

Original Article

Angelica gigas extract ameliorates allergic rhinitis in an ovalbumin-induced mouse model by inhibiting Th2 cell activation

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ABSTRACT

Background: Allergic rhinitis (AR) is a well-documented type 2 helper T (Th2) cell-mediated allergic disease that is accompanied by symptoms such as nasal rubbing, sneezing, itching, and rhinorrhea. *Angelica gigas* (AG) is traditional oriental medicine, and its dried root is widely used for the treatment of anemia, as a sedative, and as a blood tonic.

Purpose: The effects of AG on allergic diseases including AR are currently unclear; therefore, we aimed to investigate the effects of AG extract (AG-Ex) in ameliorating AR.

Study design/methods: The cytotoxicity of AG-Ex was analyzed by EZ-Cytox or MTS assay in splenocytes, differentiated Th2 cells, and human nasal epithelial cells (HNEpC). The changes of Th2 cells activation were determined by the secretion levels of cytokines and chemokines using cytometric bead array in splenocytes and differentiated Th2 cells. The expression levels of eotaxin-3 and periostin were analyzed using an ELISA. AR was induced by ovalbumin in BALB/c mice and the ameliorating effects of AG-Ex were assessed by their clinical symptoms.

Results: The secretion of Th2 cytokines such as IL-4, IL-5, and IL-13 was inhibited by the AG-Ex treatment in the splenocytes and differentiated Th2 cells. The treatment also suppressed allergic responses including the secretion of eotaxin-3 and periostin in human nasal epithelial cells (HNEpC). Moreover, the administration of AG-Ex to the OVA-induced AR mice improved their clinical symptoms, including behavioral tests, immune cell counts, histopathological analysis, and changes in serum parameters.

Conclusion: The results of this study suggest that AG-Ex ameliorates AR by inhibiting Th2 cell activation and could thus be utilized as a treatment for Th2-mediated allergic diseases in the future.

Introduction

Allergic rhinitis (AR) is a chronic hypersensitivity inflammatory disease characterized by high levels of type 2 helper T cell (Th2) cytokines, immunoglobulin E (IgE), and histamine, and the recruitment of immune cells including eosinophils, mast cells, basophils, and neutrophils. The characteristic symptoms of AR are nasal rubbing, sneezing,

itching, congestion, lacrimation, and rhinorrhea. Approximately 400 million people worldwide currently suffer from AR, and the numbers are increasing (Fröhlich et al., 2017). The treatments for AR such as anti-histamines and intranasal corticosteroids are good options for most allergic rhinitis patients, but they have limitations such as dependence on drugs, maintenance of strict adherence, and problems due to various side effects during long-term administration (Shinee et al., 2019).

Abbreviations: AG, *Angelica gigas*; AG-Ex, AG extract; APC, antigen-presenting cells; AR, allergic rhinitis; CCR3, CC chemokine receptor 3; Con A, concanavalin A; DCs, dendritic cells; DEX, dexamethasone; FBS, fetal bovine serum; H&E, hematoxylin & eosin; HNEpC, human nasal epithelial cells; HPLC, high-performance liquid chromatography; IgE, immunoglobulin E; IL, interleukin-; IP-10, interferon- γ -induced protein 10; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; NALF, nasal lavage fluid; OVA, ovalbumin; PAS, periodic acid schiff; PDA, photodiode array; RANTES, regulated on activation, normal T cell expressed and secreted; RPMI, Roswell Park Memorial Institute; SD, standard deviation; SPF, specific pathogen-free; STAT6, signaling transducer and activator of transcription 6; TCRs, T cell receptors; TFA, trifluoroacetic acid; Th2, type 2 helper T; WST, water soluble tetrazolium salt.

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Despite extensive research on this condition, an effective and practical treatment has not yet been developed.

Chronic exposure or re-exposure to external allergens, which are presented to T cells by antigen-presenting cells (APC) such as dendritic cells (DCs) and macrophages, leads to the activation of allergen-specific Th2 cells. Subsequently, this stimulates an increase in the production of Th2 cytokines (interleukin-4 (IL-4), IL-5, IL-13) (Scadding, 2014) and antigen-specific IgE production, resulting in hypersensitivity inflammation to the nasal mucosa (Eifan and Durham, 2016; Li et al., 2016). During these processes, the GATA3 signaling pathway via transcription 6 (STAT6) activation mediated by the T cell receptor (TCR) is essential for Th2 cell differentiation and maturation from immature CD4⁺ T cells. (Zheng et al., 1997; Pai et al., 2004; Goenka and Kaplan, 2011). IL-4 and IL-13 promote antigen-specific IgE production in B cells, and the IgE then binds to high-affinity IgE receptors (FcεR) expressed on the surfaces of the mast cells and basophils (Eifan and Durham, 2016; Shamji and Durham, 2017; Muluk et al., 2019). Consequently, mast cells and basophils secrete histamine, leukotriene, and prostaglandin D2 (Muluk et al., 2019). And IL-5 is well known as an important mediator for inflammation through the recruitment of eosinophils (Matucci et al., 2019; Nagase et al., 2020). Thus, IL-4, IL-5, and IL-13 are the major cytokines produced by Th2 cells in AR pathogenesis.

In addition to the Th2 cytokine, periostin is an important mediator in chronic allergic disease and has been reported to play a significant role in tissue remodeling in allergic inflammation (Ozceker et al., 2019; Chung et al., 2020; Hoshino et al., 2020). Periostin is secreted from a variety of nasal mucosal cells including epithelial cells and fibroblasts stimulated by IL-4 and IL-13 (Ishida et al., 2012). Regulated on Activation, Normal T cell Expressed and Secreted (RANTES; CCL5) and eotaxin-3 (CCL26) are known to promote the production of antigen-specific IgE by B cells and the migration of mast cells to the nasal epithelium, (Burks et al., 2019). RANTES and eotaxin-3 are mainly found in the nasal mucosa and runny noses of AR patients and are associated with mucosal edema and disease severity (Burks et al., 2019; Berghi et al., 2020). In several studies evaluating immune-modulatory drugs related to interferon-γ-induced protein 10 (IP-10; CCL10), IP-10 was found to be expressed at high concentrations in the nasal mucosa and runny noses of AR patients, suggesting that it was a potential biomarker for AR diagnosis (Mazzi and Fallahi, 2017).

Angelica gigas (AG), called dang-gui in Korea, has traditionally been used in oriental medicine as a treatment for anemia and as a sedative and blood tonic (Yan et al., 2004). Recent studies reported the therapeutic effect of AG and its components on allergic inflammation (Lee et al., 2017; Reddy et al., 2017; Ok et al., 2018; Shehzad et al., 2018). However, the effects of AG on AR have not yet been investigated. Therefore, we have investigated its biological activity with regard to the regulation of Th2 cell activation and its therapeutic activity using an OVA-induced AR mouse model.

Materials and methods

Preparation of AG extract (AG-Ex)

Dried AG was purchased from Omniherb (Yeongcheon, Korea), and voucher specimen has been deposited in the Herbarium of Korea Institute of Oriental Medicine (KIOM). AG (1 kg) was extracted with 70% ethanol (10 l) at 80 °C for 3 h using a heat-reflux extractor. The extracted solution was filtered and evaporated under reduced pressure using a rotary evaporator (N-1200A, Eyela, Tokyo, Japan) at 50 °C, and then freeze-dried using a freeze dryer (FDU-2100, Eyela, Tokyo, Japan) at −80 °C for 72 h to produce an extract powder (393.67 g; yield 39.37%).

High-performance liquid chromatography (HPLC) analysis

Four standard compounds (purity ≥ 98.0%) of the AG-Ex, chlorogenic acid (ChemFaces Biochemical, Wuhan, China), nodakenin and

decursin (Sunny Biotech, Shanghai, China), and decursinol angelate (Biopurify Phytochemicals, Chengdu, China), were used for simultaneous analysis using HPLC. HPLC grade acetonitrile and water were used as the mobile phases and were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA), and the reagent trifluoroacetic acid (TFA) was obtained from Sigma Aldrich. For quantitative analysis, the AG-Ex samples were dissolved in methanol to a concentration of 2 mg/ml and filtered through a syringe filter (0.45 μm pore size). The stock solutions (1 mg/ml) of four standard compounds were mixed and diluted with methanol to obtain a series of standard mixtures. A Waters Alliance e2695 system (Waters Corp., Milford, MA, USA) equipped with a photodiode array (PDA) detector (#2998; Waters Corp.) was used to quantitatively analyze the four compounds in the AG-Ex samples. The acquired data were processed using Empower software (version 3; Waters Corp.). The chromatographic separation of the four compounds was conducted with a gradient solvent system of 0.1% (v/v) aqueous TFA (A) and acetonitrile (B) at 30 °C using a Sunfire C18 analytical column (250 × 4.6 mm, 5 μm, Waters). The gradient conditions were set as follows: 12–25% B (0–15 min), 25–58% B (15–20 min), 58–60% B (20–35 min), 60–100% B (35–40 min), and 100% B (40–50 min). The flow rate and injection volume were 1 ml/min and 10 μl, respectively.

Precision, accuracy, and recovery

To assess the precision and accuracy of the established HPLC methods, intra- and inter-day variations were measured using the mixed standard solutions with low, middle, and high concentration levels of standard compounds. For intra-day precision and accuracy measurements, mixed standard solutions were analyzed five times a day and these analyzes were repeated for three consecutive days to evaluate the inter-day precision and accuracy. Precision was expressed as intra- and inter-day relative standard deviation (RSD) (%) and accuracy was presented as a percentage of the observed concentration for the fortified concentration. To evaluate the repeatability, AG-Ex was measured six times and the RSD (%) of the retention times and peak areas for the four compounds were used. The recovery of the four compounds was determined by adding standard solutions at three concentration levels (low, middle, and high) to 10 ml of AG-Ex (0.5 mg/ml). The recovery was carried out five times at each level and calculated according to the following equation: Recovery (%) = (Found concentration – Original concentration)/spiked concentration × 100.

Cell culture of primary splenocytes

The animal experiments for the isolation of primary splenocytes (approval number: 20–022; March 23, 2020) was approved by the IACUC of Korea Institute of Oriental Medicine (KIOM). BALB/c mice (5 weeks old, male) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The splenocytes (5 × 10⁶ cells/ml) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin; Gibco). The splenocytes were treated with the AG-Ex (10, 30, and 100 μg/ml) and stimulated with 2.5 μg/ml concanavalin A (Con A; Sigma Aldrich) for 24 h.

Differentiation and culture of Th2 cells

The animal experiments for the differentiation of Th2 cells from naïve CD4⁺ T cells (approval number: 20–070; September 23, 2020) was approved by the IACUC of KIOM. Naïve CD4⁺ T cells were isolated from the splenocytes using a Naïve CD4⁺ T Cell Isolation Kit (MACS; Miltenyi Biotec Inc., Auburn, CA, USA) and differentiated to Th2 cells using CellXVivo Mouse Th2 Cell Differentiation Kit (R&D Systems, Minneapolis, MN, USA). The differentiated Th2 cells (5 × 10⁶ cells/ml) were incubated with the AG-Ex (10, 30, and 100 μg/ml) and stimulated with a

cell activation cocktail (Tocris Bioscience, Bristol, UK) for 24 h.

Cell culture of human nasal epithelial cells (HNEpC)

HNEpC were obtained from PromoCell (Heidelberg, Germany) and maintained in Airway Epithelia Cell Growth Medium (PromoCell). The cells were treated with the AG-Ex (3, 10, and 30 µg/ml) or 1 nM of the JAK inhibitor (Cat. No. 11,598; Cayman Chemical, Ann Arbor, MI, USA) for 24 h, after stimulation of the IL-4 (7.5 ng/ml; Peprotech) and IL-13 (7.5 ng/ml; Peprotech).

Viability analysis of primary cells

The viability of splenocytes and differentiated Th2 cells was determined by water soluble tetrazolium salt (WST) assay using EZ-Cytox Cell Viability Assay Kit (Daeil Lab Service, Seoul, Korea) (Kim et al., 2018), according to the manufacturer's instructions. And the viability of HNEpC was investigated by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) (Ji et al., 2019), according to the manufacturer's instructions. The viability of the cells was compared with un-stimulated control or stimulated control.

Analysis of chemokines and cytokine secretions

The culture supernatants of splenocytes or differentiated Th2 cells were harvested after treatment of the AG-Ex for 24 h. The secretion levels of the chemokines (RANTES and IP-10) and Th cytokines (IL-4, IL-5, IL-13, IL-10) were investigated using a LEGENDplex™ Mouse Proinflammatory Chemokine Panel (BioLegend, Cat. No. 740,451) and Mouse Th Cytokine Panel (BioLegend, Cat. No. 741,044), according to the manufacturer's instructions.

Analysis of eotaxin-3 and periostin in HNEpC

The culture supernatants of HNEpC were collected after treatment of the AG-Ex for 24 h. The expression levels of eotaxin-3 and periostin were analyzed using an ELISA kit (R&D systems), according to the manufacturer's protocols.

Flowcytometry analysis

The differentiated Th2 cells (1×10^6 cells/test) were resuspended with staining buffer (BD Bioscience, Franklin Lakes, NJ, USA) and stained with the indicated antibodies; anti-CD3-FITC (Cat. No. 561,798; 0.5 µg/test), anti-CD4-BB700 (Cat. No. 566,407; 0.5 µg/test), anti-GATA3-BV421 (Cat. No. 563,349; 1 µg/test) were purchased from BD Bioscience. The population of the Th2 cells was analyzed by LSRFortessa™ X-20 flow cytometry (BD Biosciences) and FlowJo v. 10 software (FlowJo, Ashland, OR, USA).

Analysis of STAT6 activity

HEK-Blue™ IL-4/IL-13 cells were obtained from InvivoGen (Cat. No. hkb-il413, San Diego, CA, USA). The cells (5×10^4 cells/well) were plated in 48-well culture plates and stimulated with 1 ng/ml of IL-4 (Cat. No. 200-04; Peprotech, Rocky Hill, NJ, USA) for 24 h, after treatment with the AG-Ex (10, 30, and 100 µg/ml) or 100 nM of the JAK inhibitor (Cayman Chemical). Then, the 20 µl of culture supernatant was harvested and mixed with 180 µl of the QUANTI-Blue™ Solution (InvivoGen, Cat. No. rep-qbs), and it was incubated for 1 h at 37 °C. The STAT6 activity was determined by measuring optical density (OD) at 620 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Mouse model of OVA-induced AR

The animal experiment for OVA-induced AR mouse model (approval number: CNU IACUC-YB-R-2020-98, November 9, 2020) was approved by the IACUC of Chonnam National University. Animal experiment was conducted as per a previously described method with slight modification (Bui et al., 2019). BALB/c mice (6 weeks old, female, $n = 8$ /each group) were obtained from Samtacko (Osan, Korea). The mice were sensitized with 200 µl of PBS (containing 2 mg of aluminum hydroxide (Sigma Aldrich) and 50 µg of OVA (Sigma Aldrich)) by intraperitoneal (i.p.) injection at day-1, day-7, and day-14. From day 21 to day 27, the mice were exposed to 20 µl of PBS (containing 400 µg of OVA) via direct intranasal (i.n.) injections, 4 times every other day. The AG-Ex was orally administered to the mice every day at a dose of 30 or 100 mg/kg/mice from day-21 to day-27. The OVA-induced control and positive control mice were orally administered saline and 1 mg/kg of dexamethasone (DEX), respectively. At day-27, the frequencies of rubbing and sneezing behavior were counted for 5 min, and the mice were sacrificed at day-28 after anesthesia with alfaxan (Jurox Pty Ltd, Ruthersford, NSW, Australia).

Histopathological analysis of the nasal tissue

After fixation and decalcification, the nasal tissue was embedded and sectioned to 5 µm, and stained with Hematoxylin & Eosin (H&E) staining solution (Sigma Aldrich) and a Periodic Acid Schiff (PAS) staining kit (Sigma Aldrich). The nasal septum was captured using a light microscope (Olympus Corporation, Tokyo, Japan) at $\times 400$ magnification.

Collection of nasal lavage fluid (NALF)

The NALF was collected by washing from the nasal cavity to the nasopharynx with 10 ml of PBS after creating an incision in the bronchial tubes, and the total cells were measured using a Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA). Eosinophils, macrophages, neutrophils, and lymphocytes were counted using a light microscope ($\times 400$ magnification) after cytopspin (Hanil, Seoul, Korea) and staining with a Diff-Quik stain kit (Sysmex, Chuo-ku, Kobe, Japan), according to the manufacturer's instructions.

Analysis of the serum parameters

After anesthetized the mice with alfaxan, blood was collected from the abdominal vein, and centrifuged at 3000 rpm for 15 min to collect the serum. The serum levels of parameters were measured using an ELISA kit of OVA-specific IgE (Cayman Chemical), histamine (Enzo Life Science, Farmingdale, NY, USA), and IL-13 (R&D system), according to the manufacturer's instructions.

Statistical analysis

The data are presented as mean $\pm$ standard deviation (SD), and statistical analyses were performed using GraphPad Prism version 7 (GraphPad. Software, Inc., La Jolla, CA, USA). Statistical differences between the groups were analyzed by one-way analysis of variance, followed by Tukey's multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

Results

Simultaneous quantification of four compounds in the AG-Ex

The HPLC method was optimized for the simultaneous analysis of four compounds, chlorogenic acid, nodakenin, decursin, and decursinol angelate, in the AG-Ex. The UV wavelength used for quantitative

analysis was 330 nm. The four compounds were successfully separated within 35 min using mobile phases consisting of 0.1% (v/v) aqueous TFA and acetonitrile. HPLC chromatograms for the AG-Ex and the standard mixture are presented in Fig. 1a and the chemical structures of the four compounds are shown in Fig. 1b. The calibration curves for the four standard compounds were calculated using the linear relationships between the peak area (y) and concentration (x, $\mu\text{g/ml}$) of the standard solution. The concentration ranges, correlation coefficient values, limits of detection and quantification, and amounts of the four compounds are presented in Table 1.

Precision was presented as the RSD (%) of the concentrations of standard compounds in the mixed standard solutions, and repeated five times at each concentration level (low, middle, and high). The values for the intra- and inter-day precision and accuracy are shown in Table 2. The intra- and inter-day precisions for the four standard compounds were less than 2.03% and the accuracy ranged from 96.41 to 105.29%. The RSD values for the repeatability of retention times and peak areas of the four compounds ranged from 0.06 to 0.18% and 0.51 to 0.67%, respectively (Table 3). The recovery tests of the four compounds were conducted by adding three concentration levels of low, middle, and high concentrations of the standard solution to the original concentration of AG-Ex. The recoveries of the four compounds ranged from 96.45 to 107.14% and the RSD was less than 2.18% (Table 4). These results demonstrate that the established HPLC method was satisfied the precision, accuracy, repeatability, and recovery for the simultaneous analysis of the four compounds.

Inhibitory effect of AG-Ex on the activation of Th2 cells in the splenocytes

The cell viability of the splenocytes was not changed with the 100 $\mu\text{g/ml}$ AG-Ex treatment, but cell toxicity was seen with the 300 $\mu\text{g/ml}$ AG-Ex treatment (Fig. 2a and b). The secretion levels of the chemokines including RANTES and IP-10 were significantly decreased by the 100 $\mu\text{g/ml}$ AG-Ex treatment (Fig. 2c and d). The secretion of IL-4, IL-5, IL-13,

and IL-10 was also significantly reduced by the 100 $\mu\text{g/ml}$ AG-Ex treatment (Fig. 2e-h). Moreover, this AG-Ex treatment also suppressed the increased activity of STAT6 by IL-4 stimulation in a dose-dependent manner (Fig. 2i).

Suppressive effect of AG-Ex on the activation of differentiated Th2 cells

The purity of the naïve CD4^+ T cells (CD45^+ CD3^+ CD4^+ CD44^- CD62L^+ cells) was 14.4% in the total cells of the spleen and lymph nodes (Supplementary figure 1a) and this was 85.0% after isolation of the naïve CD4^+ T cells from the spleen and lymph nodes (Supplementary figure 1b). The population of the Th2 cells (CD3^+ CD4^+ GATA3^+ cells) was over 80% after their differentiation from the naïve CD4^+ T cells (Fig. 3a). However, the population of the Th2 cells did not change with the AG-Ex treatment during their differentiation (Supplementary figure 1c). Thus, we investigated the effect of AG-Ex on the activation of Th2 cells after their differentiation. Only Th2 cell cytokines were secreted after stimulation by the activation cocktail in the differentiated Th2 cells (Fig. 3b), and cell toxicity was identified when the AG-Ex treatment was 300 $\mu\text{g/ml}$ (Fig. 3c). The secretion levels of IL-4, IL-5, IL-13, and IL-10 were increased by the stimulation of the activation cocktail in the differentiated Th2 cells, and it was significantly reduced by the treatment of AG-Ex in a dose-dependent manner (Fig. 3d-g). These data suggest that the AG-Ex has a suppressive effect not on the differentiation but the activation of the Th2 cells.

Reducing effect of AG-Ex on the allergic responses in human nasal epithelial cells

The AG-Ex significantly decreased the cell viability of HNEpC at a dose of 100 $\mu\text{g/ml}$ treatment ($80 \pm 2.5\%$, $p < 0.001$) (Fig. 4a). The secretion of eotaxin-3 and periostin was observed with the increasing levels in the HNEpC after stimulation of IL-4 and IL-13 (eotaxin-3, 42.1 ± 3.3 pg/ml, $p < 0.001$; periostin, 831.9 ± 7.8 pg/ml, $p < 0.001$), and it

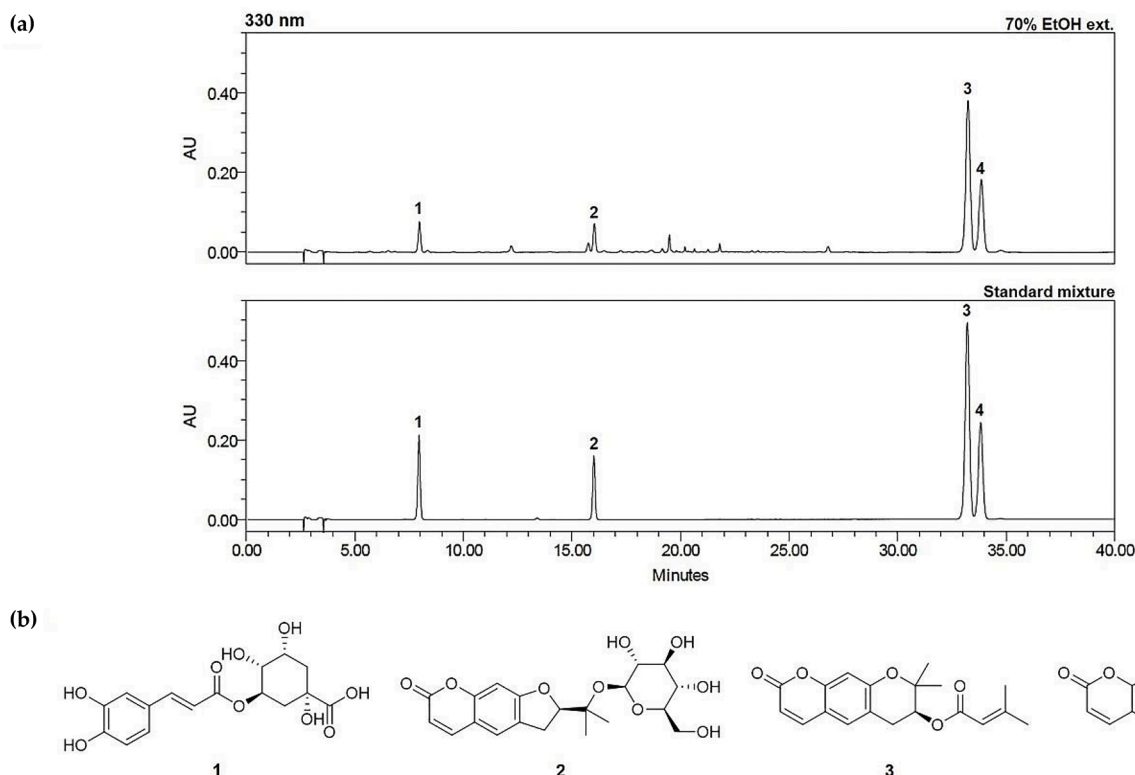


Fig. 1. Simultaneous quantification of four compounds in AG-Ex. (a) HPLC chromatograms for the AG-Ex and standard mixture at 330 nm. (b) Chemical structures of the four standard compounds. 1: chlorogenic acid, 2: nodakenin, 3: decursin, 4: decursinol angelate. AU, absorbance unit.

Table 1

Regression equation, linearity, LOD, LOQ, and content of the four analyzed compounds.

Compound	Linear range (µg/ml)	Regression equation ($y = ax + b$) ^a		r^2	LOD ^b (µg/ml)	LOQ ^c (µg/ml)	Content (mg/g)
		Slope (a)	Intercept (b)				
Chlorogenic acid	3.125–100	32,120	8894.5	1.0000	0.145	0.439	9.128 ± 0.026
Nodakenin	3.125–100	23,961	12,384.0	1.0000	0.180	0.546	11.105 ± 0.075
Decursin	12.5–400	33,865	52,054.0	1.0000	0.457	1.384	77.851 ± 0.069
Decursinol angelate	6.25–200	34,666	14,982.0	1.0000	0.552	1.672	37.608 ± 0.038

LOD, limit of detection; LOQ, limit of quantification; SD, standard deviation.

^a $y = ax + b$, y means peak area and x means concentration (µg/ml).^b LOD: $3.3 \times (\text{SD of the response} / \text{slope of the calibration curve})$.^c LOQ: $10 \times (\text{SD of the response} / \text{slope of the calibration curve})$.**Table 2**

Precision and accuracy of four compounds in AG-Ex.

Compound	Fortified conc. (µg/ml)	Intra-day ($n = 5$)			Inter-day ($n = 5$)		
		Observed conc. (µg/ml)	Precision ^a (%)	Accuracy ^b (%)	Observed conc. (µg/ml)	Precision (%)	Accuracy (%)
Chlorogenic acid	1.5	1.45	0.93	96.56	1.48	2.03	98.36
	3	3.02	0.97	100.80	2.99	0.64	99.81
	6	6.21	0.63	103.48	6.14	0.70	102.30
Nodakenin	1.5	1.47	0.94	97.90	1.48	1.23	98.93
	3	2.93	0.84	97.52	2.93	0.82	97.59
	6	6.13	0.67	102.25	6.07	0.70	101.18
Decursin	10	9.64	1.64	96.41	9.77	1.33	97.66
	20	19.79	0.84	98.93	19.84	0.77	99.21
	40	39.47	0.69	98.67	39.65	0.59	99.12
Decursinol angelate	2.5	2.57	0.59	102.88	2.54	0.91	101.75
	5	5.20	0.88	104.01	5.13	1.17	102.55
	10	10.53	0.42	105.29	10.49	0.52	104.91

^a Precision is expressed as RSD (%) = $(\text{SD}/\text{Mean}) \times 100$.^b Accuracy (%) = $(\text{Observed concentration}/\text{Fortified concentration}) \times 100$.**Table 3**Repeatability of retention times and peak areas for the four analytes ($n = 6$).

Compound	Retention time (min)		Peak area (AU)	
	Mean±SD	RSD (%)	Mean±SD	RSD (%)
Chlorogenic acid	8.09±0.01	0.18	140,025.50±831.47	0.59
Nodakenin	16.08±0.02	0.14	125,305.17±639.34	0.51
Decursin	33.21±0.02	0.06	1,353,385.00±7033.07	0.52
Decursinol angelate	33.81±0.02	0.06	666,242.00±4441.22	0.67

SD, Standard deviation; RSD, Relative standard deviation.

Table 4

Recovery of the four compounds in AG-Ex.

Compound	Original conc. (µg/ml)	Spiked conc. (µg/ml)	Found conc. (µg/ml)	Recovery ^a ± SD (%)	RSD (%)
Chlorogenic acid	4.08	1.5	5.59	100.52±1.12	1.11
		3	7.09	100.30±1.03	1.03
		6	10.31	103.83±0.45	0.43
Nodakenin	4.71	1.5	6.26	103.66±2.05	1.98
		3	7.77	102.15±1.04	1.02
		6	10.90	103.27±0.62	0.60
Decursin	38.44	10	48.26	98.27±1.25	1.27
		20	57.73	96.49±0.64	0.66
		40	81.29	107.14±0.56	0.53
Decursinol angelate	18.75	2.5	21.16	96.45±2.10	2.18
		5	23.69	98.78±2.12	2.14
		10	29.11	103.52±1.34	1.29

^a Recovery (%) = $(\text{Found concentration} - \text{Original concentration})/\text{spiked concentration} \times 100$.

was reduced with the JAK inhibitor treatment (eotaxin-3, 25.5 ± 0.8 pg/ml, $p < 0.001$; periostin, 232.7 ± 7.6 pg/ml, $p < 0.001$). Similar to the JAK inhibitor, treatment of the AG-Ex reduced the increased levels of eotaxin-3 (30 µg/ml; 29.2 ± 1.6 pg/ml, $p < 0.001$) and periostin (30 µg/ml; 570.5 ± 5.2 pg/ml, $p < 0.001$) secretion by stimulation of IL-4 and IL-13 in the HNEPC (Fig. 4b and c).

Ameliorating effect of the AG-Ex on the clinical symptoms of AR in the OVA-induced mouse model

An increased number of rubbing (8.1 ± 0.8 , $p < 0.01$) and sneezing (12.0 ± 1.3 , $p < 0.01$) was found in the OVA-induced control mice compared to the WT mice, but it was decreased with the administration of DEX (rubbing, 2.6 ± 0.5 , $p < 0.01$; sneezing, 5.7 ± 0.6 , $p < 0.01$) or AG-Ex-100 mg/kg (rubbing, 5.0 ± 0.6 , $p < 0.05$; sneezing, 7.6 ± 0.7 , $p < 0.01$) (Fig. 5a and b). The OVA-induced control mice had an increased number of eosinophils ($23.4 \pm 5.1 \times 10^5$ cells/ml, $p < 0.01$), macrophages ($12.8 \pm 5.8 \times 10^5$ cells/ml, $p < 0.001$), neutrophils ($1.1 \pm 0.8 \times 10^5$ cells/ml, $p < 0.05$), lymphocytes ($1.4 \pm 1.1 \times 10^5$ cells/ml, $p < 0.05$), and total cells ($38.8 \pm 8.1 \times 10^5$ cells/ml, $p < 0.01$) of the NALF compared to the WT mice. With the DEX or AG-Ex-100 mg/kg however, the number of eosinophils (DEX, $7.3 \pm 1.6 \times 10^5$ cells/ml, $p < 0.01$; AG-Ex, $12.5 \pm 2.3 \times 10^5$ cells/ml, $p < 0.01$) and total cells (DEX, $16.7 \pm 5.9 \times 10^5$ cells/ml, $p < 0.01$; AG-Ex, $28.9 \pm 4.8 \times 10^5$ cells/ml, $p < 0.05$) in the OVA-induced mice was significantly decreased (Fig. 5c). Moreover, OVA exposure increased the histopathological alternations, including the infiltration of immune cells and mucus production in the nasal septum of the OVA-induced control mice but it was ameliorated by the administration of DEX or AG-Ex-100 mg/kg (Fig. 5d and e).

Reducing effect of the AG-Ex on the serum parameters for the allergic response in the OVA-induced mouse model

Increased serum levels of the OVA-specific IgE (1065 ± 66.7 pg/ml, p

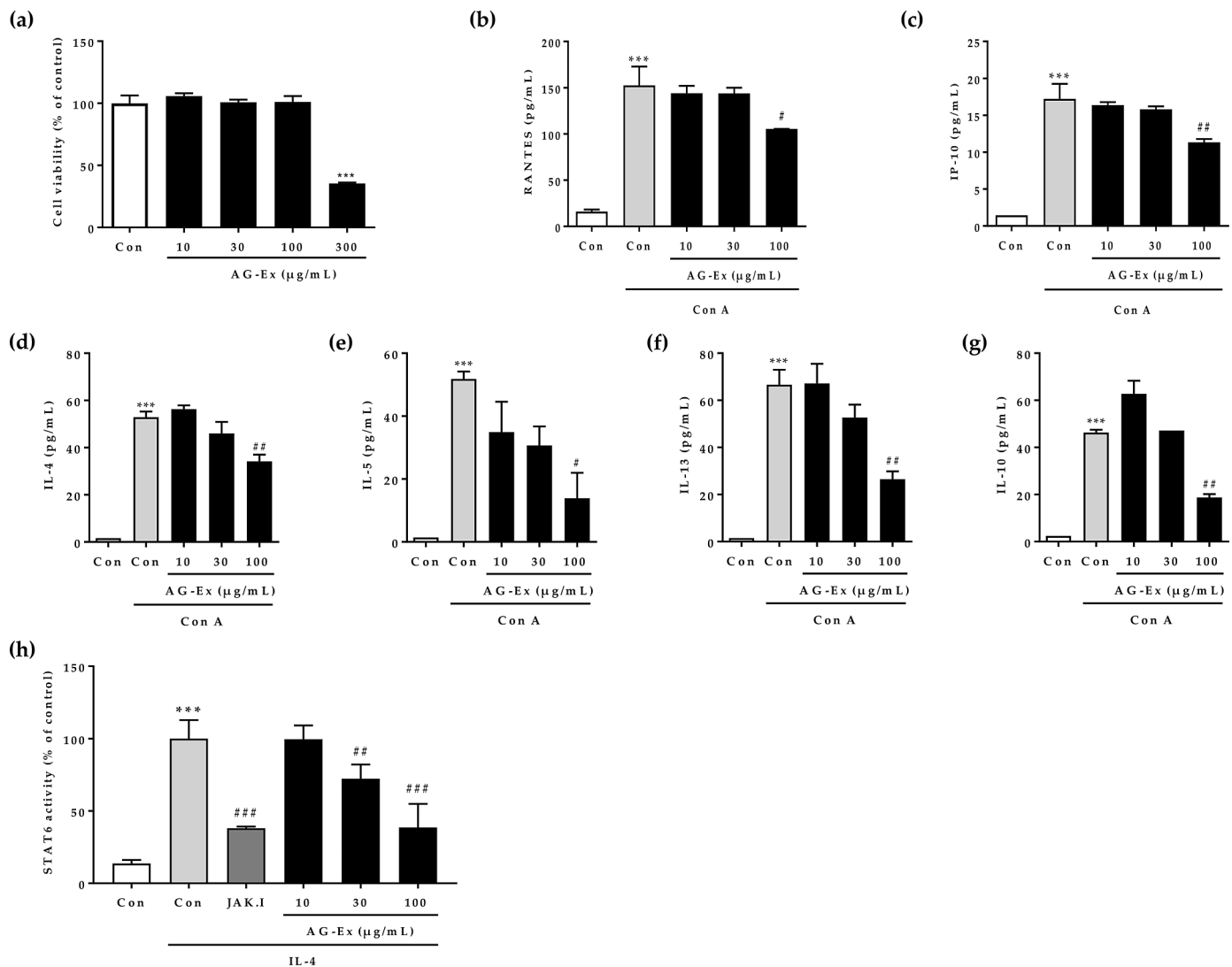


Fig. 2. AG-Ex inhibited the activation of the Th2 cells in the splenocytes. (a) The cell viability of the splenocytes was analyzed using an EZ-Cytox solution. The secretion levels of the chemokines and cytokines including RANTES (b), IP-10 (c), IL-4 (d), IL-5 (e), IL-13 (f), and IL-10 (g) were measured using LEGENDplex and flow cytometric analysis of the culture supernatants. (h) The STAT6 activity was investigated using HEK-Blue IL-4/IL-13 cells after treatment with IL-4 and the indicated concentrations of AG-Ex for 24 h. The data are presented as the mean $\pm$ SD ($n = 3$). ***, $p < 0.001$ vs. control. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ vs. control stimulated with Con A.

< 0.001), histamine (100.3 ± 21.8 pg/ml, $p < 0.001$), and IL-13 (858.3 ± 70.9 pg/ml, $p < 0.001$) were found in the OVA-induced control mice when compared to the WT mice, and the administration of DEX or AG-Ex-100 mg/kg significantly reduced the serum levels of OVA-specific IgE (DEX, 456.9 ± 51.0 pg/ml, $p < 0.001$; AG-Ex, 685.0 ± 80.8 pg/ml, $p < 0.01$), histamine (DEX, 47.6 ± 12.8 pg/ml, $p < 0.001$; AG-Ex, 56.2 ± 18.5 pg/ml, $p < 0.01$), and IL-13 (DEX, 392.0 ± 64.0 pg/ml, $p < 0.001$; AG-Ex, 519.5 ± 73.9 pg/ml, $p < 0.01$) (Fig. 6a-c).

Discussion

This study aimed to evaluate whether AG-Ex could ameliorate AR. Our results showed that AG-Ex did not inhibit the differentiation but rather the activation of Th2 cells and the secretion of IL-4, IL-5, and IL-13. AG-Ex suppressed allergic responses in the nasal epithelial cells including the expression of eotaxin-3 and periostin via the stimulation of IL-4 and IL-13. Moreover, clinical symptoms and the serum parameters of AR were restored via the administration of the AG-Ex in the OVA-induced mice. These results suggest that AG-Ex could ameliorate AR through the inhibition of Th2 cell activation and the suppression of

allergic responses in nasal epithelial cells.

It is well known that Th2 cells play an important role in allergic diseases including AR (Scadding, 2014; Li et al., 2016). Here, we first showed the inhibition of allergic chemokines and Th2 cytokine secretion in the splenocytes and the decrease of STAT6 activity in the HEK-Blue IL-4/IL-13 cells via the treatment of the AG-Ex, respectively. Second, we showed the suppression of Th2 cytokine secretion with the AG-Ex treatment in the differentiated Th2 cells. Finally, to clarify the effects of AG-Ex on the Th2 cells, we showed that there was no change in the Th2 population with the AG-Ex treatment during the differentiation of Th2 cells. These results suggest that the AG-Ex down-regulated the activation of Th2 cells, not their differentiation.

In recent studies, AR was shown to increase the production of Th2 cytokines, IgE, histamine, eotaxin-3, and periostin by immune cells and nasal epithelial cells in nasal mucosa (Reddy et al., 2017; Li et al., 2019). The secretion of Th2 cytokines including IL-4, IL-5, and IL-13 is increased by the activation of the Th2 cells due to the interaction between the APC and Th2 cells (Shamji and Durham, 2017; Matucci et al., 2019; Nagase et al., 2020). Consequently, the secreted Th2 cytokines induced the production of eotaxin-3 and periostin in the nasal epithelial

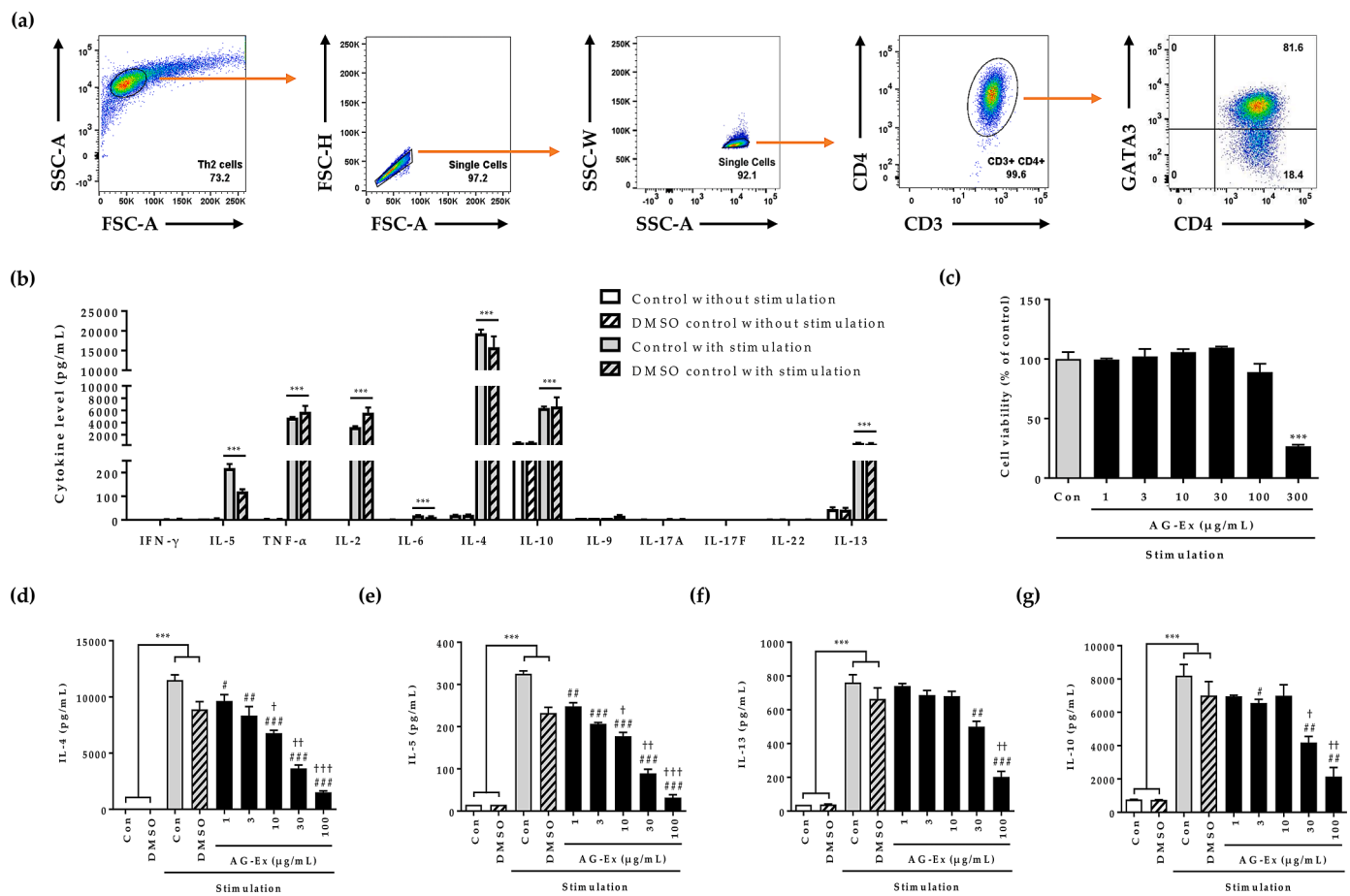


Fig. 3. The AG-Ex suppressed secretion of Th2 cell cytokines in the differentiated Th2 cells. (a) The population of Th2 cells (CD3⁺ CD4⁺ GATA3⁺ cells) was analyzed using flow cytometry. (b) The cell viability of the Th2 cells was measured using an EZ-Cytox solution. (c-g) Secretion levels of Th2 cell cytokines were determined using LEGENDplex and flow cytometric analysis of the culture supernatants. The data are presented as the mean $\pm$ SD ($n = 3$). ***, $p < 0.001$ vs. control. #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ vs. control stimulated with activation cocktail. †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ vs. DMSO control stimulated with activation cocktail.

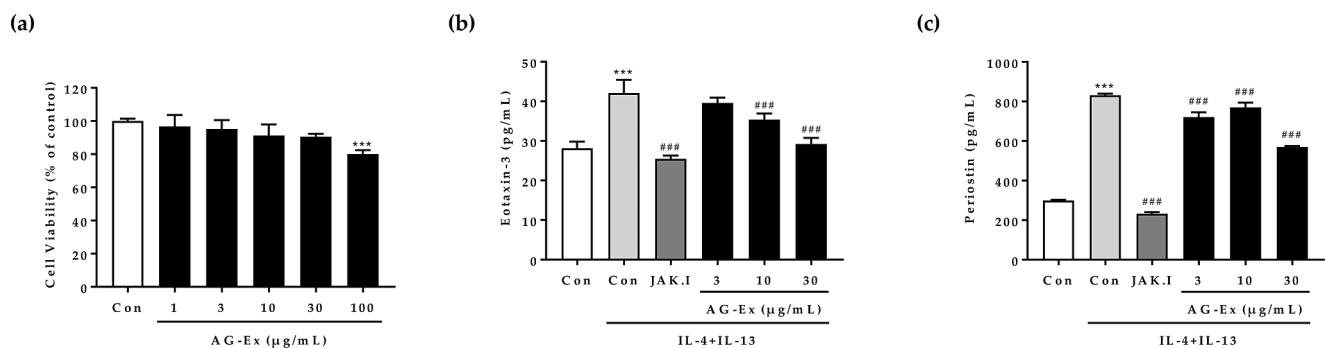


Fig. 4. AG-Ex reduced the secretion of eotaxin-3 and periostin in the human nasal epithelial cells. (a) The cell viability of HNEpC was investigated using EZ-Cytox solution. The secretion levels of eotaxin-3 (b) and periostin (c) were measured using ELISA from the cultured supernatants. The data are presented as the mean $\pm$ SD ($n = 3$). ***, $p < 0.001$ vs. control. ###, $p < 0.001$ vs. control stimulated with IL-4 and IL-13.

cells, histamine release in the mast cells, and IgE secretion in the B cells. Likewise, this study also shows the increased production and secretion of Th2 cytokines in the splenocytes and differentiated Th2 cells after activation. IL-4 and IL-13 stimulated the secretion of eotaxin-3 and periostin in the HNEpC (nasal epithelial cells). Moreover, we showed changes in the IgE, histamine, and IL-13 levels in the serum of the OVA-induced AR mouse model. Therefore, our findings suggest that the expression levels of Th2 cytokines, eotaxin-3, periostin, IgE, and histamine could be used as clinical biomarkers for AR.

Previous studies reported that APC, including DCs and macrophages, are activated by allergen uptake, which stimulates the activation of Th2 cells (Shamji and Durham, 2017; Matucci et al., 2019; Muluk et al., 2019; Nagase et al., 2020). Our study showed that the activation of Th2 cells was stimulated by ConA in the splenocytes or by the activation cocktail in the differentiated Th2 cells, respectively, and was inhibited by treatment with AG-Ex. Although, this study observed the inhibitory effect of AG-Ex on the activation of Th2 cells, further research is required to understand the regulation of Th2 cell activation through its

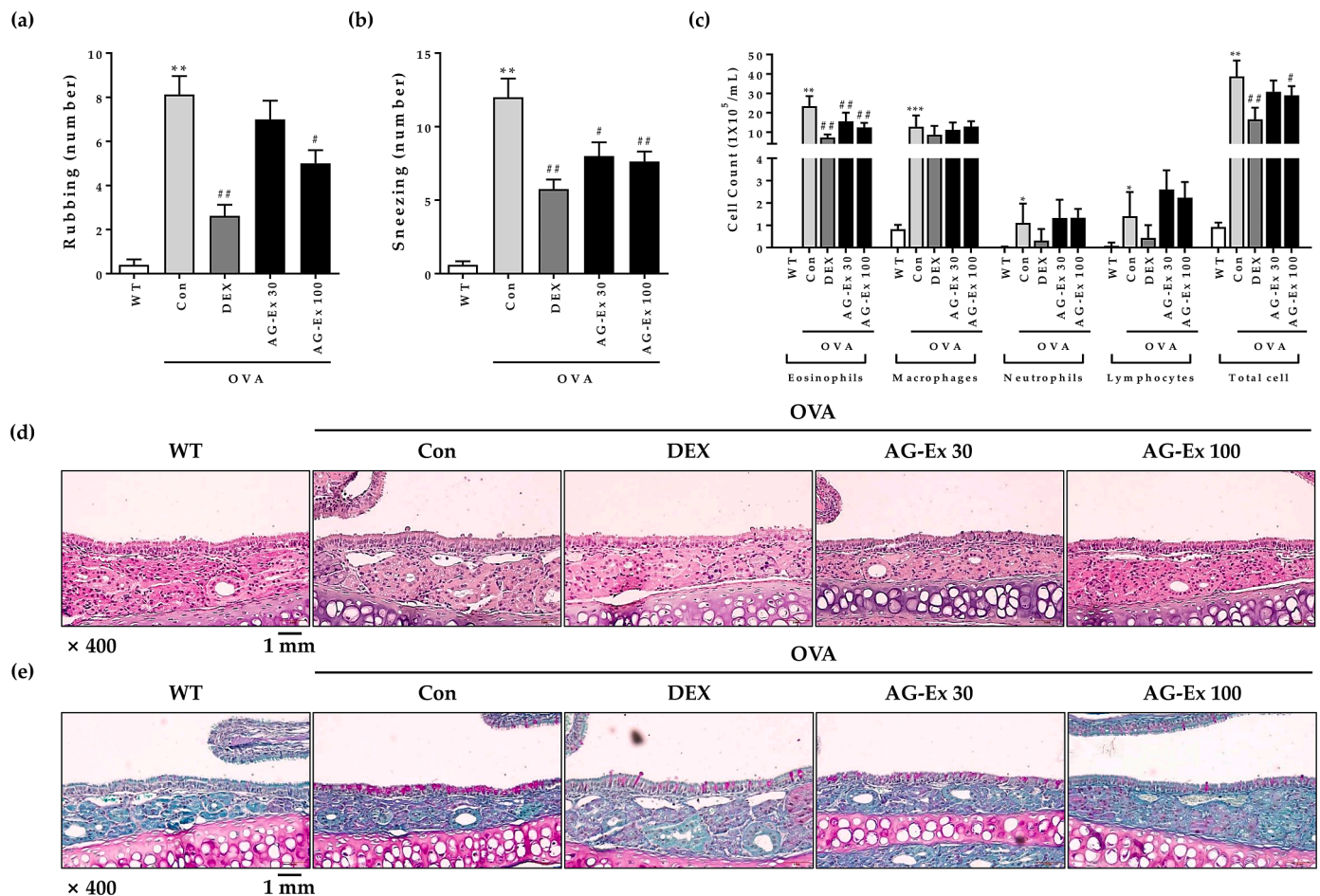


Fig. 5. AG-Ex ameliorated the clinical symptoms of AR in the OVA-induced mice model. The histological analysis was evaluated using H&E (a) and PAS (b) staining, respectively. The staining images are $\times 400$ magnification. The frequency of rubbing (c) and sneezing (d) was evaluated after the last OVA exposure. (e) The number of various immune cells was counted from the NALF. The data are presented as the mean $\pm$ SD ($n = 8$ /each group). **, $p < 0.01$; ***, $p < 0.001$ vs. WT. #, $p < 0.05$; ##, $p < 0.01$ vs. control.

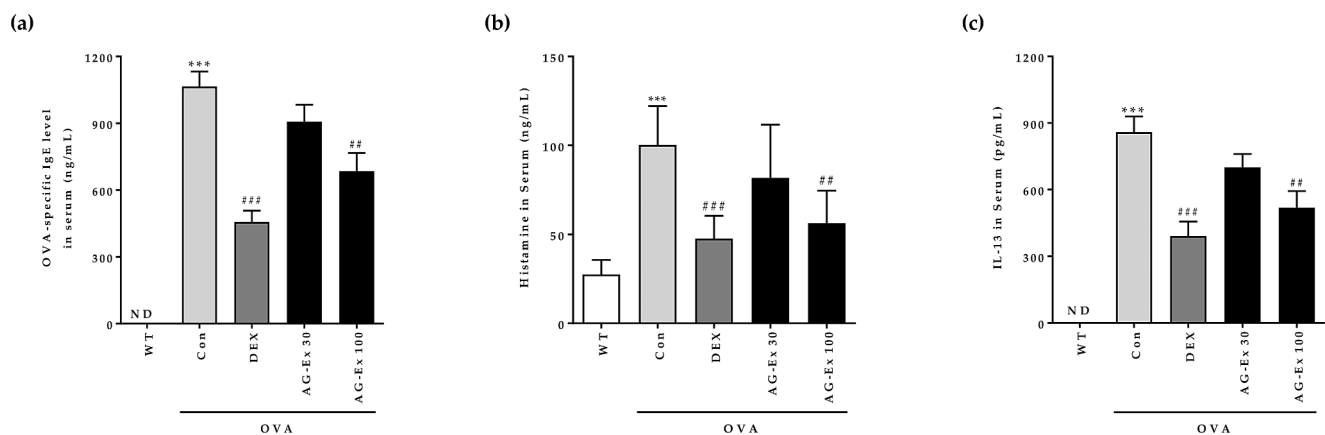


Fig. 6. AG-Ex reduced serum parameters for the allergic response in the OVA-induced mouse model. The levels of OVA-specific IgE (a), histamine (b), and IL-13 (c) were evaluated using ELISA. The data are presented as the mean $\pm$ SD ($n = 8$ /each group). ***, $p < 0.001$ vs. WT. ##, $p < 0.01$; ###, $p < 0.001$ vs. control.

interactions with the APC stimulated with allergens, to help clarify the therapeutic effects of AG-Ex.

IL-25 (IL-17E), a member of the IL-17 family of cytokines, has been reported to be an important regulator of Th2 activity and Th2 memory cell differentiation (Yao et al., 2016). Similar to IL-4, it increases the production of related cytokines through STAT6 and GATA3 activation, and is involved in increased production of IgE and effector cell

recruitment-related chemokines (Valizadeh et al., 2015). IL-17BR, a receptor for IL-25, is expressed in a variety of respiratory-related cells and regulates Th2 memory cell differentiation. In particular, IL-17BR is up-regulated by TNF- α and down-regulated by TGF- β (Lajoie-Kadoch et al., 2006). In this study, the change of TNF- α by AG-Ex did not show a significant change in splenocytes (data not shown), so the evaluation of IL-25 was not performed. However, IL-25 and its receptor are important

factors for Th2 differentiation and activity, and further studies are needed to evaluate the efficacy of AG-Ex.

Recent studies report that AG and its components have a therapeutic effect on mast-cell-mediated allergic inflammation (Lee et al., 2017; Reddy et al., 2017; Shehzad et al., 2018). Moreover, Ok et al. reported the anti-inflammatory effects of AG-Ex on atopic dermatitis models (Ok et al., 2018). In addition, it has been reported that chlorogenic acid, one of the components of AG-Ex, improves rhinitis symptoms and allergic inflammatory responses in OVA-induced AR mice by regulating Th17 cells and regulating Th1/Th2 balance (Dong et al., 2020; Shi et al., 2020). Likewise, we showed the ameliorating effects of AG-Ex on OVA-induced AR models through the inhibition of Th2 cell activation, but we suggest that further studies are necessary including the evaluation of AG-Ex components efficacy and the clinical trials in allergic diseases.

Conclusions

AG-Ex was found to have clear ameliorating effects on AR through inhibiting not the differentiation of Th2 cells but the activation of them and the allergic responses of the nasal epithelial cells. Although further studies are necessary including the more detailed mechanisms of AG-Ex components and clinical trials, this study suggests that AG-Ex has therapeutic potential for the treatment of AR, and our data could help develop new treatments for Th2 cell-mediated allergic diseases.

Author contributions

K.-Y.J. and D.H.J.; investigation, writing-original draft preparation, and writing-review and editing. B.-J.P., Y.J.K., J.Y.L., S.C., and M.-A.J.; investigation. K.H.S.; conceptualization, and writing-review and editing. T.K.; conceptualization, supervision, project administration, and writing-review and editing. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2021.153789.

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