Allium hookeri root extract regulates asthmatic changes through immunological modulation of Th1/Th2-related factors in an ovalbumin-induced asthma mouse model

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Abstract. In 2013, WHO estimated that approximately 235 million people suffered from asthma worldwide. Asthma is a hyper responsive disorder, which is related to an imbalance between the T-helper type 1 and 2 cells (henceforth, Th1 and Th2, respectively). *Allium hookeri* is a plant that is widely used for culinary purposes and also in traditional Asian medicine. The present study was conducted to elucidate the anti-asthmatic effects and mechanism of action of *A. hookeri* root extracts (AHRE) in an ovalbumin (OVA)-induced asthma mouse model. The mice were divided into five groups, namely, the control, the OVA-treated group, the dexamethasone-treated group, the 30 mg/kg AHRE-treated group, and the 300 mg/kg AHRE-treated group. The total WBC count and the differential cell count in the bronchoalveolar fluid, the level of serum IgE, the histopathological changes in the lung, and changes in the cell surface molecules, the asthma-related cytokine levels, and Th cell transcription factors were evaluated. AHRE significantly ameliorated asthmatic changes, such as the total WBC count, eosinophil count, and the level of IgE; in addition, it reduced mucus hypersecretion, epithelial hyperplasia, and eosinophil infiltration in the lungs. AHRE significantly inhibited the expression of $CD68^+$ cells and MHC class II^+ molecules, Th1 cell transcription factor (T-bet) activation, Th2 cell transcription factor (GATA-3) activation, and TNF-α in the lung tissue. Furthermore, it suppressed cell surface molecules, such as $CD4^+$and $CD8^+$; Th1-related cytokines, such as IFN-γ and IL-12p40; Th2-related cytokines, such as IL-4 and IL-5; and Th17-related cytokines, such as IL-6 and TNF-α, in a dose-dependent manner. Thus, AHRE may be considered a promising anti-asthmatic drug.

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Introduction

In 2013, the World Health Organization (WHO) reported that 235 million people suffered from asthma (1). The inadequate control of asthma is a serious problem (2). Asthma is a pulmonary disease that can lead to apnea and death due to excessive mucus production, goblet cell hyperplasia, epithelial cell shedding, basement membrane thickening, and eosinophil and lymphocyte infiltration (3). The different allergens that are the inducers of asthma can be classified into two main categories: Indoor and outdoor allergens. The indoor allergens include pet dander, domestic mites, and cockroaches, while the outdoor allergens include tobacco smoke, chemical irritants, and dust particles (4).

The re-uptake of the allergens can cause an imbalance in the T-helper (Th) cells and eventually result in asthma (3). Various studies have revealed that Th type 1 (Th1)-related cytokines [interleukin (IL)-12 and interferon (IFN)-γ], Th type 2 (Th2)-related cytokines (IL-4, IL-5, and IL-13), and other proinflammatory cytokines (IL-1β, IL-6, and TNF-α) are associated with asthma. Of these, IFN-γ has been revealed to have an important role in suppressing asthma induction by regulating the increase in the level of IgE (5) and accelerating the activation of T-bet, which is a Th1 cell transcription factor, in a positive feedback loop (6,7). IL-12 has been demonstrated to modulate the balance between Th1 and Th2 cells not only by promoting Th1 cell proliferation and inhibiting Th2 cell proliferation, but also by accelerating IFN-γ production (8,9). IL-4 has been reported to upregulate the level of IgE, increase eosinophil count (10), and

induce GATA-3 activation (11,12). IL-5 has been revealed to have several functions related to the development, activation, migration, and survival of eosinophils, in addition to augmentation of IL-6 proliferation (9,13). IL-13 has been demonstrated to not only modulate B cell activation but also induce airway remodeling, which causes excessive mucus production, goblet cell hyperplasia, epithelial cell shedding, basement membrane thickening, and eosinophil and lymphocyte infiltration in asthma (14-17). IL-6 is one of the proinflammatory cytokines which has been revealed to promote the expression of IgE and modulate the activation of Th cells. TNF-α, on the contrary, has been demonstrated to recruit granulocytes and induce fibroblast proliferation (18).

Inhaled corticosteroids are usually used for the treatment of asthma and are often administered as a combination therapy with long-acting β_2-agonist or leukotriene modulators, such as leukotriene receptor antagonist or 5-lipoxygenase inhibitor (19). However, corticosteroids exhibit several severe adverse effects on different systems, such as growth suppression in children (20); cataract and glaucoma; hypertension, water retention, and hyperlipidemia in the cardiovascular system; peptic ulcer and pancreatitis; and catatonia, decreased concentration, agitation, insomnia, and behavioral changes (21).

Effort has been escalated to identify effective and safe drugs, especially natural products, for the treatment of asthma (22-24). *Allium hookeri* is a plant belonging to the *Alliaceae* family. It is commonly used for culinary purposes in India, Thailand and China. The rhizome of this plant is used in traditional medicine for protection against hypertension (25). Recently, several biological activities of *A. hookeri* have been reported, such as antimicrobial effects (26), anti-inflammatory effects (27), and vascular growth factor regulation in allergic rhinitis (28). The anti-asthmatic effects of *A. hookeri* root extracts (AHRE), however, have not been studied yet. Therefore, this study was conducted to evaluate the anti-asthmatic activities of AHRE (30 and 300 mg/kg/day for 5 days) in an ovalbumin (OVA)-induced asthma model using experimental mice.

Materials and methods

Preparation of AHRE. Briefly, *A. hookeri* plants were collected in May 2014 from near Naju (Korea). A voucher specimen (MNUCSS-SC-01) was deposited at Mokpo National University (Muan, Korea). The roots were separated, air-dried, powdered (250 g), and extracted twice with 70% ethanol (1 l) at room temperature for 3 days. Following filtration, the ethanol was evaporated and the extract was dried and stored at -50˚C. Yield of extraction was 9% (w/w).

Animal experiments. All the experiments were approved by the Institutional Animal Care and Use Committee at Dongshin University, Naju, Korea (approval no. 2014-08-03). Eighty female BALB/c mice were purchased from Samtako Korea (Osan, Korea) and divided into five groups that received different treatment drugs as follows: i) Control, receiving vehicle (sterile tap water); ii) OVA for asthma induction; iii) OVA for asthma induction plus 1 mg/kg/day dexamethasone (positive control); iv) OVA for asthma induction plus 30 mg/kg/day AHRE; and v) OVA for asthma induction plus 300 mg/kg/day AHRE. Dexamethasone was selected as a positive control since it is broadly used for the treatment of asthma.

On days 1 and 8, all the groups of mice except the control were exposed to intraperitoneal OVA (20 μg) containing 1 mg aluminum hydroxide hydrate (both from Sigma-Aldrich; Merck KGaA) as an adjuvant in 500 μl saline. From days 21 to 25, the mice were challenged once daily with 5% OVA for 30 min using a nebulizer (NE-U17; OMRON Co. Ltd.) at a flow rate of 3 ml/min. During the same 5-day period, the other groups of mice were also treated once daily with oral doses of sterile tap water, dexamethasone, 30 mg/kg/day AHRE, and 300 mg/kg/day AHRE, respectively 1 h prior to the OVA challenge. The control group was sensitized to OVA according to the same procedure as the other groups, following which they were exposed to saline and aluminum hydroxide hydrate by a nebulizer for 5 consecutive days.

BALF and serum analysis. One day after the final treatment, the mice were anesthetized with intraperitoneal injections of 50 mg/kg Zoeltil 100 (Virbac Corporation), and the tracheas were cannulated with disposable animal feeding needles. Bronchoalveolar lavages were performed with three 0.4 ml aliquots of cold phosphate-buffered saline (PBS). The BALF samples were collected and immediately centrifuged at 900 x g for 5 min (Sorvall Legend Micro 17R; Thermo Fisher Scientific, Inc.). The cell pellets were re-suspended in PBS for WBC and differential cell counts. The total WBC and differential cell count were performed by a Hemavet Multispecies Hematology System (Drew Scientific, Inc.). The serum IgE levels were estimated using a specific mouse IgE enzyme-linked immunosorbent assay kit (cat. no. 555248; BD Biosciences) according to the manufacturer's instructions.

Histopathological examinations. The lung tissues were fixed in 10% (v/v) formaldehyde solution, dehydrated in a graded concentration of ethanol (99.9, 90, 80, and 70%), at room temperature for 1 month and embedded in paraffin at room temperature for 2 weeks. The paraffin-embedded lung tissues were then sectioned longitudinally (5 μm) and in order to analyze semi-quantitatively glycopreoteins expression they were stained with hematoxylin and eosin (H&E) at room temperature for 6 min, and with Periodic acid-Schiff stain at room temperature for 12 min. The images were captured with an Axioscope A1 microscope (Carl Zeiss AG). In order to define the change of histopathological morphology on mucous hypersecretion, epithelial cell hyperplasia, and inflammatory cell infiltration scores from 0 (none) to 3 (severe) were assigned.

Immunohistochemical examinations. Immunohistochemical examinations were performed by a method previously described (24). The de-paraffinized tissue sections were treated with 3% hydrogen peroxide in methanol for 10 min to remove the endogenous peroxidase. Antigen retrieval was carried out with sodium citrate buffer (0.1 M) using the hot plate method. The slides were incubated with normal serum to block the nonspecific bindings and then incubated for 1 h at 4˚C with different primary antibodies (diluted 1:100 to 1:200), such as rat anti-mouse CD4 monoclonal (cat. no. 14-9766;

eBioscience; Thermo Fisher Scientific, Inc.), rabbit anti-mouse CD8 polyclonal (cat. no. MBS551004; Mybiosource, Sand Diego, CA, USA), rabbit anti-mouse CD19 polyclonal (cat. no. 250585; Abbiotec), rabbit anti-mouse CD68 polyclonal (cat. no. bs-0649R; Bioss, Inc.), rat anti-mouse MHC class II monoclonal (cat. no. sc-59318cat), rat anti-mouse IFN-γ monoclonal (cat. no. sc-74104), goat anti-mouse IL-12 Ap35 polyclonal (cat. no. sc-9350), rat anti-mouse IL-12p40 monoclonal (cat. no. sc-57258), rat anti-mouse IL-4 monoclonal (cat. no. sc-73318), rabbit anti-mouse IL-5 polyclonal (cat. no. sc-7887), goat anti-mouse IL-13 polyclonal (sc-1776), goat anti-mouse IL-6 polyclonal (cat. no. sc-1265; all from Santa Cruz Biotechnology, Inc.), and rabbit anti-mouse TNF-α polyclonal antibody (cat. no. 3053R-100; BioVision, Inc.). The slides were then incubated with a biotinylated pan-specific secondary antibody for 10 min and reacted with a streptavidin-peroxidase complex for 5 min (Universal Quick HRP Kit; cat. no. PK-7800; Vector Laboratories, Inc.). The signals were detected using 3,3-diaminobenzidine tetrahydrochloride substrate chromogen solution, and the cells were counterstained with Mayer's hematoxylin. The cells were imaged using an Axioscope A1 microscope (Carl Zeiss AG). To determine the number of positively stained cells, the cells in five randomly selected non-overlapping fields (x200 magnification) of three separately immunostained lung sections per mouse (n=8/group) were counted.

Immunofluorescence analysis. In order to localize the Th1 cell transcription factor (T-bet), and Th2 cell transcription factor (GATA-3), immunofluorescence analysis was conducted in four groups of mice such as the control, the OVA for asthma induction, the OVA for asthma induction plus dexamethasone, and the OVA dexamethasone plus 300 mg/kg/day AHRE groups. The steps prior to the antibody binding step were similar to that of the immunohistochemistry method except for rabbit anti-mouse T-bet (cat. no. orb7075; Biorbyt Ltd.) or goat anti-mouse GATA-3 (cat. no. TA305795; OriGene Technologies, Inc.) were used as primary antibodies for 1 h at a room temperature. The slides were incubated for 2 h with FITC-conjugated anti-rabbit IgG (cat. no. 315-095-003; Jackson Immunoresearch Laboratories, Inc.) or Alexa Fluor 555-conjugated anti-goat IgG antibody (cat. no. A-21127; Thermo Fisher Scientific, Inc.) and the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (cat. no. 62249; Thermo Fisher Scientific, Inc.). The images were obtained using a K1-Fluo confocal microscope (Nanoscope Systems, Inc.).

Statistical analysis. The results were expressed as the mean ± standard deviation (SD). The group differences were evaluated by one-way analysis of variance, followed by Dunnett's multiple comparison post hoc test. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

AHRE significantly suppresses WBC count, especially eosinophils in bronchoalveolar lavage fluid (BALF). The WBC count, and especially the eosinophil count, in BALF were significantly increased in the OVA-treated group (Fig. 1). As revealed in Fig. 1A, the WBC count in the OVA-treated group was significantly increased, but was considerably reduced by dexamethasone treatment, which was selected as a positive control since it is broadly used for asthma treatment. AHRE reduced the OVA-induced increase in WBC count in a dose-dependent manner. The eosinophil count was also reduced by AHRE (Fig. 1B). Based on the eosinophil count in the OVA-treated group (100%), the reduction in the dexamethasone group was 53% and that in the 300 mg/kg AHRE group was 58%.

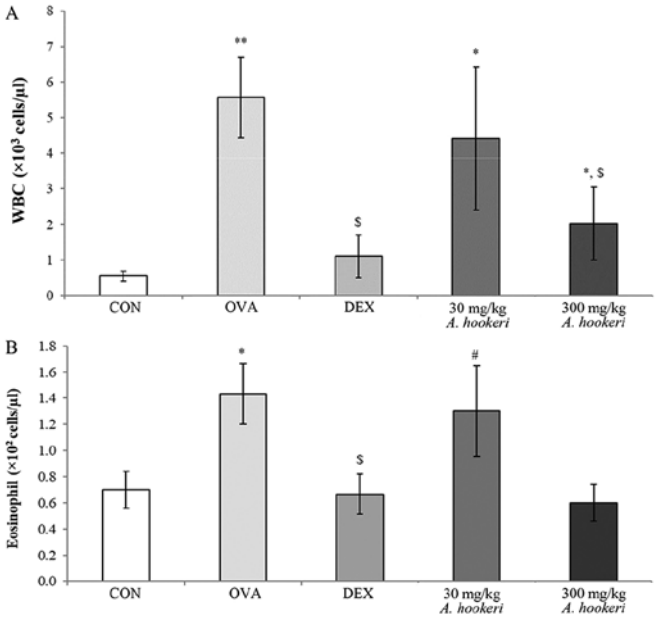

Figure 1. AHRE suppresses the OVA-induced increase in (A) WBC and (B) eosinophil count in the BALF of the OVA-induced asthma mice in a dose-dependent manner. Each bar represents the mean ± S.E.M. of six experiments. *P<0.05 compared with the control, **P<0.001 compared with the control, $P<0.05 compared to asthma induction (OVA-treated group), and #P<0.05 compared with dexamethasone. AHRE, *Allium hookeri* root extract; BALF, bronchioalveolar lavage fluid; CON, control; OVA, ovalbumin; DEX, dexamethasone.

AHRE downregulates the OVA-induced increase in serum IgE. The change in the level of IgE was similar to that of WBC (Fig. 2). The level of IgE in the OVA-treated group was significantly increased, which was reduced by dexamethasone. AHRE decreased the levels of IgE in a dose-dependent manner, and the effect of administering 300 mg/kg AHRE was similar to that of dexamethasone treatment.

AHRE ameliorates asthmatic changes in the lungs. In the lungs of the OVA-treated group, typical histopathological changes associated with asthma were observed, such as mucus hypersecretion, epithelial hyperplasia, goblet cell hyperplasia, and eosinophil infiltration near the bronchioles and vessels (Fig. 3A-b), when compared with that in the lungs of the control group animals (Fig. 3A-a). Dexamethasone ameliorated the OVA-induced morphological changes, as evidenced by the presence of only a sparse eosinophilic infiltration (Fig. 3A-c). In the lungs of the 30 mg/kg AHRE-treated group, asthma-like typical morphological changes were observed (Fig. 3A-d),

Table I. The quantitative results of histopathological changes by AHRE.

Groups	Mucous hypersecretion (0-3)	Epithelial cell hyperplasia (0-3)	Inflammatory cell infiltration (0-3)
CON	0.3±0.46	0.3±0.46	0.3±0.46
OVA	2.8±0.46[b]	2.9±0.35[b]	2.8±0.46[b]
DEX	0.4±0.52[c]	0.4±0.52[c]	0.4±0.52[c]
30 mg/kg *A. hookeri*	2.8±0.46[b,e]	2.9±0.35[b,e]	2.4±0.52[b,e]
300 mg/kg *A. hookeri*	0.5±0.53[c]	1.0±0.76[a,c]	1.4±0.52[b-d]

AHRE effectively regulated the representative asthma-related morphological changes. The change of histopathological morphology was scored from 0 (none) to 3 (severe). [a]P<0.05 vs. the control, [b]P<0.001 vs. the control, [c]P<0.001 vs. asthma induction (OVA-treated group), [d]P<0.05 vs. dexamethasone and [e]P<0.001 vs. dexamethasone. AHRE, *Allium hookeri* root extract; OVA, ovalbumin; DEX, dexamethasone.

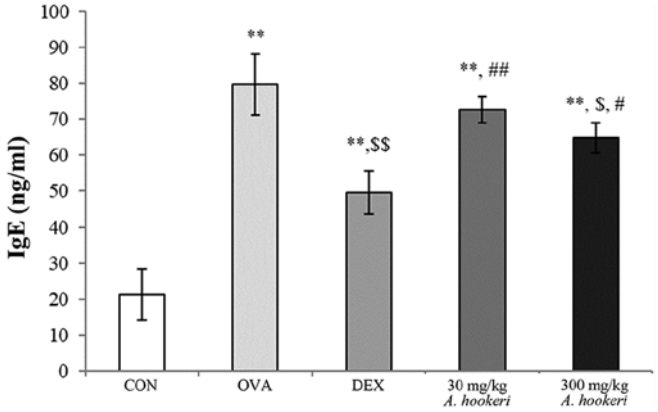

Figure 2. AHRE suppresses the OVA-induced increase in serum IgE levels of the OVA-induced asthma mice in a dose-dependent manner. **P<0.001 compared with the control, $P<0.05 compared with asthma induction (OVA-treated group), $$P<0.001 compared with asthma induction (OVA-treated group), #P<0.05 compared with dexamethasone, and ##P<0.001 compared with dexamethasone. AHRE, *Allium hookeri* root extract; CON, control; OVA, ovalbumin; DEX, dexamethasone.

whereas, in the 300 mg/kg AHRE-treated group, only a sparse eosinophilic infiltration was observed (Fig. 3A-e) similar to that in the dexamethasone-treated group (Fig. 3A-c).

In order to assess mucus secretion, Periodic-Schiff staining was performed (Fig. 3B). Mucus hypersecretion, also known as mucus plugs, was observed in the bronchioles (Fig. 3B-b) in the OVA-treated group, when compared with the control (Fig. 3B-a). Dexamethasone (Fig. 3B-c) and AHRE, in a dose-dependent manner, (Fig. 3B-d and-e) decreased the OVA-induced mucus hypersecretion. Especially, in the 300 mg/kg AHRE-treated group, mucus was almost undetected in the bronchioles and was observed only near the epithelium (Fig. 3B-e).

In order to compare the morphological change level of each group, quantitative analysis was conducted and the results revealed that AHRE effectively suppressed the OVA-induced histopathological changes in the lung (Table I).

AHRE suppresses expression of T cell-related factors, macrophages, and major histocompatibility complex (MHC) class II molecules in asthma. The immune modulatory effects of AHRE were investigated by analyzing the changes in the population of T cells ($CD4^+$ Th cells, Fig. 4A, and $CD8^+$ cytotoxic T cells, Fig. 4B), B cells ($CD19^+$ cells, Fig. 4C), macrophages ($CD68^+$ cells, Fig. 4D), and MHC class II molecules (Fig. 4E). AHRE suppressed all these cells, and especially almost completely inhibited the expression of $CD68^+$ cells (Fig. 4D-e), and MHC class II^+ molecules (Fig. 4E-e) in a dose-dependent manner. The expression of $CD4^+$ Th cells (Fig. 4A-e), $CD8^+$ cytotoxic T cells (Fig. 4B-e), and $CD68^+$ cells (Fig. 4D-e) in the 300 mg/kg AHRE-treated group was lower than in the dexamethasone-treated group (Fig. 4A-c, B-c, and D-c). The expression of $CD19^+$ B cells (Fig. 4C-e) and MHC class II^+ molecules (Fig. 4E-e) in the 300 mg/kg AHRE-treated group was similar to that in the dexamethasone-treated group (Fig. 4E-c).

AHRE suppresses the expression of Th1-related cytokines (IFN-γ and IL-12p40) but not IL-12p35. Asthma is a chronic respiratory disorder caused not only by the imbalance between Th1- and Th2-related cytokines (29,30) but also by changes in the activation of several inflammatory mediators (13,18). The changes in the asthma-related inflammatory mediators, such as Th1-related cytokines (IFN-γ, IL-12p40, and IL-12p35), (Fig. 5) and Th2-related cytokines (IL-4, IL-5, and IL-13) (Fig. 6) were evaluated. All of these factors are usually secreted and detected near the bronchioles and pulmonary vessels. In asthma, an imbalance exists between the Th1- and Th2-related factors and the quantity of Th1-related cytokines may be lower than that of the Th2-related ones. In the lungs of the OVA-treated group, the expression of the Th1-related cytokines, such as IFN-γ, IL-12p40, and IL-12p35 was increased (Fig. 5A-b, B-b, and C-b) compared to the control, whereas, the expression was significantly less in the dexamethasone-treated group (Fig. 5A-c, B-c, and C-c) compared to the OVA-treated group. AHRE decreased the expression of IFN-γ (Fig. 5A) and IL-12p40 (Fig. 5B) in a dose-dependent manner compared to the OVA-treated group. The 300 mg/kg AHRE-treated group almost completely inhibited the expression of these cytokines (Fig. 5A-e). Although the expression of IL-12p35 was affected by AHRE, a dose-dependent effect was not observed (Fig. 5C) compared to the OVA-treated group. And the activation of transcription factors that regulate T-bet and induce GATA-3 were analyzed (Fig. S1). In order to activate the Th1 cell and Th2 cell transcription factors, T-bet and GATA-3 exist in the nucleus, whereas, during the state of

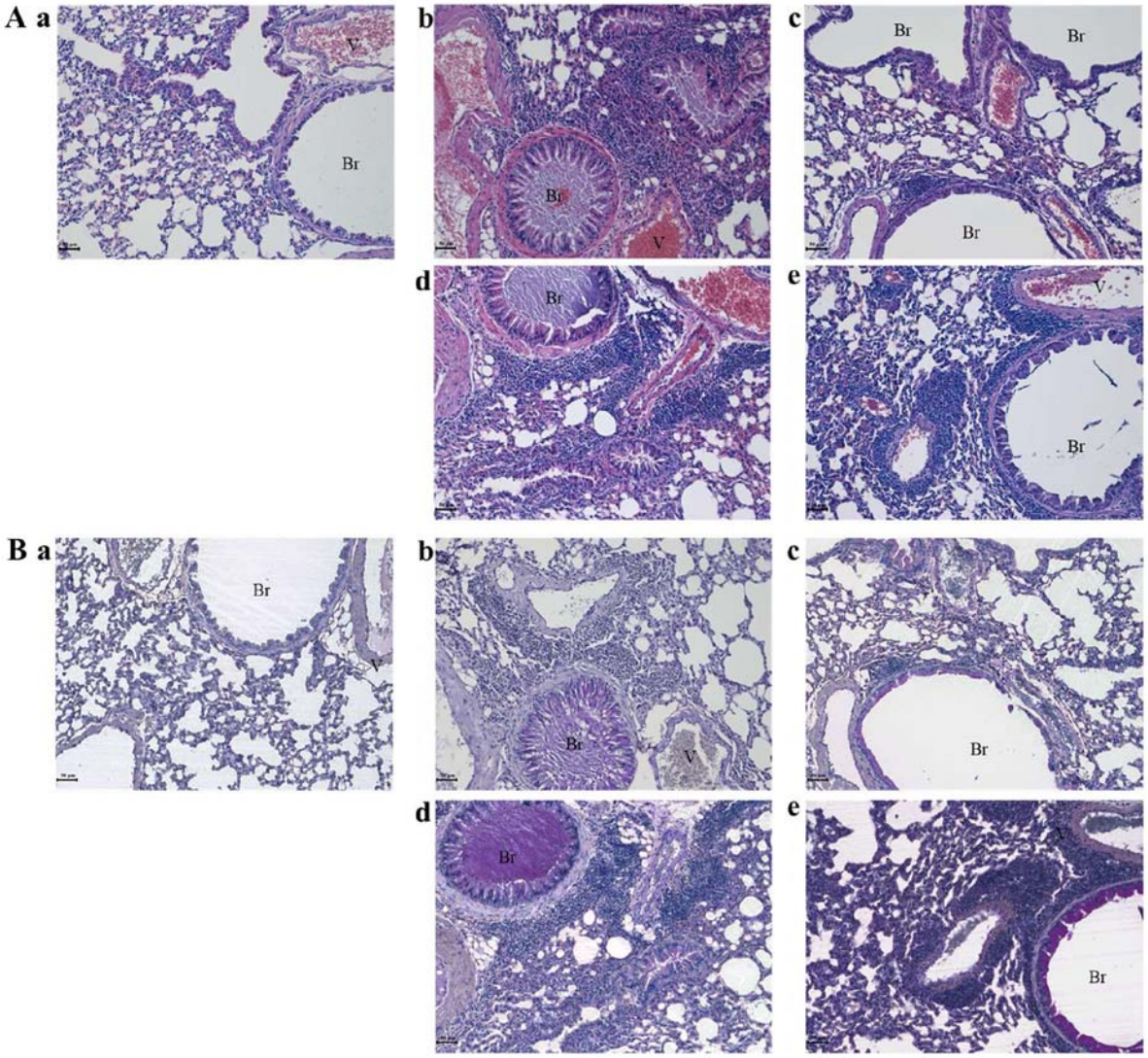

Figure 3. AHRE ameliorates OVA-induced asthmatic changes in the lungs in a dose-dependent manner. AHRE ameliorated the asthmatic changes in mouse lungs, such as mucus hypersecretion, eosinophil infiltration near the bronchioles and vessels, epithelial cell hyperplasia, and goblet cell hyperplasia as observed by (A) hematoxylin and eosin staining. (B) Periodic acid-Schiff staining confirmed a protective effect by AHRE against mucus hypersecretion (scale bar, 50 μm). Br, bronchiole; V, vessel; a, vehicle control; b, asthma induction; c, dexamethasone; d, AHRE 30 mg/kg/day; and e, AHRE 300 mg/kg/day. AHRE, *Allium hookeri* root extract; CON, control; OVA, ovalbumin; DEX, dexamethasone.

inactivation, they localize in the cytoplasm. The distribution of T-bet in the control, dexamethasone group, and 300 mg/kg AHRE group were detected in the cytoplasm (Fig. S1B-a, -c, and-d), whereas, in the OVA-treated group it was in the nucleus (Fig. S1B-b). The distribution of GATA-3 was similar to that of T-bet (Fig. 1SC-a to -d). AHRE inhibited T-bet and GATA-3 activation similar to that by dexamethasone (Fig. S1).

AHRE downregulates the expression of Th2-related cytokines (IL-4, IL-5, and IL-13). The change in the expression of the Th2-related cytokines was similar to that of the Th1-related ones (Fig. 6). Although IL-4, IL-5, and IL-13 were barely detected in the control group (Fig. 6A-a, B-a, and C-a), OVA increased the expression of these cytokines (Fig. 6A-b, B-b, and C-b) compared to the control, and dexamethasone suppressed all of them (Fig. 6A-c, B-c, and C-c) compared to the OVA-treated group. AHRE suppressed the expression of Th2-related cytokines such as IL-4 (Fig. 6A), IL-5 (Fig. 6B), and IL-13 (Fig. 6C) in a dose-dependent manner compared to the OVA-treated group. Especially, 300 mg/kg AHRE effectively suppressed the expression of IL-4 (Fig. 6A-e) and IL-5 (Fig. 6B-e) compared to the OVA-treated group, similar to that by dexamethasone.

In addition to the changes of Th1-/Th2-related cytokines the changes of proinflammatory cytokines were analyzed. The effects of AHRE on proinflammatory cytokines, such as TNF-α and IL-6 that play important roles in the immune regulation of asthma were studied (Fig. S2). TNF-α was overexpressed by OVA (Fig. S2A-b), but significantly suppressed by dexamethasone (Fig. S2A-c). The expression of TNF-α was decreased by AHRE in a dose-dependent manner. Especially, 300 mg/kg AHRE suppressed the expression of TNF-α (Fig. S2A-e) to a level similar to that achieved by dexamethasone treatment. IL-6 expression was increased by OVA treatment (Fig. S2B-b), whereas, dexamethasone inhibited it (Fig. S2B-c). Although the expression of IL-6 was suppressed by AHRE, a dose-dependent effect was not observed.

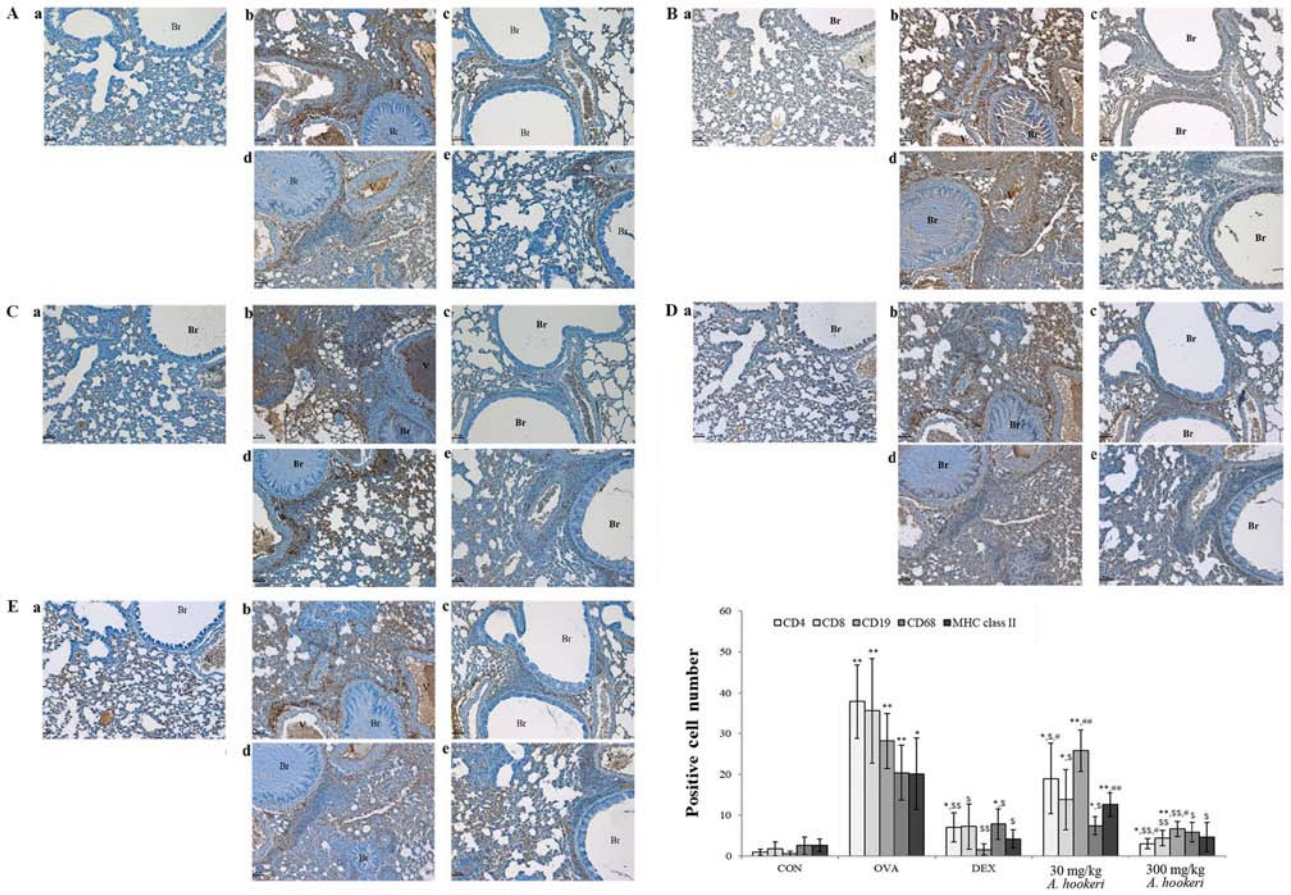

Figure 4. AHRE suppresses the OVA-induced increase in the expression of (A) $CD4^+$ Th cells, (B) $CD8^+$ cytotoxic T cells, (C) $CD19^+$ B cells, (D) $CD68^+$ cells, and (E) MHC class II^+ molecules in a dose-dependent manner. The immune positive cells were counted in five randomly selected non-overlapping fields (x200, magnification) from three separately immunostained lung sections per mouse (scale bar, 50 μm); a, vehicle control; b, asthma induction; c, dexamethasone; d, AHRE 30 mg/kg/day; and e, AHRE 300 mg/kg/day. *P<0.05 compared with the control, **P<0.001 compared with the control, $^{\$}$P<0.05 compared with asthma induction (OVA-treated group), $^{\$\$}$P<0.001 compared with asthma induction (OVA-treated group), $^{\#}$P<0.05 compared with dexamethasone, and $^{\#\#}$P<0.001 compared with dexamethasone. AHRE, *Allium hookeri* root extract; CON, control; OVA, ovalbumin; DEX, dexamethasone.

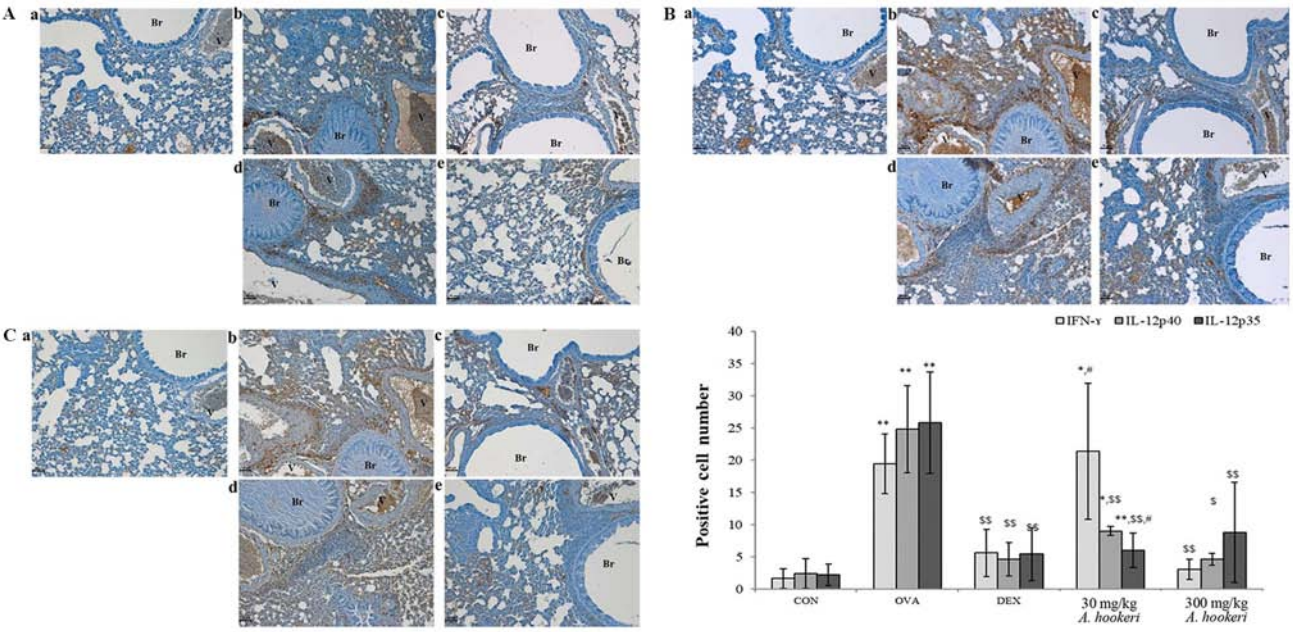

Figure 5. AHRE reduces the expression of (A) IFN-γ, and (B) IL-12p40 but not (C) IL-12p35 in the lungs. The immune-positive cells were counted in five randomly selected non-overlapping fields (x200, magnification) from three separately immunostained lung sections per mouse (scale bar, 50 μm); a, vehicle control; b, asthma induction; c, dexamethasone; d, AHRE 30 mg/kg/day; and e, AHRE 300 mg/kg/day. *P<0.05 compared with the control; **P<0.001 compared with the control; $^{\$}$P<0.05 compared with asthma induction (OVA-treated group); $^{\$\$}$P<0.001 compared with asthma induction (OVA-treated group); and $^{\#}$P<0.05 compared with dexamethasone. AHRE, *Allium hookeri* root extract; CON, control; OVA, ovalbumin; DEX, dexamethasone.

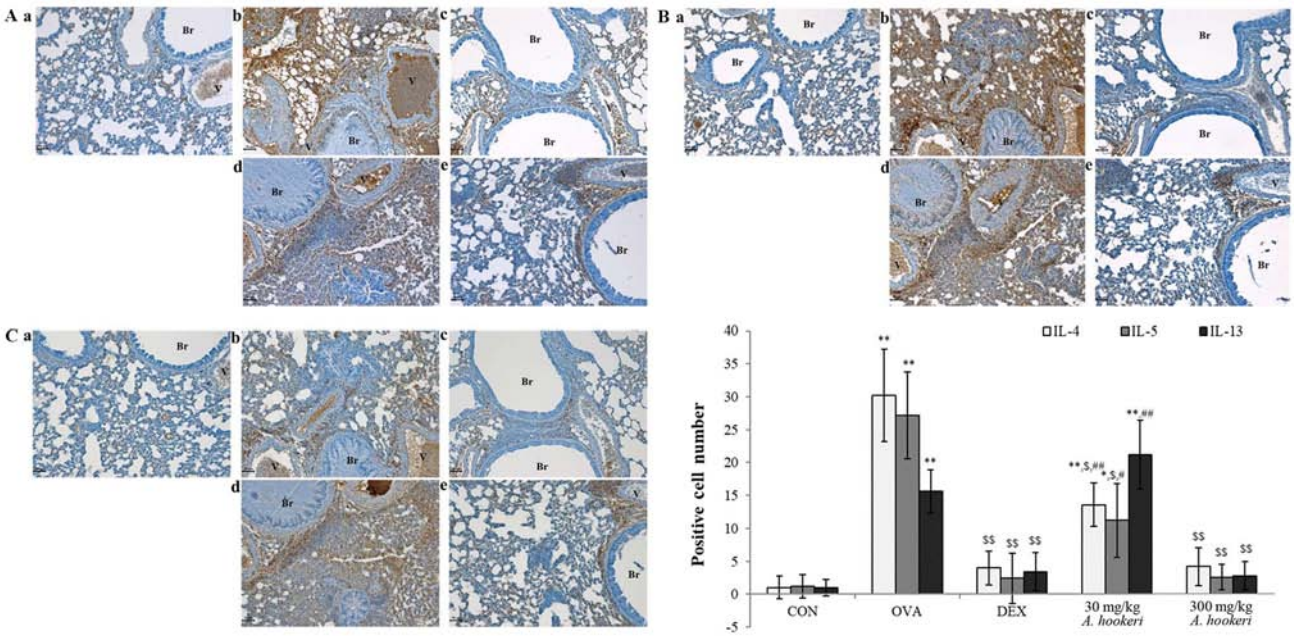

Figure 6. AHRE significantly inhibits the expression of (A) IL-4, (B) IL-5, and (C) IL-13 in the lungs in a dose-dependent manner. The immune-positive cells were counted in five randomly selected non-overlapping fields (x200, magnification) from three separately immunostained lung sections per mouse (scale bar, 50 μm); a, vehicle control; b, asthma induction; c, dexamethasone; d, AHRE 30 mg/kg/day; and e, AHRE 300 mg/kg/day. *P<0.05 compared with the control; **P<0.001 compared with the control; $^{\$}$P<0.05 compared with asthma induction (OVA-treated group); $^{\$\$}$P<0.001 compared with asthma induction (OVA-treated group); $^{\#}$P<0.05 compared with dexamethasone; and $^{\#\#}$P<0.001 compared with dexamethasone. AHRE, *Allium hookeri* root extract; CON, control; OVA, ovalbumin; DEX, dexamethasone.

Discussion

Asthma is a type 1 hypersensitivity reaction, which is caused by exposure to specific allergens and is related to the activation of IgE (29). The allergens can be classified into two main categories according to IgE dependence (31). One of the categories stimulates IgE production and includes pollens, animal dander, house-dust-mite particles, venom, and foods, such as peanuts, fish, milk, and egg. The other category is not related to IgE and includes reactions such as allergic contact dermatitis. The OVA-induced asthma model is related to IgE production. This model depicts not only a surge in the immune cell populations and inflammatory mediators but also an imbalance between the Th1- and Th2-related factors including proinflammatory cytokines (22).

The expression of macrophages (CD68^{+} cells) has been revealed to be elevated (32) in asthma. The macrophages in the respiratory system can be divided into M1 or M2 subsets according to their role and cytokine profile (33). The M1 subset is mainly related to anti-inflammatory effects and releases IL-12 (34), while the macrophage M2 subset regulates inflammation and produces TNF-α (35). The alveolar macrophage contains both the M1 and M2 subsets of macrophages. The present results revealed that AHRE suppressed CD68^{+} cell expression (M2 subset).

The MHC gene family is subdivided into class I and II in humans and mice. The MHC class II molecules initiate immune reactions related to CD4^{+} cells (Th cells), whereas, the MHC class I molecules initiate reactions related to CD8^{+} cells (cytotoxic T cells) (36-38). The MHC class II molecules have important roles in the regulation of Th cell and cytotoxic T-cell activation, and Th cell modulation (balance between Th1 cells and Th2 cells) is important in asthma. AHRE may control asthma through the downregulation of CD4^{+} cells (Fig. 4E).

Asthma is caused by various cytokines and not by any single factor (11). Its pathogenic mechanism was reported to be due to the imbalance of Th1- and Th2-related factors. IL-4 and IL-13 play key roles in asthma induction (39) and are responsible for the typical morphological changes such as mucus hypersecretion, epithelial cell hyperplasia, goblet cell hyperplasia, eosinophil infiltration, as well as the physiological changes such as B cell activation in the pulmonary system associated with asthma. AHRE suppressed the expression of IL-4 and IL-13 in a dose-dependent manner. IL-4 expression was significantly downregulated to a level similar to that of the dexamethasone-treated group. Although AHRE suppressed most of the asthma-related cytokines, such as IFN-γ, IL-12p40, IL-4, IL-5, and IL-13, the mode of action may be related to T-bet (Th1 cell transcription factor), GATA-3 (Th2 cell transcription factor), CD68^{+} cells (TNF-α producer), and T-cell recognition involving MHC class II^{+} molecules and CD68.

Recently, Jang *et al* (39) reported that AHRE suppressed inflammation in the macrophage cells, which was induced by lipopolysaccharide through nuclear factor (NF)-κB and cyclooxygenase (COX)-2 downregulation. Based on these results, it could be inferred that the hyperactivated immune cells, such as B cells, and T cells, may be decreased by AHRE owing to its downregulatory effects on NF-κB and COX-2.

Inhaled corticosteroids are extensively used for the treatment of asthma. However, they exhibit numerous adverse effects, such as growth suppression in children (20) catatonia, decreased concentration, agitation, insomnia, and abnormal

behaviors (21). Several efforts have been undertaken to identify new drugs for treating asthma, which have no or less adverse effects. In the present study, AHRE ameliorated asthma-related changes as evidenced by the Th1-/Th2-related inflammatory cytokine levels and histopathological changes. AHRE was revealed to suppress T-cell recognition, which is an important step in asthma induction, by the downregulation of $CD68^+$ and MHC class II^+ molecules.

In previous studies, we have identified ferulic acid, linoleic acid, and cinnamic acid from the roots of AHRE (40,41). Major compounds were identified as linoleic acid ethyl ester (8.02%), hexacosane (7.5%). In addition, minor compounds were identified as ferulic acid (0.17%) and cinnamic acid (1.99%). It was reported that ferulic acid controls Th2-response by decreasing lung inflammation, eosinophil infiltration, mucus production, and serum IgE level. It also decreases chemokines and cytokines, such as CCL5, CCL11, CCL20, IL-4, IL-5, IL-13, IL-25, and IL-33 in the lungs. Linoleic acid is one of the major constituents of AHRE. We previously reported that the anti-inflammatory effects of AHRE were due to the antioxidant and anti-inflammatory activities of linoleic acid (41). Xu *et al* demonstrated that cinnamic acid suppressed the production of pro-inflammatory cytokines and expression of NLRP3, caspase-1, and IL-1β mRNA proteins, and also reversed the lipopolysaccharide-induced histopathological changes in the lungs and the spleen (42).

From the results of the present study, it can be concluded that the active compounds, such as ferulic acid, cinnamic acid, and linoleic acid present in AHRE demonstrated effective anti-asthmatic activities. AHRE has been used for culinary purposes for a long time and is confirmed to be safe. Hence, AHRE can be used as a natural source drug for treating asthma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SHB and JHS revised the manuscript and conducted the animal study and analysis, CSB analyzed the samples, BK read the histopathological results, and SSC and DHP statistically analyzed the data and revised manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All the experiments were approved by the Institutional Animal Care and Use Committee at Dongshin University, Naju, Korea (approval no. 2014-08-03).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figure S1. AHRE significantly regulates the activation of T- and B-cell transcription factors. (A) The blue spots indicate the nuclei, (B) the green spots indicate the Th1 cell transcription factor T-bet, and (C) the red ones indicate the Th2 cell transcription factor GATA-3. AHRE augmented the translocations of both T-bet and GATA-3 from the nucleus to the cytoplasm, when compared with the results of the OVA-induced asthma group. (D) The images were merged with 3 images including the location of (A) the nucleus, that of (B) T-bet, and that of (C) GATA-3; a, vehicle control; b, asthma induction; c, dexamethasone; and d, AHRE 300 mg/kg/day; (scale bar, 50 μm). AHRE, *Allium hookeri* root extract; OVA, ovalbumin.

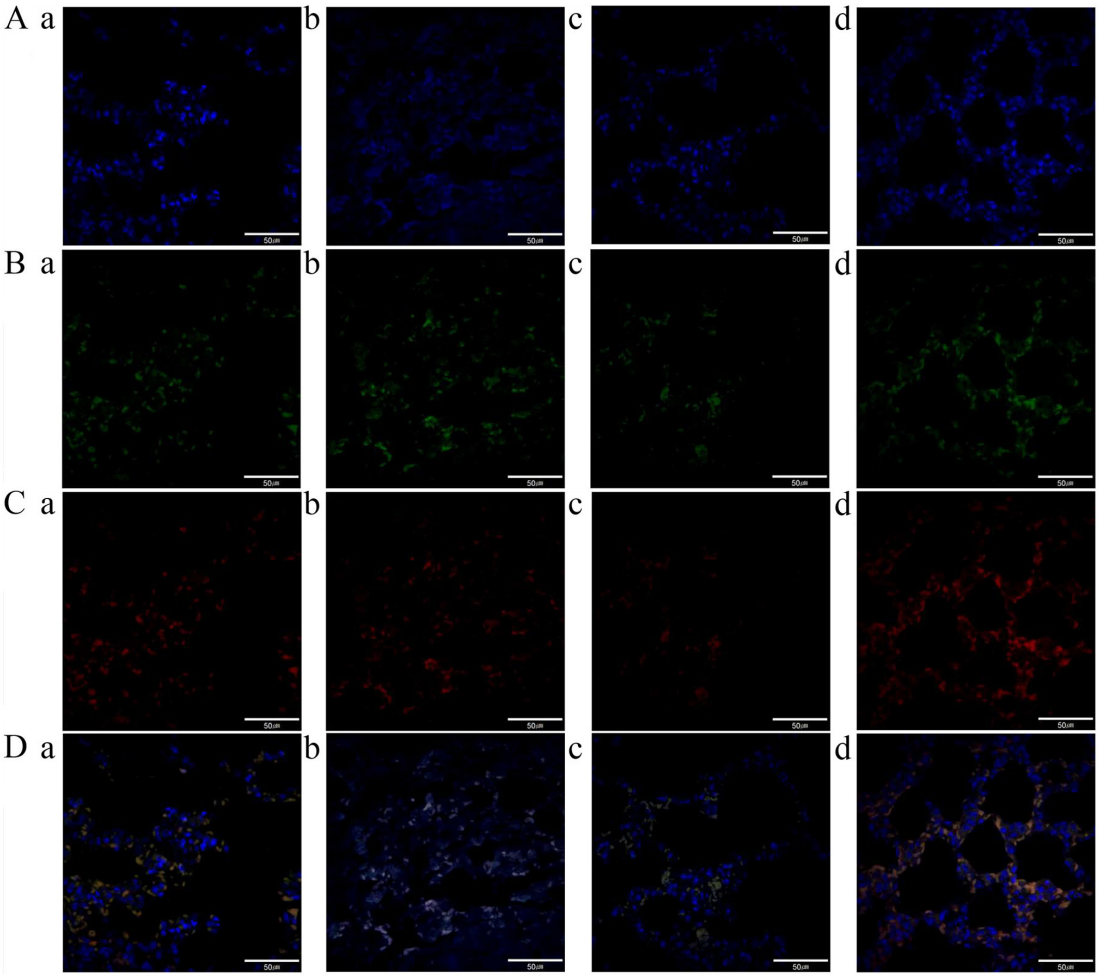
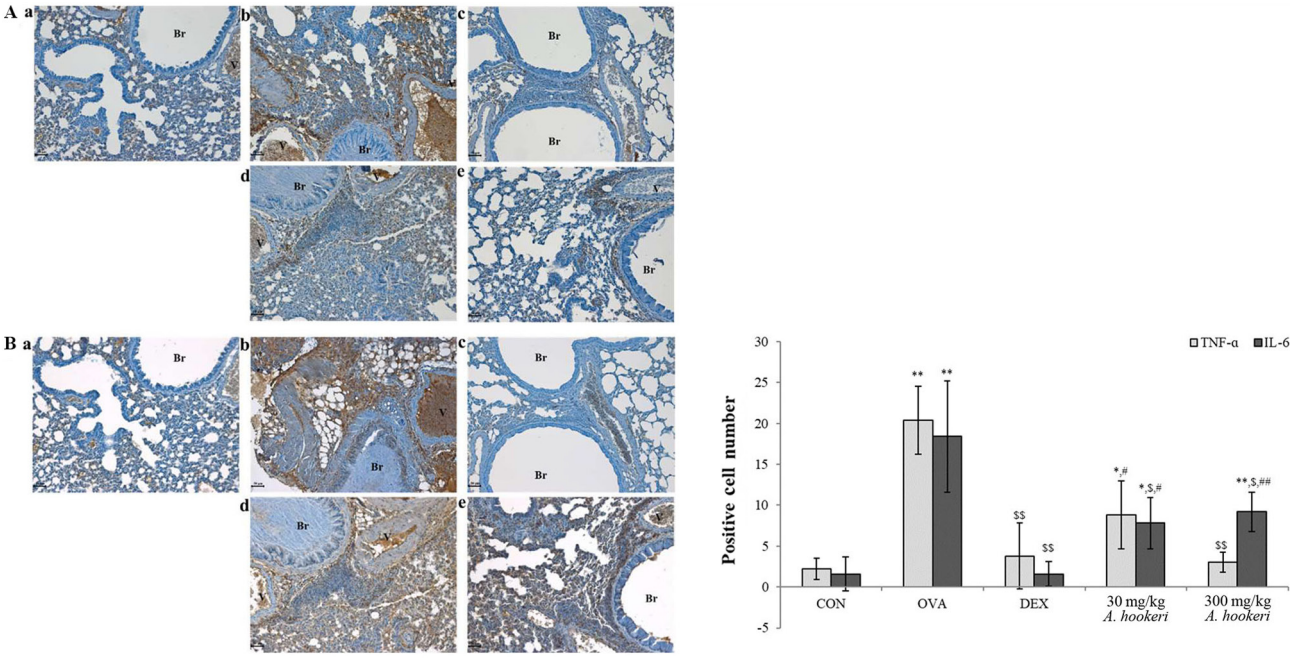
Figure S2. AHRE significantly downregulates the expression of (A) TNF-α proinflammatory cytokine but not that of (B) IL-6. The immune-positive cells were counted in five randomly selected non-overlapping fields (x200, magnification) from three separately immunostained lung sections per mouse (scale bar, 50 μm); a, vehicle control; b, asthma induction; c, dexamethasone; d, AHRE 30 mg/kg/day; and e, AHRE 300 mg/kg/day. *P<0.05 compared to the control; **P<0.001 compared with the control; $^{\$}$P<0.05 compared with asthma induction (OVA-treated group); $^{\$\$}$P<0.001 compared with asthma induction (OVA-treated group); $^{\#}$P<0.05 compared with dexamethasone; and $^{\#\#}$P<0.001 compared with dexamethasone. AHRE, *Allium hookeri* root extract; CON, control; OVA, ovalbumin; DEX, dexamethasone.