



Anthriscus sylvestris root extract reduces allergic lung inflammation by regulating interferon regulatory factor 4-mediated Th2 cell activation

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ABSTRACT

Ethnopharmacological relevance: *Anthriscus sylvestris* L. Hoffmann (AS) is a perennial plant that grows in Asia and Eastern Europe. Its dried root is used to treat conditions such as asthma, bronchitis, and cough.

Aim of the study: The present study investigated the anti-inflammatory effects of whole AS extract (ASE) on allergic lung inflammation in vitro and in vivo as well as the underlying mechanisms.

Materials and methods: We used an ovalbumin (OVA)-induced asthma mouse model and in vitro primary T helper (Th)2 polarization system. Five groups of 8-week-old female C57BL/6 mice were divided into the following groups: saline control, or OVA-induced allergic asthma with vehicle, ASE (100 or 200 mg/kg), or dexamethasone (5 mg/kg) treatment for 7 days.

Results: ASE attenuated mucus secretion in airway epithelial cells, inflammatory cell infiltration, eosinophilia, and Th2 cytokine levels in bronchoalveolar lavage fluid. Mice administered ASE showed reductions in the activated cluster of differentiation 4⁺ T cell population and GATA-binding protein-3 gene expression in the lung, and diminished Th2 cell differentiation and activation in vitro. Furthermore, ASE-treated mice showed decreased interleukin-6 and interferon regulatory factor (IRF)4 expression, with corresponding reductions in nitric oxide levels in the lungs of asthmatic mice and in stimulated RAW cells.

Conclusion: ASE exerts anti-asthmatic effects by inhibiting IRF4 expression and thereby suppressing Th2 cell activation.

1. Introduction

Allergic airway inflammation is an inflammatory disorder of the respiratory tract characterized by episodic airflow obstruction and hyper-responsiveness (Holgate, 2012). The prevalence of and economic burden associated with asthma are increasing worldwide (Pawankar, 2014). Although current standard therapies improve the symptoms and pathologic features, many patients still experience serious adverse events or become refractory to treatment (Cates et al., 2013; Wener and Bel, 2013). Patients are often treated with both conventional therapeutics and complementary alternative (including herbal) medicines (Slader et al., 2006), and a number of studies have demonstrated the efficacy of such approaches (Li, 2011; Zhang et al., 2010).

Anthriscus sylvestris (L.) Hoffmann (AS; Umbelliferae) is a perennial plant that grows in Europe, North America, Africa, Asia, and New Zealand (Hendrawati et al., 2011; Jeong et al., 2007). The dried root of

AS is used in Korean and Chinese traditional medicine for the treatment of diverse diseases including asthma, bronchitis, and cough whereas the young aerial plant parts are consumed as food (Chen et al., 2014). Although most studies on whole AS extract (ASE) have been limited to collagen biosynthesis (Lee et al., 2012) and anticancer activities (Chen et al., 2014; Jung et al., 2013), deoxypodophyllotoxin (DPT)—a major component of whole ASE—has been extensively studied (Chen et al., 2000; Guerrero et al., 2013; Ikeda et al., 1998; Kozawa et al., 1982; Sudo et al., 1998). A recent study reported that DPT inhibits allergic reactions including asthma (Lin et al., 2006, 2004).

Various cell types involved in the innate and adaptive immune systems respond to allergens and cause bronchial hyper-responsiveness, mucus overproduction, and airway remodeling (Lambrecht and Hammad, 2015). T helper (Th)2 cells play a key role in the initiation and exacerbation of asthma; activated Th2 cells secrete cytokines such as interleukin (IL)-4, -5, and -13 (Kim et al., 2010). IL-4 and -13 induce

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goblet cell hyperplasia, bronchial hyperactivity, and chemokine and immunoglobulin (Ig)E production in B cells (Ip et al., 2006; Lambrecht and Hammad, 2015; Locksley, 2010; Van der Pouw Kraan et al., 1998). Mast cells activated by interaction with antigen-specific IgE promote massive histamine degranulation, which constitutes a key event in allergic asthma (Galli and Tsai, 2012). IL-5 then drives eosinophil infiltration into the respiratory tract (Foster et al., 2001; Rosenberg et al., 2013), which contributes to asthma pathogenesis via cytotoxic granule and cytokine secretion (Isobe et al., 2012; Kato et al., 2006; Uhm et al., 2012).

Oxidative stress also enhances eosinophil infiltration (Brennan et al., 2002; Ghosh et al., 2006); eosinophils from asthma patients produce more reactive oxygen species and reactive nitrogen species (RNS) than healthy individuals that likely accelerate airway inflammation (Calhoun et al., 1992; Gleich et al., 1989; Horwitz and Busse, 1995). Nitric oxide (NO) is an RNS that is generated in the lung by NO synthase (NOS) (Stuehr, 1997). The expression of inducible (i) NOS, which plays an important role in NO production, is upregulated in the airway in asthma (Dweik et al., 2001; Khatri et al., 2003).

iNOS is activated by nuclear factor- κ B signaling induced by inflammatory cytokines such as IL-6 (Soufli et al., 2016), which is produced by lung epithelial cells (Cromwell et al., 1992; King et al., 1998) at higher levels in asthma patients than in healthy subjects (Kicic et al., 2006; Marini et al., 1992). Interferon regulatory factor (IRF)4, which is also upregulated in patients with allergic asthma (Staudt et al., 2010), plays a critical role in the transformation of cluster of differentiation (CD)4⁺ T cells into Th2 cells (Honma et al., 2008). IRF4 expression is induced by NO or IL-6 signaling via basic leucine zipper transcription factor ATF-like (Koch et al., 2013).

Based on the above observations, we speculated that ASE exerts therapeutic effects against allergic asthma by inhibiting NO/IL-6-induced IRF4 expression. To test this hypothesis, in the present study we investigated the anti-asthmatic effects of whole ASE in vitro and in vivo using a Th2 polarization system and a mouse model of allergic lung inflammation, respectively. We also examined the mechanism of action of ASE with respect to IL-6 and IRF4 expression.

2. Materials and methods

2.1. Plant materials

Dried AS roots were purchased from a Korean herbal market (Kwangmyongdang Co., Ulsan, Korea). Dr. Goya Choi, an expert herborologist of the Korea Institute of Oriental Medicine (KIOM), authenticated the specimens as *Anthriscus radix* based on macroscopic morphological characteristics. Plant materials were assigned an identification number (2-15-0652 and 2-15-0653) and preserved in the KIOM herbarium (code KIOM; Supplementary Fig. S1A). The specimens were further authenticated using sequence characterized amplified region markers as previously described (Choo et al., 2009) (Supplementary Fig. S1B).

2.2. Chemicals

Deoxypodophyllotoxin (DPT) is a member of the class of furonaphthodioxole that is (5R,5aR,8aR)-5,8,8a,9-tetrahydro-2H-furo [3',4':6,7]naphtho[2,3-d][1,3]dioxol-6(5aH)-one substituted at position 5 by a 3,4,5-trimethoxyphenyl group. It has a role as a plant metabolite, an antineoplastic agent and an apoptosis inducer. It is a lignan, a furonaphthodioxole, a gamma-lactone and a member of methoxybenzenes (National Center for Biotechnology Information, 2018). DPT (> 95%; Supplementary Fig. S2A) was purchased from the National Development Institute of Korea Medicine (Gyeongsan, South Korea). Analytical solvents were of high-performance liquid chromatography-grade and were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.3. AS extraction

AS (1.0 kg) was extracted with 70% ethanol (v/v) for 3 h at 80 °C and evaporated in vacuo. ASE yield was 14.48%. Prior to use, the lyophilized powder was dissolved in vehicle (0.25% carboxymethylcellulose or 10% dimethyl sulfoxide for in vivo and in vitro experiments, respectively).

2.4. Ultra-performance liquid chromatography (UPLC) analysis

Quantitative analysis was carried out using an UPLC system (Waters Corp., Milford, MA, USA) consisting of a binary solvent pump, sample manager, column oven, online degasser, photodiode array (PDA) detector, and QDa single-quadrupole mass detector. Data were analyzed using Empower 3 software (Waters Corp.). An HSS T3 chromatographic column was used for separation (2.1 × 150 mm, 1.7 μ m; Waters Corp.). Peaks were separated with a mobile phase of 0.05% formic acid and methanol. The isocratic mobile phase of 70% methanol was run for 5 min at a flow rate of 0.2 ml/min with a 3- μ l injection volume. Wavelengths were monitored from 200 nm to 400 nm and were detected at 290 nm. The QDa detector conditions were as follows: *m/z* range, 350–450; capillary voltage, 0.8 V; probe temperature, 600 °C; sampling frequency, 8 Hz; cone voltage, 15 V; and source and turbo temperatures, 120 °C and 45 °C, respectively. Total ion chromatography (TIC) and selective ion recording of DPT were performed at 350–450 *m/z* and 421 *m/z*, respectively, with the latter in the positive ion mode (Kim et al., 2018).

2.5. In vivo experiments

2.5.1. Induction of asthma in mice and drug administration

Female C57BL/6 mice (8 weeks old) were obtained from Daehan Biolink Co. (Seoul, Korea) and housed under specific pathogen-free conditions with free access to food and water. Chicken egg ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA) was precipitated in aluminum sulfate (alum; InvivoGen, San Diego, CA, USA) as previously described (Chun et al., 2018). The mice were divided into five groups (*n* = 7 each). To generate the asthma model, mice in four of the groups were administered 200 μ l OVA/alum (50 μ g) by intraperitoneal injection twice with a 7-day interval in between, whereas the fifth group was injected with saline. On day 13, mice in the asthma model groups were anesthetized with 2% isoflurane (Piramal Critical Care, Bethlehem, PA, USA) delivered using a Vevo Compact Anesthesia System (Fujifilm VisualSonics, Toronto, Canada) and intranasally challenged with 50 μ l OVA (25 μ g) for 4 consecutive days, followed by oral administration of vehicle, dexamethasone DEX (5 mg/kg), or ASE (100 or 200 mg/kg) (Supplementary Fig. S3). The doses used were based on the U.S. Food and Drug Administration guidelines for dose conversion between mice and humans—i.e., 100 or 200 mg/kg for mice is equivalent to 0.5 or 1 g/60 kg (16.6 mg/kg) for humans (Nair and Jacob, 2016). ASE was administered seven times from day 10 to 16 (Supplementary Fig. S3). All animal care and experimental procedures were carried out with the approval of the Institutional Animal Care and Use Committee (IACUC) of the KIOM (16-034).

2.5.2. Histological analysis

Mice were euthanized with an overdose injection of pentobarbital sodium (a short-acting barbiturate) in compliance with IACUC guidelines. Mouse lung tissue was dissected, fixed in 10% formalin solution at 4 °C, embedded in paraffin, and cut into 5- μ m sections using a microtome (Leica, Nussloch, Germany). To examine inflammatory cell infiltration, goblet cell hyperplasia, and mucus secretion in the airway epithelium, lung tissue sections were mounted on glass slides, deparaffinized, and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff solution (PAS) (both from Sigma-Aldrich). The slides were viewed with a digital slide scanner and image analyzer and

Table 1
Sequences of primers used for qRT-PCR analysis.

Genes	Sense	Antisense	Length(bp)
GAPDH	AGGTCGGTGTGAACGGATTTC	TGTAGACCATGTAGTTGAGGTCA	123
Irf4	TCCGACAGTGGTTGATCGAC	CCTCAGATTGTAGTCTGCTT	126
NOS2	GTTCTCAGCCCAACAATACAAGA	GTGGACGGGTCGATGCAC	127

photographed (3DHISTECH, Budapest, Hungary).

2.5.3. Bronchoalveolar lavage fluid (BALF) collection and quantification of inflammatory cells

We collected BALF by lavaging the lungs with 1 ml Dulbecco's phosphate-buffered saline (WelGENE, Daegu, Korea) using an endotracheal catheter (Jelco, St. Paul, MN, USA) as previously described (Kwon et al., 2017). We then recovered approximately 0.75 ml BALF; the cells were counted on a slide by cytospinning (Shandon Cytospin4; Thermo Fisher Scientific, Waltham, MA, USA) and stained with Hema 3 solution (Fisher HealthCare, Pittsburgh, PA, USA), which is similar to the Wright-Giemsa stain. After determining the leukocyte fraction, the number of each cell type was calculated by multiplying the proportions by total BALF cell count. The count was performed by a researcher blinded to the experimental grouping. To estimate cytokine and mediator levels, the BALF was centrifuged for 5 min at 4 °C, and the supernatant was stored at − 80 °C. Serum samples were obtained by centrifuging the blood collected from each mouse and were stored at − 80 °C until use.

2.5.4. Assessment of type 2 cytokine, eotaxin-3, and glycoprotein (mucus) levels in BALF

IL-4, -5, -6, and -13 levels in BALF were measured by enzyme-linked immunosorbent assay (ELISA) (Lee et al., 2017). Briefly, 11B11, TRFK5, MP5-20F3, and eBio13A, respectively, were used for cytokine capture and the biotin-conjugated antibodies BVD6-24G2, TRFK4, MP5-32C11, and eBio1316H, respectively, were used for detection. The antibodies were supplied by BD Biosciences (Franklin Lakes, NJ, USA), except for eBio13A and eBio1316H, which were from eBioscience (San Diego, CA, USA). Eotaxin-3 level was measured using the BMS5008 ELISA kit (eBioscience) according to the manufacturer's protocols. For glycoprotein (mucus) detection (Lee et al., 2017), biotinylated jacalin was purchased from Vector Laboratories (Burlingame, CA, USA).

2.5.5. Total serum IgE measurement

Total serum IgE levels were measured with an ELISA kit (product no. 439807; BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

2.5.6. Flow cytometry

Flow cytometry was performed as previously described (Kwon et al., 2013) using fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (GK1.5) and phycoerythrin (PE)-conjugated anti-CD25 (PC61) antibodies (both from BioLegend). Data were collected on a Canto II LSRFortessa X-20 instrument (BD Biosciences) and analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

2.5.7. Western blot analysis

Protein was extracted from mouse lung tissue that was lysed with radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Danvers, MA, USA). Equal amounts of protein (30 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane that was blocked in Tris-buffered saline with Tween-20 containing 5% skim milk and incubated overnight at 4 °C with primary antibodies against iNOS (2.0 µg/ml), IRF4 (0.5 µg/ml), and β-actin (2.0 µg/ml) (all from Cell Signaling Technology); Forkhead box (Fox)P3

(1 mg/ml; Abcam, Cambridge, UK); and GATA-binding protein (GATA) 3 (0.5 µg/ml; BD Biosciences). After washing, the membrane was incubated with a peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Signals were developed by enhanced chemiluminescence (GE Healthcare, Little Chalfont, UK) and detected with a ChemiDoc imager (Bio-Rad, Hercules, CA, USA). Protein band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.5.8. Quantitative real-time reverse transcription (qRT)-PCR analysis

Total RNA was extracted from homogenized lung tissue using TRIzol reagent (Ambion, Carlsbad, CA, USA) according to the manufacturer's protocol. First-strand cDNA was synthesized from 1 µg of total RNA and primers using the qPCR BIO cDNA Synthesis kit (PCR BIO Systems, London, UK).

For qRT-PCR, the prepared cDNA was amplified and detected using qPCR BIO SyGreen Blue Mix Lo-ROX (PCR BIO Systems) using specific primers (Table 1). Real-time thermal cycling and fluorescence detection were performed using Rotor-Gene 3000 (Corbett/Qiagen, Valencia, CA, USA) under the following conditions: 95 °C for 2 min, and 45 cycles of 95 °C for 10 s and 55 °C for 30 s. Gene expression levels were determined relative to that of glyceraldehyde 3-phosphate dehydrogenase mRNA.

2.6. In vitro experiments

2.6.1. In vitro Th2 polarization assay and intracellular cytokine labeling

CD4⁺ T cells (1×10^6) were purified from splenocytes by depletion of non-target cells using a naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). For in vitro Th2 polarization, purified CD4⁺ T cells were stimulated with anti-CD3/CD28 antibody (0.2 µg/ml), recombinant mouse IL-4 (40 ng/ml) and IL-2 (20 ng/ml, both Miltenyi Biotec), and anti-interferon (IFN)-γ antibody (10 µg/ml, clone: AN.18.17.24) with or without ASE in flat-bottom 12-well plates (BD Biosciences). For the neutral condition, CD4⁺ T cells were stimulated with antibody only under non-Th2-polarizing conditions. After culturing for 5 days, Th2 polarization was analyzed by measuring the levels of Th2 cytokines such as IL-5 and the fraction of GATA3⁺CD4⁺ T cells. For surface marker (CD4) and transcription factor (GATA3) labeling, polarized CD4⁺ T cells (defined as Th2 cells) were fixed and permeabilized using a FoxP3/transcription factor staining buffer kit (eBioscience) according to the manufacturer's instructions. Permeabilized cells were labeled with anti-CD4-FITC and -GATA3-PE antibodies for 30 min at room temperature. After washing, labeled cells were sorted by flow cytometry.

2.6.2. Macrophage cell culture and viability

RAW 264.7 murine macrophage cells were purchased from the American Type Culture Collection (Manassas, VA, USA; cat no. TIB-71, lot no. 3002360). Cells were grown in Roswell Park Memorial Institute 1640 medium (Gibco BRL, Gaithersburg, MD, USA) containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin sulfate at 37 °C in a humidified 5% CO₂ atmosphere.

Cell viability was determined using a cell proliferation assay kit (Promega, Madison, WI, USA). The cells were resuspended at a concentration of 5×10^4 cells/well in a 96-well tissue culture plate and were treated with various doses of ASE for 24 h at 37 °C. Cell viability

was measured using a Wallac EnVision microplate reader (PerkinElmer, Wellesley, MA, USA) at 550 nm.

2.6.3. Measurement of NO and IL-6 production by ASE-treated macrophages

RAW 264.7 cells were stimulated with 200 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) in the presence or absence of ASE for 24 h at 37 °C. NO production was measured by adding Griess reagent (Promega) to the culture supernatant and measuring the absorbance on a Wallac EnVision microplate reader at 540 nm. IL-6 level was determined using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, followed by absorbance measurement at 450 nm.

2.6.4. Western blot analysis of ASE-treated macrophages

RAW 264.7 cells were stimulated with LPS (200 ng/ml) with or without ASE for 24 h at 37 °C. Total cell lysates were extracted using RIPA buffer (Cell Signaling Technology) and western blotting was carried out with antibodies against β -actin, iNOS, and cyclooxygenase-2 (Cell Signaling Technology).

2.7. Statistical analysis

Data represent the mean $\pm$ standard error of the mean. Differences between the vehicle and drug administration groups were evaluated by analysis of variance, and $P < 0.05$ was considered significant. Analyses were performed using Prism v.6.0 software for Windows (GraphPad Inc., La Jolla, CA, USA).

3. Results

3.1. Authentication and quantitative analysis of AS

A. radix and *Peucedani radix* are frequently distributed as the same herbal medicine in Korean herbal markets (Choo et al., 2009). To identify the botanical species origin of the plant material used in the present study, we introduced genetic markers to identify commercial *A. radix*. One commercial herbal medicine (no. 1) was identified as the roots of *Peucedanum praeruptorum* Dunn, and four (nos. 2–5) were identified as authentic *A. radix* (AS roots) (Supplementary Fig. S1B). We obtained extracts of two of the latter specimens (nos. 2 and 3) for further analysis.

3.2. UPLC analysis of DPT content

In the UPLC analysis of AS, the main peak corresponding to DPT was detected at 4.1 min by the PDA (Supplementary Fig. S2B). Two peaks were detected at 4.1 and 4.4 min by TIC. The former was identified as DPT, whereas the identity of the latter is unknown. The molecular weight of DPT in the positive ion mode was 421 m/z , indicating that the compound was an $[M+Na]^+$ adduct. The DPT content was $1.82 \pm 0.02 \mu\text{g}/\text{mg}$. These findings are consistent with a previous report that DPT is the main component of ASE (Olaru et al., 2015).

3.3. ASE treatment alleviates pathological changes in lung tissue in the mouse asthma model

To evaluate the effect of ASE on inflammatory cell infiltration in a mouse model of OVA-induced asthma, mouse lung tissue samples were sectioned and stained with H&E. Compared to the saline control, asthmatic mice treated with vehicle showed extensive accumulation of inflammatory cells in the peri-bronchial areas. However, treatment with 100 and 200 mg/kg ASE and DEX (active control group) reduced inflammatory cell infiltration into the lung relative to the vehicle-treated group (Fig. 1A).

PAS-stained lung sections were examined and mucin concentrations

were evaluated in order to determine the effect of ASE treatment on mucus production by goblet cells. Vehicle-treated asthmatic mice exhibited goblet cell hyperplasia and overproduction of mucin, whereas both measures were significantly decreased in 200 mg/kg ASE-treated mice (Fig. 1B).

3.4. ASE treatment reduces inflammatory cell infiltration in BALF of asthma model mice

Eosinophilic airway inflammation, a major feature of asthma (Lambrecht and Hammad, 2015), drives the infiltration of eosinophils into the respiratory tract, which is mediated by chemotactic chemokines and IL-5 (Foster et al., 2001; Rosenberg et al., 2013). To evaluate the effect of ASE on inflammatory cell accumulation in the lung tissue of asthmatic mice, we counted the number of inflammatory cells in the BALF of ASE- and vehicle-treated asthmatic mice. The untreated group had a high number of eosinophils, whereas ASE treatment reduced the counts in a dose-dependent manner (Fig. 2A). Eotaxin-3 and IL-5 levels in BALF, which induced eosinophil recruitment in untreated asthmatic mice, were elevated compared to those in the saline control group. By comparison, the levels were lower in the BALF of mice treated with either ASE or DEX relative to untreated controls (Fig. 2B, C).

3.5. ASE decreases BALF levels of type 2 cytokines

Patients with allergic asthma show higher levels of IL-4 and -13 in their BALF than healthy individuals (Kroegel et al., 1996). We therefore measured the levels of these cytokines in the BALF of mice in the five groups and found that they were higher in vehicle-treated asthmatic than in saline-treated control mice. ASE treatment reduced BALF cytokine concentrations relative to untreated mice (Fig. 3A, C). Since IL-4 induces IgE synthesis by activating B cells and inducing their transformation into plasma cells in asthma (Locksley, 2010; Van der Pouw Kraan et al., 1998), we measured IgE levels in BALF and found that they were correlated with those of IL-4 in all groups of mice. Untreated asthmatic and ASE-treated mice showed higher and lower levels of total IgE, respectively, than the saline control group (Fig. 3B), which is consistent with the trends observed for IL-4.

An essential function of IL-13 in asthma is to promote goblet cell hyperplasia, which results in mucus production (Whittaker et al., 2002). Accordingly, IL-13 is considered as a target for asthma treatment (Hodsman et al., 2013; Oh et al., 2010; Singh et al., 2010). We found that ASE-treated mice had lower BALF levels of IL-13 than their untreated counterparts, which is consistent with the reduction in mucus production observed by PAS staining (Fig. 1B, C).

3.6. ASE suppresses Th2 cell activation and inhibits Th2-related cytokine production

Th2 cells play a key role in allergic reactions by releasing cytokines such as IL-4, -5, and -13 (Kim et al., 2010). To investigate whether the decreased activity of Th2 cells is accompanied by a reduction in cytokine production, we quantified the $\text{CD4}^+\text{CD25}^+$ T cell fraction (designated as Th2 cells) in the lungs by flow cytometry. The number of activated Th2 cells was higher in the vehicle-treated asthmatic as compared to the saline control group. Although a low dose of ASE had little effect on Th2 cell activation, at the higher dose, fewer Th2 cells were activated (Fig. 4A–C).

To confirm Th2 cell activation and inhibition of differentiation by ASE, we induced the differentiation of CD4^+ T cells into Th2 cells by establishing Th2-polarizing conditions (i.e., by adding IL-4 and -2, and anti-IFN- γ to cultures), and measured the level of the type 2 cytokine IL-5 and the size of the $\text{GATA3}^+\text{CD4}^+$ T cell population. As expected, ASE treatment inhibited IL-5 production in differentiated cells (Fig. 5A) and suppressed Th2 cell differentiation (Fig. 5B, C). On the other hand, the level of the type 1 cytokine IFN- γ was unaffected by ASE

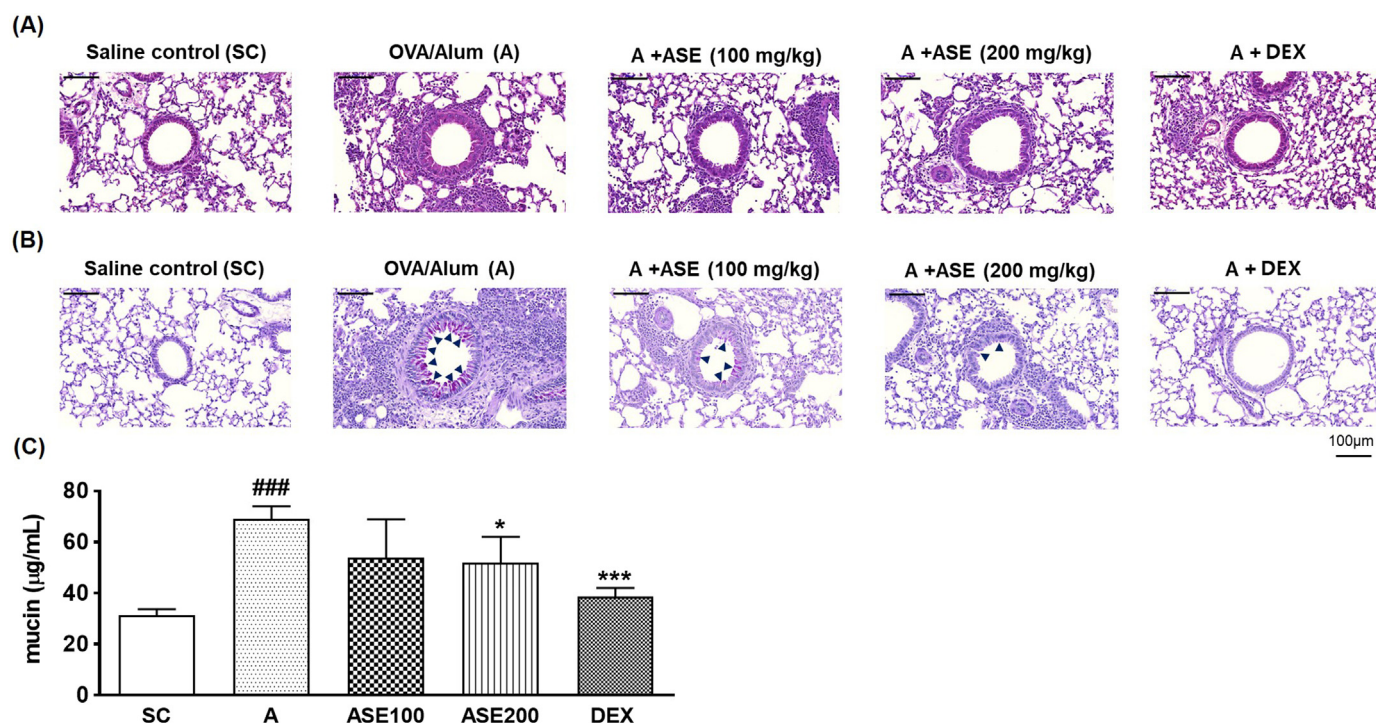


Fig. 1. Effect of ASE on histological features of the lung of asthmatic mice. (A, B) H&E (A) or PAS (B) staining of lung sections (200 $\times$ magnification). Arrowheads indicate mucus-rich areas. (C) Mucin production was measured using an image analyzer. The mouse groups were as follows: SC, saline-treated controls without OVA challenge; OVA, OVA challenge with vehicle injection; ASE100, OVA challenge with 100 mg/kg ASE treatment; ASE200, OVA challenge with 200 mg/kg ASE treatment; DEX, active control group with OVA challenge and treatment with 5 mg/kg DEX. Data are presented as the means $\pm$ standard error of the mean ($n = 7$ /group). ### $P < 0.001$ vs. SC group; * $P < 0.05$, *** $P < 0.001$ vs. OVA group.

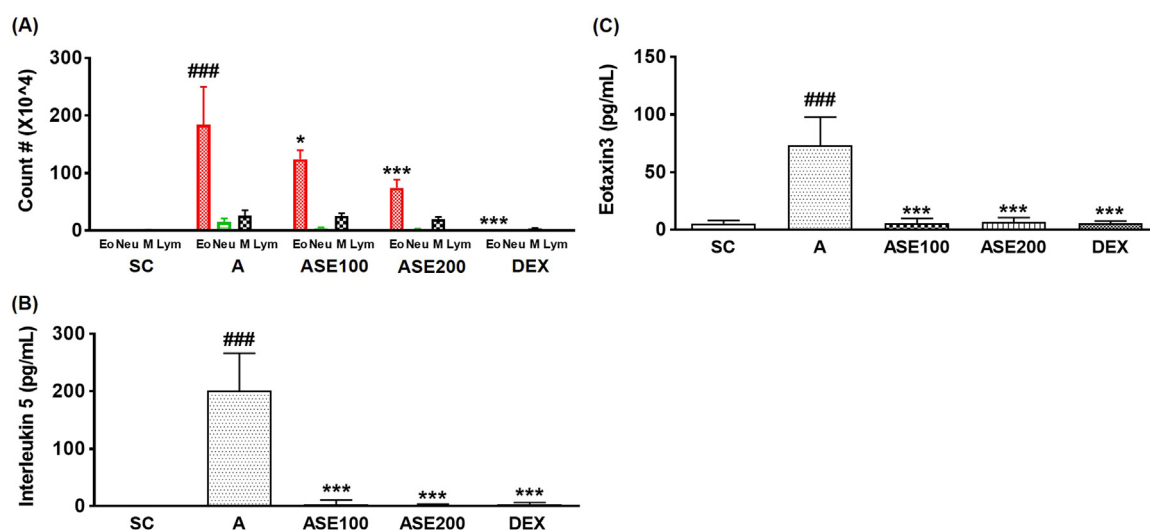


Fig. 2. (A–C) Effect of ASE on differential cell counts (A) and IL-5 (B) and eotaxin 3 (C) levels in the BALF of asthmatic mice. Cells in the BALF were isolated by centrifugation, and the number of each inflammatory cell type was counted. The mouse groups were as follows: SC, saline-treated controls without OVA challenge; OVA, OVA challenge with vehicle injection; ASE100, OVA challenge with 100 mg/kg ASE treatment; ASE200, OVA challenge with 200 mg/kg ASE treatment; DEX, active control group with OVA challenge and treatment with 5 mg/kg DEX. Data are presented as the mean $\pm$ standard error of the mean ($n = 7$ /group). ### $P < 0.001$ vs. SC group; * $P < 0.05$, *** $P < 0.001$ vs. OVA group. E, eosinophils; L, lymphocytes; M, macrophages; N, neutrophils.

(Supplementary Fig. S4). Thus, ASE mitigates the asthma phenotype by inhibiting Th2 cell activation and differentiation.

3.7. ASE inhibits IRF4-mediated Th2 cell activation by inhibiting IL-6 production in asthmatic mice

Recent studies suggest that NO may be involved in asthma; moreover, iNOS is constitutively expressed in bronchial epithelial cells of healthy individuals but upregulated in those of asthma patients (Dweik

et al., 2001; Stuehr, 1997). Furthermore, eosinophil recruitment is associated with NO production, which is regulated by iNOS expression (Soufli et al., 2016). We found here that iNOS levels in the lung were lower in ASE-treated than in vehicle-treated mice (Fig. 6A, B), which is consistent with the eosinopenia (Fig. 2A) and decreased IL-6 levels (Fig. 6C) observed upon ASE treatment.

Since IRF4 is important for Th2 cell differentiation (Staudt et al., 2010) and its expression is induced by IL-6 signaling in patients with allergic asthma (Koch et al., 2013), we assessed the IRF4 levels in the

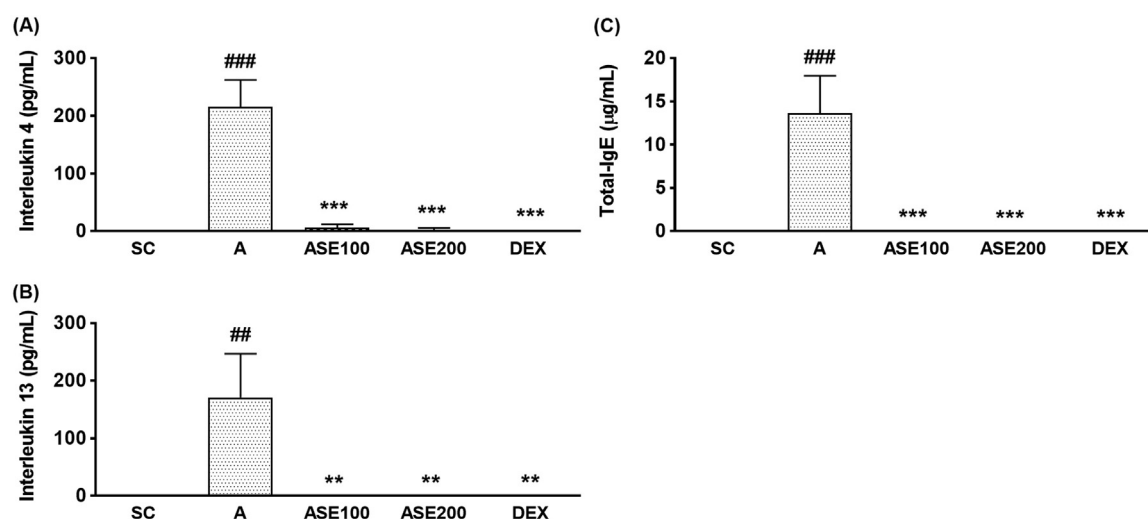


Fig. 3. Effect of ASE on type 2 cytokine and IgE levels in BALF of asthmatic mice. (A–C) IL-4 (A), IL-13 (B), and IgE (C) levels were measured by ELISA. The mouse groups were as follows: SC, saline-treated controls without OVA challenge; OVA, OVA challenge with vehicle injection; ASE100, OVA challenge with 100 mg/kg ASE treatment; ASE200, OVA challenge with 200 mg/kg ASE treatment; DEX, active control group with OVA challenge and treatment with 5 mg/kg DEX. Data are presented as mean ± standard error of the mean (n = 7/group). ^{##}P < 0.01, ^{###}P < 0.001 vs. SC group; ^{**}P < 0.01, ^{***}P < 0.001 vs. OVA group.

lungs of mice. ASE-treated groups had lower levels of IRF4 than untreated mice (Fig. 6D, E), suggesting that IL-6 is important for NO and IRF4 expression in allergic asthma. Moreover, IRF4 suppressed the expression FoxP3, which is a master transcription factor for regulatory T cell differentiation and function (Huber et al., 2008; Zheng et al., 2009). FoxP3 levels were increased by ASE treatment (Fig. 6D, E). These findings suggest that ASE treatment inhibits IL-6 production and IRF4 expression, which suppresses Th2 cell activation and enhances the regulatory T cell population, as evidenced by upregulation of FoxP3.

3.8. ASE treatment reduces inflammatory mediator expression and cytokine production by RAW 264.7 cells

Alveolar macrophages play a critical role in the aggravation of allergic asthma (Yang et al., 2012) by producing NO and IL-6 (Dweik et al., 2001; Rincon and Irvin, 2012; Stuehr, 1997). We speculated that ASE could also exert its effect via regulation of macrophage activation. To test this hypothesis, we evaluated the toxicity of ASE to RAW 264.7 cells with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cell viability was decreased by 1000 μg/ml ASE treatment. We therefore used ASE at concentrations of 125, 250, and

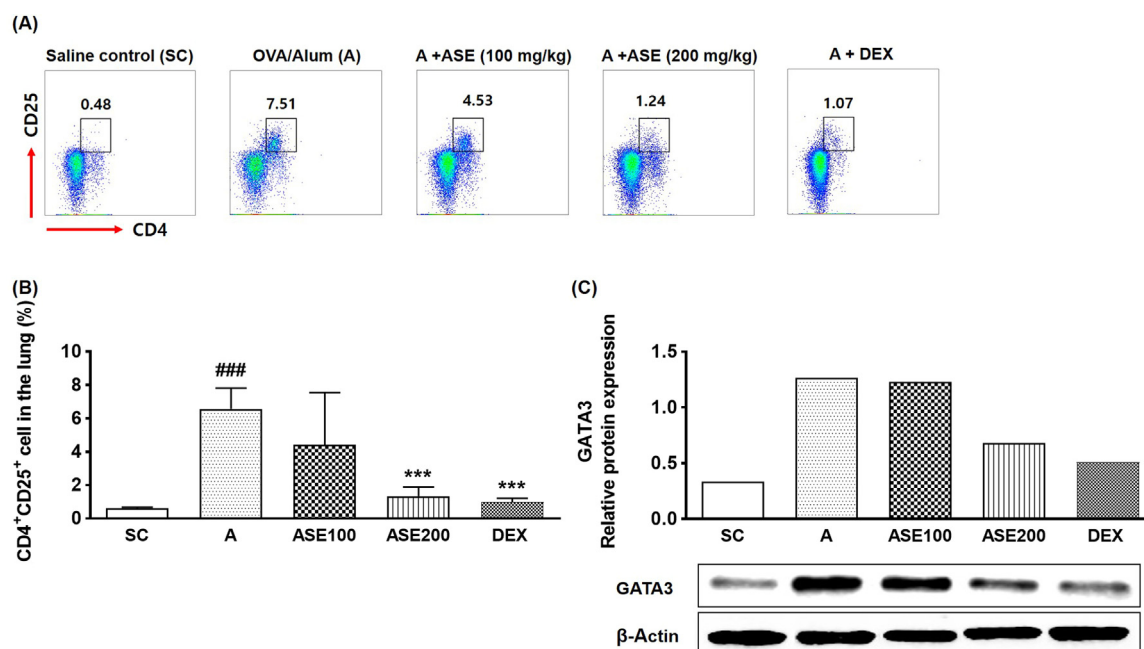


Fig. 4. Effect of ASE on Th2 cell activation. (A) The fraction of activated CD4⁺ T cells (CD4⁺CD25⁺) in the lungs was analyzed by flow cytometry. Representative data are shown. The numbers inside each plot indicate the percentage of CD4⁺CD25⁺ cells among total lung cells. (B) Histograms of the percentage of CD4⁺CD25⁺ T cells in the BALF in different groups. (C) GATA-3 expression in the lungs, evaluated by western blotting. The mouse groups were as follows: SC, saline-treated controls without OVA challenge; OVA, OVA challenge with vehicle injection; ASE100, OVA challenge with 100 mg/kg ASE treatment; ASE200, OVA challenge with 200 mg/kg ASE treatment; DEX, active control group with OVA challenge and treatment with 5 mg/kg DEX. Data are presented as means ± standard error of the mean (n = 7). ^{###}P < 0.001 vs. SC group; ^{***}P < 0.001 vs. OVA group.

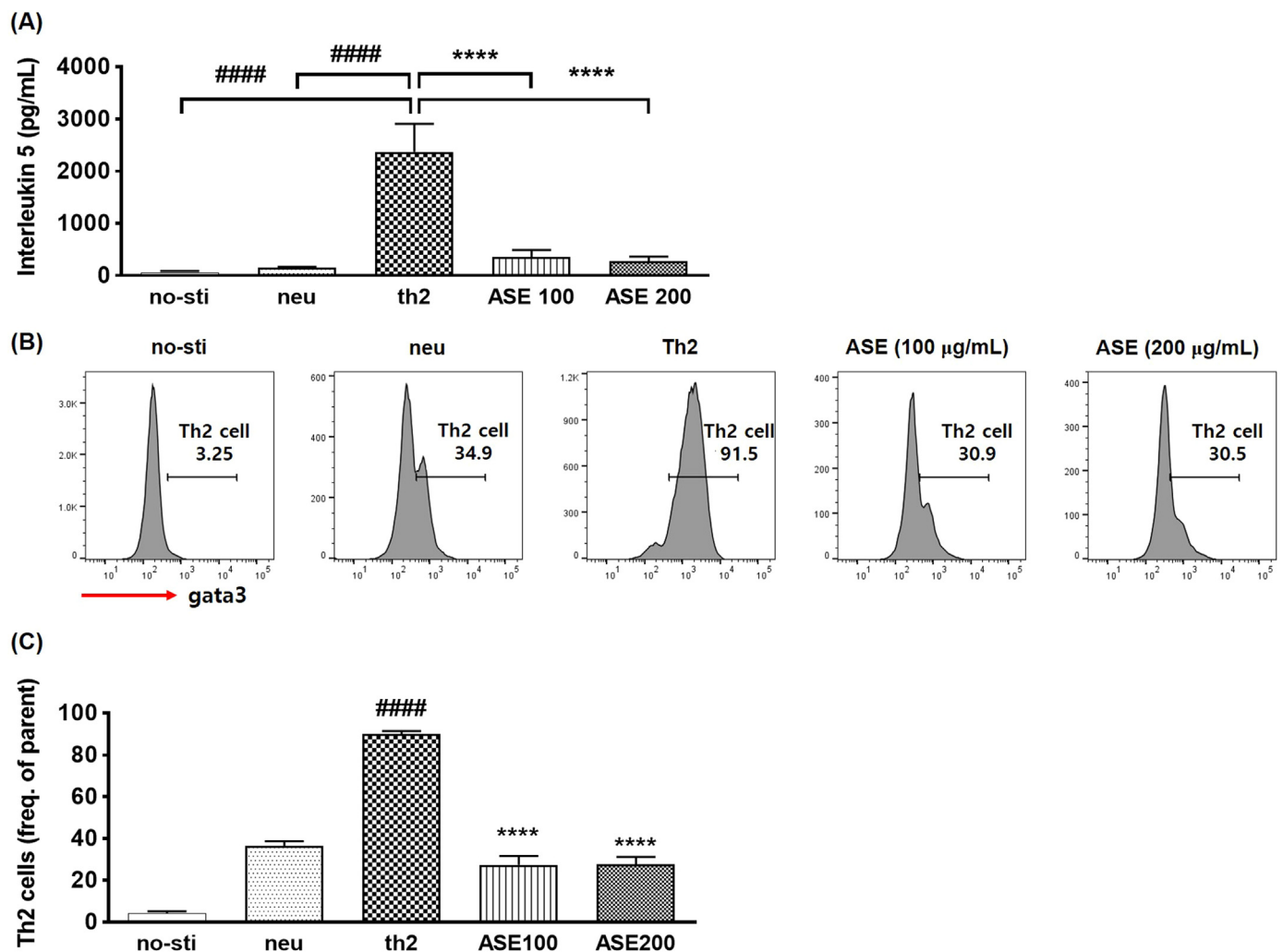


Fig. 5. Effect of ASE on Th2 cell activation in vitro. Purified naïve splenic CD4⁺ T cells were polarized in the presence of 100 or 200 µg/ml ASE by culturing under Th2-polarizing conditions. The GATA3⁺ CD4⁺ T cell fraction was analyzed by flow cytometry after intracellular cytokine labeling. (A) IL-5 levels were determined by ELISA. (B) Representative plots of a single individual from each group are shown. Numbers inside the plots indicate the abundance of GATA3⁺ in CD4⁺ T cells. (C) (–), Unstimulated cells; Neu, stimulated (anti-CD3/CD28) under non-Th2-polarizing conditions; Th2, stimulation under Th2-polarizing conditions; Th2⁺ ASE, Th2-polarizing conditions plus treatment with 100 or 200 µg/ml ASE. Data are presented as mean ± standard error of the mean (n = 3). ####P < 0.0001 vs. saline, ****P < 0.0001 vs. Th2 polarization group.

500 µg/ml for subsequent experiments (Fig. 7A) and investigated the effect of ASE on macrophage activation by evaluating NO, iNOS, and IL-6 levels. We found that ASE inhibited NO production by reducing iNOS expression (Fig. 7B, C) and dose-dependently decreased IL-6 expression (Fig. 7D). Thus, ASE inhibits macrophage activation in vivo, thereby reducing iNOS expression and IL-6 production in the lung and BALF.

4. Discussion

Allergic asthma is a multifactorial disease with numerous clinical symptoms including increased airway hyper-responsiveness, goblet cell hyperplasia, mucus secretion, and eosinophil infiltration, which are associated with disease progression (Busse and Lemanske, 2001). Despite the development of effective therapies, the economic burden due to asthma continues to escalate (Bahadori et al., 2009). Moreover, whether they are used alone or in combination, immunotherapies have side effects and limited efficacy in some patients. As such, alternative therapies including acupuncture, aromatherapy, and herbal medicine are becoming increasingly popular (Ng et al., 2003; Schäfer, 2004; Steurer-Stey et al., 2002; Ziment and Tashkin, 2000). Various medicinal herbs have been reported to be effective against asthma, with the active components having been identified in some cases (Li, 2011).

AS is a perennial herb that has been used traditionally to treat asthma and bronchitis and as an expectorant, diuretic, and cough remedy (Hendrawati et al., 2011; Jeong et al., 2007). In the present study, we investigated the anti-inflammatory effects of ASE in vitro and in a mouse model of OVA-induced asthma. The therapeutic effects of ASE on airway inflammation in mice were confirmed by the reduction of mucus secretion by airway epithelial cells and inflammatory cell accumulation, especially of eosinophils in the BALF. Moreover, ASE-treated mice showed decreased IL-5 and eotaxin-3 levels as well as reduced levels of type 2 cytokines in the BALF and total IgE.

Our results demonstrate that ASE exerts anti-asthmatic effects through a number of mechanisms. Firstly, ASE administration inhibited Th2 cell activation in vitro and in vivo, as evidenced by the diminution of the CD4⁺CD25⁺ cell population and downregulation of GATA-3 and IL-5 levels. Secondly, ASE reduced eosinophilia by decreasing iNOS and IL-6 levels in the lung and BALF, respectively. We also found that ASE treatment suppressed IL-6 and NO production by RAW 264.7 macrophages in vitro, suggesting that it modulates the innate immune response to induce eosinophilia and type 2 immunity. Finally, we identified IRF4 as a key signaling molecule that induces Th2 cell activation via upregulation of GATA-3 and suppresses regulatory T cell function by negatively regulating FoxP3 expression. IRF4 expression was

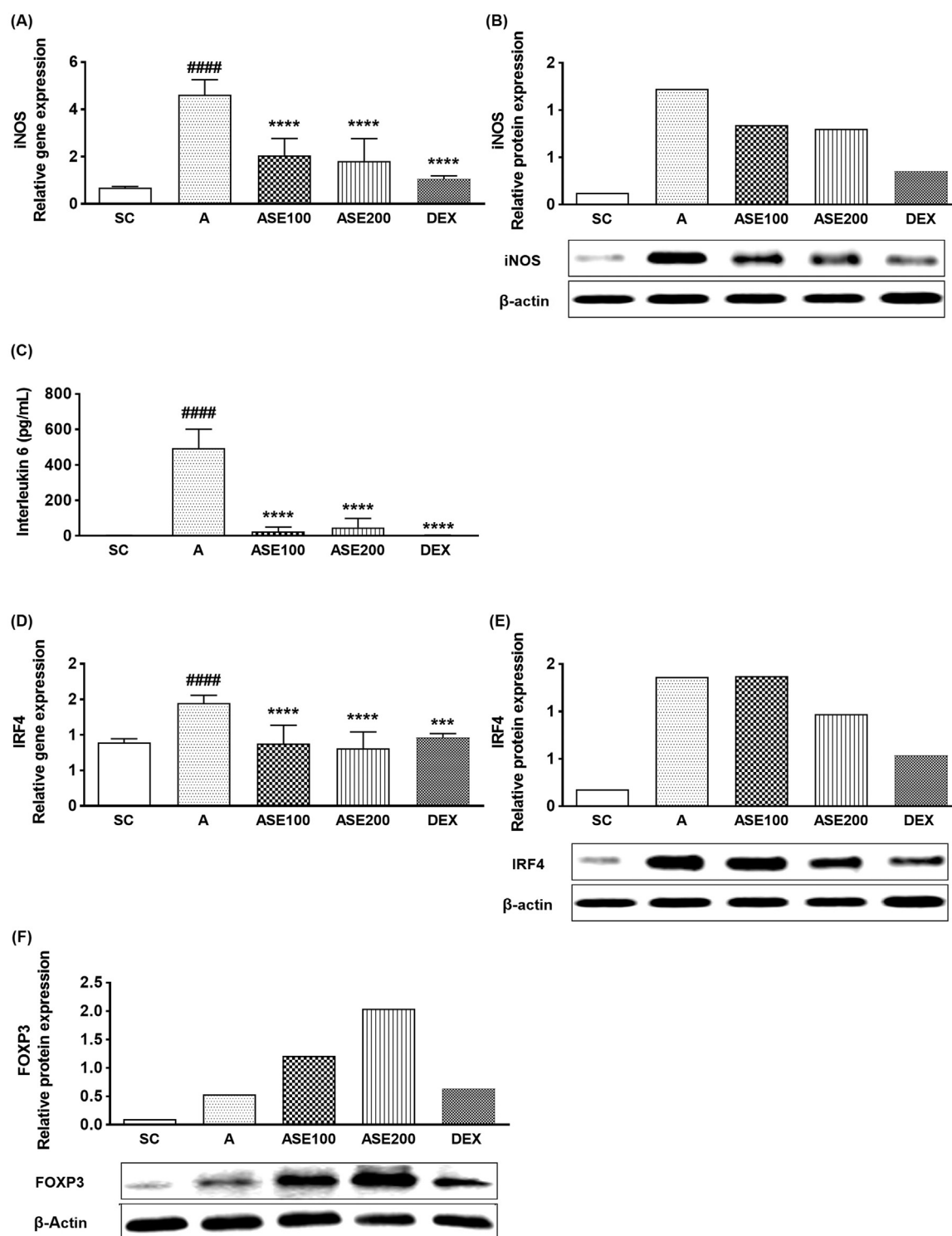


Fig. 6. Effect of ASE on IL-6 and IRF4-related immune mechanisms. (A, B, D, E) mRNA (A, D) and protein (B, E) levels of iNOS and IRF4 in lung, as determined by qRT-PCR and western blotting, respectively. (C) IL-6 levels in BALF measured by ELISA. (F) Evaluation of FoxP3 protein levels by western blotting. Protein band intensity was calculated by normalizing to the levels of β -actin and tubulin. The mouse groups were as follows: SC, saline-treated controls without OVA challenge; OVA, OVA challenge with vehicle injection; ASE100, OVA challenge with 100 mg/kg ASE treatment; ASE200, OVA challenge with 200 mg/kg ASE treatment; DEX, active control group with OVA challenge and treatment with 5 mg/kg DEX. Data are presented as mean $\pm$ standard error of the mean ($n = 7$). #### $P < 0.0001$ vs. SC group; *** $P < 0.001$, **** $P < 0.0001$ vs. OVA group.

reduced by ASE treatment in association with the decrease in GATA-3 and increase in FoxP3 expression. In other words, diminished IL-6 & NO production axis by ASE administration result in IRF4 expression in lung. Because IRF4 is pivotal signaling molecules that promote Th2 cell activation by inducing GATA3 expression and down-regulation of FoxP3,

reduction of IRF4 expression by ASE treatment is critical molecular mechanism of alleviated allergic inflammation.

Recently, innate lymphoid cell (ILC)2 and related cytokines such as thymic stromal lymphopoietin and IL-25 and -33 were shown to be critical for initiating and aggravating allergic disease through crosstalk

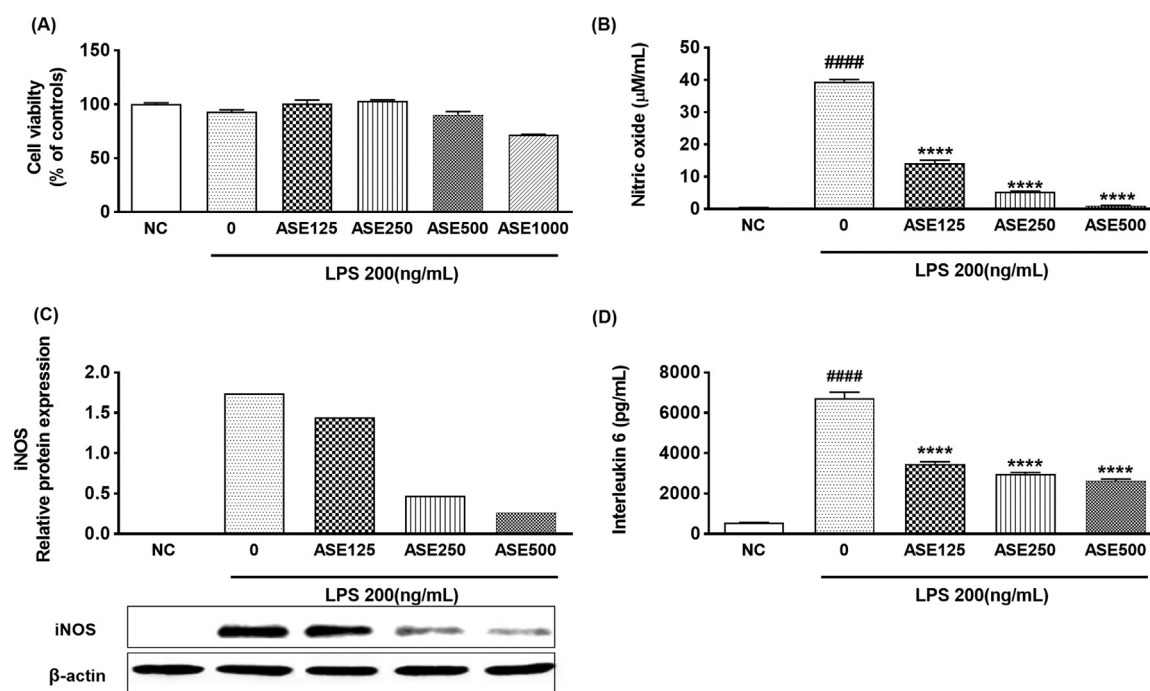


Fig. 7. Effect of ASE on macrophage activation. RAW 264.7 cells were stimulated with LPS with or without 125, 250, and 500 µg/ml ASE. (A) Cell viability was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (B) NO level was measured using Griess reagent. (C) Protein expression of iNOS, as determined by western blotting. (D) Measurement of IL-6 level by ELISA. Data are presented as the mean $\pm$ standard error of the mean ($n = 3$). #### $p < 0.0001$ vs. LPS untreated and ASE; **** $p < 0.0001$ vs. LPS only. NC, normal control.

with Th2 cells (Kwon et al., 2013; Licona-Limon et al., 2013). Specifically, ILC2 produces type 2 cytokines such as IL-5 and -13 through the action of GATA-3, which is a master transcription factor for Th2 cell differentiation (Oliphant et al., 2014). Whether ASE also exerts anti-asthmatic effects via ILC2 inhibition remains to be determined.

IRF4 expression in Th cells is induced by stimulation of T cell receptor and is important for the differentiation of naïve T cells, for instance into Th2 cells (Chapoval et al., 2010). IRF4 also directly activates dendritic cells, which are antigen-presenting cells that promote Th2 cell polarization by secreting IL-10 and -33 (Williams et al., 2013). Although we showed that IRF4-mediated Th2 activation is negatively regulated by ASE, further studies are needed to clarify whether ASE treatment promotes the IRF4-mediated activation of dendritic cells.

5. Conclusion

Asthma caused by allergic inflammation, mucus secretion from airway epithelium, eosinophil accumulation, and type 2 cytokine production by activated Th2 cells were alleviated by ASE treatment. Our results suggest that ASE acts via IL-6, a key modulator of the overall Th2 response and of IRF4 function and by suppressing NO expression in eosinophils as well as airway epithelial cells, with both IL-6 and NO being regulated by macrophages. These results demonstrate the multiple inhibitory mechanisms of ASE on allergic lung inflammation. Although additional research is needed to clarify the contributions of the active compounds in ASE and whether these exert prophylactic effects, our findings indicate that ASE has therapeutic potential for treating allergic lung inflammation.

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Conflicts of interest

The authors declare no conflicts of interest.

Author contributions

SB kim was responsible for the Conceptualization, data curation, formal analysis, investigation, writing-original draft / AY lee was responsible for the data curation, methodology, resources / JM Chun was responsible for investigation / HS Kim was responsible for methodology, resources / YS Seo was responsible for data curation, investigation / B-I Kwon was responsible for conceptualization, funding acquisition, project administration, supervision, writing- review & editing.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jep.2018.12.016.

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