

Original Article

Iristectorigenin A exerts novel protective properties against airway inflammation and mucus hypersecretion in OVA-induced asthmatic mice

Iristectorigenin A ameliorates asthma phenotype

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ABSTRACT

Background: Despite the substantial amount of efforts made to reduce morbidity and improve respiratory management, asthma control remained a major challenge for severe patients. Plant isoflavones, one of the most estrogenic compounds, are considered a potential alternative therapy for asthma. Iristectorigenin A, a naturally occurring isoflavone, is extracted from a variety of medical plants and its biological activity has not been reported previously.

Purpose: In present study, we aim to reveal the potential therapeutic role of Iristectorigenin A against acute asthmatic mice.

Study design: We established ovalbumin (OVA) induced asthmatic murine model and orally administrated Iristectorigenin A at concentration of 5 and 10 mg/kg and dexamethasone as a positive control substance.

Methods: Asthmatic murine model was established with OVA sensitization and challenge. Lung function was assessed with FinePoint Ventilation system recording lung resistance (RI) and lung compliance (C_{dyn}). White cells were sorted and counted in BALF. Histopathological assessment was conducted by H&E, PAS, and Masson's trichrome staining on paraffin embedded lung tissues. BALF content of IL-4, IL-5, IL-33, IL-13, INF- γ , IL-9 and serum IgE, IgG1 were measured using ELISA kit. Expression levels of mRNAs associated with inflammatory cytokines and goblet cell metaplasia were evaluated via quantitative RT-PCR. Protein expression levels of FOXA3, MUC5AC, SPDEF were estimated by immunohistochemistry on lung tissue, while NOTCH1 and NOTCH2 expressions were evaluated by western blotting analysis.

Results: Iristectorigenin A resulted in improved airway hyperresponsiveness (AHR) mirrored by decreased RI and increased C_{dyn}. With Iristectorigenin A, we also observed reduced number of BALF leukocytes, improved inflammatory cell infiltration in lung tissue, decreased content of BALF IL-4, IL-5, IL-33, but not IL-13, INF- γ , IL-9, and their mRNA levels, along with decreased levels of OVA-specific IgE, IgG1 in asthmatic mice. Additionally, Iristectorigenin A exhibited significant therapeutic potential on attenuating mucus production reflected by mitigated FOXA3 and MUC5AC immunostaining on the airway epithelium, as well as decreased mRNAs associated with goblet cell metaplasia. At last, a decrease in elevated expression level of NOTCH2, but not NOTCH1, in asthmatic mice lung tissue was observed by western blotting analysis.

Conclusion: Our study provides strong evidence that Iristectorigenin A can be potential therapeutic agent ameliorating airway inflammation and mucus hypersecretion in allergic asthma. This is a first research reported the potential of Iristectorigenin A as an alternative therapeutic agent.

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Introduction

Asthma is a chronic lung disease that is principally characterized by cough, wheeze, and breathlessness resulting from airway inflammation and airway hyperresponsiveness (AHR) (Wu et al., 2019). The overall asthma prevalence in China was 4.2% representing 45.7 million adults (Huang et al., 2019). Because asthma demonstrates variability in symptoms, pathological manifestation and airflow limitation, successful management of asthma involves long term continual reassessment and persistent observation to optimize disease control while minimizing side effects of medications (Pike et al., 2018). Maintenance therapy with long - and/or short- term inhaled corticosteroids (ICS) has been used for all asthma patients (Barnes, 2017). Considering the significant side effects, financial burden posed on patients and drug-related complications of ICS, Global Initiative for Asthma (GINA) guidelines recommended oral corticosteroids (OCS) used for only uncontrolled, severe asthma patients and as short-term, as low-dosed as possible (Rothe et al., 2018). However, OCS still exhibits considerable side effects in clinical treatment for asthma patients (Volmer et al., 2018). Therefore, alternative therapeutic interventions with less side-effects and less cost is needed.

The respiratory tract epithelium of healthy mammals is protected by low-level of produced mucus. Overproduction of mucus occurs in chronic airway disease, causing airway obstruction and contributing to the morbidity and mortality associated with asthma, bronchitis and cystic fibrosis (Aikawa et al., 1992; Kaliner et al., 1986). Airway goblet cell differentiation is influenced by the transcription factor SAM-pointed domain-containing ETS-like factor (SPDEF) and forkhead ortholog A3 (FOXA3), which are selectively expressed in airway goblet cells and induced by toxicants, aeroallergens, viral infections, and cytokines. SPDEF and FOXA3 are sufficient and required for goblet cell differentiation and mucus hypersecretion in the mouse airway (Chen et al., 2014; Chen et al., 2009; Park et al., 2007; Park et al., 2009). In addition to functional involvement of these transcription factors in mucus hypersecretion in asthmatic mice, recent study showed that NOTCH2 is required for inflammatory cytokine driven goblet cell metaplasia in the lung (Danahay et al., 2015). However, expressional status of NOTCH2 in OVA-induced asthmatic mice remains unclear.

Traditional Chinese Medicine (TCM) is one of the major parts of complementary and alternative medicine and has long history for treating asthma patients (Li and Brown, 2009). Traditional Uyghur Medicine (TUM) is now considered as one of the franchise of TCM in modern China which is characterized by distinct history, philosophy and medical practice from mainstream TCM (Wusiman et al., 2017). Loki Zupa is one of the medications used in TUM, constitutes of the roots of *Hyssopus cuspidatus* Boriss (Shenxiangcao), *Iris halophila* Pall (Yuanwei-gen), and honey. Our research team has previously reported its therapeutic effects on asthma patients (Lv et al., 2018), and revealed underlying pathological and molecular mechanisms in OVA-induced asthmatic mice (Wei et al., 2016; Wuniquimu et al., 2020). High Performance Liquid Chromatography (HPLC) results of Loki Zupa showed that Iristectorigenin A is one of the main components originated from roots of *Iris halophila* Pall (Called Yuan-wei-gen in Chinese) — one of the herbs of Loki Zupa (Wuniquimu et al., 2020).

Iristectorigenin A was first isolated from medical plants and shown to be cyclooxygenase inhibitor in 1999 (You et al., 1999). Since then, Iristectorigenin A has continuously reported as one of the isoflavonoids isolated from *Iris spuria* L. (Farag et al., 2009), *Belamcanda chinensis* (Kang et al., 2008), *Iris tectorum* (Qiu et al., 2009) etc. However, to best of our knowledge, no study ever reported the focused bio-activity or therapeutic potential of Iristectorigenin A. We pre-tested four compounds from Loki Zupa, include Iristectorigenin A, Iristectorigenin B from *Iris halophila*, and Aigenin, Romaniric acid from *Hyssopus cuspidatus*, and Iristectorigenin A showed some potency on amelioration of AHR in OVA-induced asthmatic mice (Supplementary Figure 1). Therefore, we hypothesized that Iristectorigenin A might has potential in improving allergic asthma and conducted systemic asthma animal

model based study. Our results revealed that Iristectorigenin A attenuates airway inflammation and airway goblet cell metaplasia in OVA-induced asthmatic mice probably by suppressing NOTCH2 expression.

Material and methods

Acute oral toxicity and determination of median Lethal dose (LD₅₀)

The LD₅₀ as an indication of acute toxicity of Iristectorigenin A were determined using Revised Limit Dose Test of the “Up and Down” procedure (UPD) according to OECD guideline (Walum, 1998). In this method, initial single limit dose of 1g/kg of Iristectorigenin A (10 ml/kg) was orally administrated individually to 5 BALB/c female mice after were fasted overnight (12 h) and within first 48 h each animal was monitored in metabolic cages and observed for instantaneous death or/and for the short-term effect. A modified 4-level Up and Down procedure was applied to determine LD₅₀ (Fig. 1). Then, daily monitoring was conducted for the next 7 days to detect any delayed toxic affects (Ebbo et al., 2020).

Animals and grouping

Our model simulates acute asthma and only one week of substance oral administration was applied in this model. Total of 60 BALB/c female mice (5-6 weeks old, 20 ± 2 g weighted) purchased from Shanghai Jiesijie Laboratory Animal Co. Ltd were recruited into our study and randomly designated into five groups, normal control/saline (NC, n=15), OVA-induced asthma (OVA/saline, n=15), Iristectorigenin A 10 mg/kg (OVA/ITG 10, n=10), Iristectorigenin A 5 mg/kg (OVA/ITG 5, n=10), and positive control group dexamethasone (OVA/Dex, n=10). Mice were housed in sterilized, pathogen free environment with controlled temperature, humidity and standard dark/light cycling. All mice exposed to free access water and standard animal diet. All experimental animal protocols were approved by the Animal Care and Use Committee of the Fudan University. Institutional Animals Care and Use Committee (IACUC) number is 2018-10-HSYY-DJC-01. Iristectorigenin A (Fig. 2 A; CAS:39012-01-6; purity > 98%) was purchased from Shanghai Winherb Medical Technology Co., Ltd.

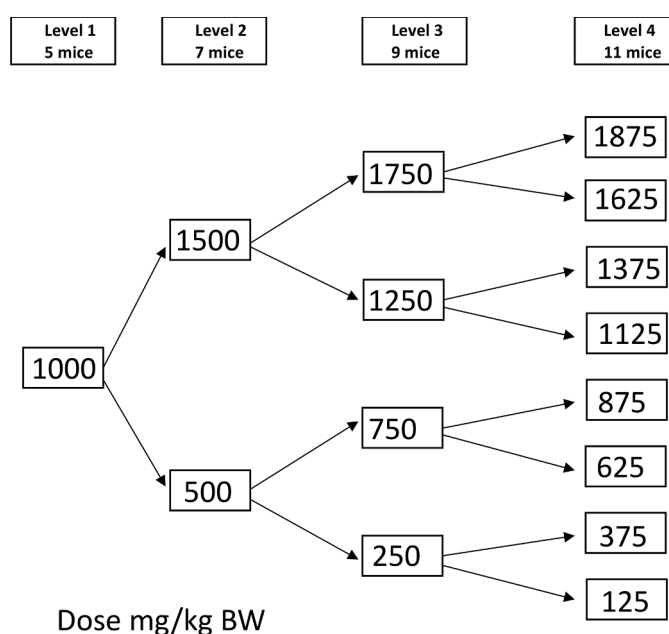


Fig. 1. The 4-level Up and Down Procedure for determining the LD₅₀ of *in vivo* Iristectorigenin A.

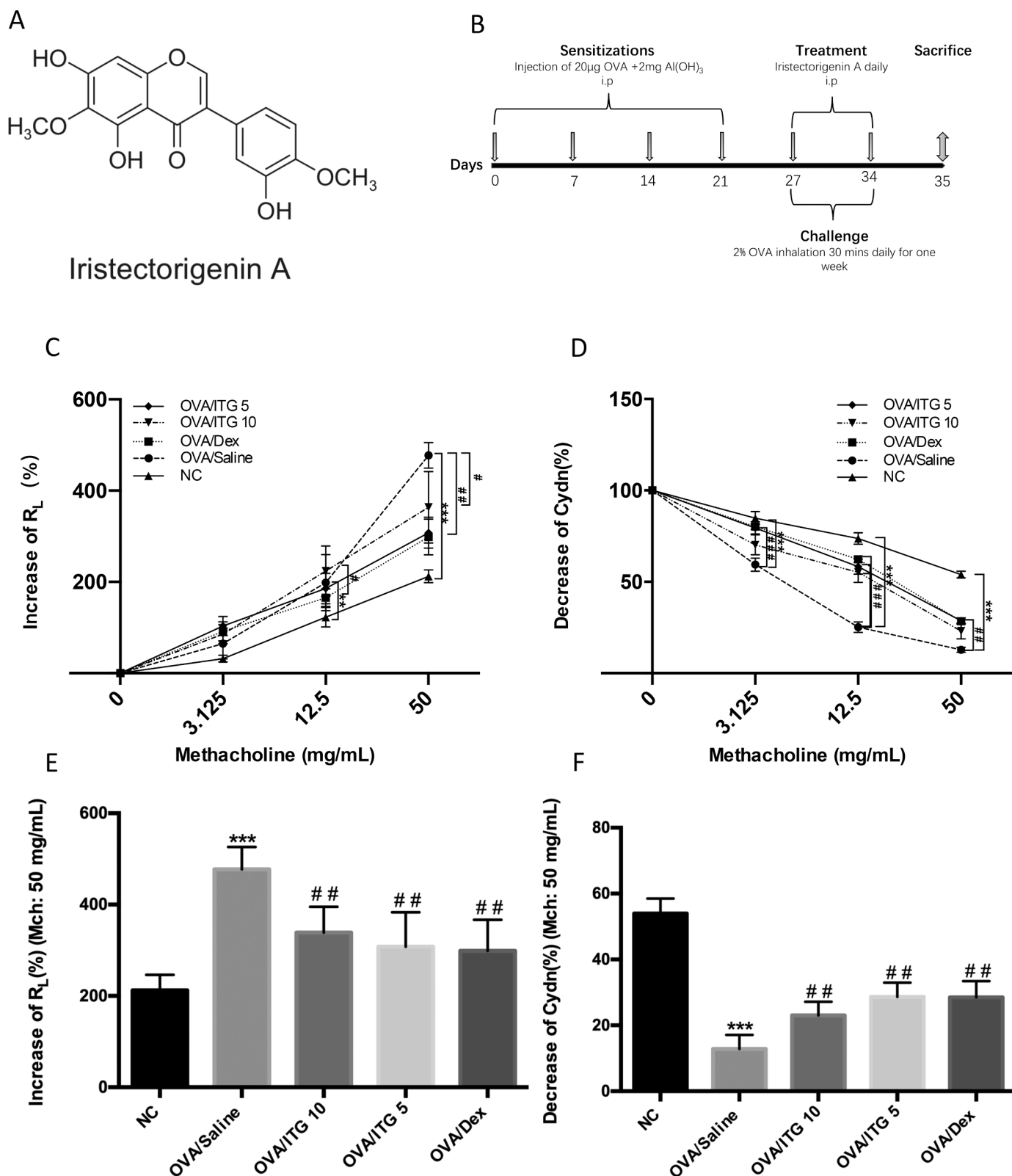


Fig. 2. Iristectorigenin A ameliorates airway hyperresponsiveness (AHR). (A) Chemical structure of Iristectorigenin A. (B) A schema for asthmatic murine model establishment with OVA sensitization and challenge. Variation of AHR was reflected by (C) increased of airway resistance (R_L) and (D) decrease of lung compliance (Cydn) responding to gradient dose of methacholine. Histogram depicting changes in (E) airway resistance (R_L) and (F) lung compliance (Cydn) at 50 mg/ml of methacholine. *** $p < 0.001$ compared to normal control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline; Statistical significance was determined using One-way ANOVA with Tukey's adjustment.

Establishment of OVA-induced asthma model and substance administration

All the mice were subjected to OVA sensitization and challenged as previously described (Hwang et al., 2017) with minor modification. Mice were sensitized four times with 20 µg of OVA (grade V; Sigma-Aldrich; St. Louis, MO) and 0.2 ml of saline solution containing 2 mg aluminum hydroxide (Thermo Scientific) by the intraperitoneal injection on days 0, 7, 14 and 21. Mice were then challenged by intranasal albumin inhalation with 2% OVA solution (w/v) or saline solution for NC group through an ultrasonic nebulizer for 30 min for seven consecutive days, from day 27 to 34 (Fig. 2B). Iristectorigenin A is dissolved in dd H₂O containing 0.5% Sodium carboxymethyl cellulose (CMC-Na; 5g CMC-NA in 100 ml of water). Mice in the normal control (NC/saline) and asthmatic model group (OVA/saline) were orally (gavage) administered with 0.2 ml of saline, while mice in the OVA/ITG 10, OVA/ITG 5, and OVA/Dex group were orally (gavage) administered with 10 mg/kg of Iristectorigenin A, 5 mg/kg of Iristectorigenin A, and 0.1 mg/kg of dexamethasone, respectively, for seven consecutive days, from day 27 to 34, 30 minutes prior to OVA challenge. Mice were subjected to lung function assessment 24 h after the final airway challenge and sacrificed with overdose pentobarbital sodium for sample collection.

Assessment of Airway Hyperresponsiveness (AHR) using FinePointe RC System

The airway hyperresponsiveness of all mice were assessed as previously described (Cui et al., 2019). Mice were anesthetized with pentobarbital sodium (50 mg/kg) by *i.p.* injection and tracheostomized using appropriate metal cannula. Then, mice were placed in a whole-body plethysmograph chamber (Buxco Electronics, Troy, NY) and connected to a ventilator by the endotracheal cannula. AHR was induced with progressively quadrupled concentrations, ranging from 0 to 50 mg/ml, of methacholine (MCh, Sigma, St. Louis, MO) administration using a nebulizer (Aeroneb). The range of base line RI was set as 0.8~1.2 as according to quantification principle of FinePoint ventilation system (Buxco). Airway resistance (RL) and lung dynamic compliance (C_{dyn}) were recorded.

Measurement of immune cells, cytokines and immunoglobulin

The collection of bronchoalveolar lavage fluid (BALF) and serum was conducted as described previously (Cui et al., 2019). BALF samples were centrifuged at 500 × g for 10 min. The cell pellet was resuspended in 100 µl of PBS. Total and inflammatory cells were counted using an automated cell counter (Hemavet 950 instrument, Drew scientific group, UK). The cell supernatant was isolated and concentration levels of selected cytokines IL-4, IL-5, and IL-13 in BALF and OVA-specific IgE, and OVA-specific IgG1 in serum were measured by enzyme-linked immunosorbent assay (ELISA) (Cayman Chemical, Michigan, USA) according to manufacturer's instructions. Cytokines IL-33, INF-γ, and IL-9 were measured by Meso Scale Discovery kit (Meso Scale Diagnostics, Rockville, MD, USA).

Histological evaluation of lung tissue morphology

The left lung lobe of the mice was dissected and placed into 4% formalin overnight at room temperature for fixation and transferred into PBS. Samples were then dehydrated with ethanol and xylene followed by paraffin embedding and cut into sections (4 µm). Each lung tissue sections were stained with hematoxylin and eosin (H&E), trichrome blue, and periodic acid-schiff (PAS). Images were acquired with a light microscope (DS-Qi2, Nikon, Japan) equipped with X 4, X 10, X 20 objective lens (Plan fluor, Nikon, Japan). Scoring and statistical analysis was performed as previously described (Ma et al., 2016).

Immunohistochemistry

Paraffin embedded lung tissue sections were deparaffinized and rinsed with degraded concentrations of ethanol and water. The endogenous peroxidase was re-activated with 3% hydrogen peroxidase. Samples were stained with primary antibody, such as rabbit polyclonal anti-FOXA3 (1: 200, Novus Biologicals, Inc. Littleton, CO, US, # NBP2-87461), anti-SPDEF (1: 100, Novus Biologicals, Inc. Littleton, CO, US, # NBP3-04686), anti-MUC5AC (1: 100, Novus Biologicals, Inc. Littleton, CO, US, # NBP2-15196). The sections were incubated with secondary antibodies (2 µg/ml, Santa Cruz Biotechnology, Santa Cruz, Calif). Images were acquired with a light microscope (DS-Qi2, Nikon, Japan) equipped with X 4, X 10, X 20 objective lens (Plan fluor, Nikon, Japan). The number of cells with positive staining were counted and area of staining was scored for statistical analysis.

Western blotting analysis

The lung tissue samples were lysed using cell lysis buffer as previously described. With 6%-12% SDS-PAGE equal amounts of protein were separated and electroblotted onto PVDA membranes (Millipore, Davers, MA). Subsequently, membranes were blocked with 5% milk in TBST at room temperature for 1 h and incubated with anti-NOTCH1 (1:1000, #ab52627; Abcam, Hangzhou, China), anti-NOTCH2 (1:1000, #D76A6; Cell Signaling Technology, Danvers, MA, USA), β-actin (Proteintech, Wuhan, China) at 4 °C overnight and followed by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. At last, the blots were visualized using an ECL detection system (Millipore, Danvers, MA, USA). For quantitative analysis, loading was normalized with β-actin and each experiment was triplicated.

Quantitative PCR analysis

Total RNA was prepared with TRIzol™ Reagent (Invitrogen, Thermo Fisher Scientific #15596018). For cDNA synthesis, RNA was reverse transcribed using PrimeScript™ RT Master Mix (Takara, #RR036A) following the standard protocol by SimpliAmp thermocycler (Applied Biosystems). Each total volume of 20 µL qPCR reaction mixture consisted of 5 µL SYBR qPCR Master Mix (Vazyme, #Q711-02/03), PCR forward primer (0.4 µM), reverse primer (0.4 µM), and 2 µL cDNA template. Primer sequences for the gene of interest is shown in Table 1. RNA was amplified and quantified using Roche 480II PCR system (Roche). The PCR thermal cycle protocol consisted of the following: 1 cycle of 95 °C for 30 seconds, 40 cycles of 95 °C for 5 seconds, and 60 °C for 30 seconds, ending with 1 cycle of 95 °C for 15 seconds, 60 °C for 60 seconds, and 95 °C for 15 seconds. Reactions were analyzed in triplicate. Expression values were normalized to the housekeeping gene β-actin.

Statistical analysis

Data was presented as means ± SEM and all the statistical analysis was performed using SPSS (Version 17.0). One-way ANOVA with Tukey's adjustment were used for determining statistical significance. *p* values <0.05 were considered significant. Graph-Pad Prism 6 (Graph-Pad Software) was performed for data visualization.

Results

Acute oral toxicity

In the Up and Down Procedure, all the 5 mice found dead within 2 h after Iristectorigenin A administration at dose of 1000mg/kg body weight (BW). Therefore, 500 mg/kg BW was set as second level dose, where 5 out of 7 mice died. Subsequently, 4 of 9 mice were died at third level with 250 mg/kg BW, which lead to fourth level with reduction to

Table 1
mRNA primers used for qRT-PCR analysis in present study

Primers	Sequences (5' to 3')
IL-1b Forward	GAGGACATGAGCACCTTCTT
IL-1b Reverse	GAGCCTGTAGTGCAGTTGTCTA
IL-4 Forward	GGTCTCAACCCCGAGCTAGTTG
IL-4 Reverse	ATGGCGTCCCTTCTCTGTG
IL-5 Forward	AACCCCTGAGTTTCAGGACTCGCCTT
IL-5 Reverse	TCTTCAGCGCTGGCCTTCAGCAA
IL-13 Forward	GGGTGACTGCGACTCGGCT
IL-13 Reverse	GTTGCTCAGCTCCTCAATAAGC
IL-33 Forward	TGGCCTCACCATAAGAAAGG
IL-33 Reverse	GGATACTGCCAAGCAAGGAT
Muc5ac Forward	TGACTCAATCTGCGTGCCTT
Muc5ac Reverse	AGGCCTTCTTTTGGCAGGTT
Spdef Forward	GACTGTGGAATTCTGGGGG
Spdef Reverse	ATTGTGGCAGGAGCAGAGAC
Foxa3 Forward	CTTGTTGGAGGTTGGGTGAG
Foxa3 Reverse	ACAGGCAAGTATTCACAGCC
Foxm1 Forward	CCTGCCAAGCAGAGTCCAGTT
Foxm1 Reverse	TGGGAGGGAATTGGAGGACT
Foxa2 Forward	TGCACTCGGCTTCCAGTATG
Foxa2 Reverse	CGTAGTAGCTGCTCCAGTGG
IL-12p35 Forward	GTCTACACTGCTGTGAAATCT
IL-12p35 Reverse	CTGTTGGAACGCTGACCATTA
Scgb1a1 Forward	ATGAAGATCGCCATCACAATCAC
Scgb1a1 Reverse	GGATGCCACATAACCGAGCTCT
Arg2 Forward	GGACTCTCGGCCCAACTAC
Arg2 Reverse	GAATGACCATCAAGGGTCTGTT

125 mg/kg BW, in which all of the mice survived (Table 2). Thus, *in vivo* LD₅₀ of Iristectorigenin A was determined as 250 mg/kg BW.

Iristectorigenin A ameliorates asthmatic Airway Hyperresponsiveness

Impaired lung function is one of the important hallmark of allergic asthma, which usually manifest itself as airway hyperresponsiveness (AHR) with increased lung resistance and reduced lung compliance (Reynolds et al., 2018).

Sensitized and nasally challenged with OVA mice that orally administrated saline solution exhibited significantly increase airway resistance (R_L) (Fig. 2C, E) and markedly reduced lung compliance (C_{dyn}) (Fig. 2D, F) compared to control mice ($p < 0.001$) responding to Mch concentration of 50 mg/ml. Airway resistance in OVA-induced asthmatic mice with Iristectorigenin A 10 mg/kg was significantly reduced compared to mice in OVA/saline group ($p < 0.05$). This reduction was more pronounced with Iristectorigenin A 5 mg/kg and dexamethasone treated asthmatic mice ($p < 0.01$). Dynamic lung compliance was significantly increased with all three treatments, Iristectorigenin A 10 mg/kg, 5 mg/kg, and dexamethasone, compared to mice treated with saline ($p < 0.01$).

Iristectorigenin A reduces number of inflammatory cells in BALF and attenuates pathologic impairment in asthmatic mice

OVA sensitization and challenge increases number of pulmonary inflammatory cells, including neutrophils, lymphocytes, monocytes, eosinophils, and basophils compared to normal control mice ($p < 0.01$).

Table 2
Mortality of mice induced by gavage administration of Iristectorigenin A and survival time corresponding to each dose treatment.

Dose (mg/kg) BW	Mortality	Survival times (h)
1000	5/5	0.28; 0.52; 1.17
500	5/7	0.48; 1.21; 1.42; 2.06
250	4/9	1.32; 0.58; 1.04; 1.54
125	0/11	>24
75	0/11	>24
25	0/11	>24

Administration of Iristectorigenin A 5 mg/kg significantly reduced the number of above inflammatory cells compared to mice in OVA/saline group ($p < 0.05$). Administration of Iristectorigenin A 10 mg/kg only reduces the number of lymphocytes ($p < 0.05$), but not neutrophil, monocytes, eosinophil, and basophil ($p > 0.05$). Dexamethasone reduces the number of neutrophil, lymphocytes, and eosinophils ($p < 0.05$) but not monocytes and basophils ($p > 0.05$) (Fig. 3 A, Supplementary Fig. 2).

H&E staining showed that OVA sensitization and challenge induced inflammatory cell infiltration and epithelial thickening in mice lung tissue, along with the significant higher inflammation score compared to normal control ($p < 0.001$). Mice treated with Iristectorigenin A 10 mg/kg, Iristectorigenin A 5 mg/kg, and dexamethasone exhibited improvement in inflammatory cell infiltration and thickness of bronchiolar epithelial layer. Inflammation score of mice lung tissue in these three groups was significantly decreased compared to those in OVA/saline group ($p < 0.05$) (Fig. 3 B, C).

Airway remodeling was evaluated by Masson's trichrome to assess the extent of subepithelial fibrosis. However, no significant alteration was observed among experimental groups (Fig. 3 B, D), therefore cannot be used for subsequent experiments on assessing subepithelial fibrosis. This may be because chronic epithelial changes, especially subepithelial fibrosis requires long-term exposure to antigen or influenza (Kumar and Foster, 2001).

The periodic acid-Schiff (PAS) staining was performed to characterize the goblet cell hyperplasia and mucus production. The number of PAS positive cells and mucus production were markedly higher with OVA sensitization and challenge than normal control ($p < 0.001$). Iristectorigenin A 10 mg/kg, Iristectorigenin A 5 mg/kg, and dexamethasone significantly reduced this phenomenon ($p < 0.01$) (Fig. 3 B, D). These results may be suggestive of protective property of Iristectorigenin A against airway inflammation and goblet cell metaplasia.

Iristectorigenin A reduces concentrations of inflammatory cytokines and OVA-specific immunoglobulin in asthmatic mice

Airway epithelial cells recognize exposed antigens or influenza and exerts immunomodulatory role by secreting various mediators (Hammad et al., 2009; Lambrecht and Hammad, 2012). To investigate the potential effect of Iristectorigenin A on the function of the airway epithelium in asthmatic mice, we investigated the inflammatory cytokines involved in asthma and OVA-specific immunoglobulin production. As expected OVA-induced asthmatic mice showed increased level of IL-4, IL-5, IL-13, and IL-33 in BALF and IgE, IgG1 in serum ($p < 0.001$). Detected concentration of INF- γ (Fig. 4 E), and IL-9 (Fig. 4 F) in BALF was very low, therefore cannot be apply to speculate any results. Administration of Iristectorigenin A 10 mg/kg, Iristectorigenin A 5 mg/kg, and dexamethasone markedly ($p < 0.001$) reduced the expression level of IL-4 (Fig. 4 A), IL-5 (Fig. 4 B), IL-33 (Fig. 4 D), IgE (Fig. 4 G), and IgG1 (Fig. 4 H), but not IL-13 ($p > 0.05$) (Fig. 4 C) in asthmatic mice.

Iristectorigenin A alters the gene expression levels of cytokines implicated with Th2 immune response and dendritic cell activation in asthmatic mice lung tissue

Type 2 immune response is well known fact that greatly implicated with allergic asthma initiation and development (Walker and McKenzie, 2018). In adult mice, conventional dendritic cell and bronchial epithelial cells manipulate the sensitization to allergen (Hammad and Lambrecht, 2015). Allergen exposure increases IL-33 level, which subsequently suppresses IL-12p35 and induces dendritic cell activation, thus promoting Th2 cell skewing (de Kleer et al., 2016) in allergic inflammation.

To assess the effect of Iristectorigenin A on allergic airway inflammation in molecular level, we investigated the expression status of genes associated with dendritic cell activation (IL-33, IL-12p35) and Th2 cytokines (IL-4, IL-5, IL-13) in mice lung tissue. OVA sensitization and

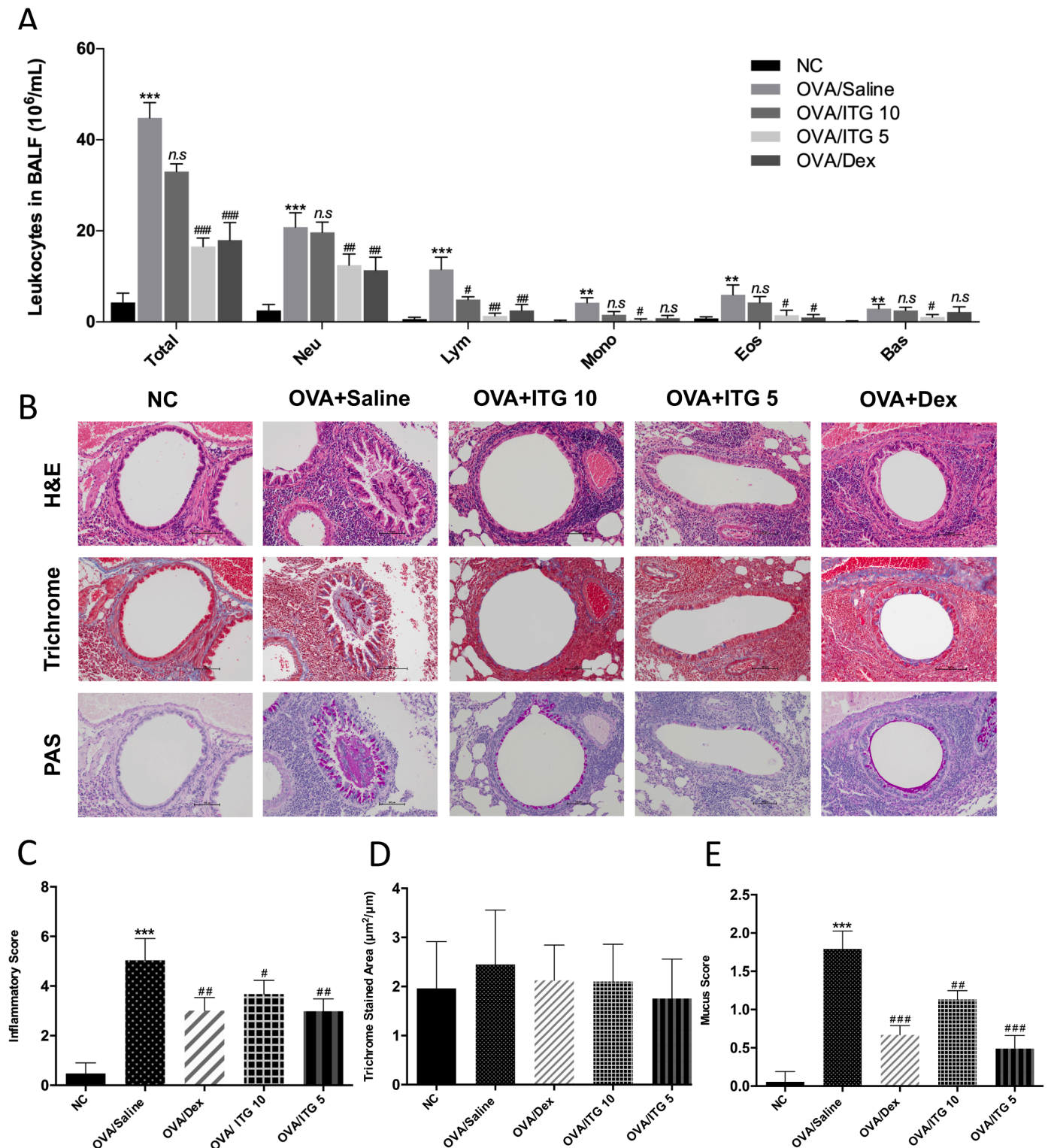


Fig. 3. Attenuated OVA-stimulated asthma phenotype following Iristectorigenin A treatment. (A) Number of leukocytes ($10^6/\text{mL}$) across five groups. (B) Representative images of histology analysis in inflammation (H&E staining), lung fibrosis (Trichrome blue staining), and mucus production (PAS staining); (Scale bar 100 μm ; 100X magnification). Histogram depicting (C) inflammation score, (D) trichrome blue staining area, and (E) mucus score. *** $p < 0.001$ compared to normal control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline; Statistical significance was determined using One-way ANOVA with Tukey's adjustment.

challenge increased mRNA expression of above genes compared to control mice ($p < 0.001$). Administration of Iristectorigenin A 5 mg/kg reduced expression level of *IL-4* (Fig. 5 A), *IL-5* (Fig. 5 B), and *IL-33* (Fig. 5 C) ($p < 0.01$) in asthmatic mice, while Iristectorigenin A 10 mg/kg decreased the expression of *IL-4*, *IL-5* ($p < 0.01$), but had no effect on

IL-33 mRNA level ($p > 0.05$). No significant change was found in *IL-13* and *IL-12p35* mRNA level with Iristectorigenin A 5 mg/kg administrated asthmatic mice ($p > 0.05$).

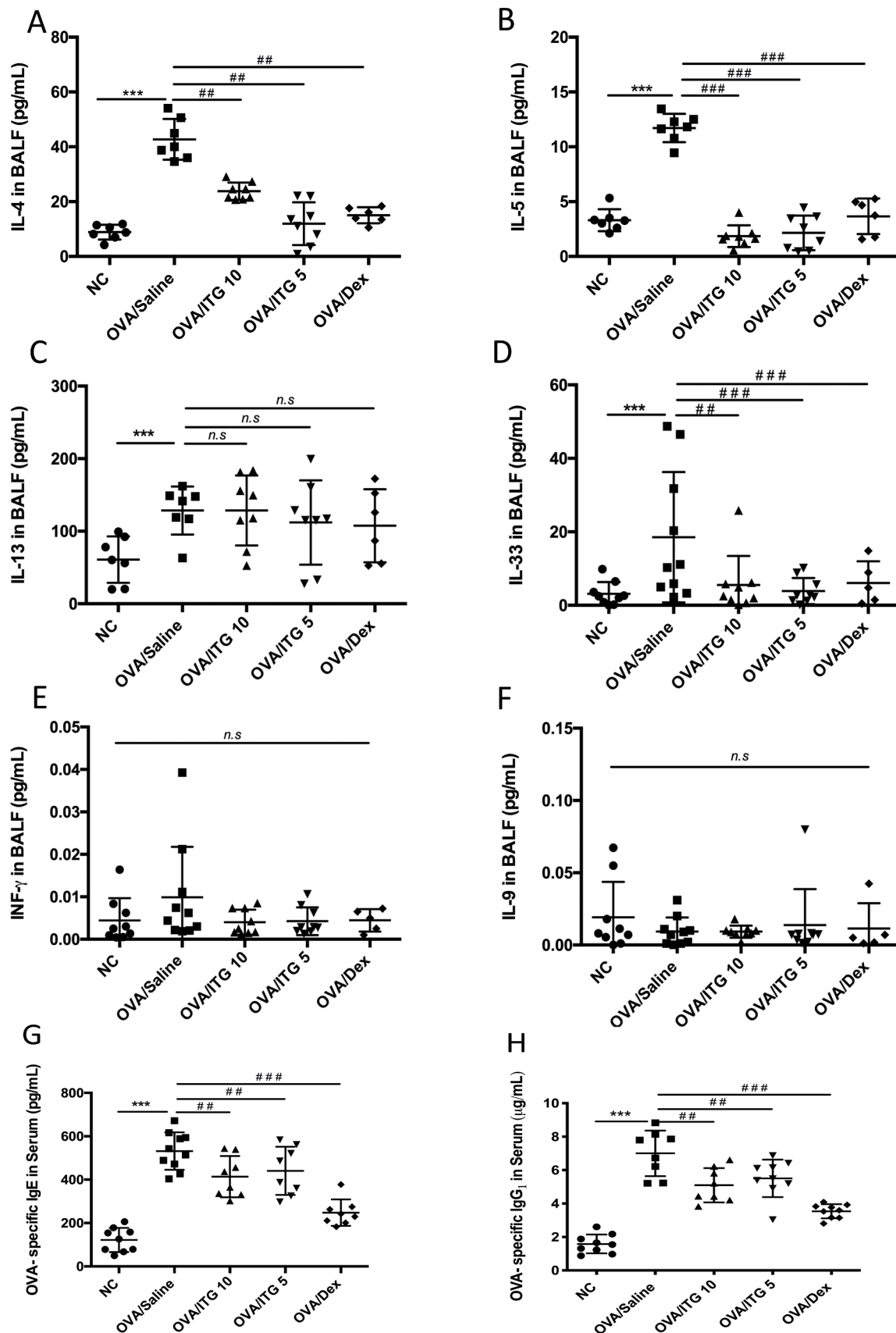


Fig. 4. Decreases content of inflammatory cytokines and OVA-specific immunoglobulin in asthmatic mice following Iristectorigen A intervention. BALF cytokine (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-33, (E) INF- γ , (F) IL-9 levels, and serum OVA-specific (H) IgE and (I) IgG1 were measured with ELISA. *** $p < 0.001$ compared to normal control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline; n.s non-significant; Statistical significance was determined using One-way ANOVA with Tukey's adjustment.

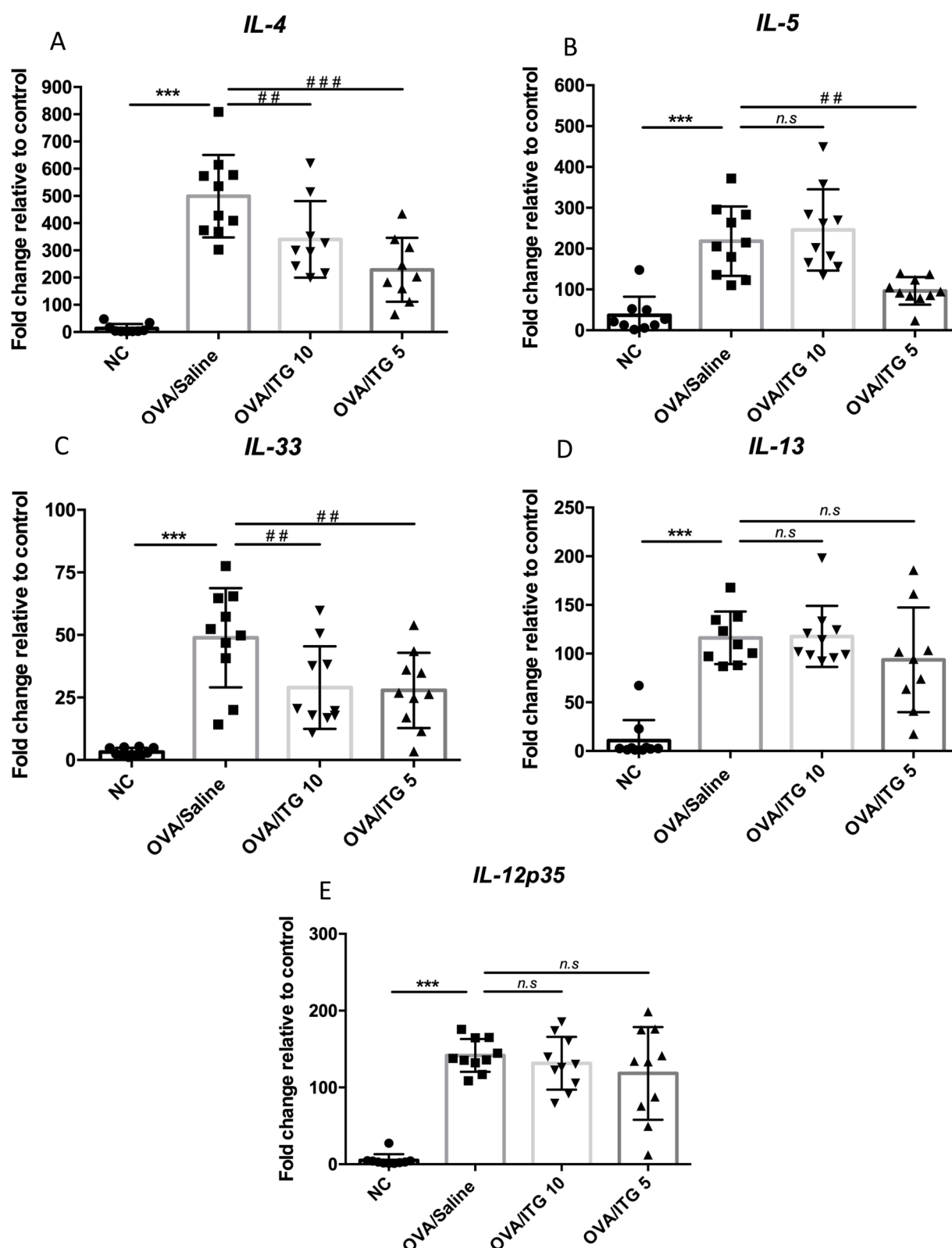


Fig. 5. Suppressed expressions of genes associated with inflammatory cytokines in lung tissue of asthmatic mice following Iristectorigenin A treatment. Expression levels of genes involved in inflammatory cytokines (A) *IL-4*, (B) *IL-5*, (C) *IL-33*, (D) *IL-13*, and (E) *IL-12p35* were measured with quantitative RT-PCR analysis. *** $p < 0.001$ compared to normal control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline; $n.s$ non-significant; Statistical significance was determined using One-way ANOVA with Tukey's adjustment.

Iristectorigenin A suppresses goblet cell-expressing proteins in bronchiolar epithelia of OVA-induced asthmatic mice

Since Iristectorigenin A showed markedly attenuated mucus production in bronchiolar airway, we further investigate the effect of Iristectorigenin A on expressions of goblet cell proteins using immunostaining. We assessed the protein abundance of MUC5AC (goblet cell proteins that are associated with increased mucus production), as well as SPDEF and FOXA3 (transcription factors that promote goblet cell metaplasia) (Fig. 6). Our results showed that FOXA3 primarily expressed on nuclei of epithelial cells in healthy lung, and such location shifted into mucus on small airway with significant abundance following OVA sensitization. Iristectorigenin A markedly reduced such abundant expression, but no difference was found between two different dosage intervention. Healthy lung exhibited null expression of MUC5AC. OVA sensitization and challenge induced substantially strong expression of MUC5AC and Iristectorigenin A mitigated MUC5AC staining in airway. However, we cannot locate expressional region of transcription factor SPDEF in airway, which led to failure of elucidating SPDEF involvement in this study. This result be of suggestive that Iristectorigenin A ameliorates airway mucus hypersecretion by influencing transcription factor FOXA3.

Iristectorigenin A reverses the genes associated with goblet cell metaplasia in OVA-induced asthmatic mice

To further clarify the effect of Iristectorigenin A on goblet cell metaplasia, we detected the mRNA expression of *Foxm1*, *Spdef*, *Foxa3*, *Agr2*, *scgb1a1*, *Muc5ac* and *Foxa2* genes by quantitative RT-PCR. OVA exposed mice showed significantly elevated mRNA expression of *Foxm1* (Fig. 7 A), *Spdef* (Fig. 7 E), *Foxa3* (Fig. 7 C), *arg2* (Fig. 7 G) and *Muc5ac* (Fig. 7 D), decreased *Foxa2* (Fig. 7 B) and *scgb1a1* (Fig. 7 F) mRNA expression ($p < 0.001$). Expressions of *Foxa2*, *Foxa3* and *Spdef* were reversed with Iristectorigenin A treatment both, 10 and 5 mg/kg dosage ($p < 0.01$). In addition, mice subjected to 5 mg/kg Iristectorigenin A administration also exhibited significantly decreased *Foxm1* and *Muc5ac* mRNA expression ($p < 0.01$), but not with 10 mg/kg dosage ($p > 0.01$). Iristectorigenin A had no effect on *scgb1a1* and *arg2* expression ($p > 0.01$).

Iristectorigenin A suppresses the NOTCH2, but not NOTCH1, expression in OVA-induced asthmatic mice lung

Our western blot analysis revealed that compared to NC mice, NOTCH2 expression was significantly higher in OVA-induced asthmatic mice lung, which was significantly reduced with Iristectorigenin A 5 mg/kg and dexamethasone administration ($p < 0.05$). However, Iristectorigenin A 10 mg/kg treated mice did not show such alterations (p

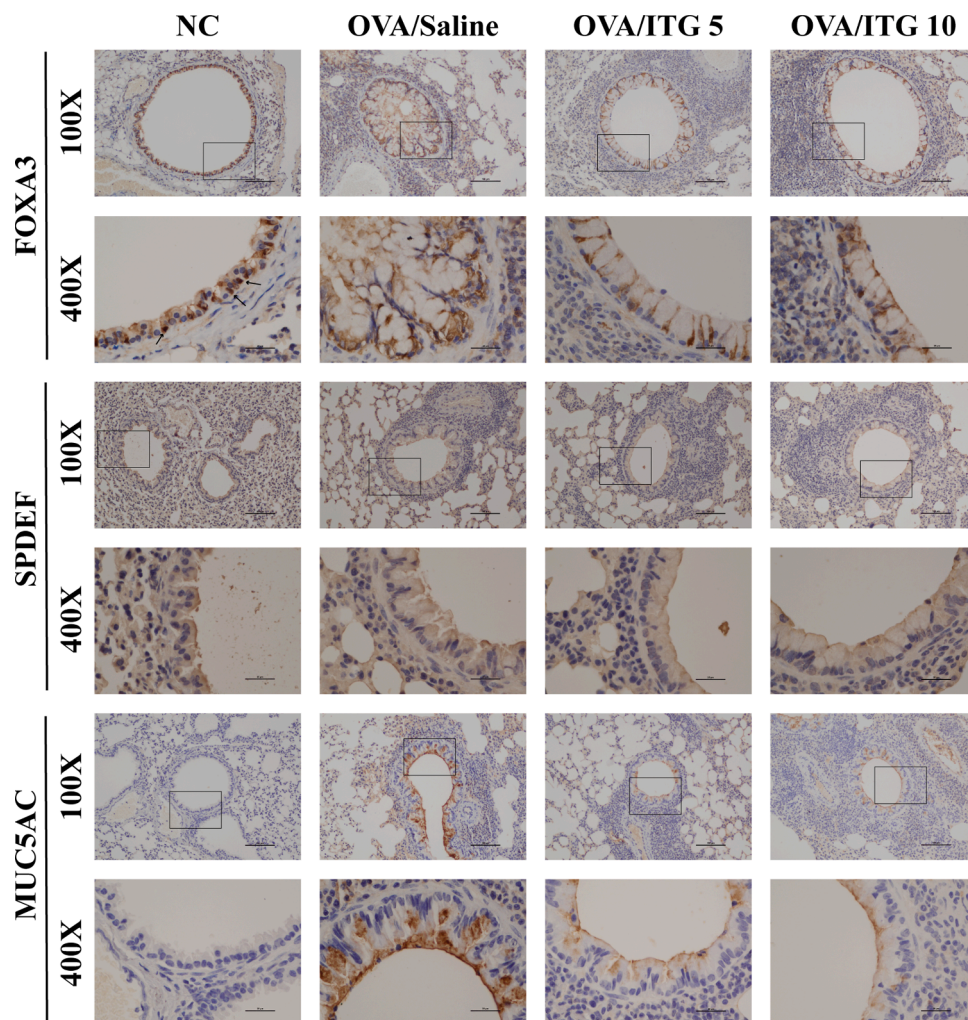


Fig. 6. Decreased mucus secretion and goblet cell in asthmatic mice following Iristectorigenin A treatment. Representative images of FOXA3, SPDEF, and MUC5AC staining with 100 X magnification (upper panel; scale bar 100 μ m) and 400 X magnification (lower panel; scale bar 35 μ m), (n=6~11 per group).

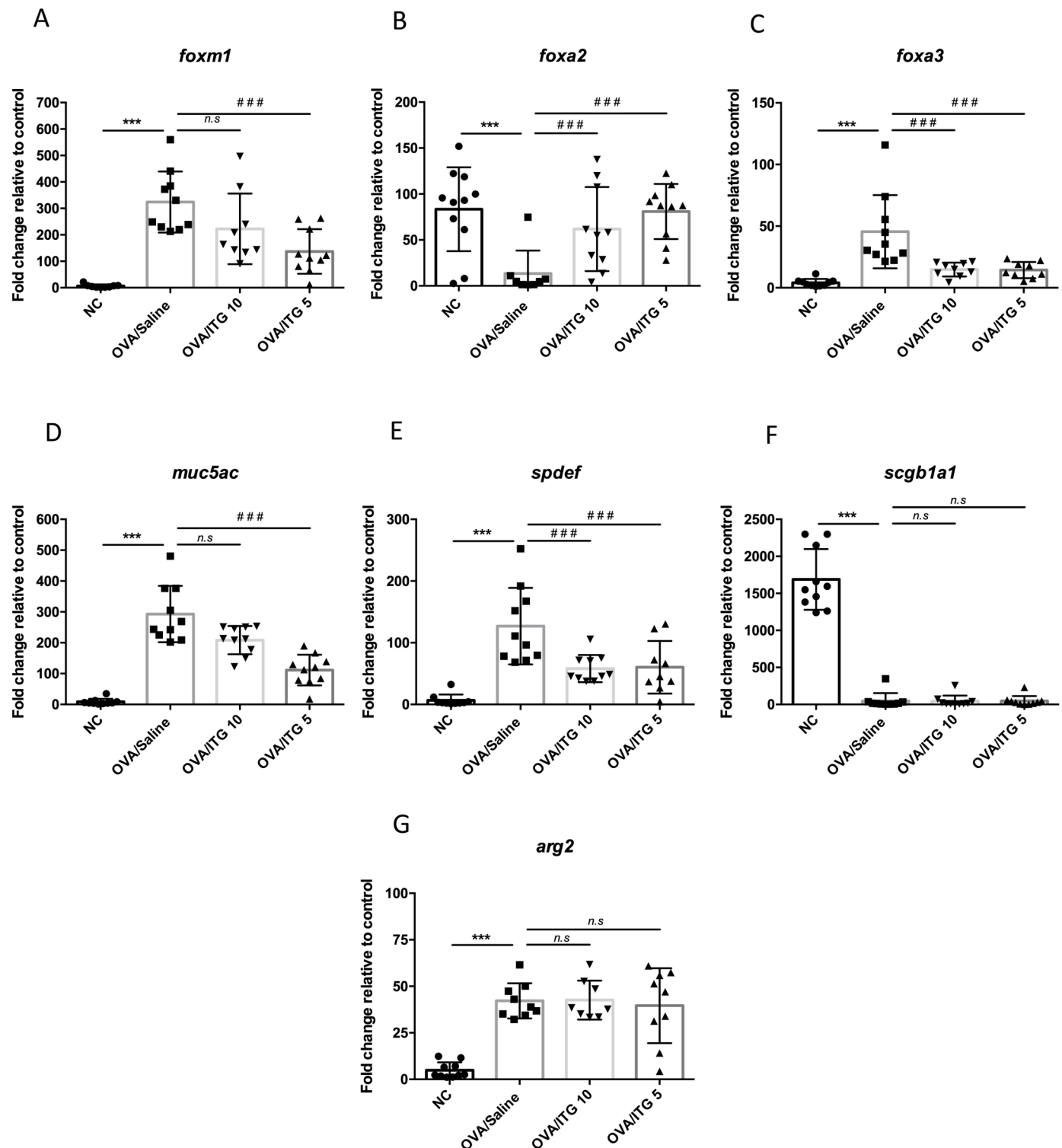


Fig. 7. Suppressed expressions of genes associated with mucus production and goblet cell metaplasia in lung tissue of asthmatic mice following Iristectorigenin A treatment. Expression levels of genes involved in goblet cells (A) *foxm1*, (B) *foxa2*, (C) *foxa3*, (D) *muc5ac*, (E) *spdef*, and (F) *arg2* were measured with quantitative RT-PCR analysis. *** $p < 0.001$ compared to normal control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline; $n.s$ non-significant; Statistical significance was determined using One-way ANOVA with Tukey's adjustment.

> 0.05; Fig. 8 B). As to NOTCH1, we found relatively low expressional content in lung tissue, and there was no obvious change among all experimental groups ($p > 0.05$; Fig. 8 A).

Discussion

Pathophysiology of asthma includes airway inflammation, airway hyper-responsiveness (AHR) and airway remodeling (Holgate et al., 2015). With well-established understanding of asthma heterogeneity and increasing number of treatment options, asthma control among

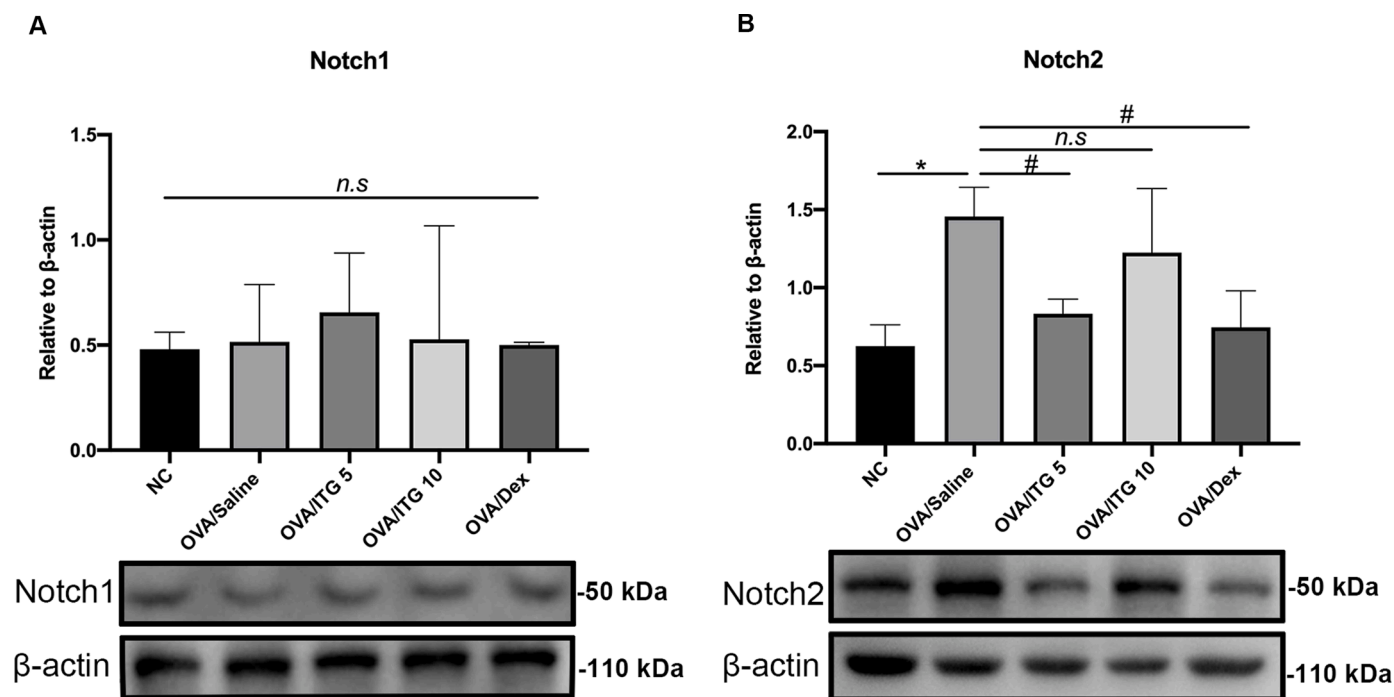


Fig. 8. Expressions of NOTCH1 and NOTCH2 in mice lung. Western blot analysis showed (A) NOTCH1 and (B) NOTCH2 expression in NC, asthmatic, Iristectorigenin A and dexamethasone treated groups. Expression level of each samples was normalized to β-actin. Each experiment was triplicated and mean value was analyzed for statistical difference. *** $p < 0.001$ compared to normal control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to OVA/Saline; n.s non-significant; Statistical significance was determined using One-way ANOVA with Tukey's adjustment.

patients with mild symptoms has met optimal clinical outcomes. However, those with severe asthma or/and unclear phenotype of asthma patients, who are poorly responsive to treatment, have still remained at high risk (Jones et al., 2018), in addition to side-effects of prescribed medicines and drug-related complications (Volmer et al., 2018). Therefore, many patients seek to alternative therapeutic interventions, such as Traditional Chinese Medicine including, herbal medicines, massage, acupuncture, diet therapy etc. (Shi et al., 2019). Plant isoflavones, one of the most estrogenic compounds, are considered chemo-protective and have potentials as an alternative therapy for various diseases, including cancer, cardiovascular disorder, osteoporosis, and asthma (Cho et al., 2019; Krizova et al., 2019; Lago et al., 2014). Iristectorigenin A, an isoflavone extracted from variety of medical plants and has been reported previously for its hepatoprotective activity (Akther et al., 2014). However, precise bioactivity and biological function of Iristectorigenin A remains unknown.

Our study revealed a novel role of Iristectorigenin A for treating airway inflammation and mucus hyper-secretion in asthmatic murine model, suggesting Iristectorigenin A as a novel, effective therapeutic agent. This proposal was projected by decreased AHR, attenuated airway inflammation and reduced mucus production in OVA stimulated asthmatic mice.

Airway hyperresponsiveness (AHR) is typical manifestation of bronchoconstriction and inflammation in response to external non-specific stimuli and is a significant characteristic of asthma (Postma and Kerstjens, 1998). The bronchoconstriction response to methacholine induces distal and focal airway closure, leading to defined ventilation defects and reduced vital capacity (Plantier et al., 2016). Loss of bronchodilator and bronchoprotector effect of deep respirations is a main contributor to AHR in asthma and evaluated by measuring airway resistance and lung compliance (Nair et al., 2017). In present study, we successfully established OVA-stimulated asthmatic mice model with pronounced AHR defined by increased airway resistance (R_L) and reduced dynamic lung compliance (C_{dyn}). With intervention of Iristectorigenin A at both dose levels, AHR has significantly ameliorated in

asthmatic mice. Although slightly improved AHR appeared at 5 mg/kg treatment with Iristectorigenin A compared to 10 mg/kg, no significant difference was found between two differential dosage interventions. In contrary to general belief that the higher dose has better effects, recent study showed that one-quarter of asthma patients in Australia associated with toxicity with inhaled corticosteroid (Barros and Gustafsson, 2011; Hew et al., 2020). In our study, the symptoms of allergic asthma were suppressed at both concentration of Iristectorigenin A. However, suppressive effect of Iristectorigenin A tend to more pronounced at concentration of 5 mg/kg. This may be implicated with minor toxicity with cumulative higher dose of Iristectorigenin A, in this case 10 mg/kg dosage. Unfortunately, in this study we did not conduct any relevant experiment to find out the reason why lower dose have better inhibitory effect. However, launching another research project focusing on optimal Iristectorigenin A dose and toxicological analysis of high dose treatment is necessary. Our study be of reasonable suggestion for future study that highest dose of Iristectorigenin A should be locked as 10 mg/kg and establishing range of lower doses below 10 mg/kg at various concentration points will be more efficient to finding optimal dose response.

Modulation of airway type 2 immune responses is mainly associated with eosinophils, basophils, mast cells, group 2 innate lymphoid cells (ILC2s), TH2 cells and IgE-producing B cells (Fahy, 2015). Our data showed well established asthmatic model characterized by pathologic inflammatory cell infiltration, airway plugging with highly secreted mucus, elevated cytokine level of IL-4, IL-5, IL-13, IL-33 in BALF, and OVA-specific IgE and IgG1 in serum, as well as increased mRNA expression of genes encode above inflammatory cytokines. Iristectorigenin A treatment showed reduced number of inflammatory cells and infiltration, pronounced mucus-free airway clearance, decreased level of IL-4, IL-5, IL-33, but not IL-13, OVA-specific IgE and IgG1, and reduced mRNA expression of IL-4, IL-5, and IL-33, but not IL-13 in asthmatic mice. These results indicate that Iristectorigenin A ameliorates airway inflammation by restricting type 2 immune responses in OVA-induced asthmatic mice.

Eosinophilic pulmonary inflammation, eosinophil-derived products,

as well as Th2 cytokines IL-13, IL-4, and IL-5, have been associated with the development of airway hyperreactivity (AHR), but the specific immunological basis underlying the development of AHR remains controversial. Recent study show that mice with targeted deletion of IL-13 failed to develop allergen-induced AHR, despite the presence of vigorous Th2-biased, eosinophilic pulmonary inflammation. However, AHR was restored in IL-13(-/-) mice by the administration of recombinant IL-13. Moreover, adoptive transfer of OVA-specific Th2 cells generated from TCR-transgenic IL-13(-/-) mice failed to induce AHR in recipient SCID mice, although such IL-13(-/-) Th2 cells produced high levels of IL-4 and IL-5 and induced significant airway inflammation (Walter et al., 2001). Therefore, in external allergen, in this case OVA, induced asthmatic model, the role of IL-13 may not be sufficient enough to cause or/and improve AHR status. In present study, Iristectorigenin A significantly reduces the IL-4 and IL-5, but not IL-13. This results suggest that Iristectorigenin A selectively effects Th2 cytokines IL-4 and IL-5 and, thus, reduces airway inflammation and improves AHR.

In addition, it has been well documented that isoflavones play vital role as phytoestrogens to induce pseudohormonal activity via binding to estrogen receptors (ER) in mammals (Barros and Gustafsson, 2011; Vitale et al., 2013), and also is of antioxidant, antimicrobial, anticancer, anti-inflammatory activities same as other flavonoids (Chacko et al., 2007; Conklin et al., 2007; Rodriguez-Roque et al., 2013). Isoflavones elicit either anti-estrogenic (antagonistic) or estrogenic (agonistic) effect, depending on ER and endogenous estrogens levels (Kuiper et al., 1998). Isoflavones block the binding of more potent estrogens, potentially playing a role in preventing hormone-related inflammation (Yu et al., 2016) and allergic mice (Yamamoto et al., 2020). Iristectorigenin A, as one of the Isoflavones, may exert anti-inflammatory activity by affecting estrogen receptors. However, this assumption needs to be scientifically proven in the future.

Airway epithelial cells (AECs) play important role in the respiratory tract of patients with allergic asthma (Benam et al., 2018; Fahy and Dickey, 2010; Lambrecht and Hammad, 2012). In normal lung, AECs mitigates inhaled allergens through mucociliary clearance mechanism and protects airway by balanced mucosal immune responses (Fahy and Dickey, 2010). However, in asthma or chronic lung disorder damaged lung results a in highly secreted mucus, leading to loss of mucociliary clearance capacity and mucus plugging of small airways (Bonser and Erle, 2017). MUC5AC and MUC5B, the polymeric gel-forming mucins, are the most abundant and common mucins of AECs (Bonser and Erle, 2017) that are commonly affected by the transcriptional factors, FOXA3 and SPDEF (Rajavelu et al., 2015), which are significant modulators of type 2 immune-associated cytokines, such as IL-4, IL-5, IL-13, IL-33 (Morris et al., 2000; Rajavelu et al., 2015). Our histology analysis (PAS positive staining) exhibited significantly increased mucus secretion from AECs, strong immunohistologic staining of FOXA3 and MUC5AC, but not SPDEF, in mice response to OVA challenge, along with elevated mRNA level of *foxm1*, *foxa3*, *muc5ac*, *spdef*, *arg2*, and decreased level of *foxa2*, *scgb1a1*. Interestingly, Iristectorigenin A treatment resulted in reduced mucus hypersecretion, decreased staining of FOXA3 and MUC5AC, and reduced mRNA level of *foxm1*, *foxa3*, *muc5ac*, and *spdef*, increased mRNA of *foxa2*. However, due to antibody specificity, our analysis failed to detect and locate SPDEF, which leads us unable to elucidate the role of SPDEF in present study.

Levels of NOTCH activity are tightly regulated by multiple mechanisms, such as post-translational modifications and endocytosis, and these can influence the temporal and cellular expression of NOTCH receptors and ligands (Zanotti and Canalis, 2016). Epithelial cell-cell communication is based on evolutionary highly conserved Notch signaling principles. Among four isotypes of Notch receptors (Notch1-4) NOTCH1 and NOTCH2 showed high expression on bone marrow-driven mesenchymal stromal cells (BMSCs) (Mangolini et al., 2018). In addition, recent study showed that Notch2 is required for cytokine-induced goblet cell metaplasia and inhibition of Notch2 prevents goblet cell metaplasia induced by a broad range of stimuli, proposing Notch 2

neutralization as a therapeutic strategy for preventing goblet cell metaplasia in airway diseases (Danahay et al., 2015). Therefore, we evaluated potent role of Notch2 in mechanisms involved in amelioration of airway mucus hypersecretion caused by Iristectorigenin A. Our results showed that NOTCH2 expression was significantly abundant in OVA-induced asthmatic mice and its expression was markedly reduced with Iristectorigenin A 5mg/kg, while no such alteration was observed in NOTCH1 expressions among all experimental groups, which might be due to surprisingly low content of NOTCH1 in mice lung tissue. However, these results can be of value for further investigating the underlying mechanism of NOTCH2 involvement in Iristectorigenin A anchored improvement of goblet cell metaplasia in airways.

Over the last decade, potential therapeutic properties of isoflavones on asthma has been well documented (Barros and Gustafsson, 2011) (Raviv and Smith, 2010). Recent clinical study demonstrated that soy isoflavones reduce exacerbation in asthmatic patients with high PAI-1-producing genotypes (Cho et al., 2019). In addition, formononetin, as one of the well-studied isoflavones, mitigates airway inflammation and oxidative stress in murine allergic asthma. Oral administration of soy isoflavone dramatically inhibited histopathologic ovalbumin-induced lung tissue eosinophil infiltration, airway mucus production and collagen deposition in lung tissues, as well as substantially decreased mRNA expression of eotaxin interleukin (IL)-5, IL-4 and matrix metalloproteinase-9, and increased mRNA expression of interferon (IFN)- γ and tissue inhibitor of metalloproteinase-1 in OVA-induced asthmatic mice (Bao et al., 2011). In present study, we demonstrate that Iristectorigenin A markedly improves airway inflammation via suppressing Th2 inflammatory cytokines IL-4, IL-5, IL-33 and leukocytes, as well as mitigates mucus hypersecretion through inhibiting transcription factor FOXA3 and mucus protein MUC5AC in the airway epithelium of OVA-induced asthmatic mice.

Conclusions

In conclusion, we provide evidence that Iristectorigenin A improves airway hyperresponsiveness, airway inflammation, goblet cell hyperplasia and mucus hypersecretion following OVA sensitized and challenge, through regulation of type 2 immune response, suppression of transcription factor FOXA3 and NOTCH2. However, our study has several limitations. First, suppressive effect of Iristectorigenin A did not increase as dose response manner. Thus, optimal dose needs to be investigated in future study with extensive range of concentration points. Second, our work still remains at preliminary level and specific molecular and cellular mechanism of Iristectorigenin A in improving asthma phenotypes still need further investigation, both *in vitro* and *in vivo*. Third, experimental subjects of our study confined to only animals. Therefore, there is a necessity for further verification of our conclusion with human samples. Despite all the limitations, this is a first study demonstrates that novel component Iristectorigenin A could be effective therapeutic agent treating allergic asthma.

Author contributions

Jingcheng Dong and Ying Wei supervised the whole research and helped to coordinate funding. Tulake Wuniquimu designed the research. Tulake Wuniquimu, Fangzhou Teng and Jingjing Qin conducted animal model establishment. Tulake Wuniquimu and Fangzhou Teng carried out data analysis. Mohammadtursun Nabijan, Muhammadjan Abdurwaki, and Mamat Nurahmat provided resources of herbal plants and information on traditional use of decoction and preparation. Jie Cui, La Yi, Weifeng Tang, and Xueyi Zhu, Shiyuan wang, Amangu helped lung function assessment. Tulake Wuniquimu carried out data visualization and manuscript writing. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy

Conflicts of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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