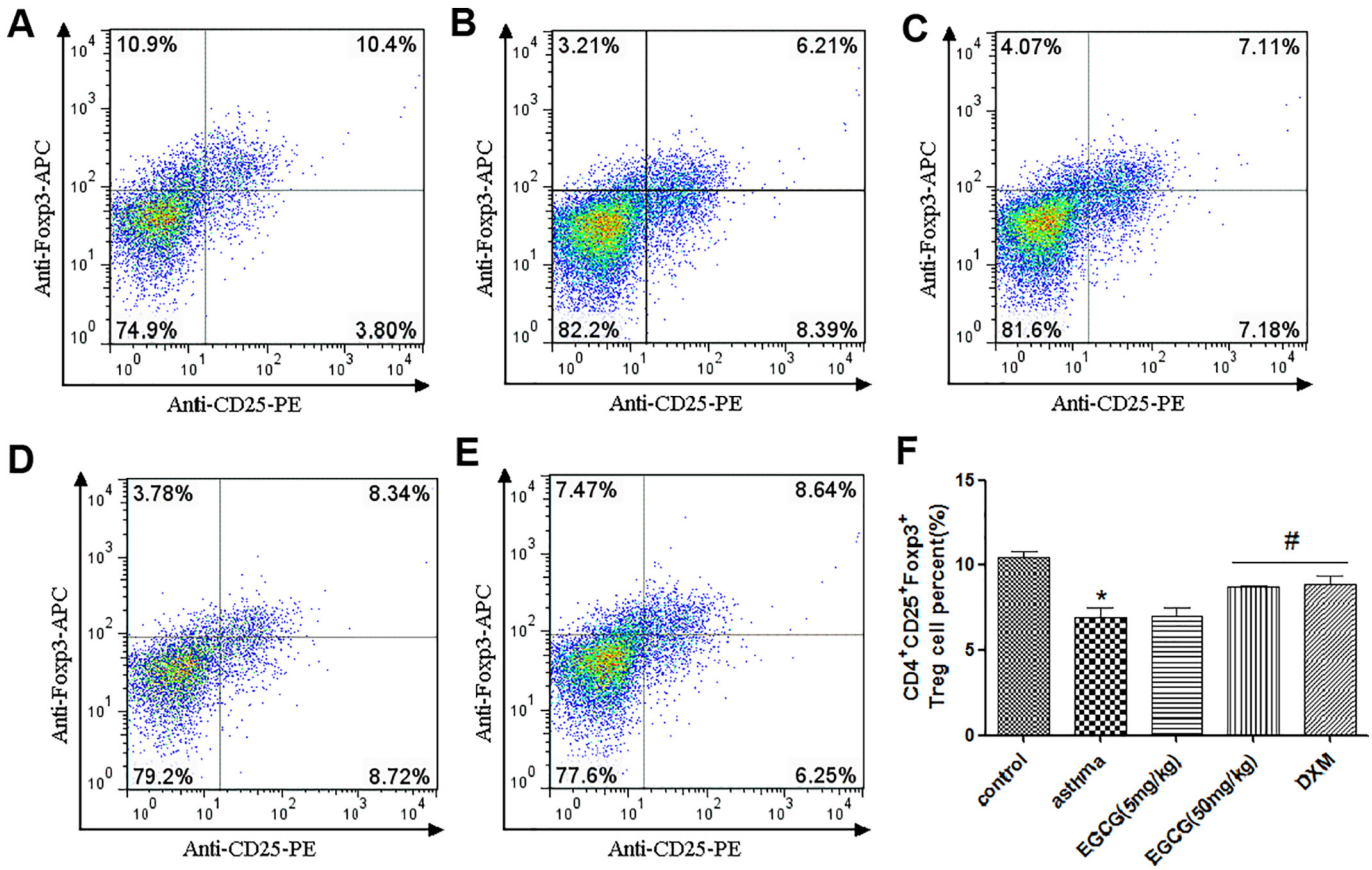

Fig. 6. Effects of EGCG on the induction of the regulatory T cells. (A) normal group, (B) asthma group, (C) EGCG (5 mg/kg) group, (D) EGCG (50 mg/kg) group, and (E) DXM group. Data are presented as means ± SD, $*P < 0.05$, compared with control group, $\#P < 0.05$, compared with asthma group.

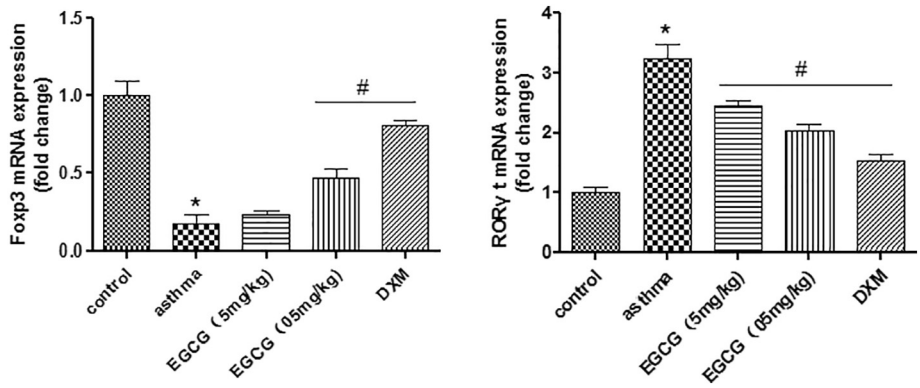

Fig. 7. Effects of EGCG on the expression of the Foxp3 gene and RORγt gene in lung tissue. Data are presented as means ± SD, $*P < 0.05$, compared with control group, $\#P < 0.05$, compared with asthma group.

through abnormal secretion of various cytokines [19]. The idea that Th1/Th2 imbalance plays a substantial role in asthma pathogenesis has been widely accepted by scholars. However, recently evidence has emerged that imbalance of Treg cells and Th17 cells may participate in allergic diseases in a variety of ways including inhibiting T cells activation and the maintenance of immune tolerance [20]. The transcription factor Foxp3 and RORγt are the specific marker of Treg cells and Th17 cells, respectively, and the expression level of Foxp3 and RORγt can reflect the number and functional activity of Treg and Th17 cells [21]. In asthma, there is a decreasing in the frequency and function of $CD4^+CD25^+Foxp3^+$Treg cells, and as well as an increasing in the frequency and function of Th17 cells [22–24].

EGCG is the major polyphenol extracted from green tea, and it has been shown that EGCG can induce a higher Treg/Th17 cell ratio during $CD4^+$ T cell differentiation. Therefore, this study assessed the immunomodulatory effects of EGCG treatment on Treg/Th17 cells in an OVA-sensitized asthmatic mouse model. The asthmatic mice had higher levels of OVA-specific IgE in the peripheral blood compared with the normal control group. In addition, there were a large number of eosinophils and a high degree of lymphocytes infiltration in the lung, which coincided with the pathological changes of asthma and indicated that the asthma model had been successfully established. Compared with asthma model, the eosinophil count in the BALF in the mice treated with EGCG and DXM was markedly reduced. Different doses of EGCG and DXM alleviated inflammatory cell infiltration in the lung and trachea and decreased AHR.

IL-10 is an anti-inflammatory cytokine that is released from Foxp3$^+$T cells. The role of IL-10 includes inhibiting the activation of mast cells, production of IgE and eosinophil survival is also associated with allergic diseases [25]. In asthma patients, IL-10 levels are generally lower [26]. Here, we showed that administration of EGCG can elevate IL-10 levels in the serum, BALF and splenocyte culture supernatant. These findings suggested that the anti-inflammatory role of EGCG could be partially mediated through an IL-10-dependent mechanism. The elevated IL-10 levels in splenocyte culture supernatant maybe attribute to the increased number and function of Treg cells following administration of EGCG, which was shown in the FACS analysis. Our findings suggested that the frequency of CD4$^+$ CD25$^+$ Foxp3$^+$Treg cells was reduced in the OVA sensitized asthmatic mice. Moreover, the expression of the Foxp3 gene in the lung tissue of OVA sensitized asthmatic mice was also reduced. After the administration of EGCG and DXM, both were obviously increased. These findings were consistent with a previous report [27].

Th17 cells and the related molecules are implicated in the induction of inflammation in asthma. RORγt was involved in the differentiation and maturation of Th17cells. Our study showed that EGCG treatment suppressed the expression of RORγt gene in lung tissue and decreased the inflammatory cytokine IL-17A levels, which may be associated with the regulatory role of EGCG on T cell differentiation.

Our study found some interesting information that EGCG treatment can attenuate AHR and airway inflammation in asthmatic mice. More importantly, the frequency of CD4$^+$CD25$^+$Foxp3$^+$Treg cells and Foxp3 expression were significantly increased by administration of EGCG. Imbalances between Treg and Th17 cells involve in contributing to the pathogenesis of asthma. CD4$^+$CD25$^+$Foxp3$^+$Treg cells play an important role in the immunoregulation of asthma, and the induction of allergen-specific Tregs has become an appealing strategy for asthma therapy. Thus, the relative proportions of Treg cells in the spleen were determined after treatment with different doses of EGCG. Our results show that high dose of EGCG regulates the imbalance in Treg/Th17 lymphocytes, but the mechanism was still unknown. Dexamethasone, which is a kind of glucocorticoids, affects the immune system by both inhibiting pro-inflammatory and activating anti-inflammatory cytokines and chemokines via the intracellular glucocorticoid receptor. Compared with the action mechanism of dexamethasone on the immune systems, the mechanism of EGCG may be different. Previous studies have proposed that EGCG is a DNA methylation inhibitor that can regulate the methylation levels of the Foxp3 gene promoter region to facilitate stable expression of the Foxp3 gene and promote the differentiation of Treg cells and help to maintain their immunosuppressive function [28,29]. An alternative hypothesis is that EGCG can interact with CD4$^+$T cells directly, to induce proliferation, differentiation and cytokine production [30]. More researches about the mechanism of EGCG regulating the balance of Treg/Th17 cells should be done in the following study.

Taken together, our studies suggest that EGCG can effectively ameliorate AHR and airway inflammation in OVA sensitized asthmatic mice likely by upregulating the frequency of CD4$^+$CD25$^+$Foxp3$^+$Treg cells, the expression of Foxp3 gene and suppressing the expression of RORγt gene. Therefore, EGCG may be a potential therapeutic agent for preventing and treating asthma.

Acknowledgments

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