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Icariside II attenuates eosinophils-induced airway inflammation and remodeling via inactivation of NF-κB and STAT3 in an asthma mouse model

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ABSTRACT

Asthma is a chronic inflammatory airway disease. Icariside II has been reported to exert anti-inflammatory effect in multiple human diseases. The present study aimed to investigate the effects and mechanisms of Icariside II on airway inflammation and remodeling in asthma. We established an asthma mouse model with ovalbumin (OVA) immunization. Histological analysis using H&E, PAS and Masson staining showed that administration of Icariside II attenuated OVA-induced airway inflammation and remodeling. Icariside II reduced the numbers of total white blood cells and eosinophils in bronchoalveolar lavage fluid (BALF). The levels of interleukin (IL)-4, IL-5, IL-13 and transforming growth factor (TGF)-β1 in peripheral blood and the expression of α-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), eotaxin-1, CC-chemokine receptor-3 (CCR-3), Toll-like receptor (TLR)-2 and TLR-4 were significantly down-regulated in lung tissues of OVA-induced mouse model. These results suggested that Icariside II inhibited eosinophil activation and thus decreased eosinophils-induced airway inflammation and remodeling in asthma. Moreover, Icariside II suppressed TGF-β1-induced cell proliferation, migration, and CTGF expression in airway smooth muscle cells (ASMCs). In both OVA-induced mouse model of asthma and TGF-β1-induced ASMCs, Icariside II decreased IκBα degradation, nuclear translocation of NF-κB p65 and STAT3 phophorylation, indicating an inactivation of NF-κB and STAT3 in the presence of Icariside II. Therefore, we demonstrate that Icariside II attenuates eosinophils-induced airway inflammation and remodeling in asthmatic mice and inhibits TGF-β1-induced cell proliferation and migration in ASMCs via suppressing NF-κB and STAT3 signalings.

1. Introduction

Asthma is a chronic inflammatory disease of the airway, which is characterized by airway inflammation, airway remodeling, airway hyperresponsiveness, goblet cell hyperplasia, and mucus hypersecretion (Mims, 2015). Increased airway smooth muscle mass is a key airway structural change (Salter et al., 2017). Airway smooth muscle cells (ASMCs) hyperplasia and hypertrophy are thought to increase airway smooth muscle mass and thus resulting in airway remodeling in asthma (Manuyakorn et al., 2013).Type 2 T helper (Th2) inflammation is common in most asthmatics, which is associated with the release of certain cytokines, such as interleukin (IL)-4, IL-5 and IL-13, and production of immunoglobulin E (IgE) and inflammatory cells such as eosinophils (Fahy, 2015). Currently, biologic therapies that targeting Th2 pathway including IgE, IL-4, IL-5 and IL-13 are increasingly being considered for the severe asthmatic patients (Abrams et al., 2018). More efforts are still necessary to devote into the exploration of phenotypic variability and pathogenesis of asthma, which provides us more therapeutic approaches to asthmatic patients.

Inflammatory cells, particularly eosinophils, are the main effectors of airway inflammatory response in asthma. Eosinophils play an important role in airway hyperresponsiveness, airway remodeling and mucus secretion in asthma, and elevated concentration of eosinophils is frequently detected in asthmatic patients (McBrien and Menzies-Gow, 2017). CC-chemokine receptor-3 (CCR-3), an important G protein-coupled receptor, is expressed on surface of eosinophils. Eotaxins are CC-chemokines that bind to CCR-3 and induce eosinophil chemotaxis (McBrien and Menzies-Gow, 2017). The expression of CCR-3 has been found increased in the bronchial tissues of patients with asthma (Ying et al., 1999). Toll-like receptors (TLRs) are transmembrane glycoproteins expressed by eosinophils, and TLR-2 and TLR-4 are reported to be involved in promoting eosinophilia (Rosenberg et al., 2013). TH2-

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derived cytokines IL-4, IL-5 and IL-13 contributes to the activation of eosinophils (Fallon et al., 2006). Besides, eosinophils promote airway remodeling by releasing various growth factors and fibrogenic mediators. Transforming growth factor-β1 (TGF-β1) is a growth factor mainly derived from eosinophils in bronchial tissues of asthmatic patients (Ohno et al., 1996). TGF-β1 level has been found increased in airway tissues, bronchoalveolar lavage fluid (BALF) and peripheral blood samples in asthmatic patients (Chu et al., 2000; Redington et al., 1997). Studies have showed that TGF-β1 contributes to tissue remodeling by promoting fibroblast proliferation and increasing collagen production (Levi-Schaffer et al., 1999; Makinde et al., 2007). Moreover, TGF-β1 is responsible for the proliferation and migration of ASMCs (Chen and Khalil, 2006). On account of the importance of eosinophils in asthma, investigation of the mechanisms underlying eosinophils-induced airway inflammation and remodeling are of clinical significance in the treatment of asthma.

Icariside II is the main bioactive pharmaceutical compound of *Herba Epimedi*, which is a traditional Chinese medicinal herb (Arief et al., 2015). Current studies have showed that Icariside II has therapeutic effects in the treatment of multiple human diseases such as cancers (Quan et al., 2017), cerebral ischemia (Deng et al., 2016), myocardial fibrosis (Fu et al., 2018), diabetic nephropathy (Tian et al., 2015) and cognitive impairment (He et al., 2018). Importantly, Icariside II has been reported to exert anti-inflammatory effects in a variety of disease models (Fu et al., 2018). For example, Icariside II attenuated cognitive deficits by reducing neuroinflammation and apoptosis in Aβ25–35-treated rats (Deng et al., 2017). A study revealed that Icariside II suppressed inflammation in diabetic rats (Yang et al., 2018). However, it is still not clear whether Icariside II has beneficial effect on eosinophils-induced airway inflammation and airway remodeling and thus ameliorates asthma, which requires further investigation.

In the present study, we established a mouse model of asthma and further investigated the effects of Icariside II on airway inflammation, airway remodeling and eosinophil activation in asthmatic mice. Besides, the effects of Icariside II on proliferation and migration of TGF-β1-induced ASMCs will be studied in vitro. To explore the mechanisms of how Icariside II functions in asthma, we further examined the activation of signal transducers and activator of transcription (STAT) 3 and nuclear factor-kappaB (NF-κB) signalings in asthmatic mice and TGF-β1-induced ASMCs.

2. Methods and materials

2.1. Animals and treatments

All male BALB/C mice aged 6–8 weeks old were obtained from Liaoning Changsheng biotechnology co. ltd., Benxi, China. Mice were randomly divided into 4 groups ($n = 12$ per group): (A) Control group, (B) Asthma group, (C) Asthma + 10 mg/kg Icariside II group, and (D) Asthma + 30 mg/kg Icariside II group. They were allowed to acclimatize under a 12/12 h light/dark cycle at 22 ± 1 °C and a relative humidity of 45–55% with free access to standard water and food for a week. All experiments in this study were approved by the Institutional Animal Care and Use Committee and animal studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

For establishment of an asthma mouse model, the mice in group B, C and D were intraperitoneally injected with 20 μg ovalbumin (OVA; Aladdin Regents, Shanghai, China) to induce allergic lung inflammation at day 0, 14, 28 and 42. Beginning on the 21st day, the sensitized mice were treated with 1% OVA via nebulization for 30 min three times per week from day 21 to 46. The mice in group C and D were administrated daily with 10 or 30 mg/kg body weight of Icariside II (Dalian Meilun Biotechnology, Dalian, Liaoning, China) by gavage from day 21 to day 49. The OVA was diluted in normal saline (Dubang Pharmaceutical, Jilin, China) without adjuvant. The mice in group B were received same volume of normal saline at the same time points and served as negative controls. After all animal treatments, peripheral blood and BALF were collected. All mice were anesthetized and decapitated to obtain lung tissues. Part of tissue was fixed in 4% paraformaldehyde (Sinopharm Chemical Reagent) and the others were snap-frozen with liquid nitrogen and stored at −70 °C for further experiments.

2.2. Primary ASMCs isolation, culture and treatments

Primary cultures of ASMCs were isolated from trachea of mice as previously described (McGraw et al., 2006). Briefly, the trachea was removed from euthanized mice and washed with 2 × concentration of penicillin-streptomycin solution. Then trachea was dissected longitudinally into 2–3 mm pieces after removal of surrounding tissues under a dissecting microscope. All pieces from a single trachea were allowed to adhere and cultured in a sterile culture flask for 4–6 h, followed by addition of 3 ml Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) containing 20% fetal bovine serum (FBS, Sigma). The cells were passaged with the use of 0.25% trypsin (Sigma) when reaching 90% confluence and cultured in DMEM supplemented with 10% FBS at 37 °C under 5% CO_2 in a humidified incubator. Cells from the third passage were used for subsequent experiments.

For cell treatment, cells were incubated in DMEM medium containing 1% FBS and treated with 10 ng/ml TGF-β1 or different concentrations (25, 50, 100, 200 μM) of Icariside II (Dalian Meilun Biotechnology). After incubation for 48 h, the cells were subjected to further detection.

2.3. BALF analysis and cell counts

Total blood white cells and eosnophils in BALF were evaluated using Giemsa staining. Briefly, cells in BALF were resuspended in 0.1 ml phosphate-buffered saline (PBS) and 10 μl of BALF was dropped onto the glass slide and covered with a glass coverslip. Slides were fixed in methyl alcohol for 15 min and dried at room temperature. Then slides were stained with Giemsa staining solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the instructions of manufacturer. Cell counts were performed under a light microscope.

2.4. Morphological analysis-Haematoxylin and eosin (H&E), Periodic acid-Schiff (PAS), Masson stainings

Formalin-fixed paraffin-embedded lung tissues were sectioned at 5 μm thickness followed by deparaffination and rehydration. Sections were stained with haematoxylin (Solarbio Science & Technology, Beijing, China) and eosin (Sangon Biotech, Shanghai, China) for observation of bronchial airway inflammation. Sections were stained with periodic acid-schiff (PAS) (Baso Diagnostic, Zhuhai, China) to evaluate bronchial airway goblet cells and mucus secretion. For Masson staining, sections were stained with haematoxylin, ponceaux-acid fuchsin and aniline blue (Sinopharm Chemical Reagent, Beijing, China) to determine bronchial airway collagen deposition. All the staining results were observed under a microscope (Olympus, Tokyo, Japan) at 200 × .

2.5. Elisa

The concentrations of IgE in BALF and IL-4, IL-5, IL-6, IL-13 and TGF-β1 in serum samples were detected using respective commercial ELISA kit (MultiSciences, Hangzhou, China) and a microplate reader (Biotek, Winooski, VT, USA) according to the instructions of the manufacturers.

2.6. Western blot

Total protein was extracted from lung tissues or cells using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime Institute of

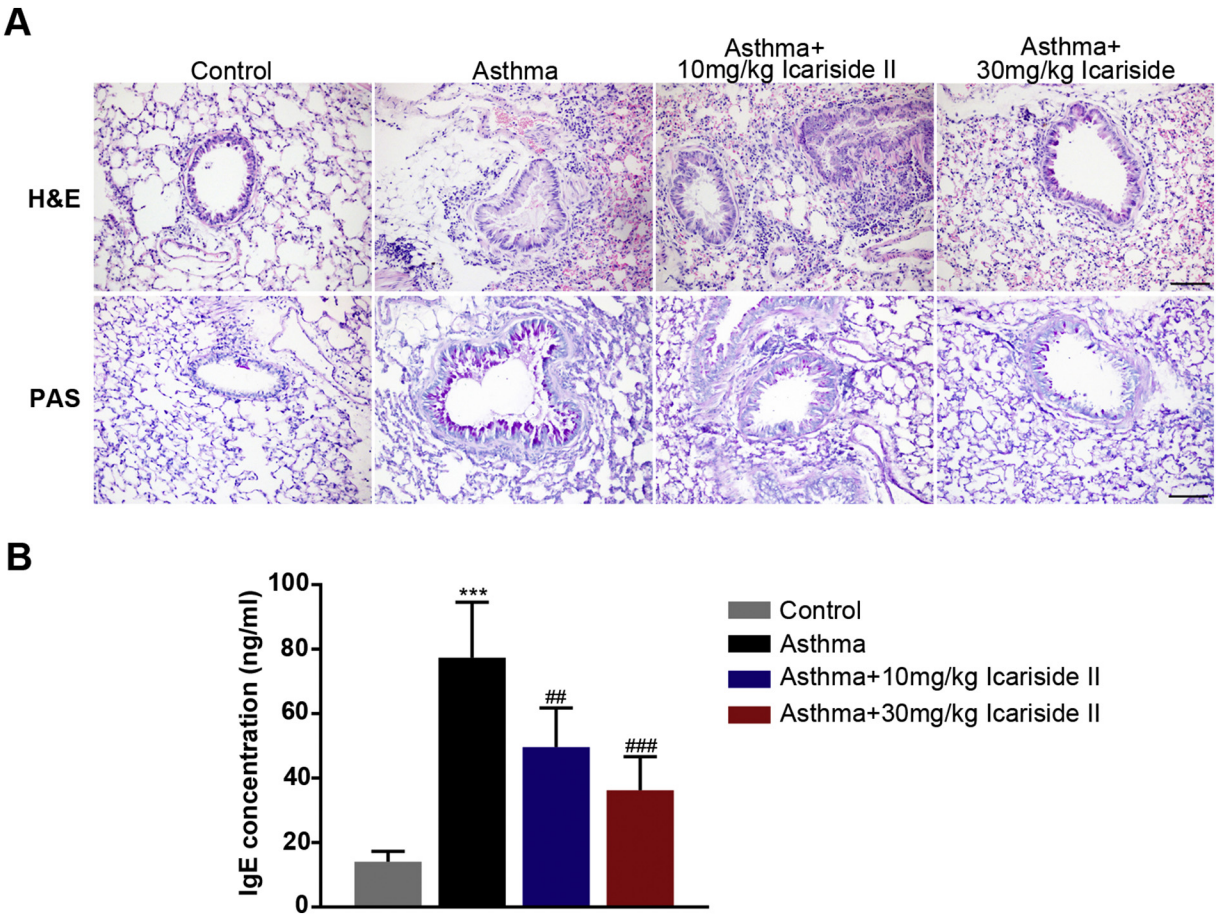

Fig. 1. Icariside II reduces airway inflammation in asthmatic mice. (A) H&E and PAS staining were performed to analyze histological alterations in lung tissues. (B) The level of IgE in bronchoalveoar lavage fluid (BALF) was evaluated by ELISA. Data were presented as mean ± SD ($n = 6$) and analyzed by one-way ANOVA. $^{***}P < .001$ vs. Control. $^{\#\#}P < .01$, $^{\#\#\#}P < .001$ vs. Asthma. Scale bar = 100 μm.

Biotechnology, Haimen, China). Equal amounts of protein (20–40 μg) were electrophoretically separated by SDS-PAGE gel and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk diluted in TBST, and then probed with primary antibodies against eotaxin-1 (1:1000; Abcam, Cambridge, MA, USA), CCR3 (1:500, Abcam), TLR-2 (1:1000, Abcam), TLR-4 (1:500; Proteintech Group, Rosemont, IL, USA), α-smooth muscle actin (α-SMA, 1:500, Proteintech), CTGF (1:500, Proteintech), inhibitory κBα (IκBα, 1:1000, Proteintech), phospho-IκBα (p-IκBα, 1:1000; Cell Signaling Technology, Danvers, MA, USA), nuclear factor (NF)-κB p65 (1:2000, Proteintech), signal transducer activators of transcription 3 (STAT3, 1:2000, Proteintech), phospho-STAT3 (p-STAT3, 1:1000, Cell Signaling Technology), Histone H3 (1:2000; Abgent, San Diego, CA, USA) or β-actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight, followed by HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (15,000, Beyotime) at 37 °C for 45 min. Histone H3 and β-actin were used as internal reference. The protein bands were visualized using ECL detection kit (Beyotime) and analyzed by Gel-Pro-Analyzer software.

2.7. Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were analyzed using NF-κB EMSA kit (Viagene, Changzhou, China) to detect the presence of NF-κB p65 in lung tissues. Briefly, equal amounts of nuclear protein (5 μg) were incubated with biotin-labeled NF-κB p65 probe and analyzed by EMSA according to the manufacture's instruction. The blots were developed by chemiluminescent detection using ECL reagent (Beyotime).

2.8. Immunohistochemistry

After deparaffination and rehydration, sections of the lung were incubated with 3% H_2O_2 (Sinopharm Chemical Reagent) for 15 min and blocked with goat serum (Solarbio) for 15 min at room temperature. Sections were then probed with primary antibody against α-SMA (1:100, Proteintech) at 4 °C overnight and incubated with HRP-labeled goat anti-rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 1 h, followed by DAB (Solarbio) reaction. Then sections were re-stained with haematoxylin. The staining results were observed under a microscope at 200 × .

2.9. Immunofluorescence

Immunostaining for α-SMA was performed to identify the isolated mouse ASMCs. Cells were seeded on glass cover slips, fixed in 4% paraformaldehyde (Sinopharm Chemical Reagent) for 15 min and permeabilized with 0.1% tritonX-100 (Beyotime) for 30 min. After blocking with goat serum (Solarbio) for 15 min, the cells were incubated with primary antibody (1:200, Proteintech) at 4 °C overnight and probed with Cy3-labeled goat anti-rabbit IgG secondary antibody (1:100, Beyotime) at room temperature for 1 h. Cell nucleus were visualized by further staining with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime). The positively-stained cells were observed under a fluorescent microscope at 200 × .

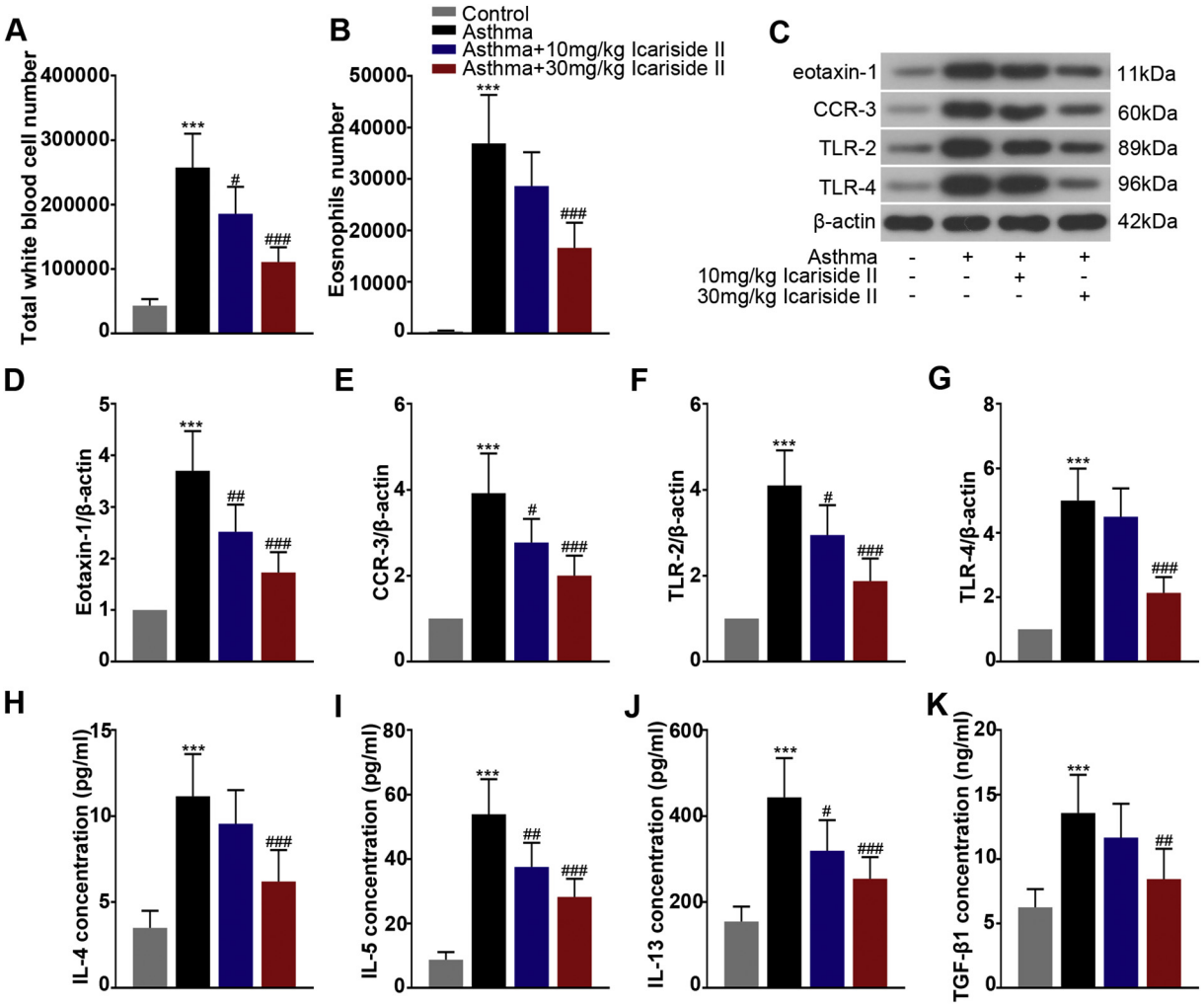

Fig. 2. Icariside II inhibits eosinophil activation in asthmatic mice. (A) Total blood white cell count in BALF. (B) Total eosinophil count in BALF. (C) Western blotting analysis of the expression of eotaxin-a (D), CC-chemokine receptor-3 (CCR3) (E), Toll-like receptor-2 (TLR-2) (F) and TLR-4 (G) in lung tissues. The protein expression was normalized to β-actin. The serum concentrations of IL-4 (H), IL-5 (I), IL-13 (J) and TGF-β1 (K) were determined using ELISA. Data were presented as mean ± SD (n = 6) and analyzed by one-way ANOVA. ***P < .001 vs. Control. #P < .05, ##P < .01, ###P < .001 vs. Asthma.

2.10. MTT

The MTT assay was performed to examine the survival of ASMCs before and after the treatment of Icariside II. Cells were seeded into 96-well plates with 200 μl DMEM medium in the presence or absence of 10 ng/ml TGF-β1 or different concentrations of Icariside II (25, 50, 100, 200 μM). After incubation for 48 h, cells of each group were subjected to MTT detection according to the manufacture's instruction. Briefly, cells were treated with 0.5 mg/ml MTT mix solution (Beyotime) for 4.5 h followed by addition of 150 μl dimethyl sulfoxide (DMSO; Sigma) to dissolve the formed violet crystals. The absorbance at 570 nm was measured using a plate reader (Biotek).

2.11. Transwell

The mobility of ASMCs was evaluated by transwell assay. Cells (4×10^3 per well) were added to the upper chamber with serum-free medium and the bottom chambers were filled with 800 μl culture medium containing 20% FBS in the presence or absence of 10 ng/ml TGF-β1 or 50 μM Icariside II. Transwell chambers were inserted into 24-well plates and incubated in a humidified incubator with 5% CO_2 at 37 °C for 48 h. The cells remained on the upper membrane surface were removed using a cotton swab, and the invasive cells through the membrane to the lower membrane surface were fixed in 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 5 min. The staining were observed under an inverted microscope at 200 × and the number of invaded cells was counted from ten random fields per well.

2.12. Statistical analysis

All data were presented as means ± SD. Differences between the groups were analyzed with GraphPad Prism 7 software using one-way-analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A $P < .05$ was considered statistically different.

3. Results

3.1. Icariside II attenuates airway inflammation in asthmatic mice

An asthma mouse model was established using OVA immunization to induce allergic lung inflammation in mice. To explore the role of Icariside II in asthma, control and OVA-induced mice were treated with 10 kg/mg or 30 mg/kg Icariside II. H&E and PAS stainings were performed to assess morphological alterations in lung tissues (Fig. 1A). H&E staining revealed that, in comparison with the controls, there were significant inflammatory cell infiltrations in peribronchial, perivascular and alveolar and marked thickening in bronchial wall in OVA-induced

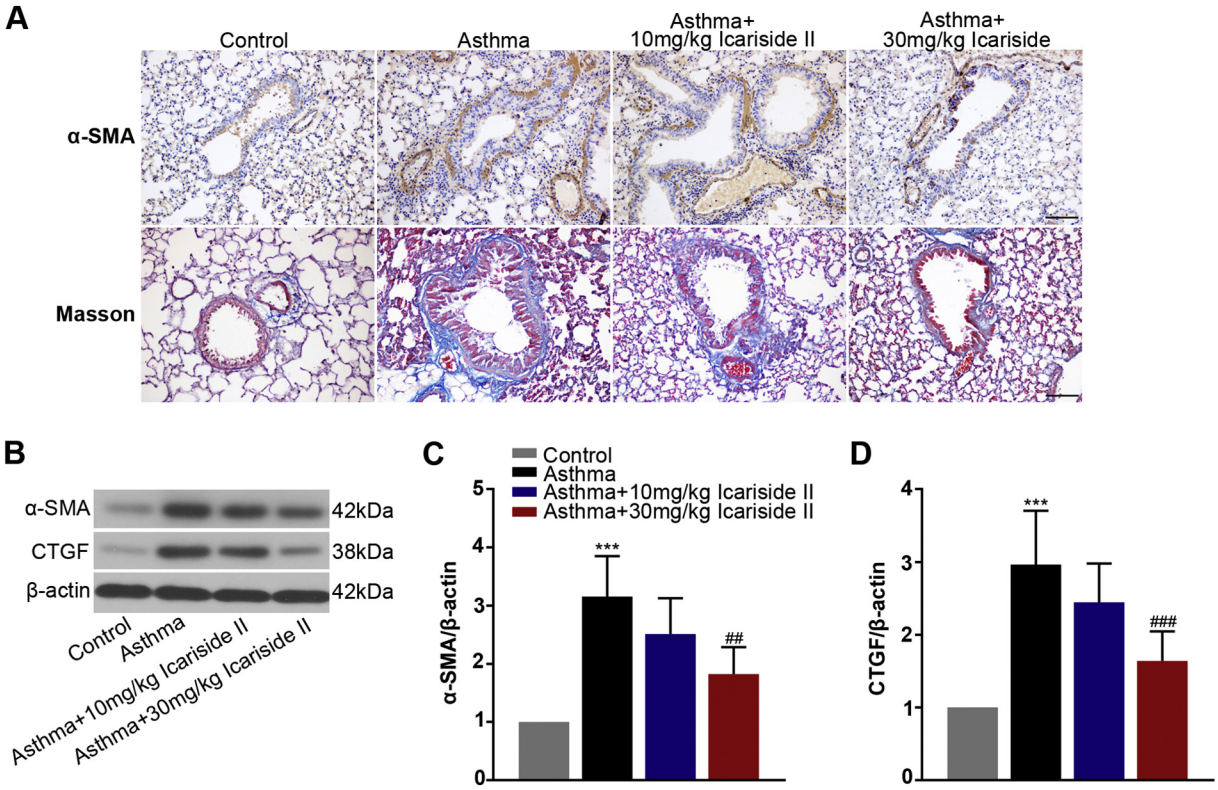

Fig. 3. Icariside II inhibits airway remolding in asthmatic mice. (A) Immunostaining for α-smooth muscle actitn (α-SMA) in lung tissues. Masson staining was performed to detect bronchial airway collagen deposition in lung tissues. (B) The expression of α-SMA (C) and connective tissue growth factor (CTGF) (D) in lung tissues was determined by western blot. Data were presented as mean ± SD (n = 6) and analyzed by one-way ANOVA. ***P < .001 vs. Control. ##P < .01, ###P < .001 vs. Asthma. Scale bar = 100 μm.

mouse model of asthma. Administration of Icariside II dose-dependently reduced infiltration of inflammatory cells and decreased bronchial wall thickness in asthmatic mice. PAS staining displayed that asthma mice exhibited bronchial airway goblet cell hyperplasia, mucus hypersecretion and increased thickness of the airway smooth muscle layer. However, OVA-induced airway inflammation and remodeling were largely mitigated by Icariside II treatment. In addition, the level of IgE in BALF was found significantly up-regulated in asthmatic mice, while down-regulated in Icariside II-treated model mice in a dose-dependent manner (Fig. 1B).

3.2. Icariside II inhibits the activation of eosinophils in asthmatic mice

Total blood white cells and total eosinophils in BALF were analyzed with the aid of Giemsa staining and cell counts were displayed in Fig. 2A-B. The number of total blood white cells and total eosinophils in BALF was significantly higher in asthmatic mice than the controls, while both were reduced after Icariside II treatment. To study the mechanism underlying Icariside II-mediated airway inflammation, the activation of eosinophils was assessed. Western blotting analysis showed that the expression of eotaxin-1, CCR-3, TLR-2 and TLR-4 was up-regulated in OVA-induced mice, while down-regulated in Icariside II-treated asthmatic mice (Fig. 2C-G). The concentrations of serum IL-4, IL-5, IL-13 and TGF-β1 were found significantly elevated in asthmatic mice and Icariside II induced dose-dependent decrease in these cytokines (Fig. 2H-K).

3.3. Icariside II inhibits airway remolding in asthmatic mice

Inmmunostaining of α-SMA displayed that α-SMA level was elevated in lung tissues in OVA-induced asthma mouse models and reduced by Icariside II (Fig. 3A). Masson staining revealed that OVA immunization increased collagen deposition in airway and lung parenchyma as well as thickened bronchial epithelium, while Icariside II treatment dose-dependently reduced collagen deposition and bronchial wall thickness in airway (Fig. 3A). In consistent with histological analysis, western blotting results showed that the expression levels of lung α-SMA and CTGF were significantly up-regulated in asthmatic mice and down-regulated by Icariside II in a dose-dependent manner (Fig. B-D).

3.4. Icariside II suppresses the activation of NF-κB and STAT3 signalings in asthmatic mice

To explore the underlying mechanism of how Icariside II functions in asthma, the activation of NF-κB and STAT3 was examined. Western blotting results showed increased p-IκBα, nuclear NF-κB p65 and p-STAT3 levels and decreased IκBα and cytoplasmic NF-κB p65 levels in lung of asthmatic mice, indicating an up-regulation of IκBα degradation, NF-κB activation and STAT3 activation (Fig. 4A-G). In contrast, the addition of Icariside II dose-dependently decreased the phosphorylated level of IκBα and increased IκBα level (Fig. 4A-B). Besides, the expression of cytoplasmic NF-κB p65 was up-regulated while nuclear NF-κB p65 expression was down-regulated in the presence of Icariside II (Fig. 4C-D). Moreover, p-STAT3 expression was reduced by Icariside II (Fig. 4E). No difference in total STAT3 level was observed following Icariside II treatment (Fig. 4F). These data suggested the inhibitory effect of Icariside II on the activation of NF-κB and STAT3 in OVA-induced asthmatic mice. Consistently, EMSA assay showed a dose-dependent inhibition of Icariside II on the nuclear NF-κB p65/DNA binding activity, indicating that Icariside II suppressed the transcriptional activity of NF-κB p65 (Fig. 4H).

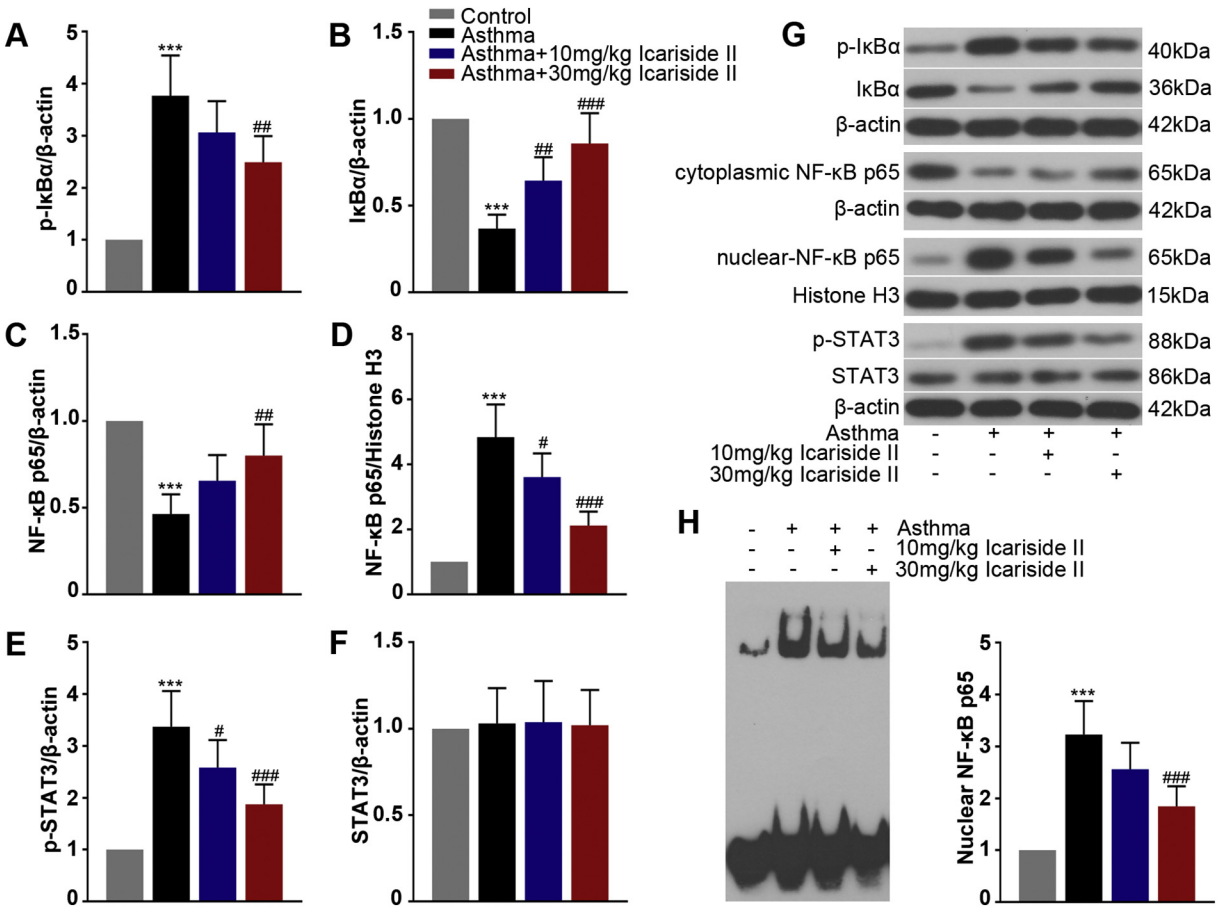

Fig. 4. Icariside II suppresses the activation of STAT3 and NF-κB signaling in asthma mice. The relative expression of phospho-IκBα (p-IκBα) (A), inhibitory κBα (IκBα) (B), cytoplasmic NF-κB p65 (C), nuclear NF-κB p65 (D), phospho-STAT3 (p-STAT3) (E) and total STAT3 (F) in lung tissues was detected by western blot (G). Histone H3 and β-actin were used as internal reference. (H) The binding activity of nuclear NF-κB p65/DNA was assessed by EMSA assay. Data were presented as mean ± SD (n = 6) and analyzed by one-way ANOVA. ***P < .001 vs. Control. #P < .05, ##P < .01, ###P < .001 vs. Asthma.

3.5. The effects of Icariside II on TGF-β1-induced cell proliferation, migration and CTGF expression in ASMCs

ASMCs were isolated from mouse trachea and cultured in DMEM supplemented with 10% FBS. Identification of the isolated ASMCs was determined by positive immunostaining for α-SMA (Fig. 5A). We further studied the influence of Icariside II in TGF-β1-induced ASMCs. The survival of TGF-β1-induced ASMCs in the presence of different concentrations of Icariside II was examined by MTT assay. The results showed that Icariside II induced a concentration-dependent decrease in TGF-β1-induced cell growth (Fig. 5B). Additionally, there was a reduction in the number of invasive cells in lung of asthmatic mice after Icariside treatment as observed in crystal violet staining and corresponding quantification (Fig. 5C-D), indicating Icariside II inhibited TGF-β1-induced cell invasiveness. In addition, TGF-β1-induced CTGF protein expression was found down-regulated in the presence of Icariside II as analyzed by western blot (Fig. 5E).

3.6. Icariside II suppresses STAT3 and NF-κB activations in TGF-β1-induced ASMCs

To further investigate the mechanism underlying the inhibitory effect of Icariside II on TGF-β1-induced cell growth, mobility and CTGF expression in ASMCs, we assessed the activation of NF-κB and STAT3. Western blotting analysis (Fig. 6A) revealed that TGF-β1 treatment resulted in an up-regulation of p-IκBα (Fig. 6B), nuclear NF-κB p65 (Fig. 6E) and p-STAT3 (Fig. F) and a down-regulation of IκBα (Fig. 6C) and cytoplasmic NF-κB p65 (Fig. 6D) in ASMCs, indicating the activation of NF-κB and STAT3 in TGF-β1-induced ASMCs. In comparison, administration of 50 μM Icariside II largely counteracted TGF-β1-induced STAT3 and NF-κB activations in ASMCs.

4. Discussion

In the present study, a mouse model of asthma was established with OVA-induced sensitization. Treatment with Icariside II decreased airway inflammation and airway remodeling though inhibition of eosinophil activation in asthmatic mice. In vitro, Icariside II suppressed TGF-β1-induced ASMCs proliferation and migration. To explore the mechanisms of the effects of Icariside II on asthma, STAT3 and NF-κB signalings were examined in vivo and in vitro. Both STAT3 and NF-κB activations were inhibited by Icariside II, sugesting that Icariside II functions in asthma via suppressing STAT3 and NF-κB signaling pathways.

Airway inflammation and airway remodeling are two major pathophysiological features of asthma (Mims, 2015). The inflammatory response in asthma includes over-production of IgE by B cells, the release of cytokines and infiltration of inflammatory cells such as eosinophils and lymphocytes (Ayakannu et al., 2019; Georas et al., 2005). Airway remodeling in asthma is characterized by smooth muscle hypertrophy and hyperplasia, goblet cell hyperplasia, mucus hypersecretion, subepithelial fibrosis and collagen deposition, increased blood vessels and inflammatory cell infiltration (Boulet, 2018; Fehrenbach et al., 2017). Therefore, control of the inflammation and tissue remodeling is the key in the treatment of asthma. Icariside II, a bioactive compound of *Herba Epimedi*, has been reported to have anti-

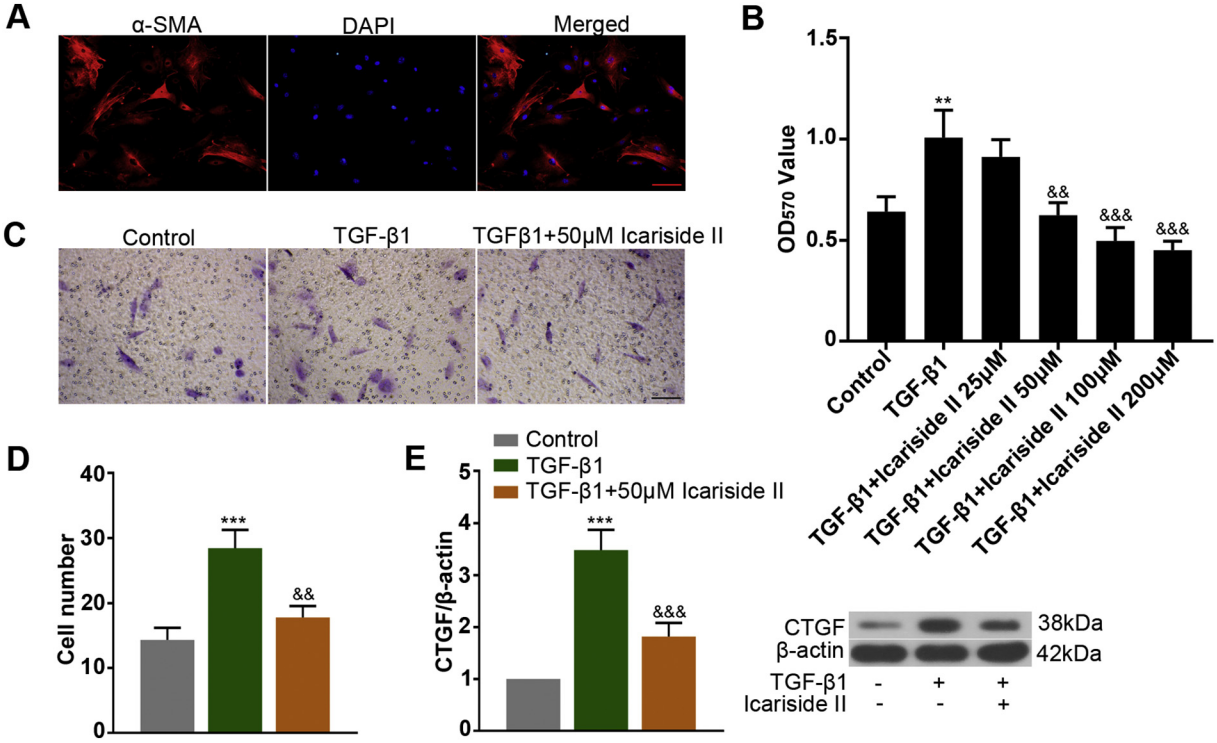

Fig. 5. The effect of Icariside II on proliferation, migration and CTGF expression in TGF-β1-induced airway smooth muscle cells (ASMCs). ASMCs were isolated from mouse trachea and cultured in DMEM supplemented with 10% FBS. (A) Immunofluorecence of α-SMA was performed to identify the isolated mouse ASMCs. (B) ASMCs were incubated in DMEM medium containing 1% FBS and treated with 10 ng/ml TGF-β1 or different concentrations (25, 50, 100, 200 μM) of Icariside II. After incubation for 48 h, cell viability was detected by MTT assay. Transwell assay was performed to determine the mobility of ASMCs in the presence or absence of 10 ng/ml TGF-β1 or 50 μM Icariside II. (C) Representative images of 0.1% crystal violet staining under an inverted microscope at 200 ×. (D) Quantification of the invasive cells. (E) The expression of CTGF at protein level was detected by western blot. Data were presented as mean ± SD (n = 3) and analyzed by one-way ANOVA. **P < .01, ***P < .001 vs. Control. &&P < .01, &&&P < .001 vs. TGF-β1. Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

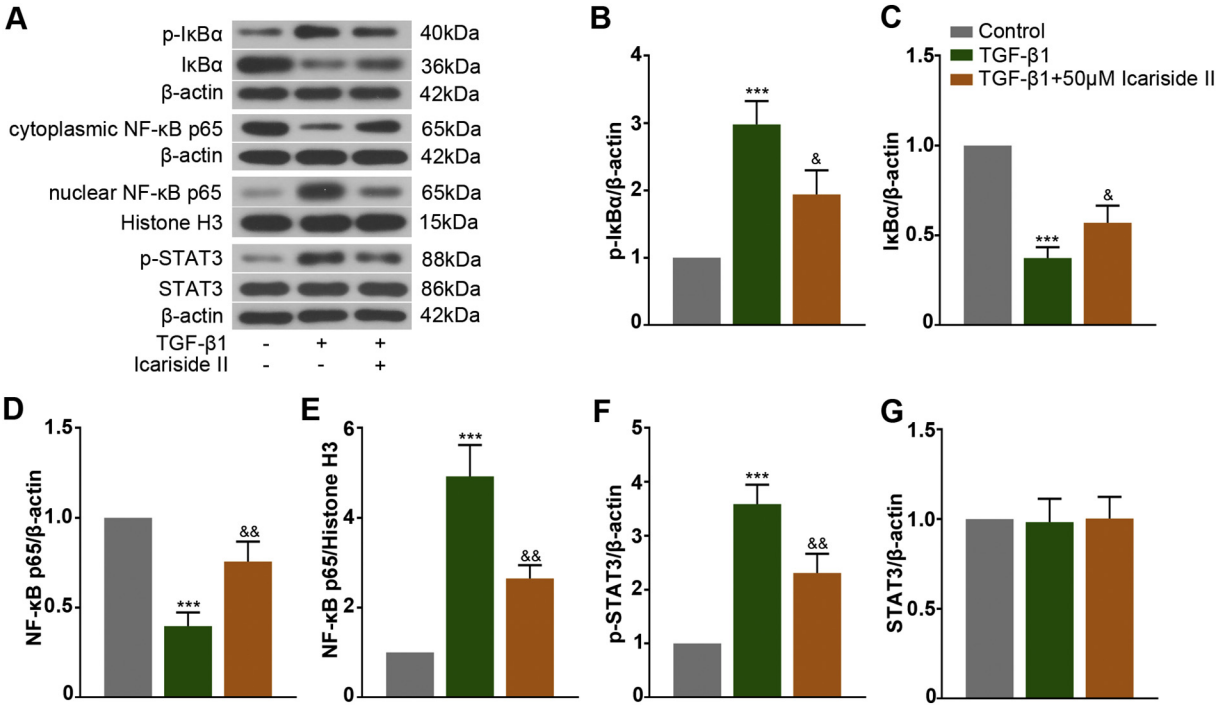

Fig. 6. Icariside II suppresses TGF-β1-induced activation of STAT3 and NF-κB signalings in ASMCs. (A) Western blotting analysis of the protein expression of p-IκBα (B), IκBα (C), cytoplasmic NF-κB p65 (D), nuclear NF-κB p65 (E), p-STAT3 (F) and STAT3 (G) in controls and TGF-β1-induced ASMCs with or without 50 μM Icariside II treatment. Data were presented as mean ± SD (n = 3) and analyzed by one-way ANOVA. ***P < .001 vs. Control. &P < .05, &&P < .01 vs. TGF-β1.

inflammatory effect in numerous human diseases (Deng et al., 2017; Zhou et al., 2019). In our OVA-induced mouse model of asthma, administration of Icariside II ameliorated asthma-induced airway inflammation and airway remodeling in lung tissues along with decreased IgE content in BALF at a dose-dependent manner. Besides, Icariside II treatment inhibited smooth muscle proliferation as evidenced by reductions in positive α-SMA immunostaining. Moreover, CTGF is a growth factor known to promote fibroblast proliferation, migration and extracellular matrix protein release (Grotendorst, 1997; Xie et al., 2005). CTGF is identified to be associated with TGF-β1 and highly expressed in patients with asthma (Kato et al., 2014). The expression of CTGF in lung was down-regulated by Icariside II accompanied by decreased TGF-β1 level in peripheral blood of asthmatic mice. The release of CTGF was reported to involve in the fibrogenic function of TGF-β1 and thus contribute to airway remodeling (Xie et al., 2005). These findings suggest that Icariside II attenuates airway inflammation and airway remodeling in asthma.

Eosinophils are considered to be involved in the pathogenesis and pathophysiology of asthma and eosinophilia contributes to asthma exacerbation (Bai et al., 2019). High eosinophils count is detected in patients with severe asthma (Yancey et al., 2019). Both numbers of total white blood cells and eosinophils were observed to be dose-dependently decreased after treatment with Icariside II in asthmatic mice, suggesting that Icariside II might attenuate asthma by inhibiting eosinophil proliferation. Moreover, IL-4, IL-5 and IL-13 are Th2 cytokines that contribute to IgE synthesis and eosinophil activation (Bagnasco et al., 2016). CCR-3 is an important chemokine receptor on eosinophils, which binds to CC chemokines such as eotaxin-1 and thus regulates eosinophil recruitment in inflammatory response in asthma (Zimmermann et al., 2000). A study showed that TLR2 and TLR-4 triggered the exacerbation of OVA-induced lung eosinophilia (He et al., 2017). In the present study, the levels of IL-4, IL-5 and IL-13 in peripheral blood and the expression of eotain-1, CCR-3, TLR-2 and TLR-4 were reduced in lung tissues of asthmatic mice following Icariside II treatment. TGF-β produced by numerous cells including eosinophils is one of the critical mediators in the regulation of airway remodeling in asthma (Halwani et al., 2011). Up-regulation of TGF-β1 level has been detected in peripheral blood, BALF and bronchial tissues from patients with asthma (Chu et al., 2000; Jiang et al., 2013). An increase in TGF-β1 level was detected in peripheral blood from asthmatic mice, which was decreased by Icariside II treatment. The previous findings together with our results indicate that Icariside II could inhibit eosinophil activation and therefore attenuates eosinophils-induced airway inflammation and remodeling in asthma.

Aberrant ASMCs including AMSCs proliferation, hypertrophy and migration are important pathological changes of asthma involved in airway remodeling development (Shifren et al., 2012). The expression of TGF-β1 was found elevated in AMSCs of asthmatic patients (Xie et al., 2007). It is reported that TGF-β1 stimulates ASMCs proliferation and migration, and thus contribute to airway remodeling (Pan et al., 2018). More attentions currently have been paid on developing treatments targeting TGF-β1-induced ASMCs proliferation for asthma and exploring its underlying mechanisms (Chen et al., 2018; Liu et al., 2018; Liu et al., 2019). We studied the effects of Icariside II in asthmatic ASMCs in vitro using TGF-β1-induced ASMCs. Treatment with Icariside II suppressed TGF-β1-induced proliferation and migration of ASMCs and CTGF expression. CTGF, an identified target gene of TGF-β1 (Xie et al., 2005), was found increased after TGF-β1 treatment in both non-asthmatic and asthmatic ASMCs and highly expressed in ASMCs from patients with asthma (Wang et al., 2018). Numerous studies revealed that inhibition of TGF-β-stimulated ASMCs proliferation and migration is a potential treatment for asthma (Fang et al., 2018; Li et al., 2015; Liu et al., 2019). Therefore, Icariside II could exert therapeutic effects on asthma via inhibition of AMSCs proliferation and migration.

Activation of NF-κB and STAT3 signalings has been demonstrated to play an important role in airway inflammation and remodeling in asthma (Huang et al., 2018). The activations of NF-κB and STAT3 were examined to explore the possible mechanisms underlying the effects of Icariside II in asthma. NF-κB is known to be a critical regulator involved in the regulation of apoptosis and inflammation. In normal condition, NF-κB is inactivated in the cytoplasm by binding to inhibitory proteins IκBs. Upon activation by stimuli, IκBs are phophorlated followed by subsequent degradation, resulting in translocation of NF-κB into the nucleus to regulate transcription of target genes (Hoesel and Schmid, 2013). Inhibition of NF-κB is emerging to be applied in anti-inflammatory therapies (D'Acquisto et al., 2002). In the present study, Icariside II treatment was showed to down-regulate IκBα and nuclear NF-κB p65 levels and up-regulate phospho-IκBα and cytoplasmic NF-κB p65 levels in both asthmatic mouse model and TGF-β1-induced ASMCs. The binding activity of NF-κB p65/DNA in nucleus was weakened by Icariside II in lung of asthmatic mice. These data implied that Icariside II might function partly via inhibiting NF-κB activation in asthma. In line with previous studies (Chen et al., 2014; Hwang et al., 2017; Sun et al., 2016), inhibition of NF-κB activation attenuated airway inflammation and remodeling in OVA-induced allergic asthmatic mice and inhibited TGF-β1-induced proliferation and migration of ASMCs. STAT3 is a cytokine-activated transcriptional factor and its activation exerts a crucial role in regulating inflammatory response (Loh et al., 2019) and promoting tumor cell proliferation (Yu et al., 2014). Once stimulated, STAT3 is phosphorylated at Tyr705 followed by translocation into the nucleus and thus transcriptionally regulates cytokine-responsive genes and cytokine production (Stritesky et al., 2011). Therefore, STAT3 activation is a potential therapeutic target for asthma (Morales et al., 2016). To further assess the effect Icariside II on STAT3 activation, we examined the expression of total STAT3 and STAT3 phosphorylation on Tyr705 in lung of asthmatic mouse model and in TGF-β-induced ASMCs. The results showed that Icariside II significantly suppressed phosphorylation of STAT3, while no difference of total STAT3 level was observed. Studies have reported that STAT3 is involved in allergic inflammation development in asthma. STAT3 induced allergic airway inflammation by mediating Th2-type cell trafficking and effector function in a mouse model of asthma (Simeone-Penney et al., 2007). Inhibition of STAT3 decreased Th2/Th17 cell production and mitigated airway inflammation and remodeling in lung of asthmatic mice (Gavino et al., 2016). Taken together, Icariside II alleviates airway inflammation and remodeling in asthmatic mice and suppresses proliferation and migration of TGF-β1-induced ASMCs via inhibition of NF-κB and STAT3 signalings.

5. Conclusion

The present study demonstrates that Icariside II attenuates asthma airway inflammation and remodeling by suppressing eosinopohil activation and inhibiting TGF-β1-induced ASMCs proliferation and migration via inactivation of NF-κB and STAT3 signalings. These findings provide insights into the effects and mechanisms of Icariside II in asthma, which could be a potential treatment for asthma.

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Declaratiojn of Competing Interest

The authors declare that there are no conflicts of interest.

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