



Root extract of *Angelica reflexa* B.Y.Lee reduces allergic lung inflammation by regulating Th2 cell activation

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ABSTRACT

Ethnopharmacological relevance: Traditionally, the roots of *Angelica reflexa* B.Y.Lee (AR) have been used to treat cough, phlegm, neuralgia, and arthralgia in Northeast Asia.

Aim of the study: The anti-asthmatic effect of AR root extract (ARE) was determined using a murine airway allergic inflammation model and the primary T cell polarization assay.

Materials and methods: To evaluate the anti-asthmatic effect of ARE, inflammatory cell infiltration was determined histologically and inflammatory mediators were measured in bronchoalveolar lavage fluid (BALF). Furthermore, the effects of AREs on Th2 cell differentiation and activation were determined by western blotting and flow cytometry.

Results: Asthmatic phenotypes were alleviated by ARE treatment, which reduced mucus production, inflammatory cell infiltration (especially eosinophilia), and type 2 cytokine levels in BALF. ARE administration to mice reduced the number of activated Th2 (CD4⁺CD25⁺) cells and level of GATA3 in the lungs. Furthermore, ARE treatment inhibited the differentiation of Th2 cells in primary cell culture systems via interferon regulatory factor 4 (IRF4) signaling.

Conclusions: Our findings indicate that the anti-asthmatic effect of AREs is mediated by the reduction in Th2 cell activation by regulating IRF4.

1. Introduction

Asthma has been increasingly recognized as a serious global public health concern affecting individuals of all age groups (Bateman et al., 2008; Dharmage et al., 2019; ISAAC, 1998; Masoli et al., 2004). Asthma is characterized by several clinical symptoms including wheezing, airflow obstruction, bronchial spasm, and dyspnea (Han et al., 2017) mediated partially by immune responses to allergens in the lung. According to recent international guidelines, patients with asthma require prolonged maintenance treatment with a cocktail of drugs including corticosteroids, a leukotriene receptor antagonist, and a long-acting β_2 -agonist (LABA) (Bateman et al., 2008). Although these drugs may alleviate the symptoms of asthma, they result in severe adverse reactions or treatment resistance (Cates et al., 2013; Wener and Bel, 2013). In

comparison, recent national surveys indicate that complementary and alternative medicine (CAMs) including herbal medicine are extensively utilized for maintaining wellness in the treatment of common chronic medical conditions (Esmail, 2007). In particular, several studies have reported the potential of CAMs in the treatment of asthma- and allergy-related disorders (Slader et al., 2006; Passalacqua et al., 2005).

The roots of *Angelica reflexa* B.Y. Lee (AR) have been used as a medicinal herb for centuries in traditional medicine. In the traditional medicine theory, inflammatory disorders of the lung are considered to be related to damp and pathogenic wind. AR, which can scatter dampness and dispel wind, is used to treat common cold, headache, neuralgia, and arthralgia (Lei and Zhang, 1998; Shin, 2002). AR has been shown to exert various biological effects including antimicrobial, anti-oxidant, anti-inflammatory, and antitumor effects (Jung et al., 2011). Although

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these findings imply the potential of AR extract (ARE) in asthma treatment, the activity of ARE and the underlying mechanism in bronchitis treatment have not yet been elucidated.

Studies in humans and mice have demonstrated that T helper type 2 (Th2) cells are involved in the pathophysiology of asthma (Zimmermann et al., 2004) and that their activation contributes to the induction of asthma (Hogan et al., 1998; Kim et al., 2019; Robinson et al., 1992). Interleukins (ILs), such as IL-4, IL-5, and IL-13, produced by Th2 cells are responsible for immunoglobulin E (IgE) and mucus production and eosinophil recruitment (Bao and Reinhardt, 2015; Deo et al., 2010). In contrast, differentiated Th1 cells predominantly produce interferon (IFN)- γ , which results in delayed hypersensitivity (Nagamachi et al., 2007).

Interferon regulatory factors (IRFs), which bind to the IFN-stimulated response element (ISRE), constitute key regulators of the immune system (Kim et al., 2019a; Taniguchi et al., 2001). IRF-4, which is induced by IL-6 in macrophages and lymphocytes, is involved in CD4⁺ T cell differentiation into effector T cells, such as Th2 and 17 (Honma et al., 2005, 2008; Koch et al., 2013; Tominaga et al., 2003). Notably, allergen challenge induces IL-6 production in antigen-presenting cells, and this in turn induces both Th2 and Th17 cell activation in asthma. Conversely, treatment with IL-6 receptor antibodies reduces the number of effector T cells via the activation of regulatory T (Treg) cells (Dienz and Rincon, 2009; Finotto et al., 2007).

In the present study, we aimed to evaluate the regulatory effects of ARE on asthmatic characteristics including goblet cell hyperplasia and immune cell infiltration using the Th2-polarization assay and a murine allergic lung inflammation model. Furthermore, we characterized the underlying mechanism of such effects by focusing on the role of ARE in modulating the expression of IL-6 and IRF4 to mediate Th2 cell polarization and activation.

2. Materials and methods

2.1. Plant material

The roots of AR, commonly called “*Kangwhal*” in Korea, were purchased from Gwangmyeongdang Co. (Ulsan, Korea) and were morphologically confirmed by Dr. Goya Choi (Herbal Medicine Resources Research Center, Korea Institute of Oriental Medicine (KIOM), Korea). The samples were further authenticated by genetic identification at the species level as previously described (Noh et al., 2018), and a voucher specimen has been deposited (Supplementary Fig. S1; Index Herbariorum code KIOM, Specimen No. 2-15-0558). To identify the AR species, we first amplified and sequenced the internal transcribed spacer (ITS) regions, a universal DNA barcode utilized for identifying plant species, from five random slices of Specimen No. 2-15-0558. We then compared the sequence identity with other ITS sequences registered in GenBank using the Basic Local Alignment Search Tool (BLAST) analysis (Kim et al., 2017; Noh et al., 2018). The BLAST analysis results confirmed that specimen 2-15-0558 constituted the roots of AR.

2.2. Preparation of ARE

The roots of AR were purchased as a dried herb from Kwangmyongdang Co. (Ulsan, Korea) and were morphologically authenticated by Dr. Goya Choi. Dried roots of AR (500 g) were extracted with 70% ethanol for 3 h using a reflux (80 °C $\pm$ 2 °C); then, the extract was concentrated and lyophilized. The yield of the dried extract was 29.14% based on the raw material.

2.3. Chemical profile of ARE

Chromatographic analysis was performed using a high-performance liquid chromatography (HPLC) system (Waters Co., Milford, MA, USA) coupled with a Waters 2695 separation module and Waters 2996

photodiode array detector. For the HPLC analysis of ARE, chlorogenic acid, ferulic acid, and nodakenin standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Xanthotoxin, bergapten, oxypeucedanin, imperatorin, osthole, and isoimperatorin were purchased from ChemFaces (Wuhan, China).

The ARE sample (0.1 g) was dissolved in 80% methanol (10 mL), and chromatographic separation was performed using an XSelect HSS T3 C₁₈ column (4.6 mm $\times$ 250 mm, 5 μ m, Waters Co.). The mobile phase consisted of acetonitrile and 0.1% formic acid in water at a flow rate of 1.0 mL/min. The gradient program was from 10% to 100% acetonitrile during 40 min. The detection wavelength was 210–400 nm and measurements were recorded at 247 nm. The injection volume was 10.0 μ L. Sample peaks were confirmed by comparison with the analytical retention time and ultraviolet wavelength of the standard peak.

2.4. In vivo experiments

2.4.1. Experimental induction of airway inflammation and drug treatment

C57BL/6 mice (8 weeks old, female) were purchased from Daehan Biolink Co., Ltd., (Chungcheongbuk-do, Korea) and maintained under specific pathogen-free conditions. Standard diet and tap water were provided ad libitum to the mice. The mice were divided into five groups of seven animals per group. The treatment groups were as follows: saline control (SC), ovalbumin (OVA)-induced asthma group (A, vehicle treatment), OVA plus ARE 100 or 200 mg/kg (ARE100 and ARE200), and OVA plus dexamethasone (DEX, 5 mg/kg).

Allergic lung inflammation was induced as previously described (Chun et al., 2018). Briefly, the mice were sensitized via an intraperitoneal injection of 200 μ L OVA/alum (50 μ g) on days 0 and 7 (Supplementary Fig. S2). Intra-nasal challenge with 50 μ L OVA (25 μ g) was initiated on day 13 for 4 days. The four asthmatic mouse groups were treated orally with the vehicle, DEX (5 mg/kg), and ARE (100 and 200 mg/kg) for 7 days until the day before euthanasia. The doses of ARE were determined based on the US Food and Drug Administration guidance (Nair and Jacob, 2016). All animal care and experimental procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) of the KIOM (16-034).

2.4.2. Histological analysis

The mice were euthanized via an intraperitoneal injection of pentobarbital sodium. The lung tissues were resected from the mice, fixed (10% formalin), paraffin-embedded, and sectioned (thickness of 5 μ m). To evaluate inflammatory status and mucus secretion, the sections were stained with hematoxylin and eosin (H&E) or periodic acid Schiff (PAS) (Sigma-Aldrich). The sections were observed using Panoramic DESK (3d Histech, Budapest, Hungary).

2.4.3. Bronchoalveolar lavage fluid (BALF) collection and differential cell counting

BALF was collected by intubating the mice using an endotracheal catheter (Jelco, St. Paul, MN, USA) and lavaging with Dulbecco's phosphate-buffered saline (PBS, 1 mL; Sciencell Research Laboratories, Carlsbad, CA, USA). The cells in the BALF were placed on a slide following cytopinning (Thermo Shandon Cytospin 4, Waltham, MA, USA) and stained with Hema-3 solution (Fisher HealthCare, Pittsburgh, PA, USA) according to the manufacturer's instructions. After determining the proportion of leukocytes, the number of different cell types, including macrophages, eosinophils, neutrophils, and lymphocytes, was calculated by multiplying their proportions with the total BALF cell count.

2.4.4. Measurement of type 2 cytokines, IL-6, eotaxin-3, glycoprotein (mucus), and total IgE levels in the BALF

IL-4, IL-5, IL-6, and IL-13 levels in the BALF were measured using a previously described enzyme-linked immunosorbent assay (ELISA) (Lee et al., 2017). The eotaxin-3 level was determined using an ELISA kit

(eBioscience, San Diego, CA, USA) as described in the manufacturer's instructions. Mucus was measured using biotinylated jacalin (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions. Levels of total IgE in the BALF were determined using an ELISA kit (BioLegend, San Diego, CA, USA) according to the manufacturer's instructions.

2.4.5. Flow cytometry

For flow cytometry, dissected cells from the lung were stained as previously described (Kwon et al., 2013) using FITC-conjugated anti-CD4, APC-conjugated anti-GATA3, PE-conjugated anti-CD25 antibodies, HorizonV500 conjugated anti-CD45 and percp-conjugated anti-CD3. The antibodies were acquired from Biolegend, except for anti-CD3 (eBioscience), and except for anti-CD45 (BD). The stained cells were analyzed using the LSRFortessa X-20 instrument (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR, USA).

2.4.6. Western blotting

The lung tissues were lysed using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling, Billerica, MA, USA). Lysates (30 µg) were separated by electrophoresis and the proteins were transferred on to nitrocellulose membranes. After blocking with skim milk, the membranes were incubated with antibodies against GATA binding protein 3 (GATA3, 0.5 µg/mL; BD Biosciences), nitric oxide synthase (iNOS, 2.0 µg/mL), forkhead box P3 (FOXP3, 1 mg/mL Abcam; Cambridge, UK), IRF4 (0.5 µg/mL), and β-actin (2.0 µg/mL; Cell Signaling Technology, Danvers, MA, USA). The membranes were then incubated with the peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX, USA). The proteins were supplemented with enhanced chemiluminescence select (GE Healthcare, Buckinghamshire, UK), and ChemiDoc (Bio-RAD, Hercules, CA, USA) was used to evaluate the bands. Band intensities were quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.4.7. Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) analysis

qRT-PCR analysis was performed as previously described (Kim et al., 2019a). Briefly, TRIzol reagent (Ambion, Carlsbad, CA, USA) was used to extract total RNA from homogenized lung tissue. First-strand cDNA was synthesized using the qPCR BIO cDNA Synthesis kit (PCR BIO Systems, London, UK). Real-time PCR was performed using qPCR BIO SyGreen Blue Mix Lo-ROX (PCR BIO Systems) in Rotor-Gene 3000 (Qiagen, Valencia, CA, USA). The primer sequences are presented in Table 1. The gene expression levels were calculated relative to *Gapdh* mRNA expression.

2.5. In vitro experiments

2.5.1. In vitro Th2 polarization assay

The Th2 polarization assay was performed as previously described (Seo et al., 2019). Briefly, splenic CD4⁺ T cells purified using a naïve CD4⁺ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) were stimulated with anti-CD3, anti-CD28, anti-IFN-γ, IL-2, and IL-4 (Miltenyi Biotec) with or without ARE treatment. For neutral conditions, CD4⁺ T cells were stimulated with only anti-CD3 and anti-CD28. After culturing for 5 days, Th2 differentiation and activation were analyzed based on IL-5 production and GATA3⁺ CD4 T cell proportion by ELISA and flow cytometry, respectively, as described in section 2.4.5.

2.5.2. Macrophage cell line culture and viability

The RAW 264.7 mouse macrophage cell line was obtained from American Type Culture Collection (cat# TIB-7, lot# 3002360; ATCC Laboratories, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum, 100 U/mL penicillin, and 50 µg/mL streptomycin. The cells were incubated in a humidified 5% CO₂ atmosphere at 37 °C and their viability was determined using the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyl tetrazolium bromide (MTT) assay with cell proliferation assay kits (Promega, Madison, WI, USA) as described in the manufacturer's instructions.

2.5.3. Measurement of nitric oxide (NO) and IL-6 levels in macrophages

Cells were stimulated with lipopolysaccharide (LPS, 200 ng/mL; Sigma-Aldrich) with or without ARE for 24 h at 37 °C. NO in the culture supernatant was analyzed using the Griess reagent (Promega), and the nitrite levels were measured using a Wallac EnVision™ microplate reader (PerkinElmer, Wellesley, MA, USA) at 540 nm. Secretion of the inflammatory cytokine IL-6 was determined as described in section 2.4.4.

2.5.4. Western blotting of ARE-treated macrophages

The treated cells were used for western blotting as described in section 2.4.6. The following primary antibodies were used: iNOS, cyclooxygenase (COX)-2 (Cell Signaling Technologies), and β-actin.

2.6. Statistical analysis

Data are presented as mean ± standard error of the mean. Differences between each group were evaluated using the one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate statistically significant results. Analyses were carried out using Prism v.6.0 (GraphPad Inc., La Jolla, CA, USA).

3. Results

3.1. Identification and ultra-performance liquid chromatography analysis of ARE

Various plant materials morphologically resembling AR are often available in Korean herbal markets as the same herbal medicine (Choo et al., 2009). Therefore, the plant species used in the present study was confirmed using a genetic marker to distinguish them from commercial AR (data not shown). The chemical components in the ARE were identified and quantified by HPLC analysis; the chromatogram is shown in Supplementary Fig. S3. The nine components of ARE, chlorogenic acid, nodakenin, ferulic acid, xanthotoxin, bergapten, oxypeucedanin, imperatorin, osthole, and isoimperatorin, were detected at approximately 7.9, 11.9, 13.0, 22.2, 24.0, 26.5, 30.6, 32.2, and 33.1 min, respectively. The chlorogenic acid content was the highest (0.443%), followed by ferulic acid (0.297%), bergapten (0.005%) xanthotoxin (0.003%), oxypeucedanin (0.001%), imperatorin (0.008%), and isoimperatorin (0.15%). Nodakenin and osthole were detected at trace levels.

Table 1
Primer sequences for quantitative real-time PCR.

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

3.2. ARE treatment alleviated lung tissue pathological changes in OVA-induced allergic lung inflammation in mice

We evaluated the effect of ARE on the infiltration of inflammatory cells in an asthmatic mouse model by staining the lung sections with H&E. The vehicle-treated asthmatic mice showed extensive inflammatory cell accumulation in the peri-bronchial areas compared with the SC mice. The ARE 100 and 200 mg/kg- and DEX-treated mice exhibited decreased inflammatory cell infiltration in the lungs compared with the vehicle-treated group (Fig. 1A).

PAS-stained lung sections and mucin concentrations in the five mouse groups were analyzed to evaluate the efficacy of ARE on mucus production due to goblet cell hyperplasia. The A group exhibited goblet cell hyperplasia and increased mucin production, whereas both parameters were substantially reduced in the ARE-treated group (Fig. 1B).

3.3. ARE administration reduced the mediators of allergic inflammation in the OVA-induced asthmatic model

To determine the effect of ARE on leukocyte accumulation in asthmatic mice, the differential inflammatory cell counts in the BALF were determined. The asthma group exhibited an increase in the eosinophil count, whereas the ARE-treated groups showed a significant dose-dependent decrease in the eosinophil count (Fig. 2A). As the BALF levels of type 2 cytokines in patients with asthma are higher than those in healthy individuals (Kroegel et al., 1996), these cytokines were measured in the BALF of the five mouse groups. Because IgE secretion through activated B cells is induced by IL-4 (Locksley, 2010; Ryzhov et al., 2004), the IgE level in BALF was measured. As expected, the IgE level in each group was correlated with the IL-4 level (Fig. 2B and E). The total IgE level in the untreated asthmatic group was significantly higher level than that in the SC group, whereas its level was relatively lower in the ARE-treated group. Notably, this observation is consistent with the IL-4 results following ARE administration. The levels of IL-5 and eotaxin-3, which induced the recruitment of eosinophils in the asthma group, were high compared with those in the SC group. Conversely, significantly lower levels of eotaxin-3 and IL-5 were

recorded in the BALF of groups treated with either ARE or DEX than in the untreated group (Fig. 2C and F). In asthma, the essential function of IL-13 is to promote the growth of goblet cells and support mucus secretion (Whittaker et al., 2002). Because of its effects on mucus hyperplasia, IL-13 is considered a therapeutic target in asthma (Ingram and Kraft, 2012; Singh et al., 2011). Here, the ARE100 and ARE200 groups exhibited significantly lower levels of IL-13 in the BALF (Fig. 2D) than the asthma group, and this was consistent with the mucus production results (mucus level in lung sections and BALF, Fig. 1B and C) following ARE administration.

3.4. ARE inhibited Th2-related cytokine production by downregulating Th2 cell activation

To investigate whether the reduction in type 2 cytokines was affected by decreased activity of Th2 cells, we measured activated helper T cell proportions ($CD4^+CD25^+$) among T cells ($CD45^+CD3^+$) in the lung tissues by fluorescence-activated cell-sorting. Activated Th2 cells were significantly increased following induction with OVA compared with that in the saline control. Moreover, ARE100 treatment did not significantly affect the activated Th2 cells, whereas ARE200 resulted in significantly less activation of Th2 cells (Fig. 3A–C).

To confirm the inhibition of Th2 cell differentiation and activation by ARE, we conducted additional *in vitro* experiments. Th2 cell differentiation was performed under Th2 polarization conditions (IL-2, IL-4, and anti-IFN- γ), and the type 2 cytokine IL-5 and GATA3 $^+$ CD4 T cell proportions were measured. As expected, ARE downregulated IL-5 production in cells under Th2 polarization conditions (Fig. 4A). Furthermore, Th2 cell differentiation was diminished by ARE (Fig. 4B and C) whereas IFN- γ , a type 1 cytokine, was not affected by ARE (Supplementary Fig. S4). Thus, we concluded that ARE reduced the asthma phenotypes by reducing Th2 cell differentiation and activation.

Isolated CD4 $^+$ T cells were cultured with or without 100 or 200 μ g/mL ARE under Th2-polarizing conditions. (A) IL-5 level was determined by ELISA. The GATA3 $^+$ CD4 $^+$ T cell population was analyzed by flow cytometry. Representative plots (B) and compiled data (C) are shown. Numbers within the panel indicate the abundance of Th2 cells among all

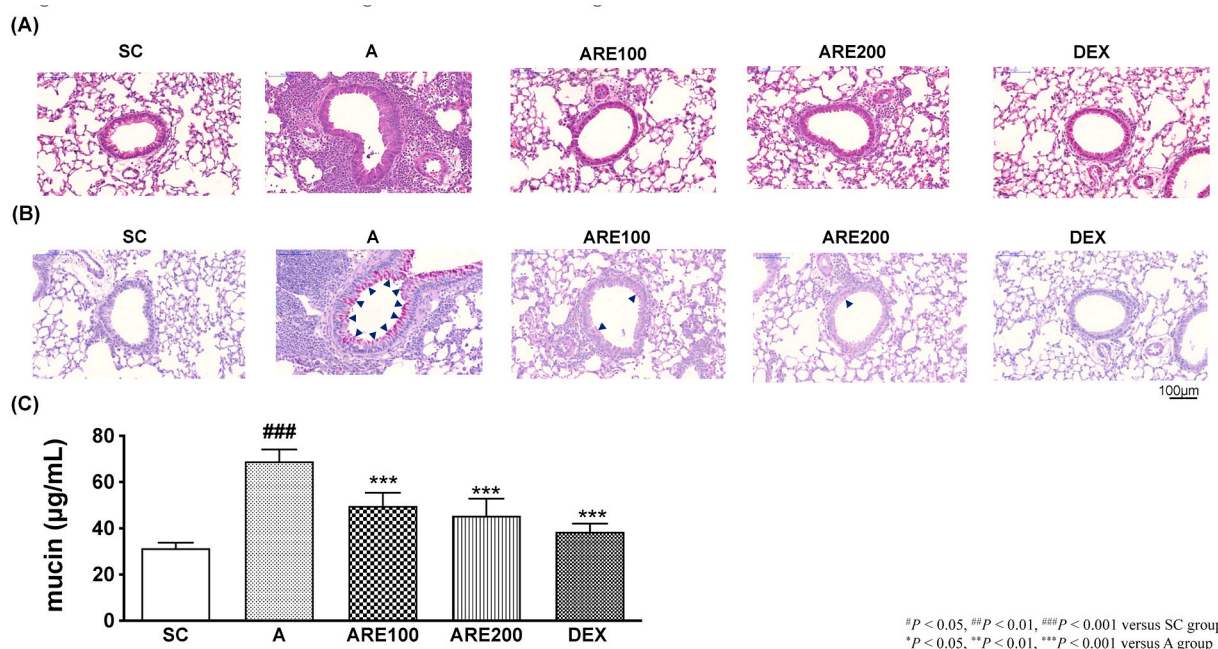


Fig. 1. Effects of ARE on the histopathological changes in the lungs of asthmatic mice. The lung sections were stained with H&E (A) or PAS (B) (200 × magnification). Arrowheads indicate mucus production. (C) Mucin production was determined using an image analyzer. SC, saline-treated controls group; A, OVA-induced asthmatic group; ARE100, asthmatic mice treated with 100 mg/kg ARE; ARE200, asthmatic mice treated with 200 mg/kg ARE; DEX, asthmatic mice treated with 5 mg/kg dexamethasone. ###P < 0.001 vs. the SC group; ***P < 0.001 vs. the A group.

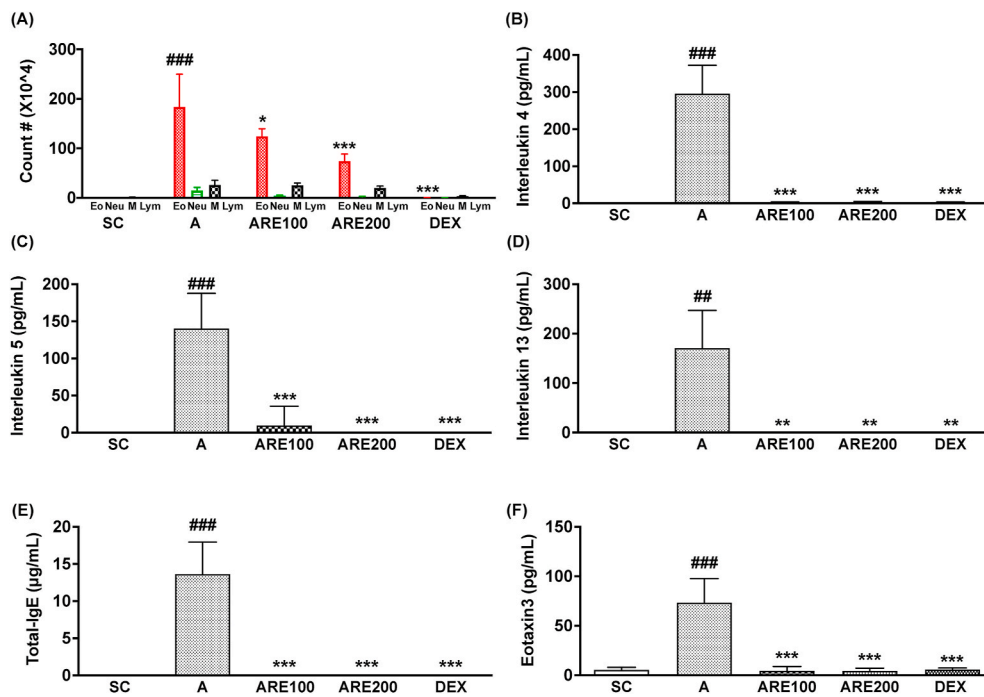


Fig. 2. Effects of ARE on inflammatory cell accumulation and expression of mediators in the BALF of asthmatic mice. (A) Cells were isolated by cytopspin and differential cell types were counted. Levels of IL-4 (B), IL-5 (C), IL-13 (D), IgE (E), and eotaxin3 (F) were measured by ELISA. SC, saline-treated control group; A, OVA-induced asthmatic group; ARE100, asthmatic mice treated with 100 mg/kg ARE; ARE200, asthmatic mice treated with 200 mg/kg ARE; DEX, asthmatic mice treated with 5 mg/kg dexamethasone. Eo, eosinophil; Neu, neutrophil; M, macrophage; Lym, lymphocyte. ^{##} $P < 0.01$, ^{###} $P < 0.001$ vs. the SC group; ^{*} $P < 0.05$, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. the A group.

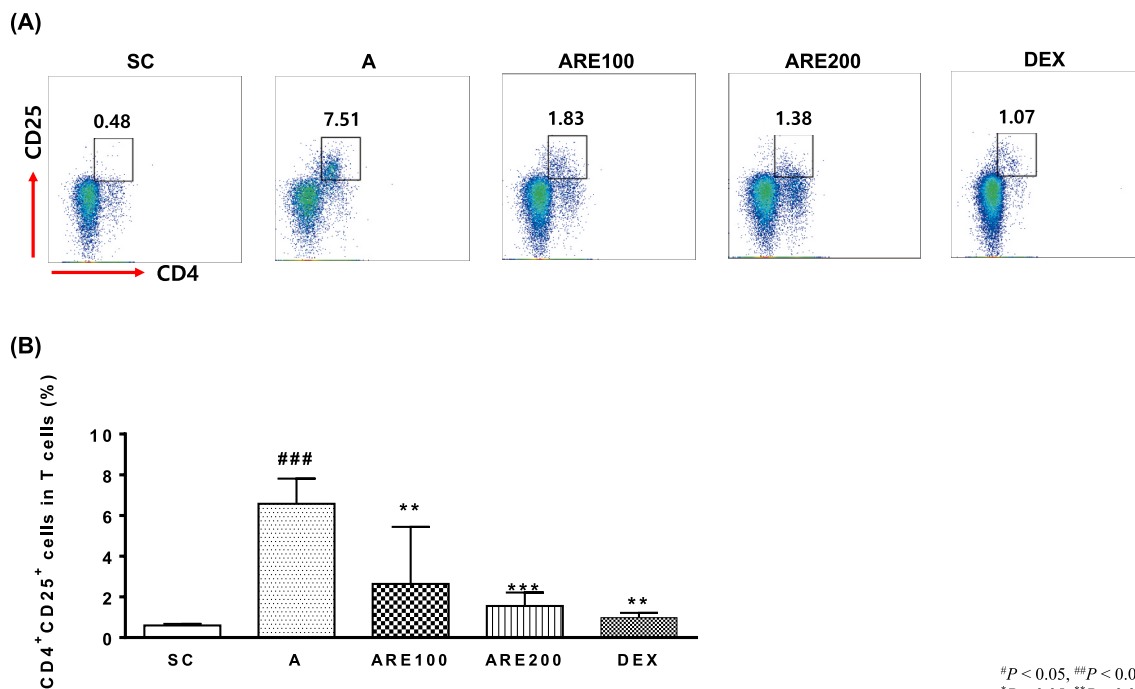


Fig. 3. Effect of ARE on Th2 cell activation in the lungs of asthmatic mice. The fraction of activated Th2 cells (CD4⁺CD25⁺) in the lungs was analyzed by flow cytometry. Representative (A) and compiled (B) data are shown. The numbers within each panel indicate the percentage of activated helper T cells among all T cells (CD45⁺CD3⁺). (C) GATA-3 expression in the lungs, as evaluated by western blotting. SC, saline-treated control group; A, OVA-induced asthmatic group; ARE100, asthmatic mice treated with 100 mg/kg ARE; ARE200, asthmatic mice treated with 200 mg/kg ARE; DEX, asthmatic mice treated with 5 mg/kg dexamethasone. ^{###} $P < 0.001$ vs. the SC group; ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. the A group.

CD4⁺ T cells. No-sti, without stimulation; Neutral, stimulated with anti-CD3 and anti-CD 28; Th2, stimulated with neu + IL2, IL-4, and anti-IFN γ ; ARE200, Th2+ AR extract 100 μ g/mL; ARE200, Th2+ AR extract 200 μ g/mL ^{###} $P < 0.0001$ vs. saline, ^{**} $P < 0.01$, ^{***} $P < 0.001$ vs. the Th2 polarization group.

3.5. ARE inhibited Th2 cell activation by downregulating IL-6-mediated IRF-4 expression

Recent studies have reported that patients with asthma exhibit highly increased levels of NO and iNOS in bronchial epithelial cells compared with healthy controls (Dweik, 2001). In addition, the

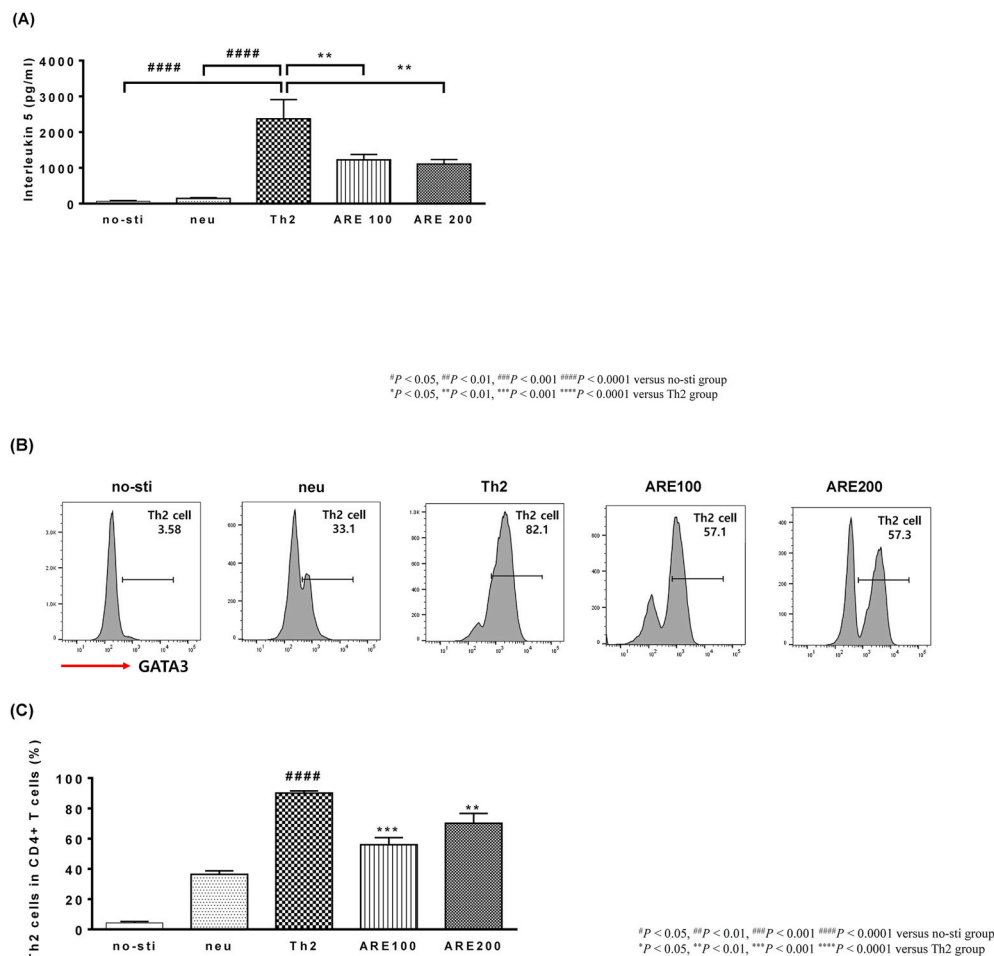


Fig. 4. Effect of ARE on Th2 cell differentiation in vitro. Isolated CD4⁺ T cells were cultured with or without 100 or 200 µg/mL ARE under Th2-polarizing conditions. (A) IL-5 level was determined by ELISA. The GATA3⁺ CD4⁺ T cell population was analyzed by flow cytometry. Representative plots (B) and compiled data (C) are shown. Numbers within the panel indicate the abundance of Th2 cells among all CD4⁺ T cells. No-sti, without stimulation; Neutral, stimulated with anti-CD3 and anti-CD 28; Th2, stimulated with neu + IL2, IL-4, and anti-INFγ; ARE200, Th2+ AR extract 100 µg/mL; ARE200, Th2+ AR extract 200 µg/mL. ###P < 0.0001 vs. saline, **P < 0.01, ***P < 0.001 vs. the Th2 polarization group.

accumulation of eosinophils is associated with nitrate level, which is regulated by iNOS (Soufli et al., 2016). Therefore, in this study, the iNOS level was determined in the lungs of ARE-treated mice; the ARE-treated groups presented a significantly lower iNOS level than the asthma group (Fig. 5A and B).

It has been reported that IRF4 expression induced by IL-6 signaling is important for Th2 cell differentiation (Koch et al., 2013; Staudt et al., 2010). Therefore, IL-6 and IRF4 expressions were determined to evaluate the mechanism underlying ARE regulatory effects in Th2 cell activation. The ARE100 and ARE200 groups exhibited significantly reduced expression of IL-6 in the BALF than the vehicle group (Fig. 5C). Furthermore, after treatment with ARE, the IRF4 expression decreased compared with that in the asthma group (Fig. 5D and E), which indicated that IL-6 might be related to NO production and IRF4 expression in asthmatic mice. In addition, IRF4 suppresses FOXP3 expression, which constitutes a key marker of Treg (Cretney et al., 2011; Zheng et al., 2009). Therefore, we assessed the expression level of FOXP3 in ARE-treated mouse lung tissue, and it was found to be substantially upregulated than the asthma group (Fig. 5F).

3.6. ARE treatment reduced inflammatory mediator and cytokine production by RAW 264.7 cells

It has been reported that alveolar macrophages are involved in the aggravation of asthma (Yang et al., 2012). In particular, macrophages contribute to allergic asthma by producing NO and IL-6 (Dweik, 2001; Rincon and Irvin, 2012; Stuehr, 1997). Therefore, we hypothesized that the regulation of macrophage activation might constitute another cellular mechanism underlying the efficacy of this extract. To verify this

hypothesis, we first measured the toxicity of ARE in a murine macrophage cell line (RAW264.7 cells). As cell viability was decreased by ARE at 1000 µg/mL, we conducted the subsequent experiments with ARE at concentrations below 500 µg/mL (Fig. 6A). Second, we investigated the effect of ARE on macrophage activation with or without ARE, and then measured the expression of NO, iNOS, and IL-6. The results indicated that ARE reduced the NO level by downregulating iNOS expression (Fig. 6B and C). ARE also significantly downregulated IL-6 in a dose-dependent manner (Fig. 6D). Therefore, we concluded that the reduced expression of iNOS and IL-6 in the lung and BALF after ARE administration might be mediated by reduced macrophage activation in vivo.

4. Discussion

Asthma is a chronic and complicated immune disorder characterized by symptoms including hyper-responsiveness, mucus hypersecretion, and lung remodeling mediated by type 2 cytokines (Endo et al., 2014; Porter et al., 2011; Rosenberg, 2013; Wüthrich, 1989). In particular, the recruitment of eosinophils into the airway constitutes a major characteristic of asthma (Lambrech and Hammad, 2015), and it is mediated by chemotactic chemokines and IL-5 (Kumar and Foster, 2012; Rosenberg, 2013).

AR, a perennial, has been traditionally used as an anti-asthma and anti-bronchitis expectorant in northeast Asian countries (Hendrawati et al., 2011; Jeong et al., 2007). Therefore, we sought to demonstrate the anti-inflammatory effects of ARE in vitro and in an OVA-induced asthmatic mouse model. The anti-asthmatic effects of ARE in the OVA-induced airway inflammation model were confirmed by the

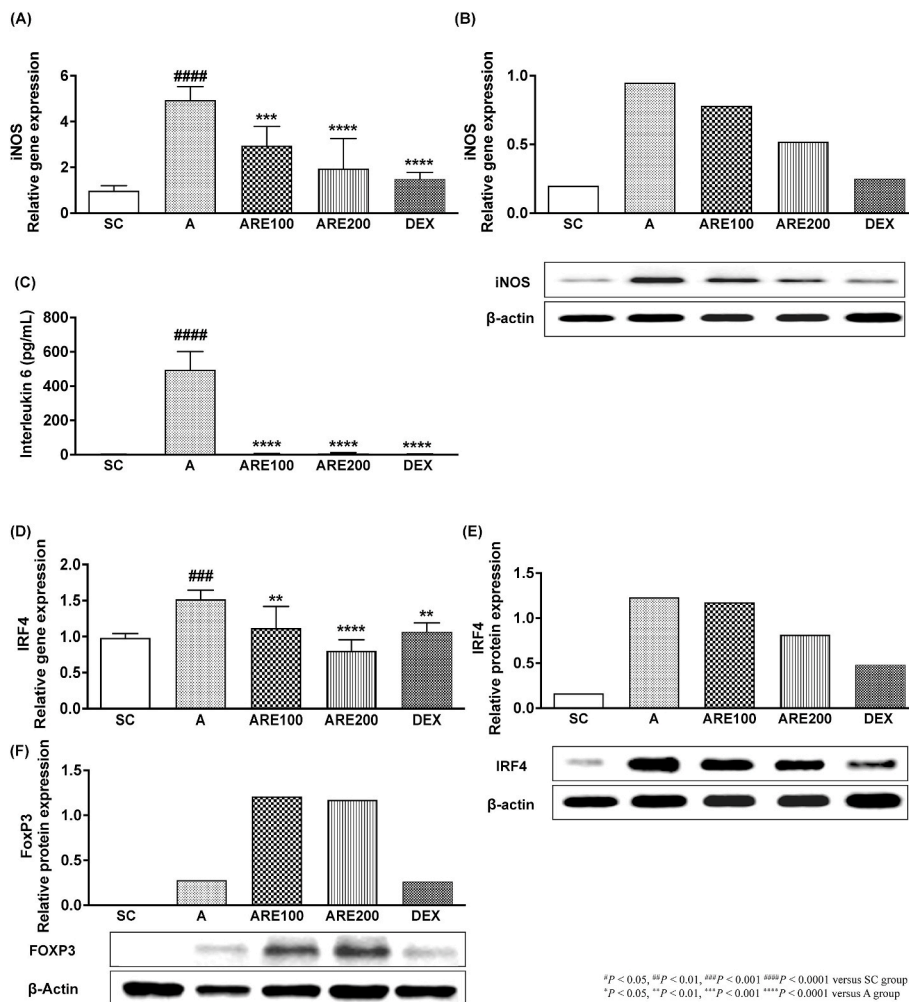


Fig. 5. Effect of ARE on IL-6- and IRF4-related immune mechanisms. mRNA (A, D) and protein (B, E) levels of iNOS and IRF4 in the lungs. (C) IL-6 level in BALF. (F) FoxP3 level. Protein expression was normalized to the level of β-actin. SC, saline-treated control group; A, OVA-induced asthmatic group; ARE100, asthmatic mice treated with 100 mg/kg ARE; ARE200, asthmatic mice treated with 200 mg/kg ARE; DEX, asthmatic mice treated with 5 mg/kg dexamethasone. ####P < 0.0001 vs. the SC group; **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the A group.

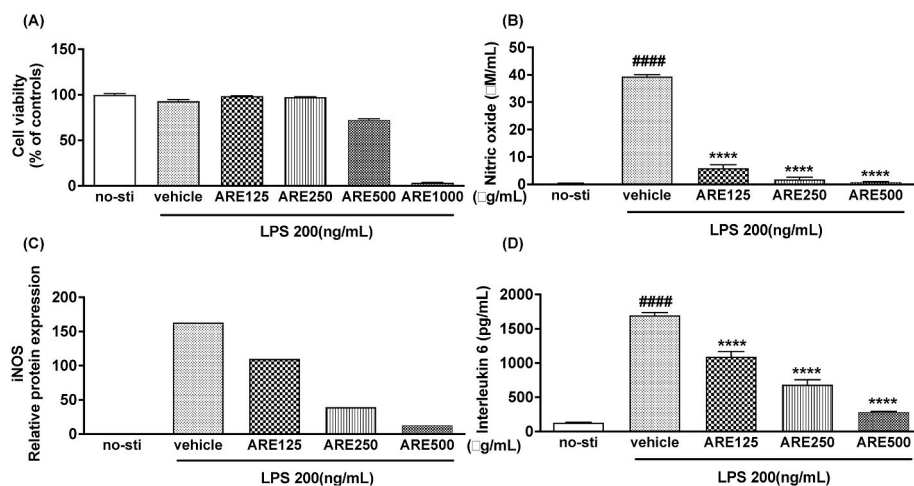


Fig. 6. Effect of ARE on macrophage activation. RAW 264.7 cells (murine macrophage cell line) were treated with LPS in the absence or presence of 125, 250, and 500 μg/mL ARE. (A) Cell viability. (B) NO level. (C) iNOS expression level. (D) IL-6 level. ####P < 0.0001 vs. no-sti (without stimulation); ****P < 0.0001 vs. lipopolysaccharide (LPS) only.

inhibition of mucus production from the airway epithelium and immune cell recruitment, including eosinophils. Moreover, the ARE-treated groups exhibited a considerable decrease in the IL-5 level and a significant difference in the eotaxin-3 level. In addition, ARE treatment significantly reduced type 2 cytokine and IgE secretion in the BALF.

Specifically, we demonstrated the anti-asthmatic effects of AR and its potential underlying mechanism as follows. Firstly, Th2 cells serve as a major mediator of allergic reactions by releasing Th2 cytokines such as IL-4, IL-5, and IL-13 (Kim et al., 2010; Kwon et al., 2017). ARE administration reduced Th2 cell activation both in vivo as determined by the

CD4⁺CD25⁺ cell population along with the GATA-3 level, and in vitro as determined by the GATA3⁺ cell proportion and IL-5 level. Secondly, we revealed the mechanism underlying reduced eosinophilia induced by ARE, which was likely mediated by the NO-IL-6 axis. Briefly, the ARE-treated group exhibited a significant reduction in the iNOS level in the lung and IL-6 level in the BALF. Furthermore, we demonstrated that ARE treatment impeded IL-6 and NO secretion by murine macrophages in vitro, indicating its effect in regulating the innate immune response associated with eosinophilia and type 2 immunity.

Finally, we revealed that IRF4 is a key signaling mediator that induces Th2 cell activation by upregulating GATA3 level and suppressing Treg cell activation by reducing FOXP3 expression. In particular, IRF4 expression was downregulated by ARE administration in accordance with the decreased GATA3 level and increased FOXP3 expression. In summary, our study demonstrated that ARE treatment inhibited IL-6-NO production and IRF4 expression, which decreased eosinophilia, macrophage activation, and Th2 cell activation as indicated by GATA3 expression, and augmented Treg cell function as determined by FOXP3 expression.

Asthma is considered an allergic disorder based on Th2 response (Jung et al., 2020). However, recent studies have classified asthma as either eosinophilic or neutrophilic (Douwes et al., 2002). In contrast to eosinophilic asthma, which is mediated by type 2 inflammation based on Th2 cells, neutrophilic asthma involves the action of Th17 cells that are crucial for tissue injury, remodeling, and autoimmunity (Oukka, 2007). Our study demonstrated that treatment with ARE substantially decreased the expression of IRF4, which is related to both Th2 and Th17 cell activation (Brüstle et al., 2007; Tominaga et al., 2003). In this study, we only demonstrated the inhibitory effects of ARE in a type 2 allergic asthma model. Therefore, further research regarding the effects of ARE on neutrophil asthma, which is related to Th17 cells, would enable to better understand the pharmacological action of ARE. Despite these limitations, our findings suggest that ARE is a potential drug candidate for treating allergic airway inflammation. Furthermore, additional research is warranted to identify the active compounds in ARE and consequently maximize its anti-asthmatic effects.

5. Conclusions

In this study, the administration of ARE to a murine model of allergic asthma attenuated inflammatory cell infiltration and mucus production in the airway. Furthermore, ARE suppressed Th2 cell production and promoted Treg cell production by regulating IRF4 expression. This study provides pharmacological evidence of the anti-asthmatic effects of AR.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2020.113752>.

Author contributions

SB Kim and YS Seo performed the experiments, analyzed data, and

wrote the manuscript; HS Kim and AY Lee prepared plant materials and conducted HPLC/MS analysis; JM Chun performed the in vivo experiments; WJ Kim and BC Moon verified the origin of the plant materials by genetic identification; B-I Kwon designed the experiments, analyzed the data, and wrote the manuscript.

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