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Regulation of Th17/Treg function contributes to the attenuation of chronic airway inflammation by icariin in ovalbumin-induced murine asthma model

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ABSTRACT

Icariin which is a flavonoid glucoside isolated from Epimedium brevicornu Maxim, has been reported to have anti-osteoporotic, anti-inflammatory and anti-depressant-like activities. In this study, we observed the effect of icariin on airway inflammation of ovalbumin (OVA)-induced murine asthma model and the associated regulatory mode on T-helper (Th)17 and regulatory T (Treg) cell function. Our data revealed that chronic OVA inhalation induced a dramatic increase in airway resistance (R_L) and decrease in the lung dynamic compliance (Cdyn), and icariin and DEX treatment caused significant attenuation of such airway hyperresponsiveness (AHR). BALF cell counts demonstrated that icariin and DEX led to a prominent reduction in total leukocyte as well as lymphocyte, eosinophil, neutrophil, basophil and monocyte counts. Histological analysis results indicated that icariin and DEX alleviated the inflammatory cells infiltrating into the peribronchial tissues and goblet cells hyperplasia and mucus hyper-production. Flow cytometry test demonstrated that icariin or DEX administration resulted in a significant percentage reduction in CD4+RORγt+ T cells and elevation of CD4+Foxp3+ T cells in BALF. Furthermore, icariin or DEX caused a significant reduction in IL-6, IL-17 and TGF-β level in BALF. Unfortunately, icariin had no effect on IL-10 level in BALF. Western blot assay found that icariin or DEX suppressed RORγt and promoted Foxp3 expression in the lung tissue. qPCR analysis revealed that icariin and DEX resulted in a notable decrease in RORγt and increase in Foxp3 mRNA expression in isolated spleen CD4+ T cell. In conclusion, our results suggested that icariin was effective in the attenuation of AHR and chronic airway inflammatory changes in OVA-induced murine asthma model, and this effect was associated with regulation of Th17/Treg responses, which indicated that icariin may be used as a potential therapeutic method to treat asthma with Th17/Treg imbalance phenotype.

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Introduction

Asthma is a common, chronic respiratory inflammatory disease affecting 1–18% of the population in different countries, and is characterized by variable symptom of wheeze, shortness of breath, chest tightness and cough, as well as by variable expiratory airflow limitation (GINA, 2014 update). It is agreed that asthma is a heterogeneous disease, with different underlying disease processes.

T-helper (Th)17 cell has been recognized as the third subset of effector CD4+ T cell distinct from the classic lineages of Th1 and Th2 cell. IL-17 is a pro-inflammatory cytokine mainly secreted from Th17 cell and is considered as its signature cytokine (Park et al., 2005). Recent evidence suggests except for Th2 cell, Th17 cell is involved in the pathogenesis of antigen-induced airway inflammation (Li and Hua, 2014; Cosmi et al., 2011). Th17 and IL-17 are described to be responsible for severe asthma (Morishima et al., 2013). IL-17 is found to have the potential to recruit and activate neutrophils and eosinophils to the airways and stimulate them and other cells to secrete inflammatory mediators (Korn et al., 2009; Iwakura et al., 2011).

Regulatory T (Treg) cell, another subtype of CD4+ T cell, has been implicated in the process of the maintenance of immune homeostasis by suppressing aberrant immune responses which are harmful

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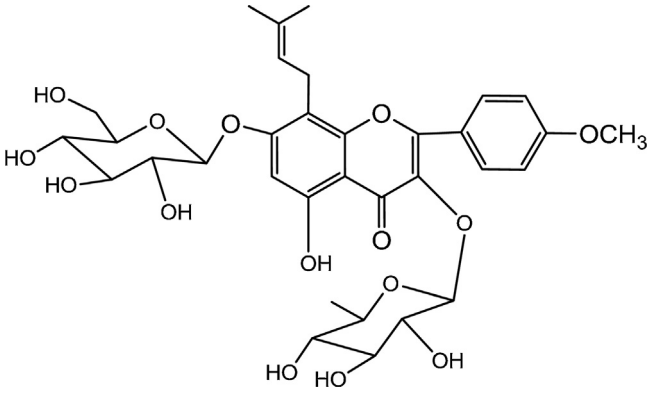


Fig. 1. Chemical structure of icariin.

to the host (Ohkura et al., 2013; Rudensky, 2011). Treg cell activity is impaired in asthma (Luo et al., 2006) and restoration of Treg cell function is documented to suppress allergic inflammation in asthma. Adoptive transfer of Treg cell into allergen-induced mice alleviates asthma manifestations (Kearley et al., 2008). Treg phenotype is altered in inflammatory lungs and abrogated by inhalation of IL-17, indicating the involvement of Th17 cell in abrogation of Treg cell-mediated tolerance in chronic allergic airway inflammation (Zhao et al., 2013).

Icariin is a flavonoid glucoside isolated from Epimedium brevicornu Maxim, which has been frequently used in traditional Chinese medicine (TCM) to treat diseases with Kidney essence deficiency. Icariin was reported to have anti-osteoporotic (Nian et al., 2009), anti-inflammatory (Xu et al., 2010) and anti-depressant-like (Pan et al., 2007) activities. Our previous studies showed that TCM formula Bu-Shen-Yi-Qi-Tang which include Epimedium brevicornu Maxim has prominent efficacy in alleviating asthma symptoms of asthma patients and attenuating inflammation in murine asthma model (Luo et al., 2014). However, whether icariin has inhibitory effect on the inflammatory changes in asthma is rarely reported. In this study, we observed the effect of icariin on airway inflammation of ovalbumin-induced murine asthma model and the associated regulatory mode on Th17 and Treg cell function.

Materials and methods

Reagents

Ovalbumin (OVA), aluminum hydroxide and methacholine (Mch) were purchased from Sigma–Aldrich Co. LLC. Icariin (purity > 98%) was purchased from Chengdu Must Biological Technology Co., Ltd. Bio-Plex kit was purchased from Bio-Rad Laboratories, Inc. PVDF membranes were purchased from Millipore. Anti-mouse RORγt and Foxp3 antibodies were purchased from abcam and cell signaling technology. Anti-mouse β-actin and HRP-conjugated IgG were purchased from KangChen Bio-Tech. Western blotting detection reagents were purchased Engreen Biosystem Co., Ltd. FITC-labeled anti-mouse CD4, PE-labeled anti-mouse RORγt and Alexa Fluor® 647-labeled anti-mouse Foxp3 were purchased from BD Pharmingen. Foxp3/Transcription Factor Staining Buffer Set was purchased from eBioscience. Mouse CD4+ T Cell Isolation Kit was purchased from Miltenyi Biotec (Fig. 1).

Animals

Female specific-pathogen-free BALB/c mice (12–16 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. and housed with food and water freely available. One hundred and twenty mice were randomly divided into six groups (20 mice/group), including the normal control (NC), OVA-induced asthma model (A), icariin (25 mg/kg, 50 mg/kg, 100 mg/kg) and the dexamethasone (DEX) groups. The protocol of the study was approved by the Committee on the Ethics of Animal Experiments of Fudan University.

OVA-induced murine asthma model establishment and treatment

As presented in Fig. 2, mice asthma model were induced by multiple sensitization with OVA and $Al(OH)_3$ suspension (1:100) in 0.2 mL normal saline on days 0, 7, 14 and 21. The sensitized mice were challenged with 3% OVA solution (w/v) for 30 min at a frequency of three times per week for 8 weeks from day 25. The NC group were sensitized and challenged with normal saline. Mice were placed in a plexiglass chamber and challenged with an ultrasonic nebulizer (402AI, Yuyue Medical equipment company, Jiangsu, China). The day before the first challenge, different doses of icariin and DEX (1 mg/kg) were oral administrated every day before OVA challenge for 8 weeks. The NC and A groups were oral administrated with normal saline in parallel. Mice were sacrificed within 24 h after the last OVA challenge and treatment.

AHR measurement

Within twenty-four hours after last OVA exposure, airway responsiveness to Mch was assessed by Buxco pulmonary mechanics with an invasive measurements as described previously (Luo et al., 2014). Increasing doses of Mch (3.125, 6.25 and 12.5 mg/mL) were used to assess airway resistance (R_L) and lung dynamic compliance (Cdyn) of mice in each group.

Inflammatory cell count and Th17/Treg examination in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was obtained and centrifuged at $800 \times g$ for 10 min. The supernatant was stored at −80 °C for cytokines assay and the cell pellet was resuspended for inflammatory cell count and classification. The leukocyte in BALF were counted and classified into five categories by hemavet 950 instrument (Drew scientific group), including neutrophil (Neu), lymphocyte (Lym), monocyte (Mon), eosinophil (Eos) and basophil (Bas).

CD4+ RORγt+ and CD4+ Foxp3+ T cells in BALF were analyzed by flow cytometry. In brief, BALF cells were stained with FITC-labeled anti-mouse CD4 antibody, then fixed and permeabilized with Foxp3/Transcription Factor Staining Buffer Set. The cells were further stained with PE-labeled anti-mouse RORγt and Alexa Fluor® 647-labeled anti-mouse Foxp3 antibodies according to the manufacturer's instructions, followed by detection with a FACSCalibur instrument (BD Bioscience).

Histological analysis

The lung tissue was obtained, fixed in 4% paraformaldehyde and then embedded in paraffin. Lung sections were cut and stained with hematoxylin–eosin (H&E) and periodic acid-schiff (PAS). The inflammatory changes and mucus secretion were analyzed by light microscope.

Cytokines determination in BALF

The supernatant in BALF was analyzed by Bio-Plex and ELISA assay according to the manufacturer's instructions.

Western blot assay

Total protein in the lung tissue was extracted by RIPA protein extraction reagent and isolated by 10% sodium dodecyl

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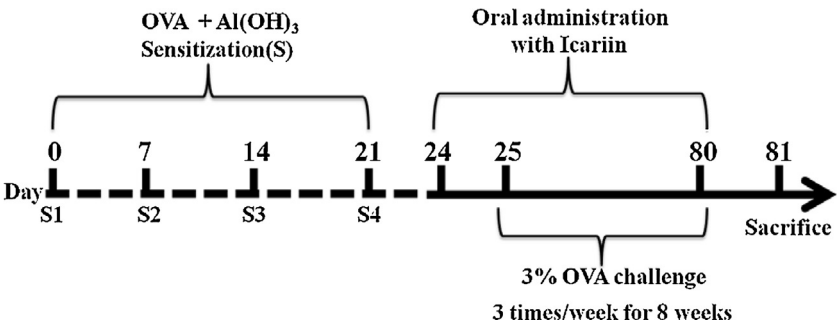


Fig. 2. Experimental protocol for establishment of the murine OVA asthma model and icariin treatment. Mice asthma model were induced by multiple sensitization with OVA and $Al(OH)_3$ suspension (1:100) in 0.2 mL normal saline on days 0, 7, 14 and 21. The sensitized mice were challenged with 3% OVA solution (w/v) for 30 min at a frequency of three times per week for 8 weeks from day 25. The NC group were sensitized and challenged with normal saline. The day before the first challenge, different doses of icariin and DEX were oral administrated every day before OVA challenge for 8 weeks. The NC and A groups were oral administrated with normal saline in parallel. Mice were sacrificed within 24 h after the last OVA challenge and treatment.

sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by electroblotting onto PVDF membranes. After electrotransfer, the PVDF membranes were blocked with 5% BSA and incubated overnight at 4 °C with primary antibodies (RORγt 1:4000 diluent, Foxp3 1:1000 diluent, β-actin 1:5000 diluent). The membranes were then incubated with HRP-conjugated IgG (1:5000 diluent) at 37 °C for 1 h and the blots were visualized and analyzed with Bio-Rad's Image Lab software.

Mouse CD4+ T cell Isolation and qPCR analysis

Mouse CD4+ T cells in spleen were isolation with magnetic activated cell sorting system according to the manufacturer's instructions and the purity of the selected CD4+ T cells were identified by flow cytometry. The isolated CD4+ T cells were >97% pure. Total RNA of the isolated CD4+ T cells was isolated and reverse transcripted into the first strand cDNA according to the manufacturer's instructions. Gene expression of RORγt and Foxp3 was measured with qPCR. The fold change of each gene was calculated using the ΔΔCt method with GAPDH gene as an internal control. The final data was presented as fold change compared to the gene expression of mice in NC group. The primers used for RORγt, Foxp3 and GAPDH were presented in Table 1.

Data analysis

Data was expressed as mean ± SD. Statistical significance of the differences were performed by one way analysis of variance (ANOVA) followed by the LSD test or Dunnett's test for multiple comparisons. A p-value of less than 0.05 was accepted as significant.

Table 1
Primer sequences used for qPCR amplification.

Gene	Sequence
Foxp3 (NM_001199347.1)	Forward primer: 5′-GGCCCTTCTCCAGGACAGA-3′ Reverse primer: 5′-GGCATGGGCATCCACAGT-3′
RORγt (NM_011281.3)	Forward primer: 5′-TGCGACTGGAGGACCTTCTA-3′ Reverse primer: 5′-TCACCTCCTCCCGTGAAAAG-3′
GAPDH (NM_008084.2)	Forward primer: 5′-CATGGCCTTCCGTGTTCCTA-3′ Reverse primer: 5′-GCGGCACGTCAGATCCA-3′

Results

Icariin suppressed AHR in OVA-induced asthma model

Hyperresponsiveness of the airways to bronchoconstrictor stimuli is a prominent feature of asthma and we assessed AHR to Mch of mice in each group. After chronic OVA inhalation, mice demonstrated obviously elevated AHR to increasing doses of Mch as compared to mice challenged with normal saline (Fig. 3). The OVA-induced asthma mice had increased R_L and decreased Cdyn

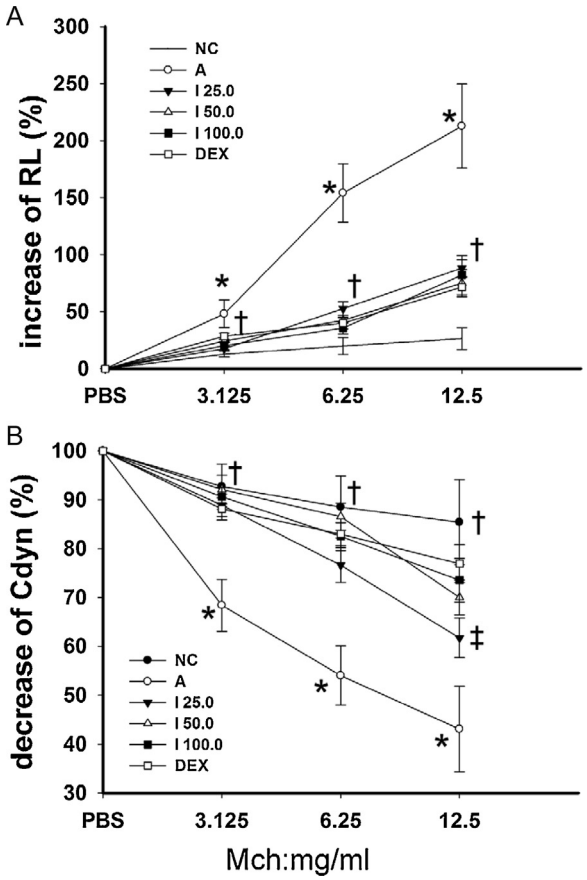


Fig. 3. Icariin suppressed AHR in OVA-induced asthma model. Airway responsiveness to Mch was assessed by Buxco pulmonary mechanics with an invasive measurements. (A) R_L changes in each group. (B) Cdyn changes in each group. Data was expressed as means ± SD values. *$p < 0.05$ vs NC group, †$p < 0.05$ vs A group, ‡$p < 0.05$ vs DEX group.

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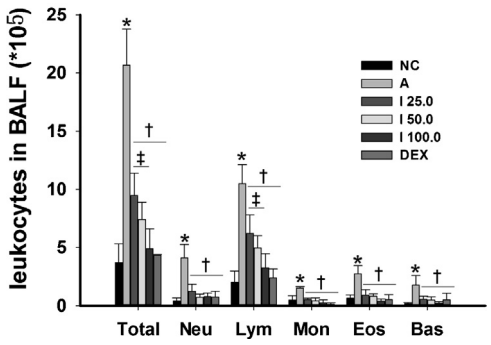


Fig. 4. Icariin inhibited inflammatory cell levels in BALF of OVA-induced asthma model. Data was expressed as means ± SD values. *$p < 0.05$ vs NC group, †$p < 0.05$ vs A group, ‡$p < 0.05$ vs DEX group.

compared to mice in NC group (Fig. 3A and B, $p < 0.05$). Icariin and DEX was effective in reducing AHR to Mch of asthma mice ($p < 0.05$), which manifested as a drop in R_L and an increase in Cdyn (Fig. 3A and B). All the three doses of icariin exerted marked inhibition on R_L compared to the asthma mice ($p < 0.05$), which had no difference with efficacy of DEX (Fig. 3A, $p > 0.05$). Furthermore, icariin also resulted in significant promotion in Cdyn of asthma mice ($p < 0.05$), and icariin (50 mg/kg and 100 mg/kg) was as effective as DEX in improvement of Cdyn (Fig. 3B, $p > 0.05$).

Icariin attenuated allergic airway inflammation in OVA-induced asthma model

Inflammatory cells count and classification in BALF were tested. Chronic OVA exposure led to increase of total leukocyte in BALF (Fig. 4, $p < 0.05$). The lymphocyte, eosinophil, neutrophil, basophil and monocyte were all increased significantly compared to mice in NC group (Fig. 4, $p < 0.05$). Icariin and DEX treatment led to a prominent reduction in total leukocyte as well as lymphocyte, eosinophil, neutrophil, basophil and monocyte counts in BALF (Fig. 4). Icariin (100 mg/kg) and DEX had the same potential in reducing total leukocyte and lymphocyte counts in BALF (Fig. 4, $p > 0.05$).

We further observed the allergic inflammatory changes in the lung tissue of mice in each group by histopathological analysis. H&E staining results revealed that a large number of inflammatory cells infiltrated into the peribronchial tissues of OVA inhalation mice compared with the NC mice (Fig. 5A). Icariin and DEX administration led to a dramatic attenuation of inflammatory infiltration around the airways (Fig. 5A). PAS staining results demonstrated a dramatically augmented goblet cells hyperplasia and mucus hyperproduction compared to the OVA-induced mice (Fig. 5B). Icariin and DEX treatment decreased accumulation of goblet cells and mucus secretion (Fig. 5B).

Icariin regulated Th17 and Treg percentages in BALF of OVA-induced asthma model

To determine whether Th17 and Treg cell were involved in the allergic inflammation in the lung, Th17 and Treg percentage changes in BALF of mice in each group were determined by flow cytometry. We found that mice in chronic OVA exposure group demonstrated markedly elevated CD4 + RORγt+ T cells (Fig. 6A and C) and decreased CD4 + Foxp3+ T cells (Fig. 6B and D) in BALF compared to mice challenged with normal saline ($p < 0.05$). Icariin and DEX administration resulted in a significant percentage reduction in CD4 + RORγt+ T cells compared to the OVA-induced asthma mice (Fig. 6A and C, $p < 0.05$). Icariin (25 and 100 mg/kg) treatment led to a dramatic percentage rise in CD4 + Foxp3+ T cells (Fig. 6B and D, $p < 0.05$).

Icariin regulated cytokines secretion in BALF of OVA-induced asthma model

TGF-β and IL-6 play a role in the induction of Th17 cell differentiation and activation (Zhou et al., 2007; Burgler et al., 2009; Yang et al., 2008). IL-17 is the signature cytokine of Th17 cell and contribute to airway inflammation in asthma. Furthermore, TGF-β and IL-10 are important in Treg cell induction (Ryanna et al., 2009). To clarify effect of icariin on these cytokines important for Th17 and Treg induction and function, we assessed these cytokines secretion in BALF of mice in each group. IL-6 and IL-17 was found elevated significantly in OVA-induced asthma model compared to the NC mice (Fig. 7A and D, $p < 0.05$). Icariin (50 mg/kg) induced a marked decrease in IL-6 level in BALF compared to the asthma model mice (Fig. 7A, $p < 0.05$). Icariin and DEX administration caused a significant reduction in IL-17 level in BALF compared to the OVA-induced asthma mice (Fig. 7D, $p < 0.05$). On the other hand, long term OVA inhalation induced a marked decrease in IL-10 level in BALF compared to mice in NC group (Fig. 7B, $p < 0.05$). DEX treatment caused a significant rise in IL-10 level compared to OVA-induced asthma mice (Fig. 7B, $p < 0.05$). Unfortunately, icariin had no effect on IL-10 level in BALF (Fig. 7, $p > 0.05$). Furthermore, we observed a dramatic increase in TGF-β level in BALF of the OVA-induced asthma model (Fig. 7C, $p < 0.05$). Icariin and DEX treatment caused a prominent reduction in BALF TGF-β level compared to the asthma model mice, and Icariin (50 mg/kg) had better potential than DEX (Fig. 7C, $p < 0.05$).

Icariin regulated Th17 and Treg transcription factor expression in lung tissue of OVA-induced asthma model

Transcription factors RORγt and Foxp3 are crucial for Th17 and Treg cell differentiation and function, respectively (Ohkura et al., 2013; Ivanov et al., 2006). To identify the role of icariin on RORγt and Foxp3 expression, we measured RORγt and Foxp3 expression in the lung tissue of mice in each group. Th17 transcription factor RORγt elevated markedly in the OVA inhalation group as compared with the NC mice (Fig. 8A and B, $p < 0.05$). Icariin (25 and 50 mg/kg) treatment suppressed RORγt expression in the lung tissue compared to the OVA-induced asthma mice (Fig. 8A and B, $p < 0.05$). DEX exerted no inhibitory effect on RORγt expression (Fig. 8A and B, $p > 0.05$). Additionally, Treg transcription factor Foxp3 expression reduced significantly in the lung tissue of asthma model mice (Fig. 8C and D, $p < 0.05$). Icariin (25 and 100 mg/kg) and DEX administration resulted in a dramatic increase in Foxp3 expression in the lung tissue (Fig. 8C and D, $p < 0.05$).

Icariin regulated Th17 and Treg transcription factor expression in isolated spleen CD4+ T cell of OVA-induced asthma model

We also determined the mRNA level of RORγt and Foxp3 in the isolated spleen CD4+ T cell from mice in each group. There was a prominent increase in RORγt (Fig. 9A) and decrease in Foxp3 (Fig. 9B) gene expression after long term OVA inhalation as compared with mice in the NC group ($p < 0.05$). Icariin and DEX treatment led to a notable decrease in RORγt (Fig. 9A) and increase in Foxp3 (Fig. 9B) mRNA level compared to the OVA-induced asthma mice ($p < 0.05$). Furthermore, the capacity of icariin (50 and 100 mg/kg) in reducing RORγt gene expression was the same to DEX (Fig. 9A, $p > 0.05$).

Discussion

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation (GINA, 2014 update). In this study, we induced an OVA-specific murine asthma model, and observed effect

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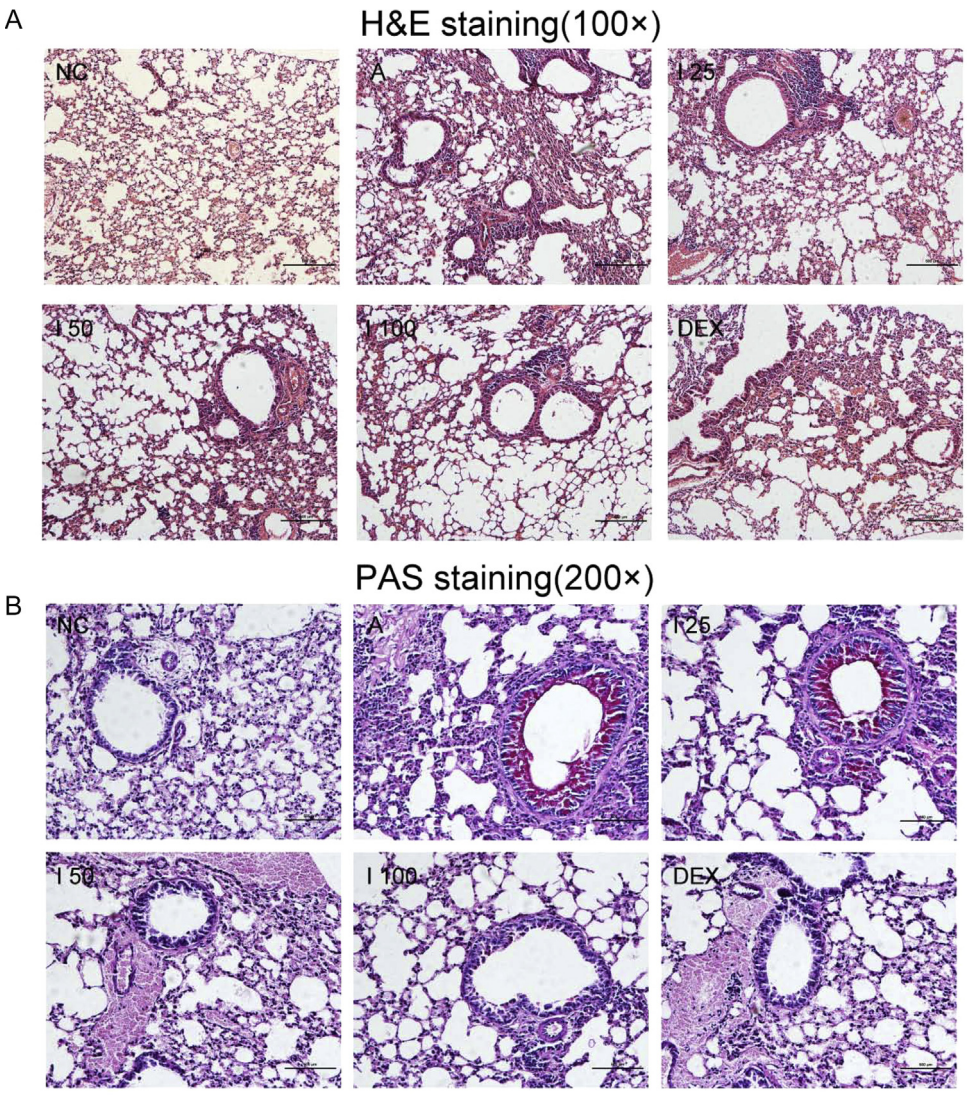


Fig. 5. Icariin attenuated airway inflammation and mucus secretion of OVA-induced asthma model. (A) The inflammatory changes of the lung measured by H&E staining. (B) The changes in mucus production measured by PAS staining. Bar = 500 μm.

of icariin on airway inflammation and the associated regulatory mode on Th17 and Treg cell function.

Consistent with previous findings, our observations revealed that AHR increased dramatically in OVA-induced asthma model, and a decline in AHR was observed in icariin and DEX treatment groups. Meanwhile, icariin and DEX caused a significant reduction in total leukocyte as well as lymphocyte, eosinophil, neutrophil, basophil and monocyte counts in BALF. Eosinophil infiltration in the airway is a hallmark of allergic asthma, and our data suggested a suppression of inflammation in asthma by icariin. Furthermore, icariin and DEX also led to alleviation of the inflammatory cells infiltrating into the peribronchial tissues and inhibition of goblet cells hyperplasia and mucus production. These data indicated the attenuation of airway inflammation by icariin. An positive interaction exists between IL-17 and eosinophilic inflammation (Dias and Banerjee, 2013). Furthermore, IL-17 was also implicated in induction of neutrophil-recruiting chemokine IL-8 secretion. The suppression of eosinophil and neutrophil counts in BALF may be related to the decline in IL-17 level by icariin.

Results obtained from cytokine analysis indicated that IL-6, IL-17 and TGF-β level in BALF declined markedly by icariin. There is mounting evidence that Th17 cell and its cytokine IL-17 play critical roles in the induction of inflammation in asthma. IL-17 is increased in asthma and IL-17-deficient mice developed reduced inflammatory responses in asthma model (Wakeland, 2014). On the contrary, Treg cell has the potential to control inflammation in asthma (Langier et al., 2012). Th17 signature cytokine IL-17 elevated in asthma (Wang et al., 2010, Zhao et al., 2010) and contributed to allergen-induced AHR (Kudo et al., 2012). TGF-β and IL-6 are reported to promote the differentiation of Th17 cell (Morishima et al., 2013; Hemdan et al., 2012). IL-6 in combination with TGF-β induces Th17 transcription factor RORγt expression, and RORγt in turn bind to the Foxp3 promoter to inhibit its transcription (Burgler et al., 2010). The inhibitory effect of icariin on IL-6, IL-17 and TGF-β secretion into BALF indicated the suppression of Th17 function by icariin. Furthermore, IL-6 has the ability to reduce Foxp3 expression (Lal et al., 2009) and cause the loss of Treg cell suppressive function (Pasare and Medzhitov, 2003). Icariin inhibited IL-6 secretion in BALF, indicating the abrogation of its suppressive effect on Treg function. Role of TGF-β in Th17 and Treg differentiation is still controversial. One report found that Foxp3 expression increased in the absence of TGF-β (Kim and Leonard, 2007). On the contrary, other reports support the role of TGF-β as an important positive regulator of Foxp3 expression (Mantel et al., 2007). As described above, we speculate that the role of TGF-β in Th17 or Treg activity may depend on the local cytokine milieu. In our study, TGF-β elevated

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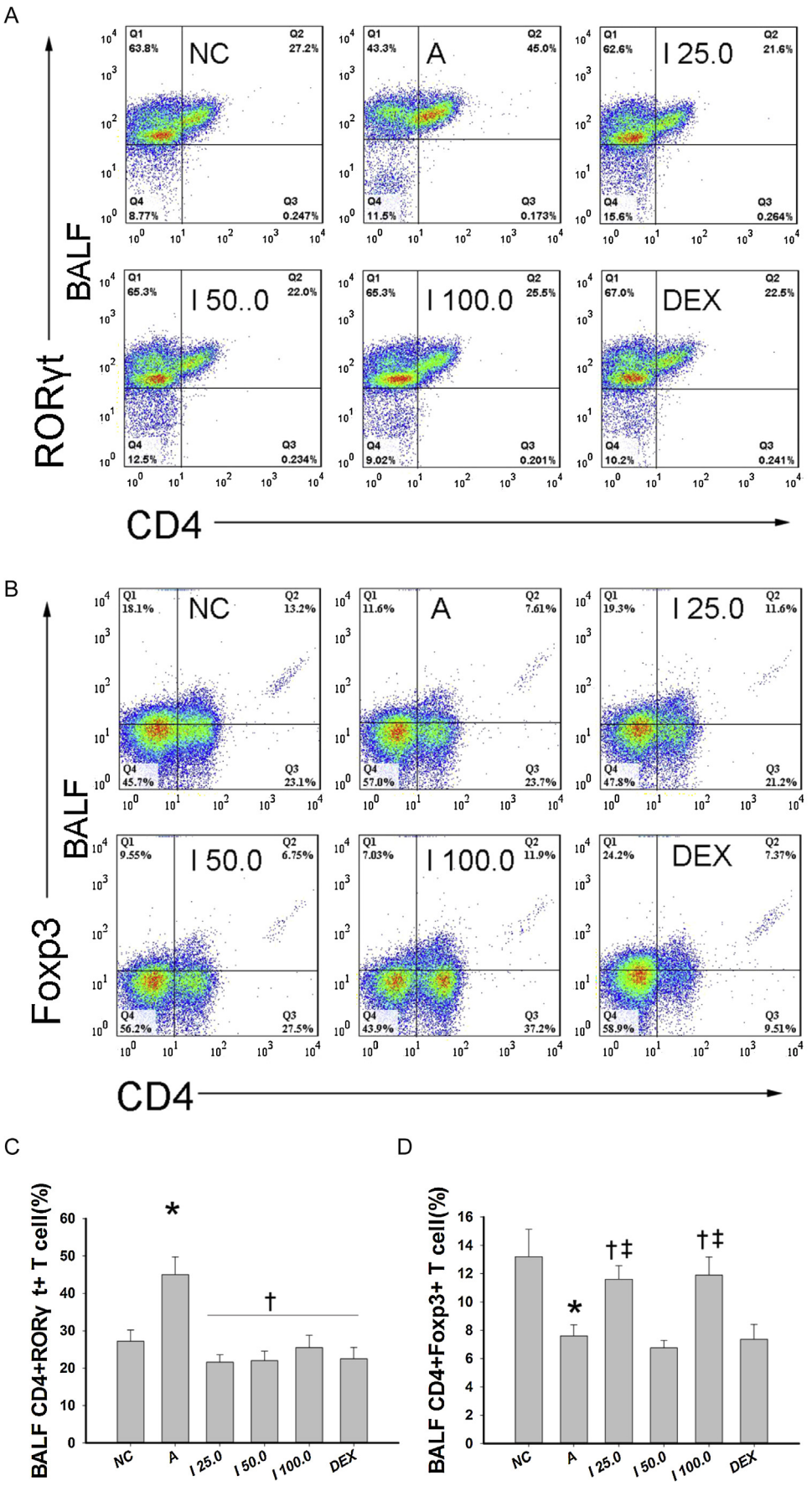


Fig. 6. Icariin regulated Th17 and Treg percentages in BALF of OVA-induced asthma model. (A) and (C) Th17 percentage changes in BALF of mice in each group. (B) and (D) Treg percentage changes in BALF of mice in each group. Data was expressed as means ± SD values. $^{*}p < 0.05$ vs NC group, †$p < 0.05$ vs A group, ‡$p < 0.05$ vs DEX group.

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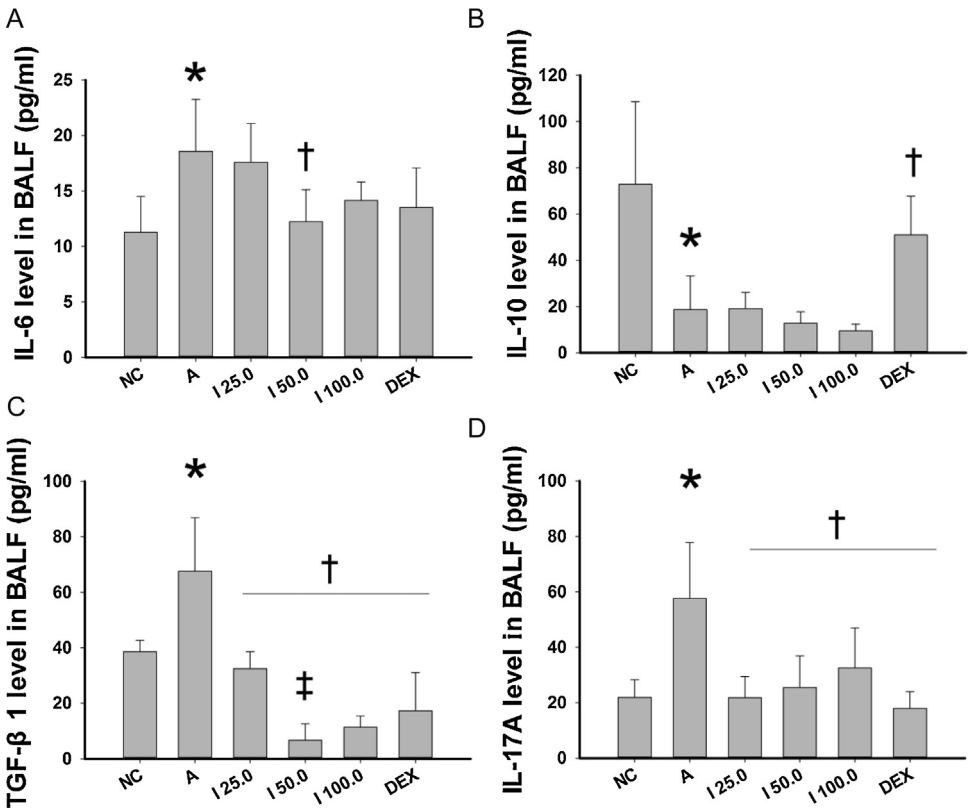


Fig. 7. Icariin regulated cytokines secretion in BALF of OVA-induced asthma model. Data was expressed as means ± SD values. *$p < 0.05$ vs NC group, †$p < 0.05$ vs A group, ‡$p < 0.05$ vs DEX group.

significantly after long term OVA inhalation, indicating that TGF-β may exist as a pro-inflammatory cytokine and may contribute to Th17 differentiation. Icariin induced a dramatic decrease in TGF-β level in BALF, suggesting the inhibition of inflammation and that it may be associated with inhibition of Th17 function. IL-10 is involved in the induction of Treg differentiation and Treg cell-mediated inhibition was dependent on the induction of IL-10 from host T cells in the lung. IL-10 treatment decreased Th17 cell function (Huber et al., 2011) and deletion of IL-10 in Foxp3-expressing CD4+ T cells promoted allergic airways disease (Rubtsov et al., 2008). However, our data demonstrated there was no obvious effect of icariin on IL-10 level in BALF, indicating a variety of regulatory pathways may exist in Treg differentiation and function.

Recent reports demonstrated that RORγt was crucial for Th17 differentiation and function (Ivanov et al., 2006). Treg cell is a subtype of CD4+ T cell with immunomodulatory or immunosuppressive activities. Foxp3 plays a key role in Treg cell development and function (Ohkura et al., 2013) and Foxp3 deficiency promotes allergic disease (Torgerson and Ochs, 2007). Foxp3+ Treg cell has the potential to migrate to inflammation sites and suppress

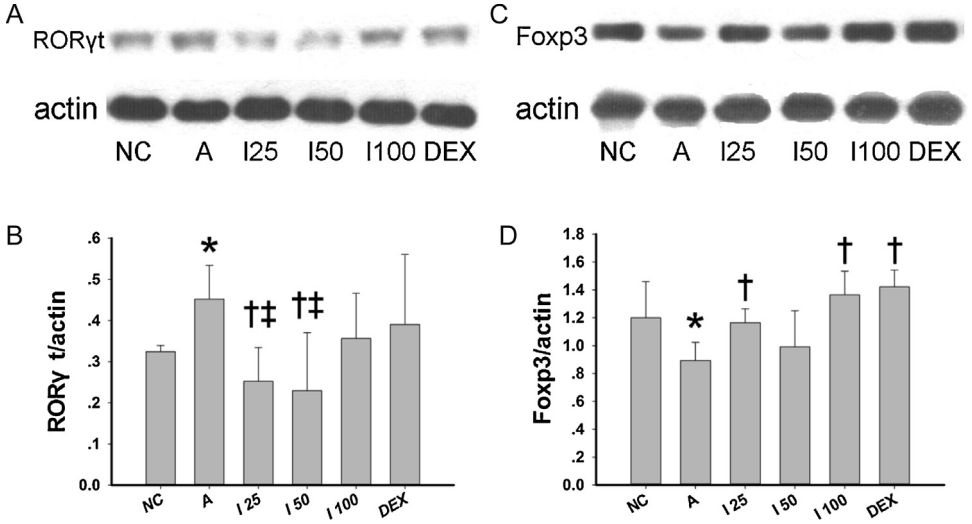


Fig. 8. Icariin regulated Th17 and Treg transcription factor expression in lung tissue of OVA-induced asthma model. Data was expressed as means ± SD values. *$p < 0.05$ vs NC group, †$p < 0.05$ vs A group, ‡$p < 0.05$ vs DEX group.

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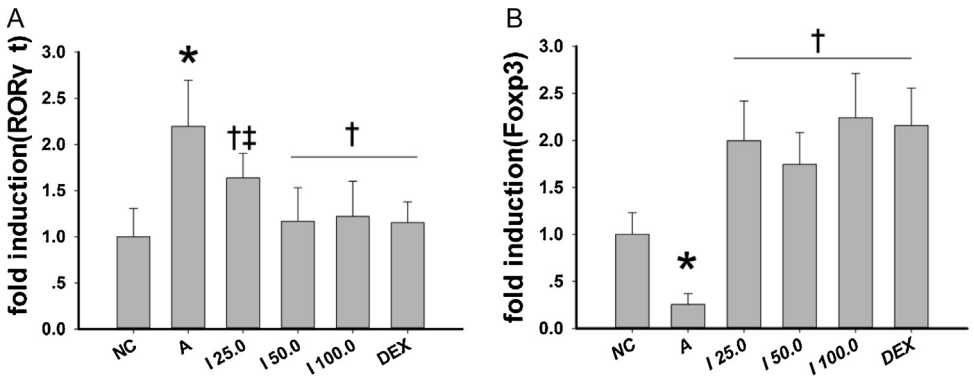


Fig. 9. Icariin regulated Th17 and Treg transcription factor expression in isolated spleen CD4+ T cell of OVA-induced asthma model. Data was expressed as means ± SD values. $^{*}p < 0.05$ vs NC group, $\dagger p < 0.05$ vs A group, $\ddagger p < 0.05$ vs DEX group.

effector Th cells such as Th17 cell (Chaudhry et al., 2009). Results from gene and protein expression analysis of CD4+ T cell from spleen and lung tissue of mice were consistent with the above published data. Icariin administration resulted in a dramatic decline in RORγt and rise in Foxp3 gene and protein expression, suggesting the regulatory role of icariin on Th17 and Treg cell function. Moreover, icariin promoted CD4 + Foxp3+ T cells and inhibited CD4 + RORγt+ T cells in BALF of OVA-induced asthma mice, which provided further evidence for the anti-inflammatory capacity of icariin through regulation of Th17 and Treg cell.

Previous studies demonstrated that icariin had anti-inflammatory activities (Xu et al., 2010) and could attenuate inflammation in murine asthma model (Luo et al., 2014). In this study, our data further revealed that icariin could alleviate AHR and chronic airway inflammation, down-regulate IL-6, IL-17 and TGF-β as well as promotion of Treg function and suppression of Th17 function. In conclusion, the present results reveal that icariin was effective in the attenuation of AHR and chronic airway inflammatory changes in OVA-induced murine asthma model, and this effect was associated with regulation of Th17/Treg responses.

Conflict of interest

The authors declare that they have no conflict of interest.

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References

Burgler, S., Ouaked, N., Bassin, C., Basinski, T.M., Mantel, P.Y., Siegmund, K., Meyer, N., Akdis, C.A., Schmidt-Weber, C.B., 2009. Differentiation and functional analysis of human T(H)17 cells. J. Allergy Clin. Immunol. 123, 588.

Burgler, S., Mantel, P.Y., Bassin, C., Ouaked, N., Akdis, C.A., Schmidt-Weber, C.B., 2010. RORC2 is involved in T cell polarization through interaction with the FOXP3 promoter. J. Immunol. 184, 6161.

Chaudhry, A., Rudra, D., Treuting, P., Samstein, R.M., Liang, Y., Kas, A., Rudensky, A.Y., 2009. CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner. Science 326, 986.

Cosmi, L., Liotta, F., Maggi, E., Romagnani, S., Annunziato, F., 2011. Th17 cells: new players in asthma pathogenesis. Allergy 66, 989.

Dias, P.M., Banerjee, G., 2013. The role of Th17/IL-17 on eosinophilic inflammation. J. Autoimmun. 40, 9.

GINA Report 2014 update. Global Strategy for Asthma Management and Prevention. [http://www.ginasthma.org/].

Hemdan, N.Y., Birkenmeier, G., Wichmann, G., 2012. Key molecules in the differentiation and commitment program of T helper 17 (Th17) cells up-to-date. Immunol. Lett. 148, 97.

Huber, S., Gagliani, N., Esplugues, E., O'Connor Jr., W., Huber, F.J., Chaudhry, A., Kamanaka, M., Kobayashi, Y., Booth, C.J., Rudensky, A.Y., Roncarolo, M.G., Battaglia, M., Flavell, R.A., 2011. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. Immunity 34, 554.

Ivanov, I.I., McKenzie, B.S., Zhou, L., Tadokoro, C.E., Lepelley, A., Lafaille, J.J., Cua, D.J., Littman, D.R., 2006. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126, 1121.

Iwakura, Y., Ishigame, H., Saijo, S., Nakae, S., 2011. Functional specialization of interleukin-17 family members. Immunity 34, 149.

Kearley, J., Robinson, D.S., Lloyd, C.M., 2008. CD4+CD25+ regulatory T cells reverse established allergic airway inflammation and prevent airway remodeling. J. Allergy Clin. Immunol. 122, 617.

Kim, H.P., Leonard, W.J., 2007. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J. Exp. Med. 204, 1543.

Korn, T., Bettelli, E., Oukka, M., Kuchroo, V.K., 2009. IL-17 and Th17 cells. Annu. Rev. Immunol. 27, 485.

Kudo, M., Melton, A.C., Chen, C., Engler, M.B., Huang, K.E., Ren, X., Wang, Y., Bernstein, X., Li, J.T., Atabai, K., Huang, X., Sheppard, D., 2012. IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat. Med. 18, 547.

Lal, G., Zhang, N., van der Touw, W., Ding, Y., Ju, W., Bottinger, E.P., Reid, S.P., Levy, D.E., Bromberg, J.S., 2009. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. J. Immunol. 182, 259.

Langier, S., Sade, K., Kivity, S., 2012. Regulatory T cells in allergic asthma. Isr. Med. Assoc. J. 14, 180.

Li, Y., Hua, S., 2014. Mechanisms of pathogenesis in allergic asthma: role of interleukin-23. Respirology 19, 663.

Luo, Z.X., Liu, E.M., Deng, B., Li, X., Chen, K.H., Wang, L.J., Huang, Y., Fu, Z., 2006. Role of Foxp3 expression and CD4+ CD25+ regulatory T cells on the pathogenesis of childhood asthma. Zhonghua Er Ke Za Zhi 44, 267.

Luo, Q.L., Nurahmat, M., Li, M.H., Sun, J., Chen, M.X., Liu, F., Wei, Y., Dong, J.C., 2014. Pharmacological investigation of a HPLC/MS standardized three herbal extracts containing formulae (Bu-Shen-Yi-Qi-Tang) on airway inflammation and hypothalamic-pituitary-adrenal axis activity in asthmatic mice. Phytomedicine 21, 1439.

Mantel, P.Y., Kuipers, H., Boyman, O., Rhyner, C., Ouaked, N., Ruckert, B., Karagiannidis, C., Lambrecht, B.N., Hendriks, R.W., Crameri, R., Akdis, C.A., Blaser, K., Schmidt-Weber, C.B., 2007. GATA3-driven Th2 responses inhibit TGF-beta1-induced FOXP3 expression and the formation of regulatory T cells. PLoS Biol. 5, e329.

Morishima, Y., Ano, S., Ishii, Y., Ohtsuka, S., Matsuyama, M., Kawaguchi, M., Hizawa, N., 2013. Th17-associated cytokines as a therapeutic target for steroid-insensitive asthma. Clin. Dev. Immunol. 2013, 609395.

Nian, H., Ma, M.H., Nian, S.S., Xu, L.L., 2009. Antiosteoporotic activity of icariin in ovariectomized rats. Phytomedicine 16, 320.

Ohkura, N., Kitagawa, Y., Sakaguchi, S., 2013. Development and maintenance of regulatory T cells. Immunity 38, 414.

Pan, Y., Kong, L.D., Li, Y.C., Xia, X., Kung, H.F., Jiang, F.X., 2007. Icariin from Epimedium brevicornum attenuates chronic mild stress-induced behavioral and neuroendocrinological alterations in male Wistar rats. Pharmacol. Biochem. Behav. 87, 130.

Park, H., Li, Z., Yang, X.O., Chang, S.H., Nurieva, R., Wang, Y.H., Wang, Y., Hood, L., Zhu, Z., Tian, Q., Dong, C., 2005. A distinct lineage of CD4T cells regulates tissue inflammation by producing interleukin 17. Nat. Immunol. 6, 1133.

Pasare, C., Medzhitov, R., 2003. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 299, 1033.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson Jr., W.R., Muller, W., Rudensky, A.Y., 2008. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity 28, 546.

Rudensky, A.Y., 2011. Regulatory T cells and Foxp3. Immunol. Rev. 241, 260.

Ryanna, K., Stratigou, V., Safinia, N., Hawrylowicz, C., 2009. Regulatory T cells in bronchial asthma. Allergy 64, 335.

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Torgerson, T.R., Ochs, H.D., 2007. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. J Allergy Clin Immunol 120, 744.
Wakeland, E.K., 2014. Hunting autoimmune disease genes in NOD: early steps on a long road to somewhere important (hopefully). J. Immunol. 193, 3.
Wang, Q., Li, H., Zhang, Z., Yao, Y., Zhou, J., 2010. Prolonged ovalbumin challenge facilitates Th17 polarization in sensitized mice. Inflamm. Res. 59, 561.
Xu, C.Q., Liu, B.J., Wu, J.F., Xu, Y.C., Duan, X.H., Cao, Y.X., Dong, J.C., 2010. Icariin attenuates LPS-induced acute inflammatory responses: involvement of PI3K/Akt and NF-kappaB signaling pathway. Eur. J. Pharmacol. 642, 146.
Yang, L., Anderson, D.E., Baecher-Allan, C., Hastings, W.D., Bettelli, E., Oukka, M., Kuchroo, V.K., Hafler, D.A., 2008. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. Nature 454, 350.
Zhao, Y., Yang, J., Gao, Y.D., Guo, W., 2010. Th17 immunity in patients with allergic asthma. Int. Arch. Allergy Immunol. 151, 297.
Zhao, J., Lloyd, C.M., Noble, A., 2013. Th17 responses in chronic allergic airway inflammation abrogate regulatory T-cell-mediated tolerance and contribute to airway remodeling. Mucosal Immunol. 6, 335.
Zhou, L., Ivanov, I.I., Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D.E., Leonard, W.J., Littman, D.R., 2007. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat. Immunol. 8, 967.

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