

Original article

Sophoricoside from *Sophora japonica* ameliorates allergic asthma by preventing mast cell activation and CD4⁺ T cell differentiation in ovalbumin-induced mice

Byung-Hak Kim^a, Sanghyun Lee^{b,*}

^a Korea Institute of Science and Technology for Eastern Medicine (KISTEM), NEUMED Inc., Seoul 02440, Republic of Korea

^b Department of Plant Science and Technology, Chung-Ang University, Anseong 17546, Republic of Korea

ARTICLE INFO

Keywords:

Airway inflammation

Allergic asthma

CD4⁺ T cell

Mast cell

Ovalbumin

Sophoricoside

ABSTRACT

Asthma is a chronic inflammatory lung disorder with continuously increasing prevalence worldwide. Novel strategies are needed to prevent or improve asthma. The aim of this study was to investigate the effects of sophoricoside from *Sophora japonica* on allergic asthma. The mature seeds of *S. japonica* contain a large amount of sophoricoside. Sophoricoside reduced allergic and asthmatic symptoms by suppressing airway inflammation and antibody-antigen reaction in mouse models. In particular, sophoricoside suppressed immune cell recruitment into the airway lumens of the lungs and production of pro-inflammatory cytokines in the bronchoalveolar lavage fluid (BALF) of ovalbumin (OVA)-induced mice. It also decreased the amounts of histamine and arachidonic acid metabolites released in OVA-induced mice and antibody-antigen stimulated mast cells. In addition, sophoricoside decreased differentiation of naïve CD4⁺ T cells into T helper type 1 (Th1), Th2, and Th17 cells. Overall, we demonstrated that sophoricoside improved allergic asthma by suppressing mast cell activation and CD4⁺ T cell differentiation.

1. Introduction

Allergic diseases are associated with hypersensitivity to external substances and are among the most common chronic diseases worldwide. Common allergic diseases include bronchial asthma, allergic rhinitis, allergic conjunctivitis, atopic dermatitis, and food allergies. Allergic asthma is a complex chronic airway inflammatory disease that causes sporadic breathing difficulties. It causes aberrant immune responses with inhalation of allergens such as house dust mites, cockroaches, animal allergens, pollens, and indoor mold. Allergic asthma has variable and recurring symptoms, such as reversible airflow obstruction, airway hyperresponsiveness (AHR), easily triggered bronchospasms, excessive T cell-mediated immune responses, mucus hypersecretion, and airway remodeling in the lung [1,2].

Recent studies have reported that allergic asthma is an aberrant anaphylactic immune response that involves a variety of immune cells, including eosinophils, T cells, B cells, mast cells, and airway epithelial cells [3,4]. In particular, activated type 2 T helper (Th2) CD4⁺ cells release type 2 cytokines, such as interleukin (IL)-4, IL-5, and IL-13, to stimulate the production of immunoglobulin E (IgE). IgE plays an

important role in inducing asthmatic inflammation by promoting mast cell activation, goblet cell hyperplasia, excessive mucus secretion, eosinophil infiltration, and AHR [2]. Although the incidence of asthma has steadily increased over the past few decades, current therapeutic approaches still largely rely on anti-inflammatory corticosteroids and bronchodilators [5]. Despite advances in diagnosis and treatment, asthma remains a serious global health problem, and many patients do not have properly controlled disease [6,7]. In fact, numerous immune cells and immune cell-driven mediators contribute to the exacerbation and progression of allergic asthma. Therefore, targeting these immune cells and mediators is a potential modality for the treatment of asthma.

Sophoricoside is an isoflavone glycoside isolated from *Sophora japonica*, a plant of the Leguminosae family. Sophoricoside was initially isolated in 1961, when its hormonal effect was reported [8]. However, few biological and pharmacological activities of sophoricoside have been reported. The activities of sophoricoside include IL-5 inhibition [9], anti-inflammation and immunosuppression [10–12], estrogenic effect [13], modulation of lipogenesis and glucose consumption [14], hepatoprotection [15], and anti-osteoporosis [16]. Interestingly, sophoricoside inhibited the bioactivities of IL-3, IL-5, IL-6, and

* Corresponding author.

E-mail addresses: bhkim@mdec.co.kr (B.-H. Kim), slee@cau.ac.kr (S. Lee).

<https://doi.org/10.1016/j.bioph.2020.111029>

Received 8 August 2020; Received in revised form 4 November 2020; Accepted 15 November 2020

Available online 27 November 2020

0753-3322/© 2020 The Authors.

Published by Elsevier Masson SAS. This is an open access article under the CC BY license

(<http://creativecommons.org/licenses/by/4.0/>).

granulocyte-macrophage colony-stimulating factor (GM-CSF) in BAF/BO3, Y16, MH60/BSF-2, and TF-1 cells, but not those of IL-1 β and tumor necrosis factor- α (TNF- α) in A375.S2 and WEHI-164 cells [17]. Sophoricoside also inhibited IL-6 bioactivity and cyclooxygenase (COX)-2 activity, but not IL-1 β and TNF- α bioactivities, inducible nitric oxide synthase (iNOS) activity, COX-2 expression, and reactive oxygen species (ROS) and nitric oxide (NO) production in RAW264.7 cells [18]. Specifically, IL-5 is the Th2 cell-driven pro-inflammatory cytokine and IL-3 is the cytokine produced from basophils, eosinophils, activated T cells and mast cells. IL-3, IL-5 and GM-CSF share the common β chain (β c) receptor, and these cytokines are closely related to allergy and asthma induced by antigen-mediated immune response [19,20].

Based on this evidence, sophoricoside was isolated from the mature seeds of *S. japonica*, and its anti-allergic and anti-asthmatic effects were demonstrated using *in vivo* mouse models. Overall, we found that sophoricoside ameliorated allergic asthma by preventing mast cell activation and differentiation of CD4⁺ T cells in ovalbumin (OVA)-induced mice.

2. Materials and methods

2.1. Plant materials and sample preparation

Dried mature seeds, leaves, stems, and roots of *S. japonica* were obtained from National Baekdudaegan Arboretum, 2019. The voucher specimen was identified by Dr. Y. S. Kim, National Baekdudaegan Arboretum and deposited at the herbarium of Department of Plant Science and Technopogy, Chung-Ang University, Anseong, Republic of Korea. Five grams of dried mature seeds, leaves, stems, and roots of *S. japonica* were repeatedly extracted with 70 % ethanol (100 mL $\times$ 3) at 80 °C for 3 h and then filtered using 0.45 μ m of polyvinylidene fluoride (PVDF) membrane filter for high-performance liquid chromatography (HPLC).

2.2. Isolation and HPLC conditions for sophoricoside analysis from *S. japonica*

Sophoricoside was isolated from the mature seeds of *S. japonica* by repeated column chromatography. To quantify sophoricoside, an Agilent 1260 Infinity II Quaternary Pump (Santa Clara, CA, USA) was used. Quantitative analysis was performed using a reverse-phase system. Separation was conducted on an INNO C18 column (25 cm $\times$ 4.6 mm, 5 μ m). Column temperature was maintained at 30 °C, and the flow rate was set at 1 mL/min. The mobile phase was comprised of (A) water (0.5 % acetic acid) and (B) acetonitrile, and the elution was conducted in a gradient system: 92 % (A)-8% (B) for 2 min; reduced to 90 % (A) for 25 min; reduced to 70 % (A) for 23 min; reduced from 70 % to 10 % for 2 min; reduced from 10 % to 0% for 8 min; and increased from 0% to 92 % (A) for 5 min. The injection volume was 10 μ L, and the UV absorbance was recorded at 270 nm.

2.3. Reagents and cell culture

HPLC grade acetonitrile and water were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). Chicken egg ovalbumin (OVA), dinitrophenol (DNP)-specific monoclonal IgE, DNP-human serum albumin (HSA), and Evans blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aluminum hydroxide (Imject™) were obtained from Thermo Fisher Scientific (Rockford, IL, USA). All other chemicals were obtained from Sigma-Aldrich unless otherwise noted. The human mast cell line HMC-1 was grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10 % fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C under humidified atmosphere containing 5% carbon dioxide (CO₂).

2.4. OVA-induced allergic airway inflammation

BALB/c mice (6–8-week old) were used for experimental study after one week of acclimation. OVA-induced allergic airway inflammation was generated using previously described modifications [21]. Briefly, mice were sensitized by intraperitoneal injection on days 0 and 14 with 200 μ L saline suspension containing 50 μ g OVA emulsified in 1.6 mg aluminum hydroxide. After one week, mice were further challenged by intranasal inhalation of 1% OVA aerosol from days 21–24 for 30 min. Dimethyl sulfoxide (DMSO, 1%) in phosphate-buffered saline (PBS, n = 6) or sophoricoside (3 or 30 mg/kg, n = 6) was intraperitoneally administered an hour before OVA challenge from days 20–25. Mice were sacrificed 24 h after final treatment, and blood and organs were collected for further experiments. All animal care and studies were conducted under specific pathogen-free conditions in accordance with the guidelines of the Institutional Animal Care and Use Committee at Chung-Ang University (Approval number: 2019–00113).

2.5. Nasal rubbing

Nasal rubbing was assessed 24 h after final administration of sophoricoside. A single event was considered each time a mouse rubbed or touched the area near its nose with its forefoot. Each mouse was acclimatized with an observation cage (one mouse/cage) for 10 min before the experiment, and nasal rubbing was counted for 10 min.

2.6. Bronchoalveolar lavage fluid (BALF) and inflammatory cells

BALF was collected by washing the airway lumens of the lungs three times with 1 mL ice cold PBS instilled into a tracheal polyethylene catheter. The fluid was centrifuged at 2000 rpm for 5 min at 4 °C. The supernatant was collected to determine histamine contents released from airway mast cells. Collected cell pellets were used to count the number of total cells and inflammatory cells, including macrophages, eosinophils, neutrophils, and lymphocytes. Total cells were counted using a hemocytometer, and differential inflammatory cells were counted using a cytospin at 500 rpm for 5 min followed by Diff-Quick (Sysmex Corporation, Kobe, Japan) or Wright-Giemsa staining (Sigma-Aldrich) according to the manufacturer's protocol. Types of inflammatory cells were determined with a differential cell counter (Hwashin Technology, Seoul, Korea) using standard morphologic criteria [22].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Samples were prepared from mice serum, BALF supernatant, and cell culture media. Total IgE, OVA-specific immunoglobulins (IgE, IgG1, and IgG2a), cytokines (IFN- γ , TNF- α , IL-4, IL-5, IL-13 and IL-17), histamine, prostaglandin D₂ (PGD₂) and leukotrienes (LTs, LTB₄ and LTC₄) were determined using an ELISA kit according to the manufacturer's protocol. Kits were obtained from Invitrogen, Cayman (Ann Arbor, MI, USA), BioLegend (San Diego, CA, USA), R&D Systems (Minneapolis, MN, USA), and Abcam (Cambridge, UK), respectively. Absorbance was measured using a Victor Nivo Multimode Microplate Reader (PerkinElmer, Boston, MA, USA).

2.8. Fluorescence-activated cell sorting (FACS)

Draining lymph node cells were prepared from the lymph nodes of OVA-induced BALB/c mice ground into single cells. To determine the population of IL-4- and IFN- γ -positive cells, draining lymph node cells or BALF cells were stained with APC-conjugated anti-IL-4 antibody, FITC-conjugated anti-IFN- γ antibody, or PE-conjugated anti-CD4 antibody (BD Pharmingen, San Jose, CA, USA) using a Cytotfix/Cytoperm kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's protocol. In addition, IgE- and IgG1-positive cells were stained with FITC-conjugated anti-IgE antibody or PE-conjugated anti-IgG1 antibody (BD

Pharmingen). Stained cells were detected by flow cytometry using the BD LSRFortessa™ cell analyzer (BD Biosciences).

2.9. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRIzol Reagent (Invitrogen), and first-strand cDNA was synthesized from 1 µg total RNA using a QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA). qRT-PCR was performed using a QuantiFast SYBR Green PCR master mix (Qiagen) with an CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). The data were analyzed by comparative C_t quantification, and the value for each sample was normalized to the value for the housekeeping *gapdh* gene. Primer sets for T-bet, GATA-3, RORγt, IFN-γ, IL-4, IL-17A, and GAPDH were obtained from Qiagen. The amplification program consisted of 1 cycle of 95 °C for 5 min followed by 25 cycles of 96 °C for 10 s, 56 °C for 20 s, and 72 °C for 20 s.

2.10. In vitro differentiation of CD4⁺ T cells

Naive CD4⁺ T cells were purified from spleens and lymph nodes of C57BL/6 mice by negative selection using T-cell enrichment columns (R&D Systems) and mouse CD4⁺ T cell isolation kits (Miltenyi Biotech, Auburn, CA, USA). *in vitro* differentiation of CD4⁺ T cells was performed as previously described [23,24]. Briefly, cells were activated by plate-bound anti-CD3 antibody in a 96-well plate (BD Biosciences) and soluble anti-CD28 (2 µg/mL) antibody. The cells were differentiated under Th1 polarizing conditions (10 ng/mL IL-12 and 10 µg/mL anti-IL-4 antibody), Th2 polarizing conditions (20 ng/mL IL-4 and 10 µg/mL anti-IFN-γ antibody), or Th17 polarizing conditions (20 ng/mL IL-6, 10 ng/mL TGF-β, 10 µg/mL anti-IFN-γ antibody and 10 µg/mL anti-IL-4 antibody). All cytokines and antibodies used for CD4⁺ T cell differentiation were obtained from BD Biosciences.

2.11. Passive cutaneous anaphylaxis (PCA)

C57BL/6 mice (6–8-week old) were locally sensitized with an intradermal injection of anti-DNP-specific IgE (100 ng/ear) into the right ear followed 24 h later by topical treatment with sophoricoside (3 or 30 mg/kg, $n = 5$). Control mice ($n = 5$) were injected with the same volume of PBS and topically treated with 1% DMSO. One hour later, the mice were intravenously challenged with soluble antigen (100 µg DNP-HSA) in 100 µL PBS containing 5% Evans blue dye. One hour after antigen challenge, the dye was extracted from each dissected ear in 0.8 mL formamide at 65 °C for overnight. The amount of extracted dye was measured spectrophotometrically at 620 nm and calculated from a standard curve established with known amounts of dye.

2.12. Histology

Tissues were fixed in 10 % neutral buffered formalin for 24 h. After dehydration, the tissues were embedded in paraffin, cut into 5 µm thick sections, and stained with hematoxylin and eosin (H&E). To determine histopathologic features, digital images were obtained using LAS microscope software (Leica Microsystems, Wetzlar, Germany).

2.13. Cell viability

Cells were seeded in 96-well plates and incubated in culture medium overnight. Cells were then further incubated with either vehicle alone or various concentrations of sophoricoside for 24–72 h. Cell viability was measured at 450 nm using a Victor Nivo Multimode Microplate Reader (PerkinElmer) after further incubation for 2–4 h at 37 °C followed by addition of 10 µL CCK-8 Reagent (Daeil Lab Service, Seoul, Korea).

2.14. Statistical analysis

Data are represented as mean ± standard error of mean (SEM) unless otherwise indicated. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Significance was determined using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc *t*-test for multiple comparisons, and *p* values < 0.05 were considered statistically significant.

3. Results

3.1. Identification and sophoricoside content of *S. japonica*

The structure of sophoricoside from *S. japonica* was assigned by nuclear magnetic resonance (NMR) and mass spectrometry (MS) analyses (data not shown). Structural elucidation and identification were carried out with MS using a JEOL JMS-600W mass spectrometer (Tokyo, Japan), and ¹H- and ¹³C-NMR spectra were created using a Bruker AVANCE 500 NMR spectrometer (Rheinstetten, Germany). Fast atom bombardment (FAB)-MS and NMR data were compared with values in the literature [25]. The content of sophoricoside in seeds, leaves, stems, and roots of *S. japonica* by HPLC analysis are shown in Fig. 1. Sophoricoside was present in the highest amounts in seeds (3.302 mg/g DW), followed by leaves (0.048 mg/g DW), but it was present in trace amounts in stems and roots of *S. japonica* (Table 1).

3.2. Sophoricoside reduces allergic and asthmatic features in OVA-induced mice

IL-3 and IL-5 are positively correlated with allergic airway inflammation [26,27] and sophoricoside inhibited their bioactivities [19], suggesting that allergic airway inflammation can be controlled by sophoricoside. To demonstrate this possibility, an allergic airway inflammation model was established in mice by sensitization and challenge with OVA (Fig. 2A). The model was used to evaluate whether sophoricoside suppresses allergic and asthmatic symptoms. Nasal rubbing was considered a visible allergic symptom on day 26 before sacrificing mice. Induction of allergic asthma increased nasal rubbing compared with the normal group, and the behavior was decreased with administration of sophoricoside (Fig. 2B). Histologic analyses showed increased infiltrating inflammatory cells in the airways and thickened airway walls in OVA-induced mice compared with normal mice. The administration of sophoricoside reduced this infiltration and thickening without cytotoxic effects in livers and spleens (Fig. 2C). These results indicate that sophoricoside has anti-allergic and anti-asthmatic activities achieved through suppression of airway inflammation.

3.3. Sophoricoside decreases recruitment of immune cells into the allergic airway

Airway inflammation, a predominant feature of allergic asthma, recruits immune cells such as eosinophils, macrophages, neutrophils, and lymphocytes [28]. The number of various immune cells in BALF was counted to evaluate the anti-inflammatory effect of sophoricoside. Upon OVA-challenge, the number of total cells and immune cells, including macrophages, eosinophils, neutrophils, and lymphocytes, was dramatically increased compared with normal control mice. The number of recruited immune cells was effectively decreased by the administration of sophoricoside in OVA-challenged mice (Fig. 3). The results indicate that sophoricoside decreases recruitment of immune cells into regions of allergic airway inflammation.

3.4. Sophoricoside decreases release of pro-inflammatory cytokines

Immune cells produce many inflammatory mediators that exacerbate allergic airway inflammation by inducing histamine release through

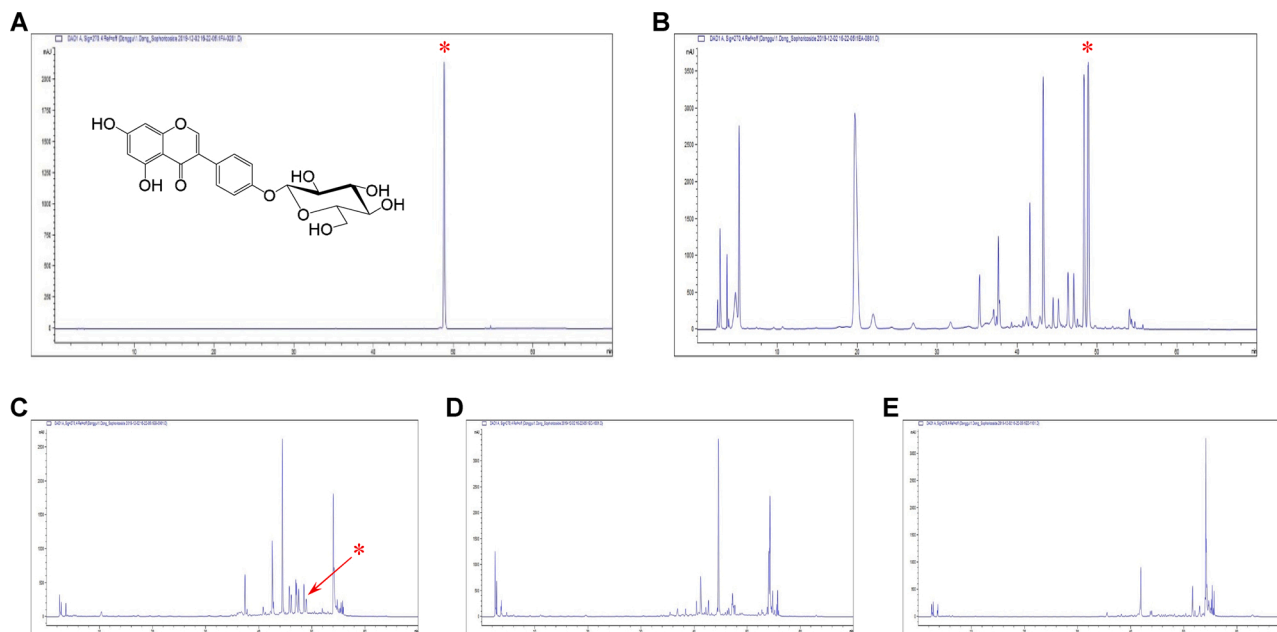


Fig. 1. Chemical structure of sophoricoside and HPLC chromatograms. (A) Chemical structure of sophoricoside and its HPLC chromatogram. (B–E) HPLC chromatograms of EtOH extracts of seeds (B), leaves (C), stems (D), and roots (E) of *S. japonica*. * indicates sophoricoside.

Table 1

Sophoricoside content in seeds, leaves, stems, and roots of *S. japonica*.

Parts	Sophoricoside amounts	
	mg/g extract	mg/g DW
Seeds	35.805 ± 0.655	1.302 ± 0.024
Leaves	1.222 ± 0.042	0.048 ± 0.002
Stems	trace	trace
Roots	trace	trace

mast cell activation [29,30]. Analysis of the released pro-inflammatory cytokines showed consistent results regarding recruitment of total cells and immune cells. Sophoricoside effectively decreased the amount of released pro-inflammatory cytokines, including IFN- γ , TNF- α , IL-4, IL-5, IL-13, and IL-17, compared with the serum of OVA-challenged mice (Fig. 4A). Interestingly, the inhibitory effect of sophoricoside on Th2 and Th17 cell-specific cytokines (IL-4, IL-5, IL-13, and IL-17) was stronger than that of Th1 cell-specific cytokines (IFN- γ and TNF- α). Consistently, the population of both IFN- γ - and IL-4-positive cells decreased in both draining lymph nodes and BALF cells, and the population of IL-4-positive cells was more effectively decreased than that of IFN- γ -positive cells (Fig. 4B and C). These results indicate that sophoricoside has anti-inflammatory activity and may have a therapeutic effect on Th cell subsets, in particular Th2 and Th17 cells.

3.5. Sophoricoside decreases secretion of immunoglobulins in OVA-induced mice

Elevated levels of immunoglobulins, specifically IgE, are key upstream players in allergic asthma. Therefore, anti-IgE therapy is a recommended therapeutic strategy for improving severe allergic asthma [31,32]. The levels of total IgE and OVA-specific IgE, IgG1, and IgG2a in mice serum were measured. Higher levels of all immunoglobulins were observed in OVA-challenged mice than normal control mice. Conversely, the administration of sophoricoside effectively decreased levels of all immunoglobulins (Fig. 5A–D). Consistent results were also observed by FACS analysis in BALF cells showing that elevated populations of IgE- and IgG1-positive cells with OVA-induction were significantly decreased by sophoricoside treatment (Fig. 5E and F).

These results indicate that sophoricoside can successfully reduce secretion of immunoglobulins, including IgE, and may improve the symptoms of allergic asthma.

3.6. Sophoricoside reduces allergic response in PCA mice

To further demonstrate the anti-allergic effect of sophoricoside by reducing IgE release and mast cell activation, acute allergic skin response was induced in a PCA mouse model by passive sensitization with intradermal injection of DNP-specific IgE into mice ears and intravenous challenge with soluble DNP-HSA antigen containing 5% Evans blue dye (Fig. 6A). Topical application of sophoricoside decreased mast cell-mediated leakage of blood vessels, resulting in decreased amount of diffused dye (Fig. 6B). Dye intensity was decreased by about 24.6 ± 2.4 % and 90.9 ± 1.0 % with 3 and 30 mg/kg sophoricoside treatment, respectively, compared with DNP-specific IgE-induced PCA mice (Fig. 6C). The increased ear swelling also reduced from 16.5 ± 1.9 μ m to 14.2 ± 1.2 and 9.4 ± 0.8 μ m with 3 and 30 mg/kg sophoricoside, respectively, compared with DNP-specific IgE-induced PCA mice (Fig. 6D). These results indicate that sophoricoside enacts an anti-allergic effect through suppression of IgE-mediated antibody-antigen reactions.

3.7. Sophoricoside decreases release of histamine and arachidonic acid metabolites

Mast cells are responsible for immediate allergic reactions initiated by cross-linking of allergens to high-affinity receptor-bound surface IgE. Released histamine and lipid metabolites, such as PGD₂ and LTs, from IgE-primed mast cells induce allergic reactions and inflammation by recruiting immune cells such as neutrophils, eosinophils, basophils, and Th2 cells [33,34]. Therefore, the amounts of released histamine were measured to determine mast cell activation and degranulation in OVA-induced mice. OVA-induction leads to dramatically elevated histamine release in BALF compared with normal mice, and the amounts were markedly decreased by sophoricoside treatment (Fig. 7A). Sophoricoside also decreased the amount of LTC₄, an arachidonic acid metabolite associated with allergy and asthma, in BALF of OVA-induced mice (Fig. 7B). The release of histamine and arachidonic acid

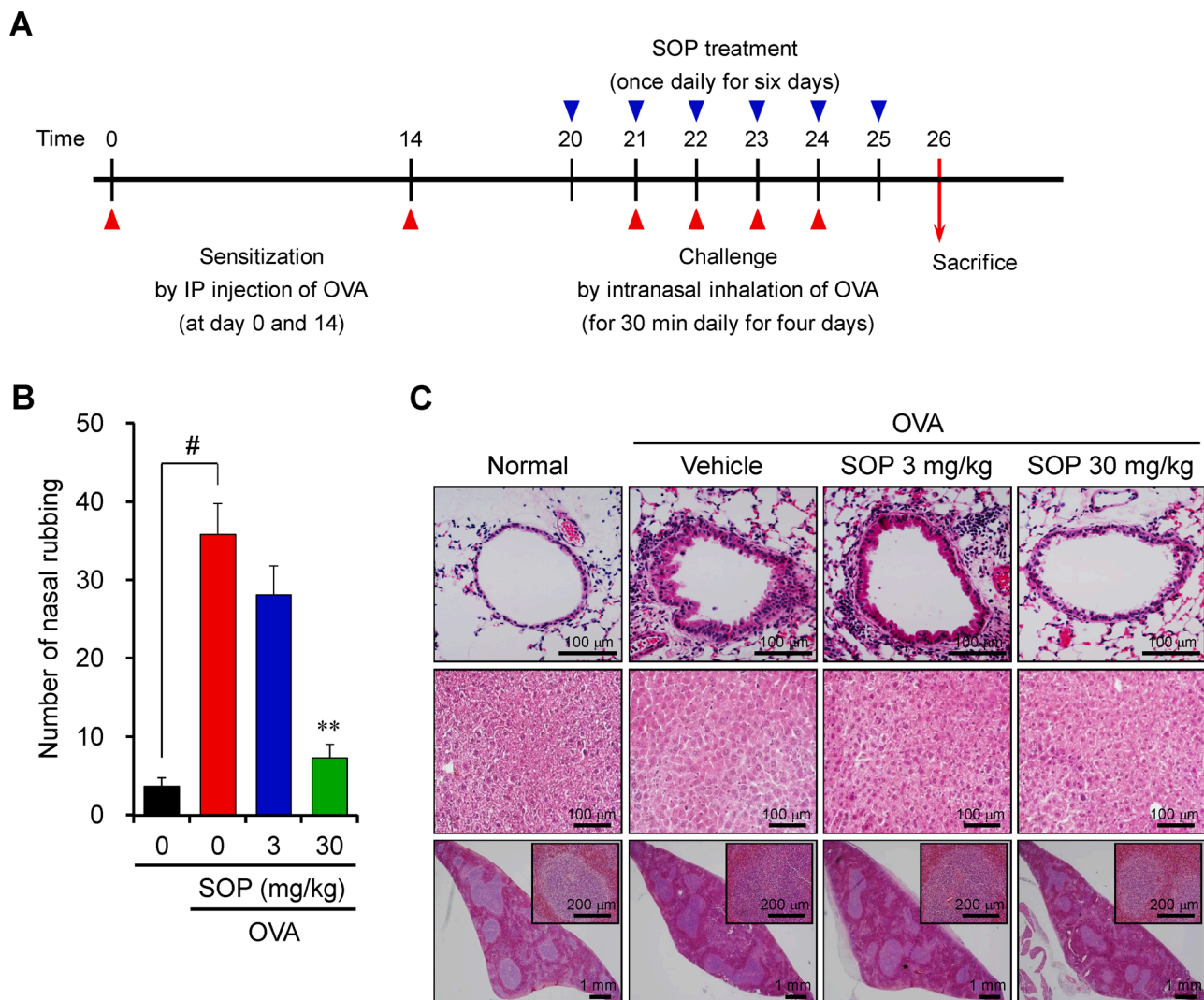


Fig. 2. Sophoricoside reduces allergic airway inflammation in OVA-induced mice. (A) Experimental scheme for OVA-induced allergic airway inflammation in BALB/c mice ($n = 6$ per each group). (B) Number of nasal rubbing events was determined for 10 min on the day 26. (C) Histology of lung (upper), liver (middle), and spleen (bottom) was assessed with H&E staining. Scale bars: 100 or 200 μ m or 1.0 mm. # $p < 0.005$ versus vehicle-treated group; ** $p < 0.005$ versus OVA-induced group. SOP, sophoricoside.

metabolites, including PGD₂, LTB₄, and LTC₄, in HMC-1 cells was further observed after activation with anti-DNP-IgE plus DNP-HSA. Consistent results were seen with OVA-induced mice, and treatment with sophoricoside effectively decreased histamine release in activated HMC-1 cells (Fig. 7C). In addition, sophoricoside treatment decreased the amounts of PGD₂, LTB₄, and LTC₄ compared to untreated HMC-1 cells (Fig. 7D–F) without affecting cell viability (Fig. 7G). These results indicate that sophoricoside inhibits allergic reactions by preventing release of histamine and arachidonic acid metabolites via inhibition of mast cell activation.

3.8. Sophoricoside suppresses CD4⁺ T cell differentiation

Differentiated CD4⁺ T cell driven airway inflammatory responses also play an important role in allergen-mediated allergic asthma. In particular, Th2 cells promote IgE-mediated sensitization, airway hyperreactivity, and eosinophilia by producing Th2 cell-specific cytokines [35,36]. Fig. 4 shows results indicating that sophoricoside inhibited production of Th cell-specific cytokines in serum of OVA-challenged mice, suggesting that sophoricoside inhibits CD4⁺ T cell differentiation. The cytotoxic activity of sophoricoside was evaluated before determining the effect of sophoricoside on CD4⁺ T cell differentiation.

Sophoricoside exhibited weak to no cytotoxic activity in cultured mouse CD4⁺ T cells *in vitro* at concentrations of up to 300 μ M for 24–72 h (Fig. 8A). To confirm whether sophoricoside affects CD4⁺ T cell differentiation, qRT-PCR analysis was performed to determine expression of Th cell lineage-specific master regulators in cultured naïve CD4⁺ T cells under the appropriate differentiation conditions. Sophoricoside markedly suppressed mRNA levels of T-bet, GATA-3 and ROR γ t, which are the master regulators for Th1, Th2, and Th17 cell subsets (Fig. 8B). In addition, the mRNA levels of Th1 lineage-specific cytokine IFN- γ , Th2 lineage-specific cytokine IL-4, and Th17 lineage-specific cytokine IL-17A were effectively decreased by sophoricoside treatment (Fig. 8C). Consistent with the mRNA levels of master regulators and cytokines, sophoricoside significantly decreased the amounts of released IFN- γ , TNF- α , and IL-17A (Fig. 8D). These results indicate that sophoricoside might improve Th cell-mediated airway inflammatory responses, including allergic asthma, by inhibiting CD4⁺ T cell differentiation.

4. Discussion

Asthma causes serious health and socioeconomic problems worldwide because it affects a large portion of the population, from children to adults. The prevalence, hospitalization rate and mortality of asthma

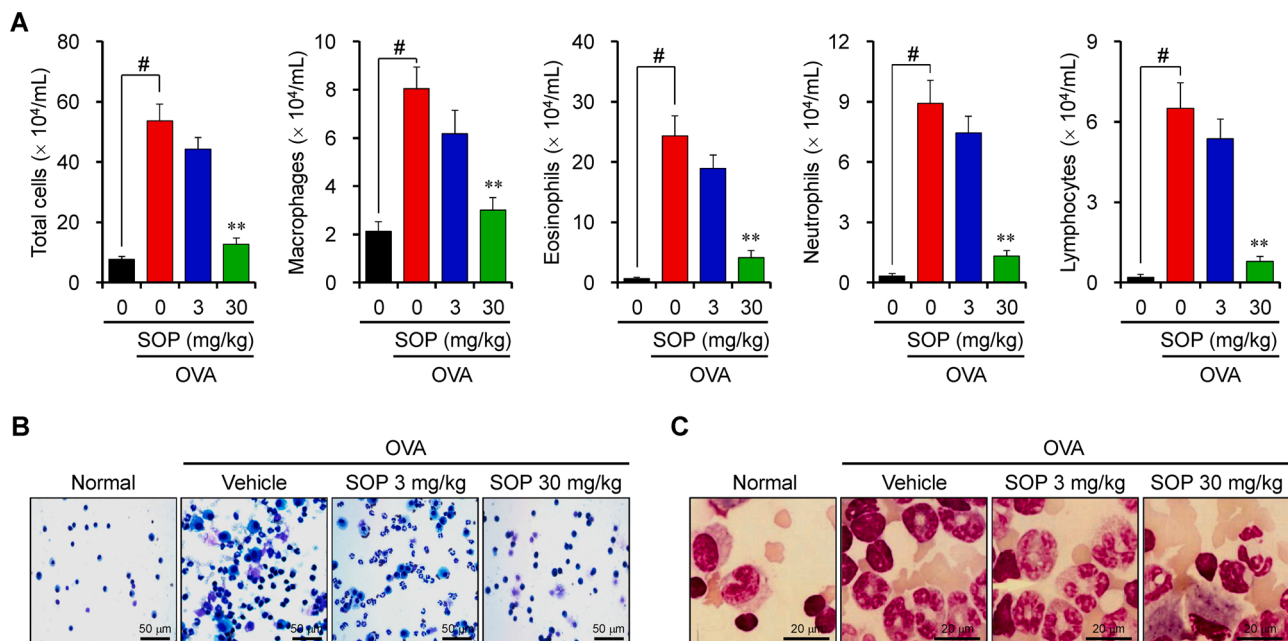


Fig. 3. Sophoricoside inhibits recruitment of immune cells into allergic airway in OVA-induced mice. (A) Cells prepared from BALF were applied to a slide by cytopinning and the number of total cells, macrophages, eosinophils, neutrophils, and lymphocytes was determined by counting within a 1 mm² area. [#] $p < 0.005$ versus vehicle-treated group; ^{**} $p < 0.005$ versus OVA-induced group ($n = 6$). (B and C) Cells prepared from BALF were stained with either Diff-Quick or Wright-Giemsa solution, and total cells (B) and eosinophils (C) were observed under light microscope. Scale bars: 50 or 20 μm . SOP, sophoricoside.

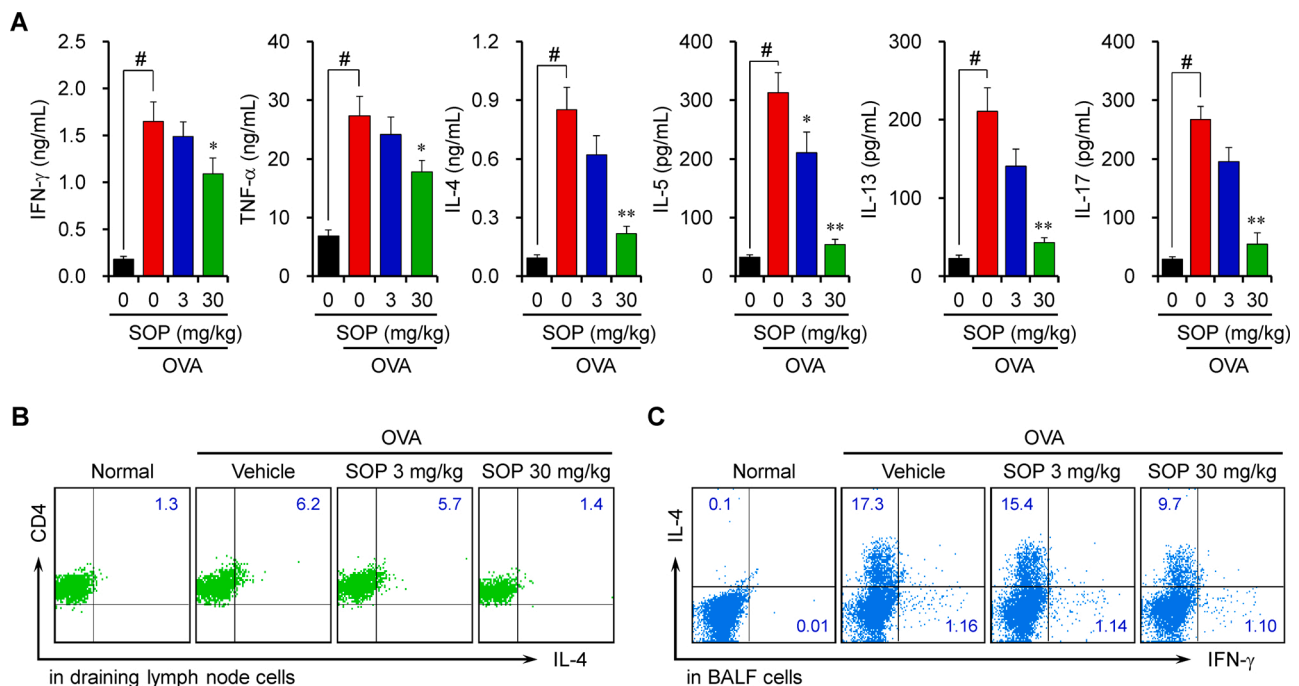


Fig. 4. Sophoricoside inhibits release of pro-inflammatory cytokines in OVA-induced mice. (A) Amounts of pro-inflammatory cytokines were measured by ELISA from serum of OVA-induced mice. [#] $p < 0.005$ versus vehicle-treated group; ^{*} $p < 0.05$ and ^{**} $p < 0.005$ versus OVA-induced group ($n = 6$). (B and C) Draining lymph node cells (B) and BALF cells (C) were stained with PE-conjugated anti-CD4 antibody, APC-conjugated anti-IL-4 antibody, or FITC-conjugated anti-IFN- γ antibody, and populations of CD4⁺, IL-4⁺, and IFN- γ -positive cells were counted by FACS analysis. SOP, sophoricoside.

have markedly increased over the past half century [37]. Over the past few decades, asthma treatment has focused on relieving inflammation and bronchoconstriction in the respiratory system with corticosteroids, leukotriene modifiers, and anti-cholinergic agents. Recently, novel targeted therapies have been developed based on improved pathophysiological and biologic understanding. The targets mainly include IgE, Th2

cell subset-derived cytokines, C-X-C motif chemokine receptor 2 (CXCR2), C-C motif chemokine receptor 3 (CCR3), and tyrosine kinases [38]. In this study, we demonstrated the anti-allergic and anti-asthmatic effects of sophoricoside that suppress mast cell activation and CD4⁺ T cell differentiation.

Sophoricoside is an isoflavone substance containing genistein

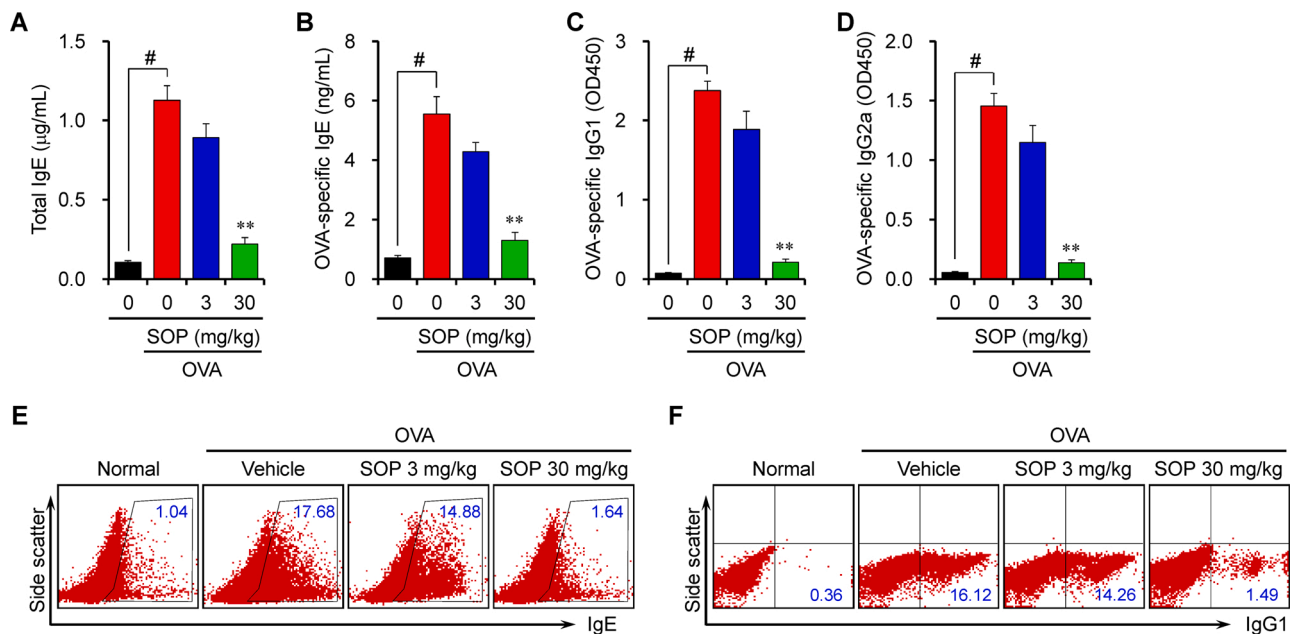


Fig. 5. Sophoricoside inhibits secretion of immunoglobulins in OVA-induced mice. (A–D) Amounts of total IgE (A), OVA-specific IgE (B), OVA-specific IgG1 (C), and OVA-specific IgG2a (D) were measured by ELISA from serum of OVA-induced mice. [#] $p < 0.005$ versus vehicle-treated group; ^{**} $p < 0.005$ versus OVA-induced group ($n = 6$). (E and F) Total cells prepared from BALF were stained with FITC-conjugated anti-IgE or PE-conjugated anti-IgG1 antibody, and populations of IgE- and IgG1-positive cells were counted by FACS analysis. SOP, sophoricoside.

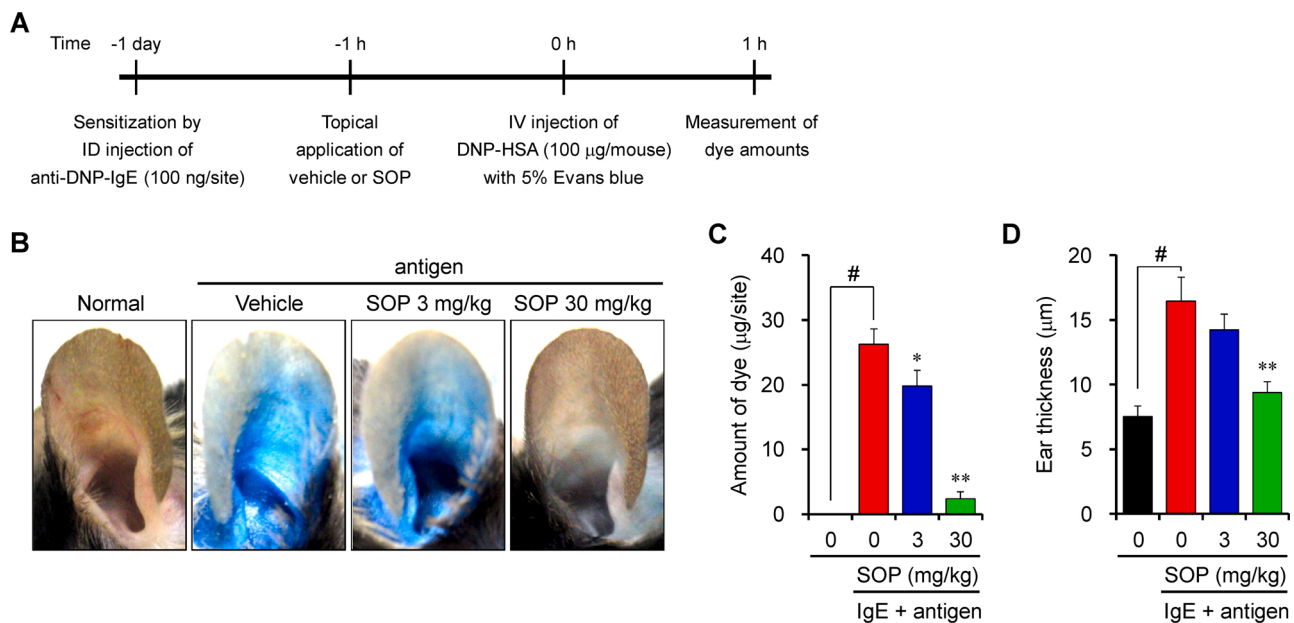


Fig. 6. Sophoricoside inhibits allergic response in PCA mice. (A) Experimental scheme for PCA experiment in C57BL/6 mice. (B–D) Mice were passively sensitized with 100 ng anti-DNP IgE. Twenty-four hours after intradermal injection of anti-DNP IgE, sophoricoside was topically applied to mouse ears 1 h before intravenous injection of 100 μg DNP-HSA containing 5% Evans' blue dye. One hour after DNP-HSA injection, images of the ears (B), amounts of Evans blue dye (C), and ear swelling (D) were measured. [#] $p < 0.005$ versus vehicle-treated group; ^{*} $p < 0.05$ and ^{**} $p < 0.005$ versus IgE/antigen-induced group ($n = 5$). SOP, sophoricoside (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

glycoside with a glucose moiety at position 4. Isoflavones are widely contained in soybeans and soy protein products. They play an important role in maintaining human health via prevention of various chronic human diseases, including inflammatory disorders. Sophoricoside has been isolated from *S. japonica*, a tree native to Korea and China of the Leguminosae family. We isolated sophoricoside from the different parts of *S. japonica* to determine its content of sophoricoside and its novel pharmacological efficacies. We found that sophoricoside was present in

the highest amounts in seeds (3.302 mg/g DW), followed by leaves (0.048 mg/g DW), and it was present in trace amounts in the stems and roots of *S. japonica*. Since initial isolation and hormonal effect of sophoricoside were reported [8], there have been few reports of its biological activities. We were interested in the inhibitory effects of sophoricoside on IL-3, IL-5, and IL-6 bioactivities [9,17] and its anti-inflammatory and immunosuppressive effects [10–12]. Specifically, IL-3, IL-5, and IL-6 are important pro-inflammatory cytokines produced from immune cells,

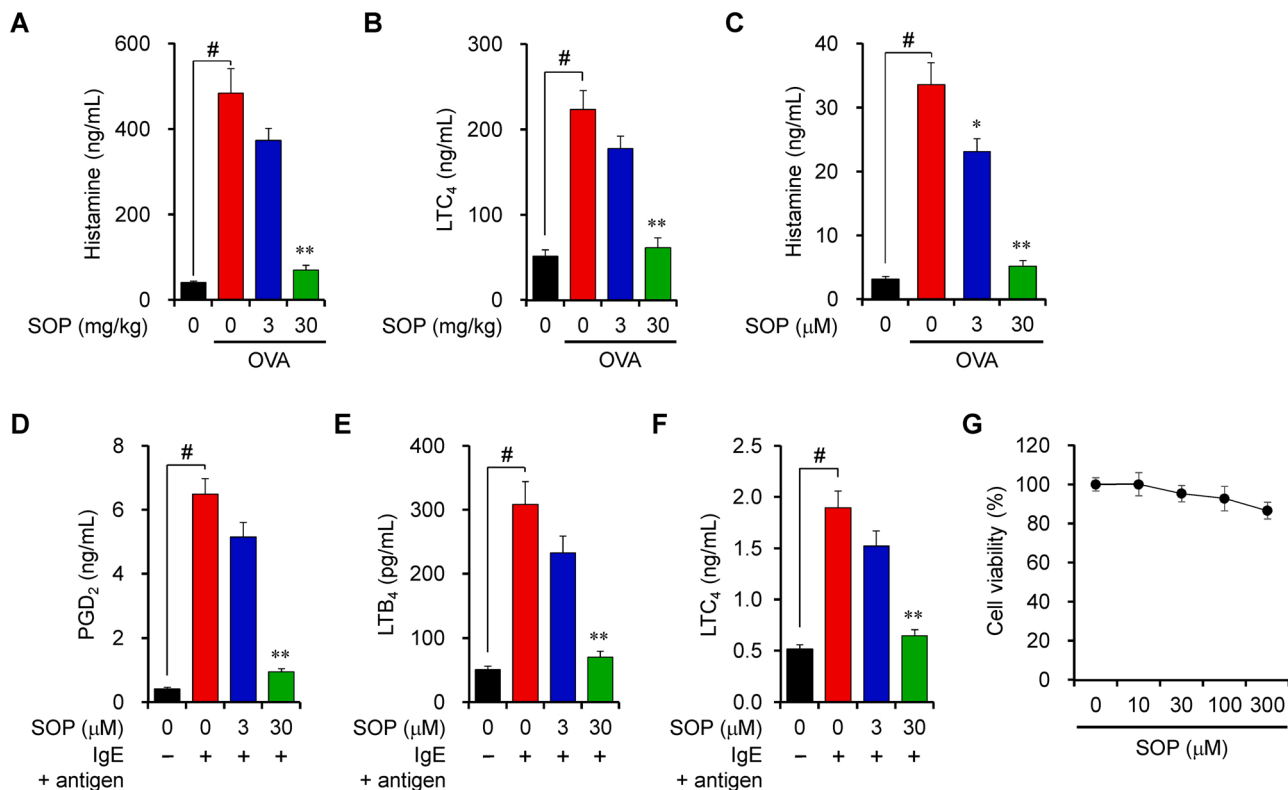


Fig. 7. Sophoricoside inhibits release of histamine and arachidonic acid metabolites. (A and B) Amounts of histamine (A) and LTC₄ (B) were measured by ELISA from BALF. [#]*p* < 0.005 versus vehicle-treated group; ^{**}*p* < 0.005 versus OVA-induced group (*n* = 6). (C–F) HMC-1 cells were sensitized with 1 μg/mL anti-DNP-IgE overnight. Cells were washed twice and treated with sophoricoside for 30 min, followed by activation with DNP-HAS (10 μg/mL). Amounts of histamine (C), PGD₂ (D), LTB₄ (E), and LTC₄ (F) were measured by ELISA. [#]*p* < 0.005 versus vehicle-treated group; ^{*}*p* < 0.05 and ^{**}*p* < 0.005 versus IgE/antigen-induced group (*n* = 3). (G) HMC-1 cells were incubated with various concentrations of sophoricoside for 24 h, and their viability was measured. SOP, sophoricoside.

such as Th2 cells, basophils, eosinophils, activated T cells and macrophages that play an essential role in the pathogenesis of inflammatory, allergic, and asthmatic diseases [39]. Allergen-induced allergic and asthmatic diseases are mainly associated with type 2 inflammation, which is induced by pro-inflammatory cytokines produced from Th2 cells [40]. In fact, sophoricoside treatment strongly inhibited the releasing of Th2 and Th17 cytokines, including IL-4, IL-5, IL-13 and IL-17, than that of Th1 cytokines, such as IFN- γ and TNF- α , by suppressing the recruitment of immune cells such as eosinophils, neutrophils, lymphocytes and macrophages into the allergic airways, thereby inhibiting allergic and asthmatic features in OVA-induced mice. These results suggested that sophoricoside may affect Th2 cell-mediated allergic and asthmatic diseases.

Allergic and asthmatic diseases are commonly induced by the sensitization to and challenge with foreign antigens. The patterns of airway inflammation are caused by immune cells such as eosinophils, neutrophils, basophils and lymphocytes controlled by CD4⁺ T cell subsets, including Th1, Th2, and Th17 [41]. Since asthmatic inflammation is considered a hallmark of Th2 cells, we generated acute allergic and asthmatic airway inflammation by intraperitoneal sensitization and intratracheal challenge with OVA in BALB/c mice. OVA is widely used to induce experimental asthma in animals. OVA-induced asthmatic airway inflammation features are mediated from T cell antigen receptor (TCR)-driven Th2 type immune responses [42]. The main symptoms are characterized by influx of immune cells into the lungs, excessive production of mucus and AHR in the airway, and elevated levels of serum IgE [21,43]. The mouse model generated by OVA showed elevated allergic and asthmatic symptoms, indicating well-induced airway inflammation in the mice. Sophoricoside treatment exhibited effective anti-allergic and anti-asthmatic activities through reduced nasal rubbing, recruitment of inflammatory cells into the lungs, and increased

levels of IgE, pro-inflammatory cytokines, histamine, and arachidonic acid metabolites. Sophoricoside also effectively decreased allergic responses such as ear swelling and mast cell-mediated leakage of blood vessels in IgE-antigen reaction-induced PCA mice. The results obtained from *in vivo* models indicate that sophoricoside is a promising reagent for preventing allergy and asthma.

Mast cells and eosinophils are the main effector cells of allergic inflammation. Activated mast cells contribute to secretion of histamine and newly-synthesized lipid metabolites that are responsible for production of various cytokines, chemokines, and growth factors, including IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-11, IL-13, GM-CSF, TNF- α , transforming growth factor-beta (TGF- β), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) [44,45]. IgE and Th2 subset signature cytokines IL-4 and IL-13 play an important role in maintaining mast cell homeostasis, and released mast cell metabolites recruit Th2 cells and eosinophils to inflammatory sites to promote allergic process [46–48]. To evaluate whether sophoricoside influences mast cell activation, we determined the amounts of released histamine and arachidonic acid metabolites, such as PGD₂, LTB₄ and LTC₄, in OVA-induced mice and human mast cells stimulated with IgE plus antigen. Sophoricoside decreased the levels of released histamine and arachidonic acid metabolites in OVA-induced mice and mast cells, indicating an inhibitory effect of sophoricoside on mast cell activation and reduced allergic responses. Histamine and arachidonic acid metabolites released from IgE-primed mast cells recruit immune cells, such as eosinophils, neutrophils, basophils, and Th2 cells [33,34]. Based on these signaling networks, the correlation between mast cell activation, immune cell recruitment, and CD4⁺ T cell differentiation is tightly correlated with exacerbation of allergic inflammation. In this pathogenic circumstance, sophoricoside reduced allergic airway inflammation by inhibition of positively

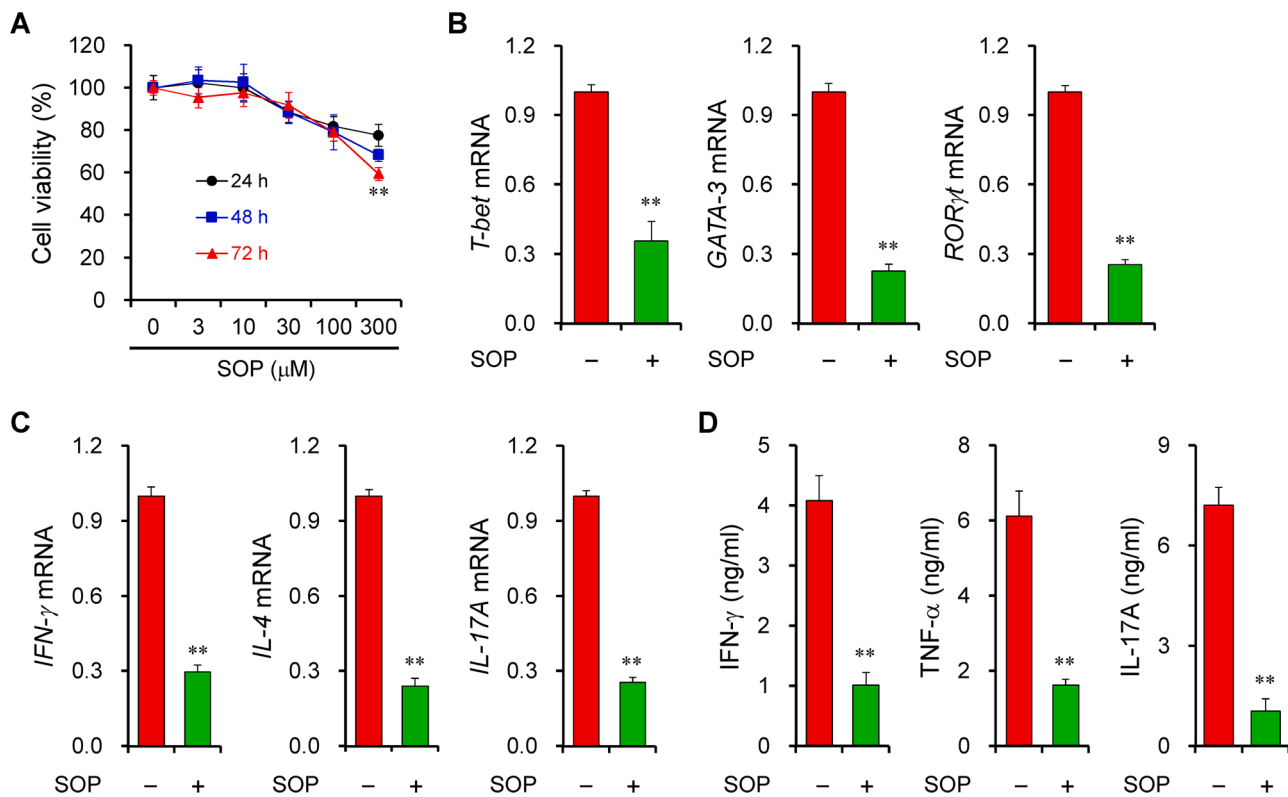


Fig. 8. Sophoricoside inhibits CD4⁺ T cell differentiation. (A) CD4⁺ T cells were incubated with various concentrations of sophoricoside for 24–72 h, and their viability was measured. (B–D) Naïve CD4⁺ T cells were differentiated under appropriate differentiation conditions for 5 days in the presence or absence of sophoricoside (30 μM), as described in Materials and Methods. Total RNA was isolated, and qRT-PCR was performed to analyze levels of CD4⁺ T cell lineage-specific master regulators (B) and cytokines (C). Cytokine amounts were measured by ELISA using cultured supernatants (D). ***p* < 0.005 versus differentiated group (*n* = 3). SOP, sophoricoside.

correlated these signaling loops.

Naïve CD4⁺ T cell-derived effector subsets mainly include Th1, Th2, Th17, and Treg, which play important roles in direct immune responses. Over-activated these effector cells aggravate allergic airway inflammation by producing lineage-specific signature cytokines and recruiting immune cells to inflammatory sites [39,41,49]. Interestingly, sophoricoside more strongly inhibited the release of Th2 and Th17 specific cytokines, such as IL-4, IL-5, IL-13, and IL-17, compared to Th1 specific cytokines, such as IFN-γ and TNF-α, in an OVA-induced mouse model. Consistent results were also observed with *in vitro* differentiation experiments of naïve CD4⁺ T cells into Th1, Th2, and Th17 cell subsets. Sophoricoside also inhibited the mRNA levels of master regulators, T-bet, GATA-3, and RORγt, for Th1, Th2, and Th17 cell subsets, respectively, as well as the production and mRNA levels of Th cell subset-specific cytokines. The inhibitory effects of sophoricoside on the expression and secretion of master regulators and cytokines associated with Th2 and Th17 cell subsets were slightly stronger than those on the Th1 cell subset.

Based on our results, sophoricoside treatment effectively reduced allergic and asthmatic responses in OVA-induced allergic airway inflammation and DNP-specific IgE-induced PCA models. These pharmacological activities of sophoricoside were associated with the inhibition of mast cell activation and CD4⁺ T cell differentiation into Th2 cells, consequently decreased the recruitment of immune cells into the allergic airways, secretion of inflammatory cytokines and immunoglobulins, and releasing histamine and arachidonic acid metabolites. In particular, it also effectively inhibited the differentiation of CD4⁺ T cells into Th1 and Th17 cell subsets. These cell subsets play important roles in inducing organ-specific and systemic autoimmune disorders and cancers [50,51]. Therefore, these results suggest that sophoricoside can be used as a potential reagent for the treatment of allergic diseases, various types

of autoimmune disorders and cancers.

5. Conclusion

In present study, we isolated sophoricoside from mature seeds of *S. japonica* to investigate its anti-allergic and anti-asthmatic activities in animal models. There was higher sophoricoside content in mature seeds than in leaves, stems, and roots. Sophoricoside reduced allergic and asthmatic responses in OVA-induced allergic airway inflammation and PCA models by inhibiting mast cell activation and CD4⁺ T cell differentiation. Our results imply that sophoricoside may be a useful therapeutic candidate for treating allergic diseases, including allergic asthma.

Funding

This work was supported by grants from Natural Product Institute of Science and Technology (www.nist.re.kr), Anseong 17546, Republic of Korea.

Declaration of Competing Interest

There is no conflict of interest to declare.

References

- [1] J.V. Fahy, Type 2 inflammation in asthma-present in most, absent in many, *Nat. Rev. Immunol.* 15 (2015) 57–65.
- [2] A. Ray, M. Raundhal, T.B. Oriss, P. Ray, S.E. Wenzel, Current concepts of severe asthma, *J. Clin. Invest.* 126 (2016) 2394–2403.
- [3] J.S. Marshall, R. Warrington, W. Watson, H.L. Kim, An introduction to immunology and immunopathology, *Allergy Asthma Clin. Immunol.* 14 (2018) 49.
- [4] A. Esmailzadeh, S. Tahmasebi, S.S. Athari, Chimeric antigen receptor -T cell therapy: applications and challenges in treatment of allergy and asthma, *Biomed. Pharmacother.* 123 (2020), 109685.

- [5] A. Papi, C. Brightling, S.E. Pedersen, H.K. Reddel, Asthma, *Lancet* 391 (2018) 783–800.
- [6] J. Quirt, K.J. Hildebrand, J. Mazza, F. Noya, H. Kim, Asthma, *Allergy Asthma Clin. Immunol.* 14 (2018) 50.
- [7] E. Zinellu, B. Piras, G.G.M. Ruzittu, S.S. Fois, A.G. Fois, P. Pirina, Recent advances in inflammation and treatment of small airways in asthma, *Int. J. Mol. Sci.* 20 (2019) E2617.
- [8] M. Gabor, The hormonal effect of an isoflavone derivative (sophoricoside), *Kiserl. Orvostud.* 13 (1961) 133–134.
- [9] B. Min, S.R. Oh, H.K. Lee, K. Takatsu, I.M. Chang, K.R. Min, Y. Kim, Sophoricoside analogs as the IL-5 inhibitors from *Sophora japonica*, *Planta Med.* 65 (1999) 408–412.
- [10] B.H. Kim, E.Y. Chung, B.K. Min, S.H. Lee, M.K. Kim, K.R. Min, Y. Kim, Anti-inflammatory action of legume isoflavonoid sophoricoside through inhibition on cyclooxygenase-2 activity, *Planta Med.* 69 (2003) 474–476.
- [11] S.J. Kim, G.Y. Lee, J.W. Jung, S.R. Oh, E.M. Ahn, S.H. Kim, S.H. Hong, J.Y. Um, The ameliorative effect of sophoricoside on mast cell-mediated allergic inflammation in vivo and in vitro, *Molecules* 18 (2013) 6113–6127.
- [12] H.K. Lee, H.S. Kim, Y.J. Kim, J.S. Kim, Y.S. Park, J.S. Kang, D.Y. Yuk, J.T. Hong, Y. Kim, S.B. Han, Sophoricoside isolated from *Sophora japonica* ameliorates contact dermatitis by inhibiting NF- κ B signaling in B cells, *Int. Immunopharmacol.* 15 (2013) 467–473.
- [13] A.M. El-Halawany, M.H. Chung, H.M. Abdallah, T. Nishihara, M. Hattori, Estrogenic activity of a naringinase-treated extract of *Sophora japonica* cultivated in Egypt, *Pharm. Biol.* 48 (2010) 177–181.
- [14] C. Wu, H. Luan, S. Wang, X. Zhang, R. Wang, L. Jin, P. Guo, X. Chen, Modulation of lipogenesis and glucose consumption in HepG2 cells and C2C12 myotubes by sophoricoside, *Molecules* 18 (2013) 15624–15635.
- [15] W. Li, Y. Lu, Hepatoprotective effects of sophoricoside against fructose-induced liver injury via regulating lipid metabolism, oxidation, and inflammation in mice, *J. Food Sci.* 83 (2018) 552–558.
- [16] H.M. Abdallah, A.M. Al-Abd, G.F. Asaad, A.B. Abdel-Naim, A.M. El-halawany, Isolation of antiosteoporotic compounds from seeds of *Sophora japonica*, *PLoS One* 9 (2014), e98559.
- [17] J. Yun, C.K. Lee, I.M. Chang, K. Takatsu, T. Hirano, K.R. Min, Y. Kim, Differential inhibitory effects of sophoricoside analogs on bioactivity of several cytokines, *Life Sci.* 67 (2000) 2855–2863.
- [18] B.H. Kim, E.Y. Chung, J.C. Ryu, S.H. Jung, K.R. Min, Y. Kim, Anti-inflammatory mode of isoflavone glycoside sophoricoside by inhibition of interleukin-6 and cyclooxygenase-2 in inflammatory response, *Arch. Pharm. Res.* 26 (2003) 306–311.
- [19] B.N. Lambrecht, H. Hammad, J.V. Fahy, The cytokines of asthma, *Immunity* 50 (2019) 975–991.
- [20] M. Dougan, G. Dranoff, S.K. Dougan, GM-CSF, IL-3, and IL-5 Family of Cytokines: Regulators of Inflammation, *Immunity* 50 (2019) 796–811.
- [21] M. Casaro, V.R. Souza, F.A. Oliveira, C.M. Ferreira, OVA-induced allergic airway inflammation mouse model, *Methods Mol. Biol.* 1916 (2019) 297–301.
- [22] C. Saltini, A.J. Hance, V.J. Ferrans, F. Basset, P.B. Bitterman, R.G. Crystal, Accurate quantification of cells recovered by bronchoalveolar lavage, *Am. Rev. Respir. Dis.* 130 (1984) 650–658.
- [23] B.H. Kim, I. Oh, J.H. Kim, J.E. Jeon, B. Jeon, J. Shin, T.Y. Kim, Anti-inflammatory activity of compounds isolated from *Astragalus sinicus* L. In cytokine-induced keratinocytes and skin, *Exp. Mol. Med.* 46 (2014) e87.
- [24] H.G. Lee, N.C. Cho, A.J. Jeong, Y.C. Li, S.J. Rhie, J.S. Choi, K.H. Lee, Y. Kim, Y. N. Kim, M.H. Kim, S.K. Ye, B.H. Kim, Immunomodulatory activities of the benzoxathiole derivative BOT-4-one ameliorate pathogenic skin inflammation in mice, *J. Invest. Dermatol.* 136 (2016) 107–116.
- [25] J. Wu, X. Yang, J. Ge, Y. Zhang, L. Wu, J. Liu, X. Zhang, Biotransformation of sophoricoside in *Fructus Sophorae* by the fungus *Schizophyllum commune*, *Bioresour. Technol.* 111 (2012) 496–499.
- [26] K.L. Asquith, H.S. Ramshaw, P.M. Hansbro, K.W. Beagley, A.F. Lopez, P.S. Foster, The IL-3/IL-5/GM-CSF common receptor plays a pivotal role in the regulation of Th2 immunity and allergic airway inflammation, *J. Immunol.* 180 (2008) 1199–1206.
- [27] M. Dougan, G. Dranoff, S.K. Dougan, GM-CSF, IL-3, and IL-5 family of cytokines: Regulators of inflammation, *Immunity* 50 (2019) 796–811.
- [28] S. Saglani, C.M. Lloyd, Novel concepts in airway inflammation and remodeling in asthma, *Eur. Respir. J.* 46 (2015) 1796–1804.
- [29] R. Pawankar, M. Hayashi, S. Yamanishi, T. Igarashi, The paradigm of cytokine networks in allergic airway inflammation, *Curr. Opin. Allergy Clin. Immunol.* 15 (2015) 41–48.
- [30] K. Hirose, A. Iwata, T. Tamachi, H. Nakajima, Allergic airway inflammation: key players beyond the Th2 cell pathway, *Immunol. Rev.* 278 (2017) 145–161.
- [31] T.A. Platts-Mills, The role of immunoglobulin E in allergy and asthma, *Am. J. Respir. Crit. Care Med.* 164 (2001) S1–S5.
- [32] E.E. Perez, J.S. Orange, F. Bonilla, J. Chinen, I.K. Chinn, M. Dorsey, Y. El-Gamal, T. O. Harville, E. Hossny, B. Mazer, R. Nelson, E. Secord, S.C. Jordan, E.R. Stiehm, A. A. Vo, M. Ballow, Update on the use of immunoglobulin in human disease: a review of evidence, *J. Allergy Clin. Immunol.* 139 (2017) S1–S46.
- [33] D.D. Metcalfe, R. Pawankar, S.J. Ackerman, C. Akin, F. Clayton, F.H. Falcone, G. J. Gleich, A.M. Irani, M.W. Johansson, A.D. Klion, K.M. Leiferman, F. Levi-Schaffer, G. Nilsson, Y. Okayama, C. Prussin, J.T. Schroeder, L.B. Schwartz, H.U. Simon, A. F. Walls, M. Triggiani, Biomarkers of the involvement of mast cells, basophils and eosinophils in asthma and allergic disease, *World Allergy Organ. J.* 9 (2016) 7.
- [34] E. Schaubberger, M. Peinhaupt, T. Cazaes, A.W. Lindsley, Lipid mediators of allergic disease: pathways, treatments, and emerging therapeutic targets, *Curr. Allergy Asthma Rep.* 16 (2016) 48.
- [35] M.F. Ling, A.D. Luster, Allergen-specific CD4(+) T cells in human asthma, *Ann. Am. Thorac. Soc.* 13 (2016) S25–S30.
- [36] L.M. Muehling, M.G. Lawrence, J.A. Woodfolk, Pathogenic CD4⁺T cells in patients with asthma, *J. Allergy Clin. Immunol.* 140 (2017) 1523–1540.
- [37] S.C. Dharmage, J.L. Perret, A. Custovic, Epidemiology of asthma in children and adults, *Front. Pediatr.* 7 (2019) 246.
- [38] M.E. Wechsler, Current and emerging biologic therapies for asthma and COPD, *Respir. Care* 63 (2018) 699–707.
- [39] J. Zhu, H. Yamane, W.E. Paul, Differentiation of effector CD4 T cell populations (*), *Annu. Rev. Immunol.* 28 (2010) 445–489.
- [40] G.M. Gauvreau, A.I. El-Gammal, P.M. O'Byrne, Allergen-induced airway responses, *Eur. Respir. J.* 46 (2011) 819–831.
- [41] B.N. Lambrecht, H. Hammad, The immunology of asthma, *Nat. Immunol.* 16 (2015) 45–56.
- [42] D.M. Durrant, D.W. Metzger, Emerging roles of T helper subsets in the pathogenesis of asthma, *Immunol. Invest.* 39 (2010) 526–549.
- [43] S.T. Holgate, S. Wenzel, D.S. Postma, S.T. Weiss, H. Renz, P.D. Sly, Asthma, *Nat. Rev. Dis. Primers* 1 (2015) 15025.
- [44] Y. Minai-Fleminger, F. Levi-Schaffer, Mast cells and eosinophils: the two key effector cells in allergic inflammation, *Inflamm. Res.* 58 (2009), 631–628.
- [45] K.D. Stone, C. Prussin, D.D. Metcalfe, IgE, mast cells, basophils, and eosinophils, *J. Allergy Clin. Immunol.* 125 (2010) S73–S80.
- [46] J. Leung, K.R. Beukema, A.H. Shen, Allergic mechanisms of eosinophilic oesophagitis, *best pract. Res. Clin. Gastroenterol.* 29 (2015) 709–720.
- [47] J.J. McLeod, B. Baker, J.J. Ryan, Mast cell production and response to IL-4 and IL-13, *Cytokine* 75 (2015) 57–61.
- [48] H.U. Simon, S. Yousefi, N. Germic, I.C. Arnold, A. Haczku, A.V. Karaulov, D. Simon, H.F. Rosenberg, The cellular functions of eosinophils: collegium international allergologicum (CIA) update 2020, *Int. Arch. Allergy Immunol.* 181 (2020) 11–23.
- [49] M. Akdis, C.A. Akdis, Mechanisms of allergen-specific immunotherapy: multiple suppressor factors at work in immune tolerance to allergens, *J. Allergy Clin. Immunol.* 133 (2014) 621–631.
- [50] V. Dardalhon, T. Korn, W.K. Kuchroo, A.C. Anderson, Role of Th1 and Th17 cells in organ-specific autoimmunity, *J. Autoimmun.* 31 (2008) 252–256.
- [51] J. Tabarkiewicz, K. Pogoda, A. Karczmarczyk, P. Pozarowski, K. Giannopoulos, The role of IL-17 and Th17 lymphocytes in autoimmune diseases, *Arch Immunol Ther Exp (Warsz)*. 63 (2015) 435–449.