



Mangiferin suppresses allergic asthma symptoms by decreased Th9 and Th17 responses and increased Treg response

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ABSTRACT

Mangiferin is the major bioactive ingredient in the leaves of *Mangifera indica* L., Aqueous extract of such leaves have been traditionally used as an indigenous remedy for respiratory diseases including cough and asthma in Traditional Chinese Medicine. Mangiferin was shown to exert its anti-asthmatic effect by modulating Th1/Th2 cytokines imbalance via STAT6 signaling pathway. However, compelling evidence indicated that subtypes of T helpers and regulatory T cells other than Th1/Th2 were also involved in the pathogenesis of asthma. In current study, we investigated the effects of mangiferin on the differentiation and function of Th9, Th17 and Treg cells in a chicken egg ovalbumin (OVA)-induced asthmatic mouse model. Mangiferin significantly attenuated the symptoms of asthma attacks, reduced the total number of leukocytes, EOS and goblet cells infiltration in lung. Simultaneously, treatment with mangiferin remarkably decreased the proportion of Th9 and Th17 cells; reduced the levels of IL-9, IL-17A; inhibited the expression of PU.1 and ROR γ t in lung. However, the proportion of Treg cells, the expression of IL-10, TGF- β 1 and Foxp3 were increased by mangiferin. Our data suggest that mangiferin exerted anti-asthmatic effect through decreasing Th9 and Th17 responses and increasing Treg response in OVA-induced asthmatic mouse model.

1. Introduction

Asthma is a complex respiratory inflammatory disorder (Papi et al., 2018; Scherzer and Grayson, 2018). CD4⁺ T-lymphocytes (CD4⁺ T) and their produced cytokines play important roles in the pathogenesis of allergic airway inflammation, including asthma (Tiddens et al., 2000; Ling and Luster, 2016). CD4⁺ T cells are able to differentiate into distinct subsets depending on different stimuli (Russ et al., 2013),

including CD4⁺ helper T-cell type 1 (Th1), CD4⁺ helper T-cell type 2 (Th2), CD4⁺ helper T-cell type 17 (Th17), regulatory T (Treg) cells, follicular TH (TFH) cells and the more recently described CD4⁺ helper T-cell type 9 (Th9) cells (Jones et al., 2012). Disruption the balance of Th1/Th2 cells is traditionally considered to be the key mechanism in asthma pathogenesis (Perez-Mazliah and Langhorne, 2014; Assaf et al., 2017). However, clinical and experimental observations indicated that Th2-targeted therapies have not been as effective as expected over the

Abbreviations: AHR, airway hyper-responsiveness; BALF, bronchoalveolar lavage fluid; Cldyn, dynamic compliance; DEX, dexamethasone; ELISA, enzyme-linked immunosorbent assay; EOS, eosinophils; H&E, hematoxylin and eosin; IHC, immunohistochemistry; OVA, ovalbumin; PAS, periodic acid-schiff; PBS, phosphate buffer saline; Re, resistance of expiration; Ri, resistance of the lung; Th1, CD4⁺ helper T-cell type 1; Th2, CD4⁺ helper T-cell type 2; Th9, CD4⁺ helper T-cell type 9; Th17, CD4⁺ helper T-cell type 17; Treg, regulatory T cells

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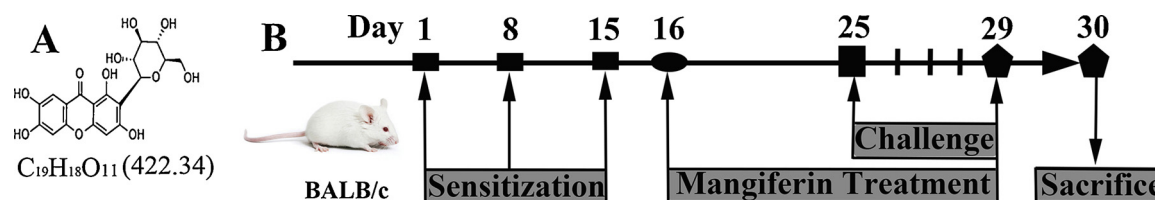


Fig. 1. Experimental drug and scheme for development of OVA-induced asthmatic mouse model. (A) Chemical structure of mangiferin; (B) Treatment schedule. Animals were sensitized with OVA-Alum injections at day 1, 8 and 15, then challenged by repeated exposure to an aerosol of OVA (1%) from day 25 to 29. Mice were orally treated with mangiferin (100 mg/kg and 200 mg/kg) from Day 16 to 29. Saline was used as vehicle and dexamethasone (1.25 mg/kg) was used as reference drug for positive control. All the animals were euthanized at day 30.

past few years (Kim et al., 2010). In addition, many non-Th1, Th2 cytokines, such as IL-17, IL-9, IL-25, transforming growth factor (TGF)- β and neutrophils, have been frequently found in the lungs of asthma patients (Farahani et al., 2014; Chenuet et al., 2017; Koch et al., 2017). IL-9, mainly secreted by Th9 subsets, stimulates the proliferation of activated T cells, promotes the proliferation and differentiation of mast cells and increases production of IgE by B cells (Kearley et al., 2011). IL-17, produced from Th17 cell, is known to play a regulatory role in allergic airway disease in animal models (Sergejeva et al., 2015), treatment for exogenous IL-17A also decreases eosinophils (EOS) recruitment and bronchial hyperreactivity in asthmatic animal model (Schnyder-Candrian et al., 2006). IL-10, secreted by Treg cell, is potent inhibitor of pro-inflammatory cytokines such as IL-1, IL-6 and IL-4, which increases Th2 responses (Hawrylowicz and O'Garra, 2005). These observations imply that imbalance of Th1/Th2 cells may not be the sole or main players in the pathogenesis of asthma, and other T-helper subsets, such as Th9, Th17 and Treg cells, may play equal or more important roles in asthma.

Mangiferin, a natural C-glycoside xanthone (1,3,6,7-Tetrahydroxy-2-[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]xanthen-9-one) (Fig. 1A, PubChem CID 5281647), is abundantly present in *Mangifera indica* L. leaves. It possesses several health endorsing properties such as anti-oxidant (Pardo-Andreu et al., 2006), anti-allergic (Rivera et al., 2006), anti-cancer (Sadhuhan et al., 2018; Deng et al., 2018), and immunomodulatory (Khare and Shanker, 2016; Imran et al., 2017; Lim et al., 2016). The aqueous extracts of mango leaves have been traditionally used as an indigenous remedy for respiratory diseases including cough and asthma in Traditional Chinese Medicine, however, the molecular mechanism is still unclear. Rivera DG et al (Rivera et al., 2011) reported that mangiferin could exert the anti-asthmatic effect by decreasing the level of Th2 cytokines IL-4 and IL-5 in BALF and lymphocyte culture supernatant. In our previous study, we found that mangiferin exhibited anti-asthmatic effect by rebalancing Th1/Th2 cytokine via STAT6 signaling pathway (Guo et al., 2014). To our surprise, levels of other non-Th1/Th2 cytokines were also altered upon mangiferin treatment. For instance, mangiferin decreased the levels of cytokines including Th9-related IL-9, Th17-related IL-17, while increased the level of Treg-related IL-10 in serum in antibody array analysis. This promoted us to examine whether mangiferin may regulate the differentiation of Th9, Th17, and Treg cells.

In current study, we investigated the effects of mangiferin on the differentiation and function of Th9, Th17 and Treg cells in an OVA-induced asthmatic mouse model.

2. Materials and methods

2.1. Chemicals and reagents

Chicken egg ovalbumin (OVA, No.A5503), Methacholine (O-acetyl- β -methyl-choline chloride, MCH, No.1396364), Aluminum hydroxide Gel (No.1017502), Hematoxylin (No.H9627), Eosin (No.E4009) and periodic acid-Schiff Kit (No.395B), RIPA buffer (R0278) were purchased from Sigma (St Louis, USA). leukocyte activation cocktail (BD

Biosciences, No.550583), Intracellular Fixation & Permeabilization Buffer Set (88-8824-00, eBioscience, No.4347572), anti-CD4 (FITC) (RM4-5, eBioscience, No.4304296), anti-IL-9 (PE) (D9302C12, eBioscience, No.561463), anti-IL-17A (PE) (eBio17B7, eBioscience, No.4306419), anti-CD25(APC) (BC96, eBioscience, No.17025941), anti-Foxp3 (PE) (MF23, eBioscience, No.560408), PE-Rat IgG1, APC-Rat IgG1, κ isotype control were purchased from BD Biosciences (Franklin Lakes, New Jersey, USA). Anti-Foxp3, anti-ROR γ t antibodies were purchased from Abcam (Cambridge, UK). Anti-PU.1, anti-GAPDH, Anti-mouse IgG HRP-linked, Anti-Rabbit IgG HRP-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for IL-9, IL-10, IL17-A, TGF- β were purchased from Cusabio (Wuhan, China). Polyvinylidene fluoride (PVDF) was purchased from Millipore (Massachusetts, USA). Micro BCA Protein Assay Kit (No.23235), Halt Protease and Phosphatase Inhibitor Cocktail (No.78444), Trizol reagent (No.15596018), High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (4374966), Restore Western Blot Stripping Buffer (No.21059), Tween 20 (No.85113), SYBR green (No.A25742) were purchased from ThermoFisher Scientific (Waltham, USA). Biotinylated secondary antibody, Streptavidin-HRP (horseradish peroxidase) and 3,3N-Diaminobenzidine Tetrahydrochloride were purchased from (DAKO, Carpinteria, CA).

Mangiferin (98.39%, HPLC), as previous describe (Guo et al., 2014), were obtained from Guangxi Key Laboratory of Pharmacodynamic Studies of Traditional Chinese Medicine (Nanning, China). Dexamethasone was purchased from Zhejiang Xianju Pharmaceutical co., LTD (Hangzhou, China, No.140708).

2.2. Animals

Specific pathogen-free female BALB/c mice (18 ± 2 g, 4-5weeks old) were provided from Hunan SJA Laboratory Animal Co., Ltd (Changsha, China), and maintained in a ventilated room at an ambient temperature of $22^\circ\text{C} \pm 2^\circ\text{C}$ and a 12 h diurnal light cycle. The mice were allowed to approach OVA-free food and water ad libitum, and acclimatize for two weeks before the experiments. All procedures were approved by the Institutional Animal Ethics Committee (IAEC, No. 201606015), Guangxi Medical University. The animal study design followed ARRIVE guidelines (Kilkenny et al., 2010).

2.3. OVA-induced asthma model and experimental groups

Sensitization and challenge were performed as previously described (Guo et al., 2014). The animals were randomized into five groups (12 mice/group) as follows: normal control group (no OVA induction); OVA-induced asthma model group; Mangiferin treated experimental groups (with doses of 100 mg/kg and 200 mg/kg); and dexamethasone (DEX) treated group.

As shown in Fig. 1B, mice were sensitized by intraperitoneal injections of 20 μg OVA and 0.15 ml aluminum hydroxide gel in 0.2 ml phosphate buffer saline (PBS, pH 7.4) at day 1, 8 and 15. The sensitized mice were then challenged by repeated exposure to an aerosol of OVA

Table 1

Scoring system used to assess symptoms of asthma attacks.

Score	Symptoms of asthma attacks
0	No symptoms of asthma attacks
1	Scratching ears, sneezing or coughing, mild abdominal breathing
2	Irritating cough, shortness of breath, obvious abdominal breathing, restlessness.
3	Dyspnea, cyanosis, excite bow back or prone, unresponsive, incontinence, poor color and dull coat.

(1%) for five consecutive days (day 25–29). Remaining one group mice, served as normal control group, were received intraperitoneal injections of 0.2 ml PBS (pH 7.4) containing 0.15 ml aluminum hydroxide gel and were challenged with PBS alone.

To evaluate its protective effect, mangiferin (100 mg/kg and 200 mg/kg) were orally administered into OVA-induced asthma model mice for 14 consecutive days (from Day 16 to Day 29). Saline were orally administered into normal mice as vehicle control. Dexamethasone (1.25 mg/kg) was orally administered into OVA-induced asthma model mice as reference drug and positive control. All specimens were processed 24 hours after last allergen challenge (thus on Day 30).

2.4. Evaluation on the symptoms of asthma in mice

The respiratory symptoms and systemic reactions, including facial itching, sneezing or choking, shortness of breath, restlessness, cyanosis, abdominal breathing, proneness, hair color and other changes in mice inhaled OVA was evaluated. The symptoms of asthma attacks was scored at last allergen challenge (on Day 29) by a subjective scale of 0–3 points (Yun et al., 2017), as described in Table 1.

2.5. Lung function evaluation

The airway hyper-responsiveness (AHR) to methacholine was determined using an AniRes2005 Lung Function System (version 3.5; Bestlab Technology Co; China) at 24 h after the final aerosol challenge according to the manufacturer's instructions. Briefly, mice were anesthetized with 50 mg/kg pentobarbital sodium and connected to a computer-controlled ventilator via the tracheal cannula. The time of expiration/inspiration and the respiratory rate were preset at 1.5:1 and 90/min, respectively. The resistance of the lung (Ri), resistance of expiration (Re), and lung dynamic compliance (Cldyn) were recorded to evaluate the reaction of mice to PBS or four doses of methacholine (0.025, 0.05, 0.1, and 0.2 mg/kg body weight); this compound was injected into the jugular vein at 5 min intervals every each concentration using a fine needle.

2.6. Collection of bronchoalveolar lavage fluid (BALF) and blood samples

24 h after the last challenge, BALF and blood samples were collected as described previously (Guo et al., 2014). Briefly, the blood samples were collected via the retroorbital plexus. Serum samples were separated by centrifugation (1500 g, 10 min at 4 °C) and kept at –80 °C until analysis for the cytokines. Then mice were euthanized and 0.6 mL cold phosphate buffered saline (PBS) per mouse was instilled into the lungs via a small syringe after tracheotomy was performed. BALF was collected after 30 s of PBS instillation, and repeated twice. Ice-cold BALF samples were centrifuged (800 g, 10 min at 4 °C) to separate suspended cells and supernatant. The pellet was resuspended in 300 µl of ice-cold PBS, centrifuged onto slides and stained with Wright-Giemsa. The slides were quantified for differential cells counts by counting a total of 300 cells per slide at 40 magnification. The total number of BALF cells was counted using a haemocytometer.

2.7. Histological examination of lungs

The inflammatory changes in lung tissues from each animal were assessed via histology. The excised lungs were fixed in 4% paraformaldehyde, dehydrated by exposure to increasing concentrations of ethanol, embedded in paraffin, sectioned at 4 µm thickness, and then stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). The density of leukocytes and goblet cells was used to evaluate the extent of goblet cell hyperplasia. Sections were imaged with microscopy.

2.8. Flow cytometry analysis of Th9, Th17 and Treg cells in spleen

Spleen samples were collected in RPMI 1640 media and a single cell suspension was prepared for immunostaining. To analyze of Treg, the cells were surface-stained with anti-CD4 (10 µg/ml), anti-CD25 (5 µg/ml) at 4 °C in the dark for 30 min. Following surface staining, the cells were fixed and permeabilized with the BD cytofix/cytoperm kit at 4 °C in the dark for 30 min, after washed and blocked with 1 % FBS, the cell pellet were stained with anti-Foxp3 (5 µg/ml) antibody at 4 °C in the dark for 30 min. To analyze intracellular cytokine production of Th17 and Th9, the cells were stimulated and isolated with leukocyte activation cocktail for 6 h. Cells were surface-stained with anti-CD4 (10 µg/ml) antibody at 4 °C in the dark for 30 min. Following surface staining, the cells were fixed and permeabilized with the BD cytofix/cytoperm kit at 4 °C in the dark for 30 min, after washed and blocked with 1 % FBS, the cells were intracellular staining with anti-IL-9 (5 µg/ml), anti-IL-17A (5 µg/ml) antibodies at 4 °C in the dark for 30 min. Background fluorescence was assessed using appropriate κ isotype control mAbs. The stained cells were fixed with 1% paraformaldehyde and analyzed with flow cytometer (C6 FlowCytometer System, BD, USA).

2.9. Quantitative real-time PCR analysis of mRNA levels of cytokines in lungs

The lung samples (50 mg) were homogenized, total RNA was extracted with the Trizol reagent and reverse-transcribed to complementary DNA with a high capacity cDNA reverse transcription kit, according to the manufacturer's protocol. Subsequent QPCR was performed using SYBR green reagent on the ABI 7300 Real-time PCR system (Applied Biosystems, CA, USA). The 15 µl reaction mix consisted of 1.2 µl 10-fold diluted first-strand cDNA, 7.5 µl 2 × SYBR green, 0.3 µl 10 µM forward and reverse primer, and 5.7 µl diethyl pyrocarbonate (DEPC)-treated water. mRNA levels were measured by the cycle threshold ($2^{-\Delta\Delta CT}$) method and were normalized to GAPDH levels. All primers of IL-9, IL-10, IL-17, GAPDH and TGF-β were purchased from Invitrogen (California, USA), listed in the Table 2.

2.10. ELISA analysis of cytokines levels in serum and BALF samples

The levels of IL-9, IL-10, IL-17, and TGF-β in serum and BALF were

Table 2

Sequences of primers used for real-time PCR.

Gene	Primer	Sequences(5'-3')
IL9	Forward	TCTTCAGTTCGTGCTGGGC
IL9	Reverse	CAGCTGCATTTTGACGGTGG
IL10	Forward	ATAACTGCACCCACTTCCCA
IL10	Reverse	TTGTCCAGCTGGTCCTTTGTT
IL17A	Forward	ATCAGGACGCGCAACATGA
IL17A	Reverse	TTGGACACGCTGAGCTTTGA
TGF-β1	Forward	GCTGCGCTTGACAGATTAAA
TGF-β1	Reverse	CACCTCAGGCGTATCAGTGGG
GAPDH	Forward	TATGTCGTGGAGTCTACTGGT
GAPDH	Reverse	GAGTTGTCATATTTCCTCGTGG

measured using commercial ELISA kits according to the manufacturer's instructions (Cusabio Life Science Inc., Wuhan, China). In brief, the serum samples were diluted 10 times in appropriate buffer and BALF sample were individually evaluated in duplicate. Add 100 µl of standard and sample per well, cover with the adhesive strip, incubate for 2 h at 37 °C. Then remove the liquid of each, add 100 µl of Biotin-antibody (1x) to each well, cover with a new adhesive strip, incubate for 1 h at 37 °C. Aspirate each well and add 100 µl of HRP-avidin (1x) to each well, cover the microtiter plate with a new adhesive strip. Incubate for 1 hour at 37 °C. Repeat the aspiration/wash process for five times, add 90 µl of TMB Substrate to each well, incubate for 20 minutes at 37 °C, Add 50 µl of Stop Solution to each well, gently tap the plate to ensure thorough mixing, determine the optical density of each well within 5 minutes, using a microplate reader set to 450 nm.

2.11. Immunohistochemistry (IHC) analysis of PU.1, ROR-γt, and Foxp3 in lungs

For immunohistochemistry analysis, paraffin-embedded sections of lung lobes were deparaffinized in xylene, rehydrated in alcohol, and incubated with 3% hydrogen peroxide for 10 min and then in 5% BSA blocking solution for 20 min. Slides were incubated overnight at 4 °C with anti-PU.1 (1:50), anti-ROR-γt (1:2000) or anti-Foxp3 (1:100) antibody in blocking solution. After washing with PBS, the slides were incubated with Biotinylated secondary antibody for 20 min, Streptavidin-HRP (horseradish peroxidase) for 20 min, and 3,3'-Diaminobenzidine Tetrahydrochloride for 10 min. The slides were then washed and counterstained with hematoxylin. Slides were imaged with microscopy.

2.12. Western-blot analysis of the protein levels of PU.1, ROR-γt and Foxp3 in lungs

The expressions of PU.1, ROR-γt and Foxp3 in lung tissues were detected by western blot. The lung samples were homogenized in tissue lysis reagent containing phosphatase and protease inhibitors. Protein concentrations were determined by BCA protein assay. 50 µg protein samples were separated on 12% reducing SDS-PAGE and then transferred to PVDF membrane. Membranes were blocked with 5% skim milk and immunoblotted overnight at 4 °C with the indicated concentrations of primary antibodies, include anti-PU.1(1:1000), anti-ROR-γt (1:1000) or anti-Foxp3 (1:200) antibody. After washing, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:5000) for 1 hour at room temperature. Signals were visualized by chemiluminescence using a ChemiDoc (Bio-Rad Laboratories). The membranes were stripped with Restore Western Blot Stripping Buffer and reprobed with Anti-GAPDH (1:2000) antibody to show equal loading of proteins from each lane.

2.13. Statistical analysis

All data points were performed in technical duplicates or triplicates, and experiments were repeated independently at least twice and reported as means ± standard deviation of the biological replicates, and analyzed with SPSS version 17.0 software. To analyze the asthma symptoms differences among certain groups of mice, multiple samples' rank-sum test (Kruskal-Wallis test) was applied. The Mann-Whitney U-test was performed to compare the mean ranks of the scores assessing asthma symptom between the two groups of participants. To adjust the level of statistical test, the original standard of $P < 0.05$ has to be changed to $P < 0.0125$ (Given that $0.05/4 = 0.0125$), a value of $P < 0.0125$ was considered to be statistically significant. For the other experiments, one-way ANOVA followed by LSD or Dunnett's T3 multiple comparisons test was used ($P < 0.05$, with statistical significance).

Table 3
Mangiferin alleviated the symptoms of asthma.

Groups	Scores				Median
	0	1	2	3	
Normal control group ^a	8	4	0	0	0
OVA-induced asthma model group	0	1	4	7	3
Mangiferin(100 mg/kg) treated groups ^a	0	8	2	2	1
Mangiferin(200 mg/kg) treated groups ^a	2	5	3	2	1
Dexamethasone treated group ^a	5	5	1	1	1

Chi-Square: 23.592.

^a $P < 0.0125$ compared with OVA-induced asthma model group.

3. Results

3.1. Mangiferin alleviated the symptoms of asthma attacks

We first examined whether mangiferin may relieve the symptoms of asthma. The mice in the normal control group breathed relatively smoothly, occasionally sneezing or coughing. When challenged with OVA, OVA induces further deterioration of symptoms in asthmatic mice which developed irritability, scratching their ears, sneezing or coughing, shortness of breath, obvious tucked-up abdomen, and aggravating with increased numbers of stimulation. The cyanosis of the nose, ears and toe were obvious, excite bow back or prone, unresponsive, poor color and dull coat. Mice in the OVA-induced asthma model group treated with mangiferin displayed significant alleviation in the symptoms. Dexamethasone, as a positive control for anti-inflammatory effects, showed comparable effectiveness in alleviating the symptoms of asthma as mangiferin. In addition, the symptoms of asthma attacks were scored and the median score in model group was around 3, indicating severe asthma symptoms in model group. This score was reduced from 3 to 1 when the mice were treated with mangiferin and dexamethasone (Table 3).

3.2. Mangiferin restored the lung function of asthma mice

The result of lung function was shown in Fig. 2. As the dosages of Methacholine gradually increase, the inspiratory and expiratory resistance in OVA-induced asthma model group increased significantly compared with the control group ($P < 0.01$) (Fig. 2A, B, D), while the Cldyn (Fig. 2C, E) decreased remarkably ($P < 0.01$). Compared with the model group, mangiferin notably reduced the expiratory and inspiratory resistance ($P < 0.01$ or 0.05) (Fig. 2A, B, D), and restored the Cldyn slightly at the both dosages ($P < 0.05$) (Fig. 2C, E).

3.3. Mangiferin decreased the infiltration of leukocytes and eosinophils (EOS) in BALF

Here we further confirmed the role of mangiferin in OVA-induced mouse models of allergic asthma. Compared to the normal control group, OVA induced a significant infiltration of leukocytes into BALF and the number of EOS in leukocytes markedly increased ($P < 0.01$). Compared with the model group, mangiferin notably reduced the number of leukocytes and EOS in a dose-dependent manner ($P < 0.01$) (Fig. 3A–B).

3.4. Mangiferin reduced airway inflammation and mucus hypersecretion in lung

We evaluated if mangiferin could attenuate airway inflammation by H&E staining and reduce mucus secretion by PAS staining on lung tissues. Compared to normal control group, OVA challenges in OVA-induced asthma model group resulted in severe peri-bronchiolar and alveolar inflammation, indicated by excessive inflammatory cell

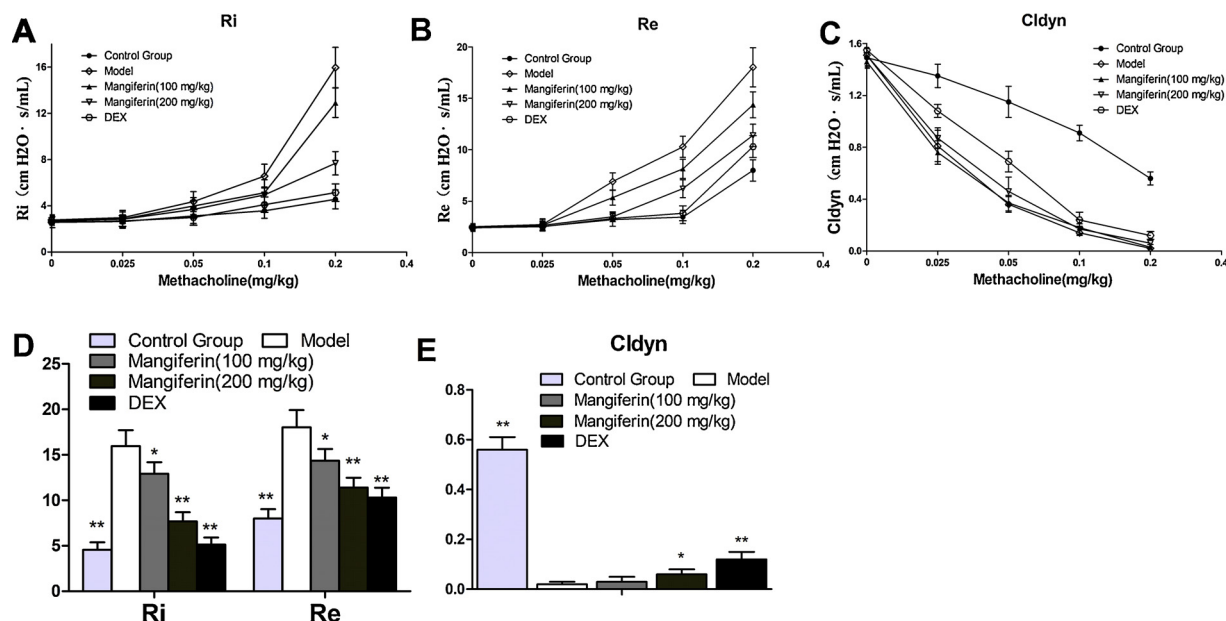


Fig. 2. Mangiferin restored the lung function of asthma mice. The airway hyperresponsiveness was evaluated using Ri, Re and Cldyn by AniRes 2005 lung function system. Ri (A), Re (B) and Cldyn (C) to double multiplication concentration of methacholine (0, 0.025, 0.05, 0.1 0.2 mg/kg) and bar plot of Ri, Re (D) and Cldyn (E) to 0.2 mg/kg of methacholine. The data are represented as the means $\pm$ SDs ($n = 12$). Compared with model group: * $P < 0.05$; ** $P < 0.01$.

infiltration surrounding small airways and vasculature, as well as alveolar septa, increased goblet cells and mucus production. In OVA-induced asthma model mice treated with low-dose (100 mg/kg) of mangiferin, we found that infiltration of leukocytes and mucus secretion were significantly attenuated compared to nontreated OVA-induced asthma model mice. More interestingly, few inflammatory cells and goblet cells were found in the lungs of high-dose (200 mg/kg) of

mangiferin and dexamethasone treated groups (Fig. 3C–D). These results suggested that mangiferin significantly attenuated OVA-induced allergic inflammation, manifested with reduced infiltration of inflammatory cells in airway and alveolar septa, reduced hyperplasia and mucus secretion of goblet cells.

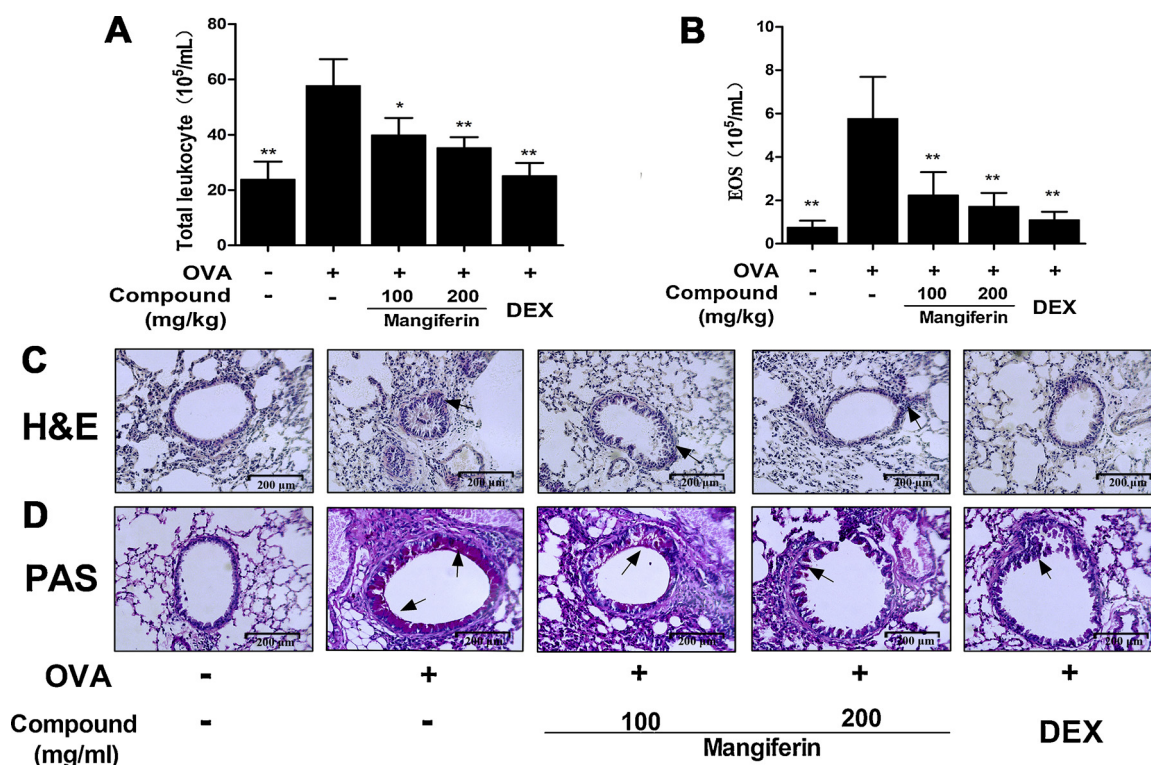


Fig. 3. Mangiferin alleviated the airway inflammation and eosinophils infiltration in lung. BALF was collected at 24 h after the last OVA challenge. The total leukocyte (A) and EOS (B) in BALF were counted. Histological assessment of airway inflammation and mucus secretion in lung tissues was made in each group. A representative figure (magnification 400 $\times$) of lung tissue stained with H&E solution (C) and PAS solution (D). The data are represented as the means $\pm$ SDs ($n = 12$). Compared with model group: * $P < 0.05$; ** $P < 0.01$.

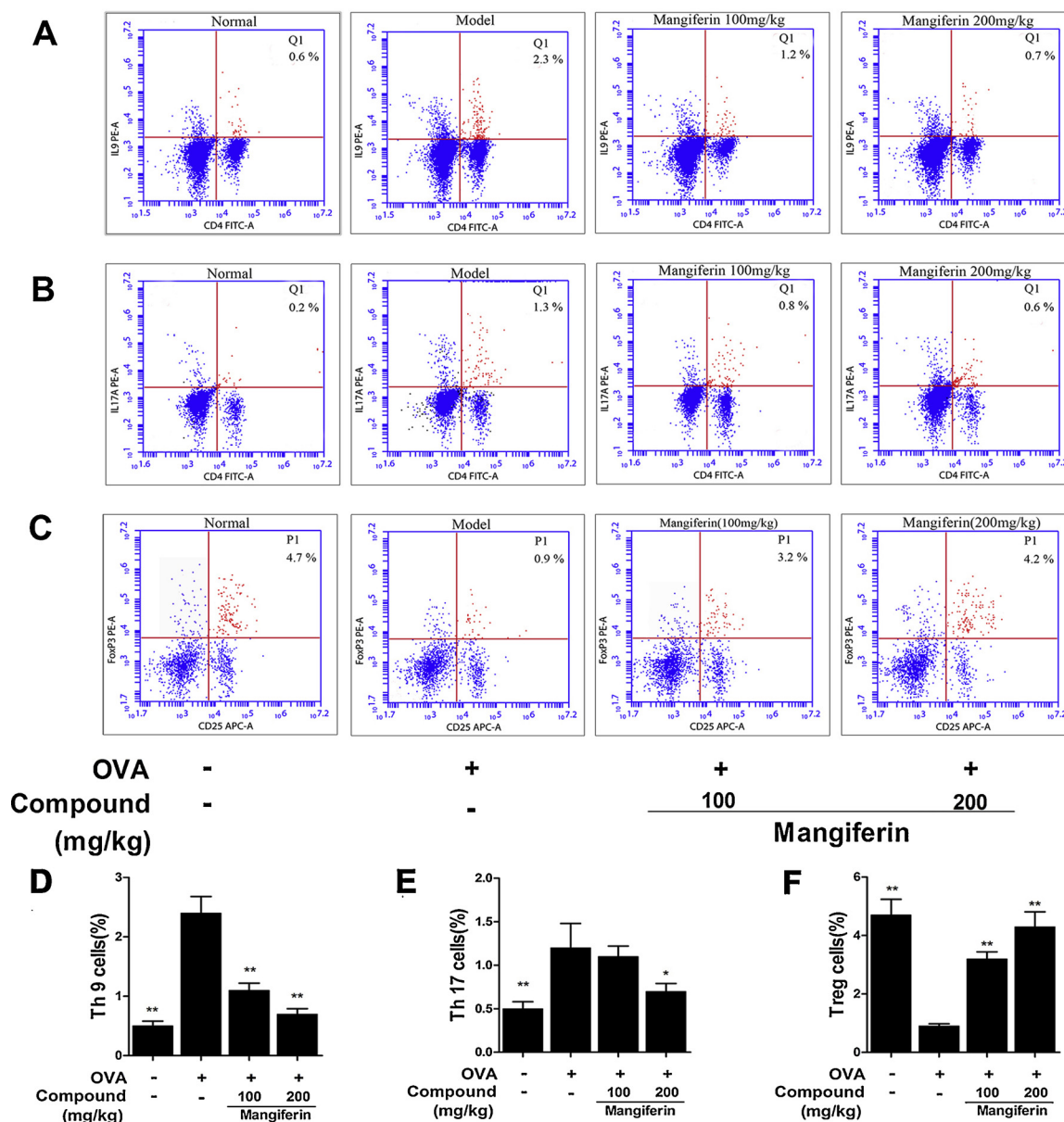


Fig. 4. Mangiferin attenuated the imbalance of Th9 /Treg and Th17/ Treg cells in spleen. The percentages of Th9, Th17 and Treg cells in CD4⁺ T cells in the spleen were analysis by flow cytometry. (A–C) Representative diagram showing the ratio of Th9, Th17 and Treg cells in the spleen; (D–F) Bar plot of the average percentage of Th9, Th17 and Treg cells. The data are represented as the means $\pm$ SDs (n = 12). Compared with model group: * $P < 0.05$; ** $P < 0.01$.

3.5. Mangiferin altered the population of Th9, Th17, and Treg in spleen

We detected decreased levels of IL-9 and IL-17 and increased level of IL-10 with protein array analysis in serum samples from mangiferin treated OVA-induced asthmatic mouse model (Guo et al., 2014). This promoted us to examine the population of Th9, Th17, and Treg cells in the spleen. Flow cytometry analysis showed that the proportion of Th9 and Th17 cells was escalated and the proportion of Treg cells was decreased in OVA-induced asthmatic mouse model group, compared to control group. After the intervention of mangiferin, the proportion of Th9, Th17, and Treg cells was restored (Fig. 4).

3.6. Mangiferin had reciprocal effects on Th9, Th17, and Treg related cytokines expression in lung, serum and BALF

To better understand the roles of mangiferin on Th9, Th17, and Treg cell related cytokines, we measured the mRNA (Fig. 5) and protein levels (Fig. 6) of IL-9, IL-10, IL-17A, and TGF- β 1 in the lung, serum and

BALF respectively. Compared to control group, the mRNA and protein levels of IL-9 and IL-17A were significantly increased in OVA-induced asthmatic model group ($P < 0.01$), while the levels of IL-10 and TGF- β 1 were markedly decreased ($P < 0.01$ or 0.05). Interestingly, mangiferin significantly impaired the increased levels of IL-9 and IL-17A ($P < 0.05$) and restored the decreased levels of IL-10 and TGF- β 1 ($P < 0.01$ or 0.05) in OVA-induced asthmatic model group. These results suggest that mangiferin had reciprocal effects on Th9, Th17, and Treg cells related cytokines expression in OVA-induced asthmatic mouse model.

3.7. Mangiferin regulated the expression of PU.1, ROR γ t and Foxp3 in lung tissue

PU.1, ROR γ t, and Foxp3, as the specific transcription factors, play vital roles in the differentiation of Th9, Th17 and Treg cells. So we measured the expressions of PU.1, ROR γ t, and Foxp3 in lung tissue with both Western blot and IHC. As shown in Fig. 7D–G, western blot

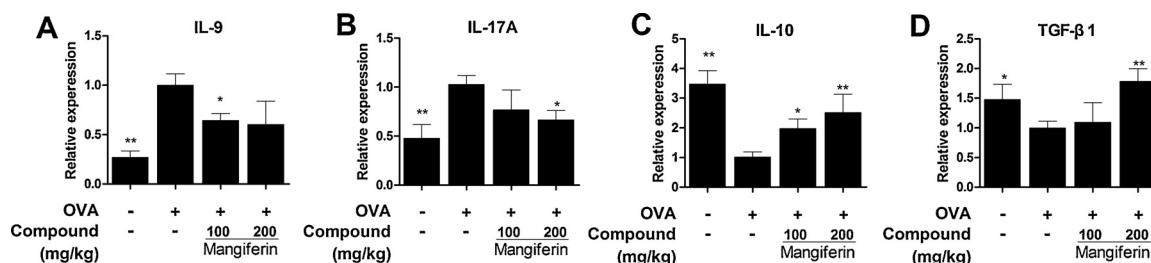


Fig. 5. Mangiferin Regulated the mRNA levels of IL-9, IL-17A, IL-10 and TGF- β 1 in lung tissues. The relative expression of IL-9, IL-17A, IL-10 and TGF- β 1 mRNA in lung tissues were analyzed with real-time quantitative PCR. The data are represented as the means $\pm$ SDs (n = 12). Compared with model group: *P < 0.05; **P < 0.01.

revealed excessively high levels of PU.1 and ROR γ t and low level of Foxp3 in OVA-induced asthmatic mouse model compared with normal control group. However, mangiferin significantly suppressed the expression of PU.1 and ROR γ t ($P < 0.01$ or 0.05) and elevated the expression of Foxp3 ($P < 0.05$ in high-dose group). IHC revealed protein expression changes were similar as WB ones (Fig. 7A–C). Compared with normal control group, the positive expressions of PU.1 and ROR γ t significantly increased, but that of Foxp3 obviously decreased in model group. Mangiferin could restore the abnormal expressions of PU.1, ROR γ t and Foxp3 in lungs in asthmatic mouse model.

4. Discussion

Asthma is a complex chronic respiratory inflammatory disease, which is associated with AHR and tissue remodeling of the airway structure (Murdoch and Lloyd, 2010). The syndrome of asthma is a broad spectrum of presentations and clinical courses, including cough, restlessness, and others (Blakey et al., 2013). In this study, we found that animals developed asthmatic symptoms of sneezing or coughing, shortness of breath, tucked-up abdomen and so on in OVA-challenged mouse model. Mangiferin treatment significantly alleviated these symptoms and decreased AHR, indicating that mangiferin exerted protective effect against allergic asthma.

In recent years, our research group did lots of studies on mangiferin, including pharmacology, pharmacokinetics and toxicology of mangiferin (Wang et al., 2017; Wei et al., 2016; Luo et al., 2017; Pan, 2017). All of these studies demonstrated that a wide margin of safety was expected for orally administered mangiferin. In this study, we selected

two doses of mangiferin (100 and 200 mg/kg) based on the results of previous study in which three doses (50, 100, and 200 mg/kg) of mangiferin were used (Guo et al., 2014). We found that 100 and 200 mg/kg showed better therapeutic effects than 50 mg/kg in most cases from our experiences. Therefore, we selected doses of 100 and 200 mg/kg for the current study and no animal death was found with both doses in this study.

EOS, as end-stage effector cells in asthma, could release an array of products that damaged epithelium, induced mucus production, bronchoconstriction and airway remodeling (Hogan et al., 2008; Akuthota et al., 2008). EOS inflammatory response has been indicated as the hallmark of airway inflammation in asthma (Possa et al., 2013). In current study, we demonstrated that mangiferin significantly attenuated asthma attacks in OVA-induced asthmatic animals with reduced number of EOS in BALF, less infiltration of leukocytes and mucus secretion in lungs compared to asthma model group. These results suggested that mangiferin could alleviate the pathological changes of lung tissue, reduce bronchial mucosa in lung and attenuate airway inflammation in asthma. Recently, it was reported that STAT6 signaling was critical for the responses of eosinophils during the development of allergic airway disease. STAT6 deficient eosinophils fail to migrate to the lung during the development of allergic airway inflammation (Stokes et al., 2015). Global STAT6 deficient mice are unable to recruit inflammatory cells to the lungs, including eosinophils in allergic airway disease (Hoshino et al., 2004). Our previous study (Guo et al., 2014) showed that mangiferin inhibited increased protein expression of STAT6 and levels of p-STAT6 in asthma mice lung tissue, which suggested mangiferin could reduce the infiltration of EOS via inhibiting

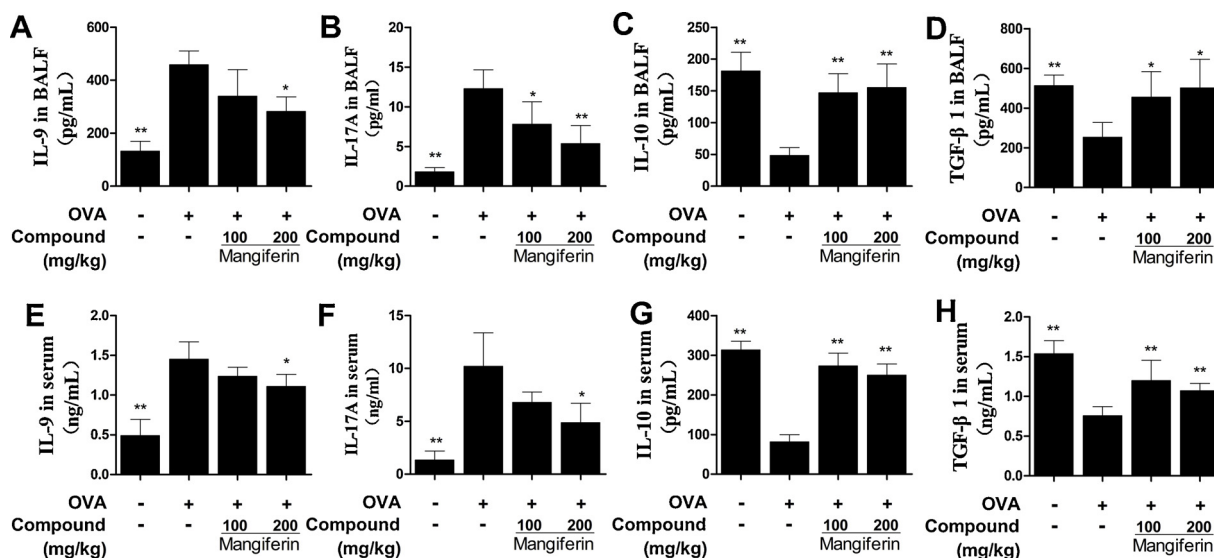


Fig. 6. Mangiferin had reciprocal effects on the secretion of IL-9, IL-17A, IL-10 and TGF- β 1 in BALF and serum. BALF and serum were collected at 24 h after the last OVA challenge. The levels of IL-9, IL-17A, IL-10 and TGF- β 1 in BALF (A–D) and serum (E–H) were measured using ELISA kits and indicated in each panel. The data are represented as the means $\pm$ SDs (n = 12). Compared with model group: *P < 0.05; **P < 0.01.

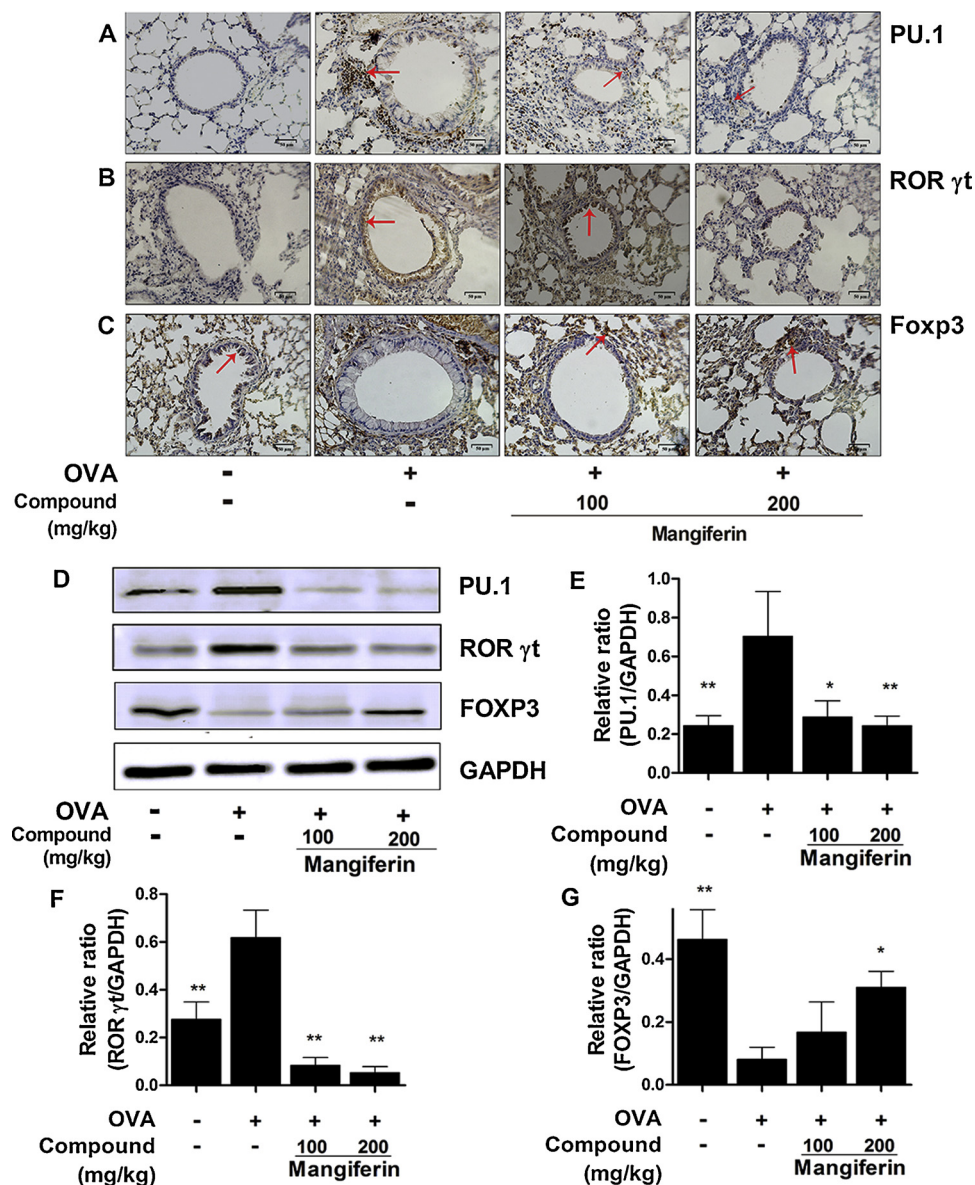


Fig. 7. Mangiferin regulated the expression of PU.1, ROR γ t and Foxp3 in lung tissue. (A–C) The expressions of PU.1, ROR γ t and Foxp3 in lung tissue were detected by immunohistochemistry. A representative picture is shown (magnification 400 $\times$). (D) The levels of PU.1, ROR γ t and Foxp3 in lung tissue were measured by western blot. (E–G) Bar plot of the average relative ratio of PU.1, ROR γ t and Foxp3. The data are represented as the means $\pm$ SDs (n = 12). Compared with model group: * P < 0.05; ** P < 0.01.

STAT6 signaling pathway.

The cells are important regulators of the adaptive immune response. The cells were characterized as either Th1, Th2, Th9, Th17, Th22 and Treg subtypes, based on the cytokines they produce. Asthma is originally characterized by Th2 type inflammation, leading to airway hyperresponsiveness and tissue remodeling (Maddox and Schwartz, 2002; Ferreira, 2004; Kikkawa et al., 2012). In the last several years the complexity of T-cell subtypes and highly heterogeneous and the potential role of airway structural cells in the immunopathology of asthma has increased (Lloyd and Saglani, 2013). The balance between effector Th2 and Th1 cells, and novel T-cell subsets, including Treg cells, Th17, Th9, and Th22, have been described, and there is much interest in how to redress this equilibrium. Our previous study showed that the levels of other non-Th1/Th2 cytokines such as IL-9, IL-17 and IL-10 were also altered upon mangiferin treatment. This promoted us to examine whether mangiferin may regulate the differentiation of Th9, Th17, and Treg cells. Therefore, in this study we used FACS to determine the differentiation of Th9, Th17 and Treg in spleen. Generally, it is the best

to detect above T cell subsets in lung tissue. However, we failed to detect them in lungs due to not being able to collect enough numbers of T cells in lungs. Considering spleen is the secondary immune organ where the lymphocytes do their jobs, we detected the T cell subsets in spleen, which was also employed to evaluate anti-asthma effects in other similar published articles (Qin et al., 2017; Ma et al., 2014; Yang, 2019).

Th9 cells are a subset of T cells that develop from naive T cells and mainly produce the cytokine IL-9. Functionally, Th9 cells promote allergic responses, resulting in enhanced pathology mediated by the specific recruitment and activation of mast cells in the lung. Th9 cell numbers are increased in the peripheral blood of asthma subject compared with healthy body (Jones et al., 2012). Similarly, IL-9 was also involved in the immunopathology of asthma via influencing a variety of distinct cell types like T cells, B cells, mast cells and airway epithelial cells (Koch et al., 2017). Enhanced expression of IL-9 had been verified in the lungs of asthma in mouse asthma model (Stokes et al., 2015). IL-17A (Park et al., 2005; Cosmi et al., 2011), which stimulates airway

epithelial cells to produce pro-inflammatory mediators. Emerging evidence suggested that Th17 cytokines including IL-17A, IL-17F, and IL-22 increased airway neutrophils infiltration, induced airway mucous cell metaplasia, and had pleotropic effects on airway smooth muscle with narrowed airway (Newcomb and Peebles, 2013). In the present study, we showed that mangiferin reduced the ratio of Th9 and Th17 cells, decreased the mRNA and protein levels of IL-9, IL-17A in OVA-induced asthmatic mouse model. These findings suggested that mangiferin might exert anti-asthmatic effect by inhibiting the differentiation and function of Th9 and Th17 cells.

PU.1, an ETS-family transcription factor, promotes polarization of Th9 subset through direct binding on IL-9 promoter (Jabeen et al., 2013; Kaplan, 2017). The gene expression of PU.1 is up-regulated in asthma patients. ROR γ t, a unique lineage-specific transcription factor, is selectively expressed in Th17 cells and regulates differentiation of Th17 cells (Ivanov et al., 2006; Korn et al., 2009). Overexpression of ROR γ t promoted Th17 differentiation both in vitro and in vivo (Yang et al., 2008; Korn et al., 2009; Blakey et al., 2013). Our data showed that mangiferin markedly inhibited expression of PU.1 and ROR γ t in lungs, which strongly implied that mangiferin might tune the expression of transcription factors of PU.1 and ROR γ t to regulate the differentiation and function of Th9, Th17 cells.

Treg cells are a subset of CD4⁺ CD25⁺ T lymphocytes that were originally termed "suppressor cells" (Gershon, 1975). Genetic and immunological evidence reinforce the idea of a pivotal role for Treg cells in anti-inflammatory to allergens and preventing allergic disorder (Chatila et al., 2000; Wu et al., 2017; Zhu et al., 2017). Previous study indicate that Treg cells may interfere with asthma at different stages, such as sensitisation, allergic inflammation, airway remodeling and AHR (Martin-Orozco et al., 2017). Suppressive function of Treg cells is mediated by multiple mechanisms that involve the release of inhibitory cytokines, such as TGF- β and IL-10 (Noack and Miossec, 2014). It has been reported that TGF- β affects airway remodeling during late phase of lung inflammation in asthma. Treg cell activity is regulated by a specific transcription factor namely forkhead/winged helix (Foxp3), which is required for the differentiation of Treg cells and is often used as a specific marker for Treg cells (Chen et al., 2011). Our study showed that mangiferin enhanced the differentiation of Treg by up-regulating the expression of Foxp3 with increasing the secretion of TGF- β 1 and IL-10, which suggested that the protective effect of mangiferin might be associated with the regulation of Treg cells.

To sum up, our study revealed that mangiferin possesses anti-asthma activity, manifested with ameliorating the symptoms of asthma and pathological changes in lungs and bronchi. We further demonstrated that mangiferin regulated the balance of Th9, Th17, and Treg cells in OVA-induced asthmatic mouse model via tuning the expression of transcription factors of PU.1, ROR γ t, and Foxp3, which orchestrate the secretion and activities of cytokines of IL-9, IL-17A, IL-10, and TGF- β 1 to attenuate asthma symptoms. Taken together, these results may conclude the therapeutic effect of mangiferin on inflammatory diseases and immunologic disorders of asthma.

Author contributions

HWG, CXY contributed to designing the work. CXY, MC, GHH, HBY and ZHS wrote the manuscript, analyzed and interpreted of data. MC, GHH, TJL, DZ, WPL, QFL and HYZ performed the experiments. HWG, JGD, JZ and YL provided reagents/materials/analysis tools. All authors read and approved the final version.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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