



Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Apigenin protects ovalbumin-induced asthma through the regulation of Th17 cells

Jiamin Li, Bingfeng Zhang

College of Chemistry & Bio-engineering, Yichun University, Yichun, Jiangxi 336000, People's Republic of China

ARTICLE INFO

Article history:

Received 22 June 2013

Accepted in revised form 9 September 2013

Accepted 14 September 2013

Available online xxxx

Keywords:

Apigenin

Asthma

Th17 cells

ABSTRACT

The aim of the study was to investigate the anti-asthmatic effects of apigenin and the possible mechanisms. Asthma model was established by ovalbumin-induced asthma. A total of 50 mice were randomly assigned to six experimental groups: control, model, dexamethasone (2 mg/kg) and apigenin (5 mg/kg, 10 mg/kg). Airway resistance (Raw) was measured, histological studies were evaluated by hematoxylin and eosin (HE) staining, OVA-specific serum and BALF IgE levels and Th17 cytokines were evaluated by enzyme-linked immunosorbent assay (ELISA), Th17 cells were evaluated by flow cytometry (FCM), and protein level of ROR γ t was measured by western blotting. Our study demonstrated that apigenin inhibited OVA-induced increases in Raw and eosinophil count; interleukin (IL)-6, TNF- and IL-17A levels were recovered. Histological studies demonstrated that apigenin substantially inhibited OVA-induced eosinophilia in lung tissue and airway tissue. Flow cytometry studies demonstrated that apigenin substantially inhibited Th17 cells. Western blotting studies demonstrated that apigenin substantially inhibited ROR γ t protein level. These findings suggest that apigenin may effectively ameliorate the progression of asthma and could be used as a therapy for patients with allergic asthma.

© 2013 Published by Elsevier B.V.

1. Introduction

Allergic diseases of the airway such as asthma or allergic rhinitis are on the rise throughout the world, especially in western countries [1]. One of the most important diseases is bronchial asthma, that is defined as a chronic inflammation of the bronchial airways and characterized by reversible airway obstruction, increased mucus production and infiltration of the airway with eosinophils, neutrophils, mast cells and T-lymphocytes [2]. Type 1 allergies are caused by characteristic immune responses to allergens, primarily mediated by Th2 cells. Th2 cells synthesize high levels of interleukin IL-4, IL-5, and IL-13, which leads to the production of allergen-specific immunoglobulin (Ig) E and the release of mediators from mast cells [3]. Th1 cells suppress Th2 immune responses by secreting interferon IFN- γ . IL-4 induces class switching in IgG1 and IgE, whereas IFN- γ is involved in IgG2 α class switching. Accordingly, estimation of IgE production and Th1/Th2 cytokine balance is an important tool in the evaluation of asthma [4]. Another T lymphocyte subset, namely Th17 cells, has been recently

discovered and it may be involved in the pathogenesis of several immune-mediated disorders [5]. Th17 cells are characterized by the production of various cytokines, including IL-17, IL-6, TNF- α , and IL-22. IL-17 is involved in tissue pathology in autoimmune disorders [5]. Furthermore, anti-IL-17 reduces eosinophil infiltration in experimental murine asthma model [6]. IL-17 induces recruitment and increases survival for airway macrophages [7]. These facts have initially suggested that a regulatory role for IL-17 [8] and Th17 cell is very important in autoimmune disorders. Th17 cells expressing retinoic acid receptor-related orphan receptor γ t (ROR γ t) play crucial roles in induction of autoimmune tissue injuries and inflammation by producing cytokines such as IL-17, tumor necrosis factor (TNF)- α , and IL-6.

The flavonoids comprise a family of common phenolic plant pigments that have been identified as anticarcinogens and antioxidants. In the earlier studies, a variety of flavonoids exhibited profound immunoregulatory activity [9,10].

Apigenin has demonstrated anti-inflammatory, anticarcinogenic, and free radical-scavenging activities in a variety of in vitro systems [11]. Additional apigenin has demonstrated

anti-asthma through the regulation of GATA-3 gene [12]. The purpose of this study was to determine whether apigenin exerts an anti-inflammatory effect via regulation ROR γ t gene.

2. Materials and methods

2.1. Chemicals and reagents

Apigenin (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), aluminium hydroxide (Pierce Biotechnology, Rockford, USA), 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 Yichun University. A total of 50 female BALB/c mice obtained from Experimental Animal Center of Yichun University. 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 7 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), apigenin treatments (5 mg/kg, 10 mg/kg). Sensitization, challenge treatment protocols for the different groups in this study are summarized in (Fig. 1). Mice (except control group) were sensitized by intraperitoneal (ip) injection of 10 μg ovalbumin and 1 mg aluminium 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), apigenin treatments (5 mg/kg, 10 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 $^{-1}$, 0.25 mg ml $^{-1}$, 0.5 mg ml $^{-1}$, and 1 mg ml $^{-1}$). 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 [13]. BAL fluid was centrifuged (250 g, 4 $^{\circ}\text{C}$, for 5 min) and each supernatant was collected and stored at -70°C . BAL fluid (100 μl) was placed on a slide and centrifuged (200 g, 10 min, at 4 $^{\circ}\text{C}$) 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 $^{\circ}\text{C}$. The fixed tissues were embedded in paraffin and cut into 4- μm 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 [14]. The



Fig. 1. Experimental protocol for induction of airway inflammation, and treatment with apigenin, using a mouse model.

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 BLAF cytokines IL-6, TNF- and IL-17A

The concentrations of IL-6, TNF- and IL-17A in the BAL fluid were measured by ELISA according to the manufacturer's instructions (R&D, Minneapolis, MN, USA). All assays were performed in triplicate. The concentration of each protein was calculated from the standard curve.

2.8. Measurement of OVA-specific serum and BALF levels of IgE

OVA-specific serum and BALF IgE levels were determined by enzyme linked immunosorbent assay (ELISA) using samples collected 48 h after the last OVA challenge, as described previously [15]. In brief, a 96-well microtitre plate was coated with OVA (10 mg/ml), and then treated with mouse sera followed by biotin-conjugated rat anti-mouse IgE (Pharmingen, San Diego, CA). Then, avidin horseradish peroxidase (HRP) solution was added to each well. Units are optical density readings at 405 nm.

2.9. Flow cytometric analysis of 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 Th17 analysis cells were incubated with fluorescein isothiocyanate (FITC) antihuman CD4 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-mice IL-17A (eBioscience) for Th17 detection.

2.10. Statistical analysis

All values were expressed as the mean $\pm$ S.D. 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 apigenin 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 ($P < 0.05$) with concentrations of 0.125–1.000 mg ml⁻¹ methacholine. OVA-sensitized and -challenged mice treated with apigenin, displayed a reduced mean Raw upon exposure to 0.5–1.0 mg ml⁻¹ compared with the response of the OVA-sensitized and -challenged group. Positive control mice

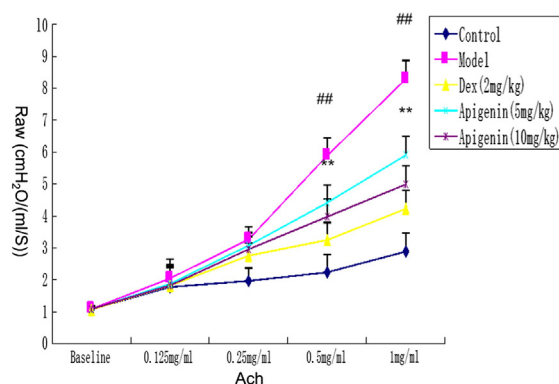


Fig. 2. Effect of apigenin on airway Resistance (Raw), Raw was measured 24 h after the last OVA challenge in mice exposed to incrementally greater doses of methacholine (0.125–1 mg ml⁻¹). Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; compared with model: * $P < 0.05$, ** $P < 0.01$.

given with dexamethasone showed a Raw decrease similar to that achieved using apigenin.

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

We examined changes in total cell levels in the BAL fluid to determine the effects of apigenin on asthma. Suppression of eosinophilia by apigenin in OVA-challenged mice was estimated by counting the 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, to levels greater than those of total leukocytes, in the BAL fluid of OVA-challenged mice. OVA-sensitized and-challenged mice treated with apigenin displayed significantly reduced leukocytosis, a fall in absolute eosinophilia, and decreased lymphocytosis, upon asthma induction. The positive drug control, dexamethasone, exerted similar suppressive effects on leukocyte influx into BAL fluid.

3.3. Effect of apigenin 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 marked infiltration of inflammatory cells into perivascular and connective tissues, 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 apigenin was significantly attenuated compared with the level seen in OVA-challenged mice as shown in Figs. 4 and 5.

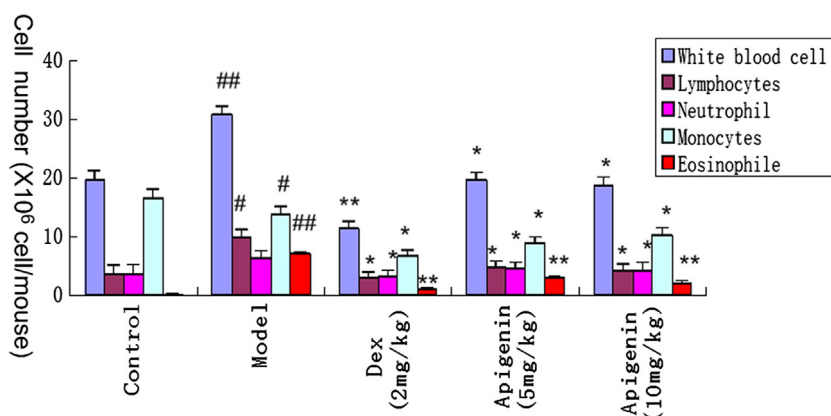


Fig. 3. Effect of apigenin on OVA-induced eosinophilia and BAL fluid composition. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; compared with model: # $P < 0.05$, ## $P < 0.01$.

3.4. Effect of apigenin on cytokine levels in BAL fluid

To determine whether apigenin influenced cytokine secretion into BAL fluid, IL-6, TNF- 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-6, TNF- and 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 apigenin, the rises in cytokine levels were significantly suppressed, compared with the concentrations seen in the OVA-challenged group. Specifically, apigenin (10 mg/kg).

3.5. Effect of BA on OVA-specific serum and BALF levels of IgE

The cross-linking of allergen-specific IgE on the surfaces of mast cells upon allergen challenge is relevant to the initiation of the early asthmatic reaction. The serum and BALF levels of OVA-specific immunoglobulins were measured 24 h after the final airway challenge. We determined that sensitization and challenge with OVA resulted in increased serum and BALF levels of OVA-specific IgE, when compared with the control mice. The treatment of sensitized mice with apigenin resulted in reductions in OVA-specific IgE (Fig. 7).

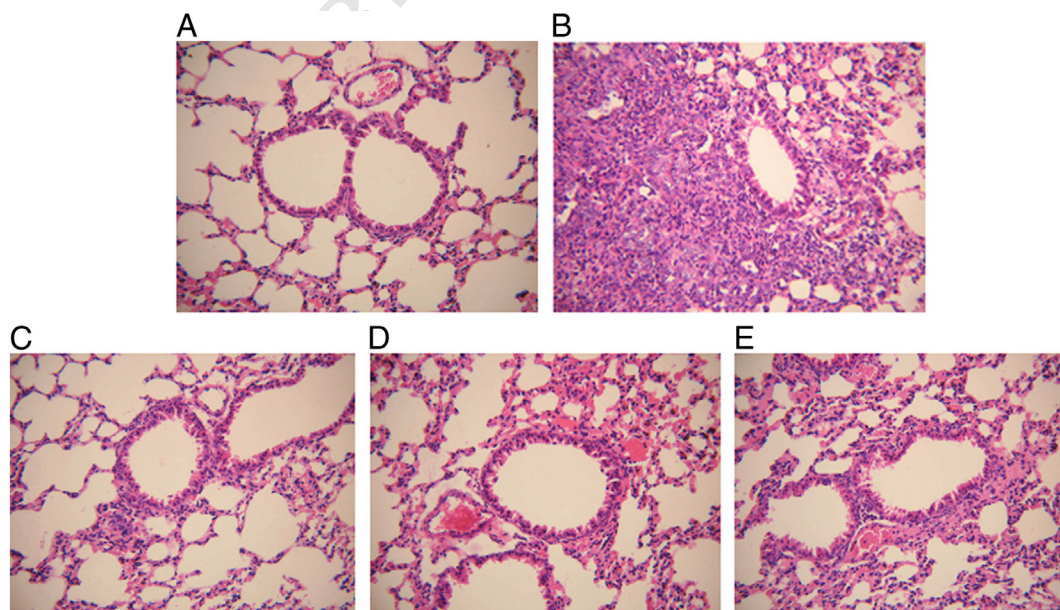


Fig. 4. Pathological changes of lung tissues observed by HE staining (Light microscopy, $\times 200$): A (control); B (model); C [Dexamethasone (2 mg/kg)]; D [apigenin (5 mg/kg)]; E [apigenin (10 mg/kg)].

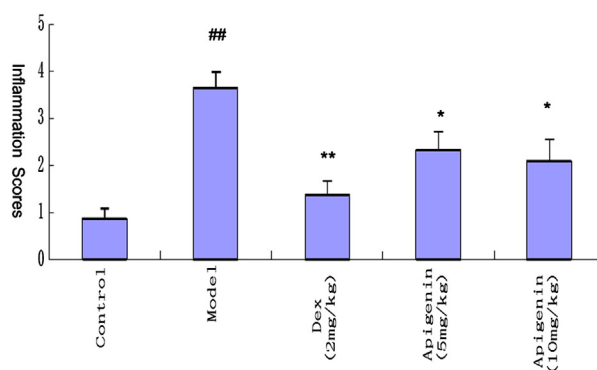


Fig. 5. Scoring of the extent of inflammation via quantitative analysis of inflammatory cell infiltration in lung sections. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; compared with model: * $P < 0.05$, ** $P < 0.01$.

3.6. Circulating Th17 cells

As shown in Figs. 8 and 9, the frequencies of Th17 cells in model group ($4.8 \pm 0.2\%$) were higher than those in the normal group ($1.2 \pm 0.22\%$), while in dexamethasone group and treatment groups were significantly lower than those in model group ($P < 0.05$).

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 β_2 adrenergic agonists. Herbal medicines are commonly used to this end in the Orient, 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 apigenin, a natural extract, 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 [16]. AHR is a measure of the bronchial constriction commonly observed in individuals with asthma. We monitored the effects of apigenin on AHR by measuring Raw. These experiments disclosed that apigenin 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 [17]. A rise in the number of eosinophils in the BAL fluid is a characteristic of asthma. The present results clearly demonstrate that apigenin significantly reduces eosinophil numbers in the BAL fluid and lung tissue.

The pathogenesis of asthma is not entirely clear and apigenin may also have additional effects on the other immune cells. Th17 cells are considered to take an important

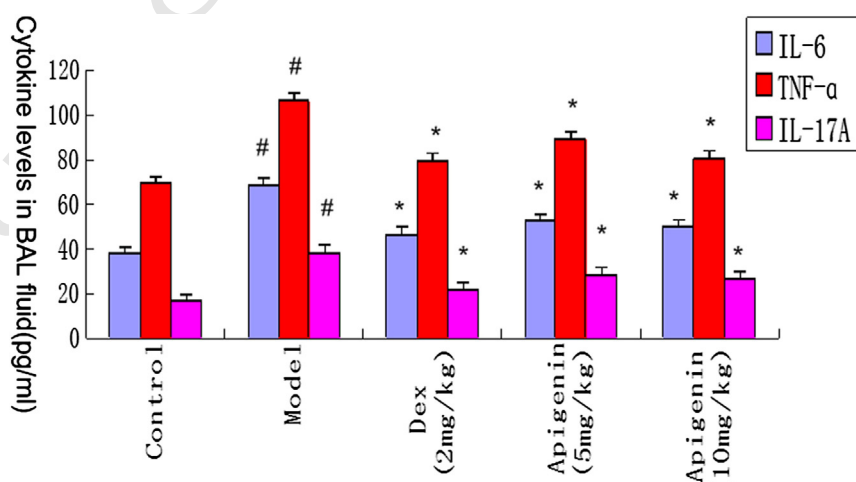


Fig. 6. Effect of apigenin on TNF- α , IL-6 and IL-17A levels in BALF. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; compared with model: * $P < 0.05$, ** $P < 0.01$.

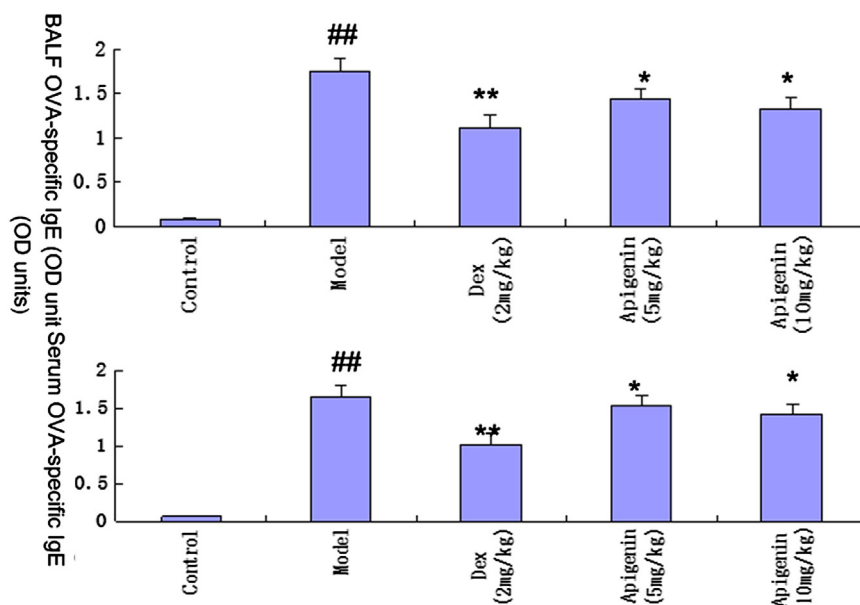


Fig. 7. Effects of apigenin on serum and BALF OVA-specific IgE antibody levels. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; compared with model: * $P < 0.05$, ** $P < 0.01$.

role and are implicated in the pathogenesis of a number of inflammatory diseases.

IL-17, a recently identified cytokine expressed in eosinophils, neutrophils, and monocytes has attracted considerable interest because it is involved in both the immune and inflammatory responses. The biological functions of IL-17

include regulation of neutrophil-dominated inflammation; the cytokine promotes granulopoiesis, neutrophil accumulation, and neutrophil activation in the lung [18,19].

Our results show that apigenin can significantly suppress IL-17A, Th17 cells in an OVA-induced mouse model, and inhibited upon treatment with apigenin. These findings

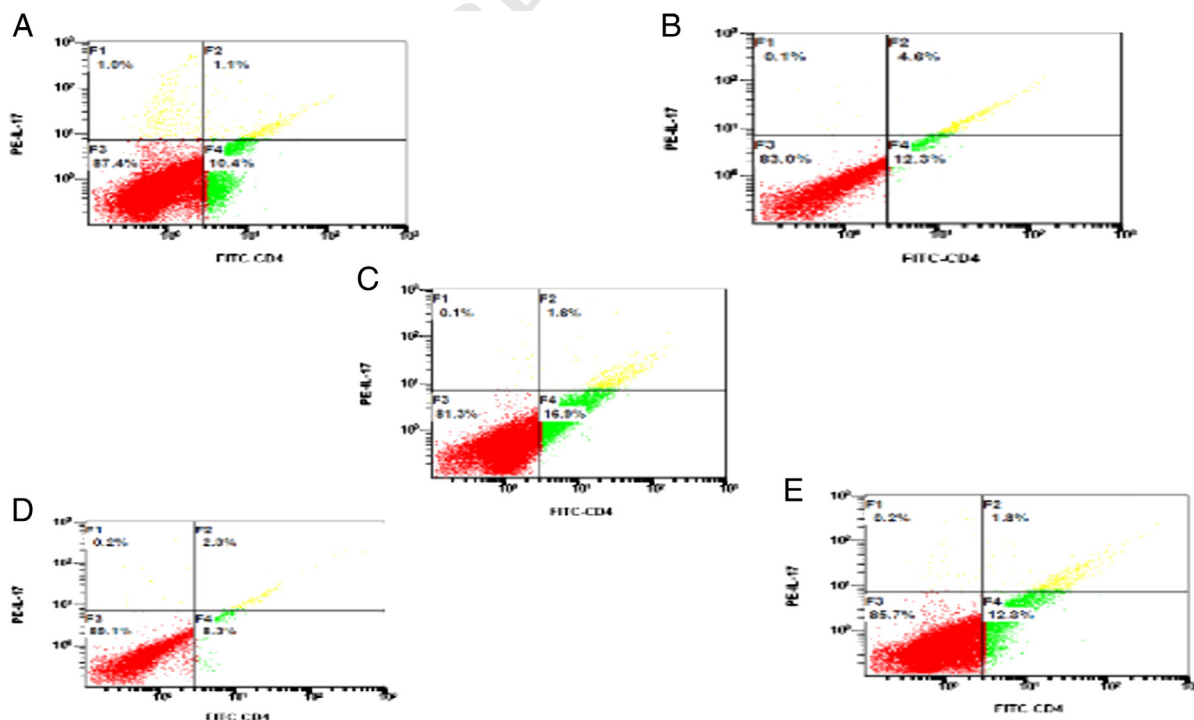


Fig. 8. Effect of apigenin on Th17 cells.

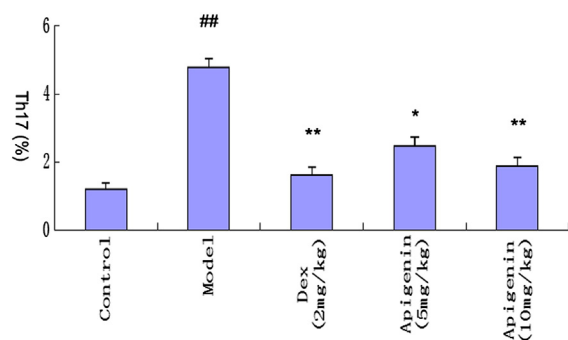


Fig. 9. Effect of apigenin on Th17 cells. Values are expressed as means $\pm$ SDs. Compared with control: * $P < 0.05$, ** $P < 0.01$; Compared with model: * $P < 0.05$, ** $P < 0.01$.

338 suggest that apigenin may effectively ameliorate the progres-
351 sion of asthma and could be used as a therapy for patients with
Q4 allergic asthma.

353 References

- 354 [1] Beasley R, Crane J, Lai CK, Pearce N. Prevalence and etiology of asthma. *J*
355 *Allergy Clin Immunol* 2000;105:466–72.
- 356 [2] Busse WW, Lemanske Jr RF. Asthma. *N Engl J Med* 2001;344:350–62.
- 357 [3] Mosmann TR, Moore KW. The role of IL-10 in cross regulation of
358 TH1 and TH2 responses. *Immunol Today* 1991;12:A49–53.
- 359 [4] Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander
360 J. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2
361 responses in the absence of IL-4 or IL-13-mediated signaling. *J*
362 *Immunol* 1999;163:6448–54.
- 363 [5] Schmidt-Weber CB, Akdis M, Akdis CA. Th17 cells in the big picture of
364 immunology. *J Allergy Clin Immunol* 2007;120:247–54.
- [6] Hellings PW, Kasran A, Liu Z, Vandekerckhove P, Wuyts A, Overbergh L, 365
et al. IL-17 orchestrates the granulocyte influx into airways after 366
allergen inhalation in a mouse model of allergic asthma. *Am J Respir* 367
Cell Mol Biol 2003;28:42–50. 368
- [7] Sergejeva S, Ivanov S, Lotvall J, Linden A. IL-17 as a recruitment and 369
survival factor for airway macrophages in allergic airway inflammation. 370
Am J Respir Cell Mol Biol 2005;33:248–53. 371
- [8] Schnyder-Candrian S, Togbe D, Couillin I, Mercier I, Brombacher F, 372
Quesniaux V, et al. IL-17 is a negative regulator of established allergic 373
asthma. *J Exp Med* 2006;203:2715–25. 374
- [9] Rogers J, Perkins I, van Olphen A, Burdesh N, Klein TW, Friedman H. 375
Epigallocatechin gallate modulates cytokine pro-duction by bone 376
marrow-derived dendritic cells stimulated with lipopolysaccharide or 377
muramyl dipeptide, or infected with *Legionella pneumophila*. *Exp Biol* 378
Med (Maywood) 2005;230:645–51. 379
- [10] Ahn SC, Kim GY, Kim JH, Baik SW, Han MK, Lee HJ, et al. Epigallocate- 380
chin-3-gallate, constituent of green tea, suppresses the LPS-induced 381
phenotypic and functional maturation of murine dendritic cells 382
through inhibition of mitogen-activated protein kinases and NF- 383
kappaB. *Biochem Biophys Res Commun* 2004;313:148–55. 384
- [11] Kim HP, Mani I, Iversen L, Ziboh VA. Effects of naturally-occurring flavonoids 385
and biflavonoids on epidermal cyclooxygenase and lipoxygenase from 386
guinea-pigs. *Prostaglandins Leukot Essent Fatty Acids* 1998;58:17–24. 387
- [12] Choi Jun-Rim, Lee Chang-Min, Jung In Duk, Lee Jun Sik, et al. Apigenin **Q8**
protects ovalbumin-induced asthma through the regulation of GATA-3 389
gene. *Int Immunopharmacol* 2009;9:918–24. 390
- [13] Djukanovic R, Roche WR, Wilson JW. Mucosal inflammation in asthma. 391
Am Rev Respir Dis 1990;142:434–57. 392
- [14] Duan W, Chan JH, Wong CH. Anti inflammatory effects of mitogen- 393
activated protein kinase inhibitor U0126 in an asthma mouse model. *J* 394
Immunol 2004;172:7053–9. 395
- [15] Jain VV, Kitagaki K, Businga T, Hussain I, George C, O'shaughnessy P, et al. 396
CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model 397
of chronic asthma. *J Allergy Clin Immunol* 2002;110:867e72. 398
- [16] Wenzel SE. Asthma: defining of the persistent adult phenotypes. *Lancet* 399
2006;368:804–13. 400
- [17] Elsnér J, Kapp A. Regulation and modulation of eosinophil effector 401
functions. *Allergy* 1999;54:15–26. 402
- [18] Park HZ, Li XO, Yang SH, Chang R, Nurieva YH, Wang Y, et al. A distinct 403
lineage of CD4 T cells regulates tissue inflammation by producing 404
interleukin 17. *Nat Immunol* 2005;6:1133–41. 405
- [19] Kolls JK, Linde'n A. Interleukin-17 family members and inflammation. 406
Immunity 2004;21:467–76. 407
408
409