

Ethanol extract of *Veronica persica* ameliorates house dust mite-induced asthmatic inflammation by inhibiting STAT-3 and STAT-6 activation

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ABSTRACT

Veronica persica is a flowering plant belonging to the family Scrophulariaceae. Here, we aimed to evaluate the pharmacological activity of the ethanol extract of *Veronica persica* (EEVP) in an airway inflammation model. We examined airway responsiveness to aerosolized methacholine, serum immunoglobulin (Ig)E levels, and total cell numbers in the lung and bronchoalveolar lavage fluid (BALF). Histological analysis of the lung tissue was performed using hematoxylin–eosin, Masson trichrome, or periodic acid-Schiff staining. Fluorescence-activated cell sorting analysis in the lung and BALF was applied to clarify the changes in immune cell types. Enzyme-linked immunosorbent assay and quantitative real-time polymerase chain reaction were applied to investigate cytokine levels and gene expression related to airway inflammation. STAT-3/6 phosphorylation was examined in primary bronchial/tracheal epithelial cells using western blot analysis. EEVP significantly suppressed total IgE levels and methacholine-induced increase of Penh value in the HDM-challenged mouse model. EEVP also attenuated the severity of airway remodeling in lung tissues and decreased eosinophil and neutrophil infiltration in the lungs and BALF. EEVP significantly reduced the production of cytokines in BAL and splenocyte culture medium, and the expression of mRNAs related to airway inflammation in the lung tissue. EEVP suppressed IL-4/13-induced STAT-3/6 phosphorylation in the epithelial cells. We showed for the first time that EEVP effectively inhibits eosinophilic airway inflammation by suppressing the expression of inflammatory factors for T cell activation and polarization, and inhibits MCP-1 production of bronchial/tracheal epithelial cells by suppressing STAT-3/6 activation. EEVP may be a potential pharmacological agent to prevent inflammatory airway diseases.

1. Introduction

Asthma is a chronic inflammatory disease of the respiratory tract characterized by airway hyperresponsiveness, mucus hyperproduction and production, and airway wall remodeling in response to allergens such as house dust mite (HDM) or air pollution [1]. Inflammatory cells, including type 2 helper T (Th2) cells, eosinophils, and mast cells, are principal cells recruited to bronchiolar lesions during the development of asthma [2,3]. As regulators of the inflammatory response to asthma, Th2 cells produce several inflammatory cytokines (IL-4, IL-5, IL-13,

IL-33, or TNF- α) to stimulate leukocyte recruitment and activation, especially eosinophils, at the inflammatory site [4–7]. These activated immune cells further induce leukocyte inflammation, increase immunoglobulin E (IgE) and goblet cell hyperplasia, and increase airway mucus secretion to aggravate pathological features of asthma. It has been suggested that mast cells are linked to airway hyperresponsiveness in the airway smooth muscles, but eosinophils are associated with airway fibrosis [3,8]. The main treatment for asthma in the clinic is inhaled glucocorticoid in combination with bronchodilator drugs, beta-agonists in particular [9,10]. Selective immunomodulators to

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inhibit Th2 cells or Th2 cytokine release have also been suggested as a new therapy for asthma with clinical benefit and few significant side effects [11,12].

There have been many reports to demonstrate the anti-asthmatic effect of botanical drugs or herbal plants used in traditional medicine [13]. *Veronica persica* is a flowering plant belonging to the family Scrophulariaceae, widely distributed in Europe and Asia. It has been traditionally used to treat genital diseases, such as reduction of testis swelling or leukorrhea, in Korea. Phytochemical studies of *Veronica p.* have demonstrated that they contain mainly iridoid glucosides, especially benzoic and cinnamic acid, some phenylethanoids, and flavonoid glycosides [14]. Several pharmacological studies have also reported its free radical scavenging activity [15], anti-inflammatory effect on lipopolysaccharide-stimulated nitric oxide production in peritoneal macrophages [16], anti-cancer activity against KB and B16 melanoma cells [16], and anti-nociceptive effect on carrageenan-induced hind paw edema in rats [17]. However, the pharmacological effect of *Veronica p.* on allergen-induced airway inflammation has not been evaluated. Therefore, in this study, we aimed to explore the potential inhibitory effect of the ethanol extract of *Veronica p.* (EEVP) on lung inflammation in a murine model HDM-induced allergic asthma.

2. Materials and methods

2.1. Preparation and characterization of plant material

The *Veronica persica* (Plantaginaceae) was obtained from Prof. Byung Kil Choo (Chonbuk National University, Jeonju, Korea), and plant material was identified of the Korea Institute of Oriental Medicine (KIOM). *Veronica p.* (10 g) (voucher number, KIOM 52) was extracted twice in 70 % ethanol (100 mL) at boiling for 2 h in a total three times, and then concentrated under a vacuum freeze dryer. To determine major constituents of the ethanol extract of *Veronica p.* (EEVP) (10 mg/mL), we used ultra-performance liquid chromatography/quadrupole-orbitrap mass spectrometry (UPLC-MS/MS) as reported previously [18]. Briefly, chromatographic analysis was performed on a Dionex UltiMate 3000 system, and chromatographic separation was achieved with a C18 column (Acquity BEH, 100 × 2.1 mm, 1.7 μm) using gradient elution (0.1 % formic acid in water and acetonitrile). MS analysis was conducted on a Q-exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA), and data acquisition was performed in ddMS2 mode under negative ionization. Five iridoid glycosides, including aucubin, loganic acid, verproside, catalposide, and picoside II, were identified (Table 1 and Fig. 1). The retention time and mass spectrums of three phytochemicals were compared with those of reference standards (Targetmol, Wellesley Hills, MA, USA), whereas verproside and catalposide were identified by the mass to charge ratio (m/z) as reported previously [19].

2.2. Animal experiments

Male BALB/c mice (Orientbio Inc., Seongnam, Korea), 7-week old (20–25 g) were housed under standard laboratory conditions for mice (21 ± 2 °C, 50 ± 5 % humidity, and a 12/12-h light/dark cycle). Food and water were provided ad libitum. The protocol was reviewed and

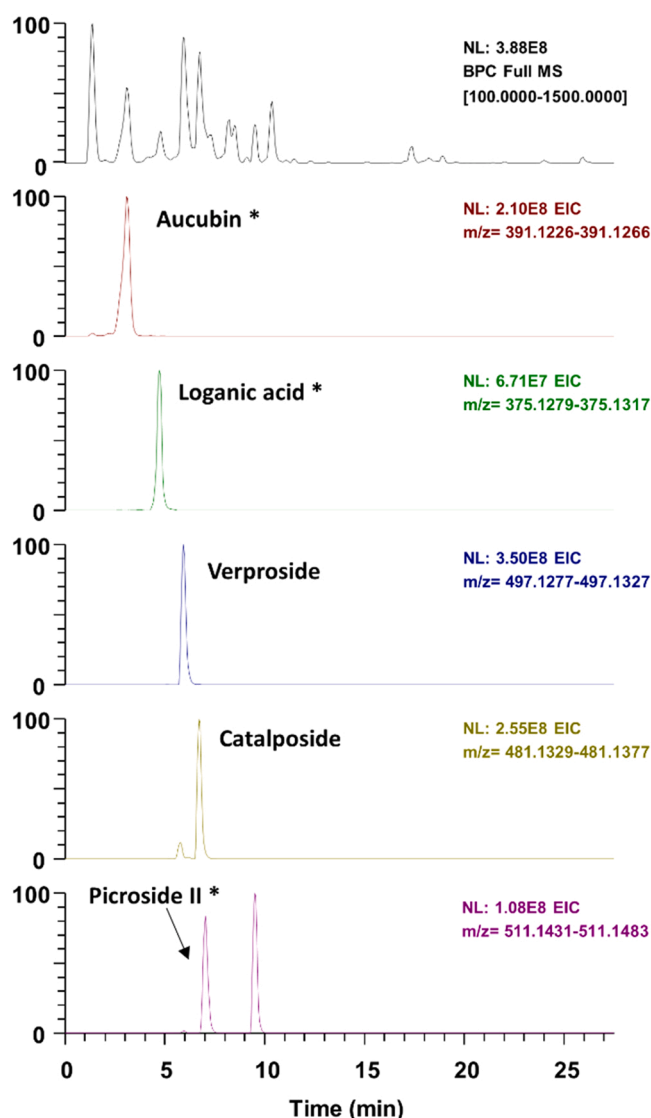


Fig. 1. Base peak and extracted ion chromatograms of EEVP using UPLC-MS/MS analysis. *, Compared with the retention time and mass spectral data of reference standards.

approved by the Committee for Animal Welfare at Daejeon University, Republic of Korea (IACUC no.: DJUAR2021-003). This study was conducted following the Guidelines of the Institutional Animal Care and Use Committee of Daejeon University (Daejeon, Republic of Korea).

2.3. HDM-sensitized asthma model

HDM-sensitized asthma mouse model was established as followed previous studies [20,21]. Briefly, the mice were immunized via intraperitoneal injection with 25 μg of HDM extracts (Dermatophagoides

Table 1
Phytochemicals identified in EEVP using UHPLC-MS/MS.

No	Rt (Min)	Calculated (m/z)	Estimated (m/z)	Adducts	Error (ppm)	Elemental composition	MS/MS fragments (m/z)	Identifications
1	3.13	391.1246	391.1249	M + HCO ₂	0.84	C ₁₅ H ₂₂ O ₉	183, 165, 139	Aucubin
2	4.71	375.1297	375.1299	M-H	0.57	C ₁₆ H ₂₄ O ₁₀	213, 169, 151, 125	Loganic acid
3	5.88	497.1301	497.1304	M-H	0.69	C ₂₂ H ₂₆ O ₁₃	497, 335, 221, 153	Verproside [19]
4	6.72	481.1352	481.1354	M-H	0.47	C ₂₂ H ₂₆ O ₁₂	205, 137	Catalposide [19]
5	7.05	511.1457	511.1458	M-H	0.08	C ₂₃ H ₂₈ O ₁₃	235, 167	Picoside II

Rt, retention time; MS/MS, quadrupole-orbitrap mass spectrometry; m/z , mass to charge ratio; ppm, EEVP, ethanol extract of *Veronica persica*; UHPLC, ultra high-performance liquid chromatography.

farinae, Dfb, BioStir, Japan) and 2 mg of aluminum potassium sulfate (Sigma-Aldrich, St. Louis, MO, USA) in 200 μ L of PBS on days 0, 7, and 14. Blood was collected from the orbital vascular plexus on day 15 to evaluate the anti-HDM antibody level. HDM-sensitized mice were divided into six groups (n = 10 per group) as follows: (1) Normal control mice; (2) HDM-sensitized control mice; (3) HDM-sensitized dexamethasone (3 mg/kg)-treated mice; (4) HDM-sensitized EEVP (50 mg/kg)-treated mice; (5) HDM-sensitized EEVP (100 mg/kg)-treated mice; (6) HDM-sensitized EEVP (150 mg/kg)-treated mice. The normal control mice were sensitized with 0.9 % saline. The mice received allergen challenges via intratracheal (i.t.) instillation of 100 μ g of HDM in 100 μ L of PBS under anesthesia on days 27, 28, and 30 (Fig. 2A).

2.4. Penh value measurement

Penh value was measured 24 h after the last HDM challenges on day 30. The mice were given increasing doses of aerosolized methacholine (3.125, 6.25, 12.5, and 25 mg/mL) under anesthesia. Airway hyper-responsiveness was then monitored for 30 min using a Buxco System (Biosystem XA; Data Sciences International Inc., St. Paul, MN, USA) after each nebulization and stabilization period. Airway obstruction curves were converted into enhanced pause (Penh) values [22].

2.5. Collection of bronchoalveolar lavage fluid (BALF), lung tissue, and mesenteric lymph node cells

Tracheotomy was performed via cannula insertion into the trachea. BALF was obtained via intranasal instillation of Fetal bovine serum-free Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Rockford, IL, USA) into the lungs, and lung lavage via tracheal cannulation with 1 mL of 0.9 % saline. Total and differential BALF cell numbers were

counted using 0.04 % trypan blue staining. For cytological examination, cells were prepared with Cytospin (Hanil Science, Daejeon, Korea), fixed, and stained with a modified Diff-Quick stain. Lungs were removed from the mice, minced, sectioned, and incubated with shaking for collagenase digestion (1 mg/mL collagenase IV and 2 mg/mL dispase; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 40 min. The cell suspension was then filtered through a 70 μ m cell strainer and recentrifuged at 450 $\times$ g for 20 min to collect the cells. Isolated mesenteric lymph node (MLN) cells were incubated with red blood cell (RBC) lysis buffer (Thermo Fisher Scientific, Rockford, IL, USA), and filtered with a cell strainer. The total cell number was also determined using 0.04 % trypan blue staining (Sigma-Aldrich, St. Louis, MO, USA).

2.6. Splenocyte culture

Mouse spleens from each group were pooled and mashed through a cell strainer into the culture dish for a single-cell suspension of splenocyte for HDM-induced Th1 or Th2 cytokine measurement. After removing RBC using RBC lysis buffer, the cells were washed with PBS, seeded onto HDM peptide (10 μ g/mL)-coated wells, and cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) for 48 h. The splenocyte culture medium was removed and analyzed for IFN- γ , IL-4, IL-5, and IL-10 using enzyme-linked immuno-sorbent assay (ELISA) (BioSource, Rockford, IL, USA).

2.7. Histological analysis

Lungs and trachea were fixed in 10 % neutral formalin and embedded in epoxy. The blocks were sliced into 4- μ m sections using a Cryostat Microtome (Leica, Wetzlar, Germany). Hematoxylin and eosin (H&E), Mason trichrome (M-T), periodic-acid-Schiff (PAS), and alcian blue PAS (AB-PAS) staining were performed for collagen deposition,

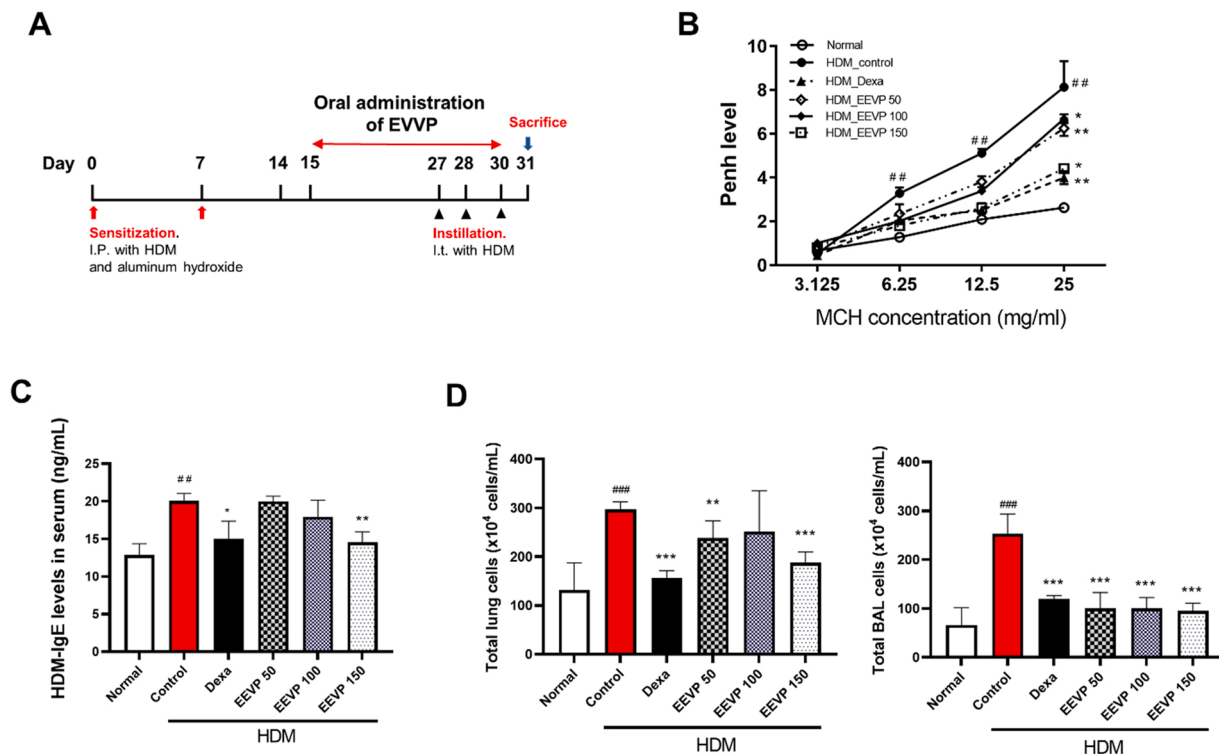


Fig. 2. Effects of EEVP on HDM-induced asthmatic inflammation in mice. (A) The experimental scheme. (B) airway responsiveness to aerosolized methacholine dosage (Penh level). (C) IgE level in serum. (D) Total lung and BAL cell numbers in the lung and BALF, respectively. Normal, normal mice; Control, HDM-sensitized mice; HDM-Dexa, HDM-sensitized dexamethasone (3 mg/kg)-treated mice; HDM-EEVP 50, HDM-sensitized EEVP (50 mg/kg)-treated mice; HDM-EEVP 100, HDM-sensitized EEVP (100 mg/kg)-treated mice; HDM-EEVP 150, HDM-sensitized EEVP (150 mg/kg)-treated mice. ##p < 0.001 vs normal group; *p < 0.05 and **p < 0.01 vs HDM-sensitized control group.

goblet cells, and trachea, respectively. To analysis the goblet cell hyperplasia, immune cell infiltration and collagen deposition, two to four specimens of the stained histological preparations of the left lobe were selected. To determine the severity of inflammatory cell infiltration, peribronchial cell counts and the extent of mucus production and goblet cell hyperplasia in the airway epithelium were blindly quantified with a five-point grading system according to the previous study [23].

2.8. Flow cytometric analysis

The number of cells from the lungs, BAL, and MLN cells were adjusted to 5×10^4 cells for immunofluorescence staining. Cells were incubated with PE-anti-CD3e (553 064, BD Pharmingen, San Diego, CA, USA), FITC-anti-CD8 (553 031, BD Pharmingen, NJ, USA), PE-anti-CD4 (553 047, BD Pharmingen), PE-cy5.5-anti-CD69, PE-anti-Gr-1, (553 128, BD Pharmingen), and FITC-anti-neutrophil (ab55453, Abcam) (Cambridge, UK) antibodies for 30 min and then fixed in 4 % paraformaldehyde with repeated PBS washing at 4 °C. Two-color flow cytometry analysis was performed using the FACSCalibur device with CellQuest software (BD Biosciences, CA, USA). After analyzing the cell frequency as a percentage (%), the absolute number of total cells was calculated by applying the total number of cells.

2.9. Cytokine measurements

The level of IgE in the serum, that of CXCL-1, MIP-2, IL-17, and TNF- α in BALF, and that of IFN- γ and Th2 cytokines (IL-4, IL-5, and IL-10) in splenocyte culture media was measured using an antibody-linked mouse ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

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

Total RNA was isolated using RNazol/chloroform phase separation protocol according to the manufacturer's instructions (RNazolB: Tel-Test Co. Inc., Friendswood, TX, USA). After checking RNA purity and concentration (NanoDrop spectrophotometer, Thermo Fisher Scientific), 5 μ g of total RNA was reverse-transcribed using a First Strand cDNA synthesis kit (Amersham Pharmacia, Piscataway, NJ, USA). The generated cDNAs were amplified using TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA, USA) in an ABI 7500 RT-PCR Instrument (Applied Biosystems). PCR primer sequences from Bioneer (Bioneer, Daejeon, Korea) are listed in Table 2. Relative mRNA expression of the target gene was calculated using the $\Delta\Delta C_t$ method and represented as fold changes.

Table 2
Primer sequences for qRT-PCR.

Gene	Primer sequence
<i>Trpv1</i>	F 5'-CATCTTCACACGGCTGCTTAC-3' R 5'-CAGACAGGATCTCTCCAGTGAC-3'
<i>Trpa1</i>	F 5'-TGAGATCGACCGGAGT-3' R 5'-TGCTGAAGGCATCTTG-3'
<i>Il-5</i>	F 5'-AACCCCTTACTGAACTCAGATTGTTAG-3' R 5'-TAAGTCAGTTTAAATGCTTAGGG-3'
<i>Il-13</i>	F 5'-ATGCCCAACAAAGCAGAGAC-3' R 5'-TGAGAGAACCGGAGCTGT-3'
<i>Cox-2</i>	F 5'-GGGTGTCCCTTCACTTCTTTCA-3' R 5'-TGGGAGGCACTTGCAATTGA-3'
<i>Muc5ac</i>	F 5'-AGAATACTTTCAGGACCCCTGCT-3' R 5'-CACCAGTGCTGAGCATACTTTT-3'
<i>Nos2</i>	F 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' R 5'-GGCTGTGAGGCTCGTGGCTTTGG-3'
<i>Tnf-α</i>	F 5'-GGCTTTCCGAATTCACCTGGAGCCT-3' R 5'-CCCGGCGCTTCCAAATAATACATTCATA-3'
<i>Gapdh</i>	Probe_VIC 5'-TGCATCCTGCACCACCACTGCTTAG-3'

F, forward; R, reverse.

2.11. Immunohistochemistry staining

The lung tissues were fixed with 4 % paraformaldehyde, embedded in paraffin, and cut into 4- μ m sections using Cryostat (Leica, Wetzlar, Germany). After deparaffinization, the sections were permeabilized with 0.5 % Nonidet P-40, blocked with 2.5 % bovine serum albumin, incubated with the primary antibodies for CD4 and TRPA1, and then with the secondary antibody. Stained sections were observed and quantified under a fluorescence microscope (Zeiss LSM 510, Carl Zeiss, Germany).

2.12. Cells culture and cell cytotoxicity

Primary bronchial/tracheal epithelial cells (HBECs) were purchased from ATCC (Manassas, VA, USA). The cells were cultured in Airway Epithelial Cell Basal Medium supplemented with Bronchial Epithelial Cell Growth kit (ATCC) in a humidified incubator containing 5 % CO₂ at 37 °C. Recombinant human IL-4 and IL-13 were purchased from Peprotech (Rocky Hill, NJ, USA). Cell cytotoxicity was determined using the cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Cells were allowed to attach to 96-well plate for overnight and treated with several concentrations of EEVP (0–300 μ g/mL), and incubated for 24 h. Then, pre-warmed 20 μ L of assay reagent was added to each well and the cells were incubated at 37 °C for 2 h. the absorbance was detected at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, United States).

2.13. Measurement of MCP-1 level

The cells in 96-well plate were pre-incubated with sample at indicated concentrations (30 100 300 μ g/mL) for 1 h and stimulated with 20 ng/mL of IL-4/IL-13 for 48 h at 37 °C. After stimulation, the supernatant was collected and centrifuged at 4000 rpm for 5 min to remove the particulates. The levels of secreted pro-inflammatory chemokines in stimulated HBECs were detected using the LEGENDplex™ Human inflammation Panel (BioLegend, San Diego, CA, USA) as described in previous study [24].

2.14. Western blot analysis

HBECs were pre-incubated with 300 μ g/mL of sample for 1 h and treated with 20 ng/mL IL-4 and 20 ng/mL IL-13 at 37 °C. The cells were lysed with cold radioimmunoprecipitation assay buffer (Biosesang, Seongnam, Korea) containing Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). 10 % Mini-PROTEAN TGX Pre-cast Protein Gels electrophoresis and Trans-Blot Turbo transfer packs with PVDF (Bio-Rad, Hercules, CA, USA) were used for protein separation. All antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) except p-STAT6 antibody (Tyr641) (Thermo Fisher Scientific, Waltham, MA, USA). p-stat 3 antibody (Tyr705) (Cell Signaling Technology, Danvers, MA, USA) was used for western blot analysis. Dilution ratio for primary or secondary antibodies was 1–1000 or 4000, respectively. Membrane blocking or washing solution was 5 % skim milk or TBST (Sigma-aldrich, St. Louis, MO, USA) for 1–2 h. Chemiluminescent bands at the expected molecular weight were detected using Super-Signal® West Femto Chemiluminescent Substrate under the ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA).

2.15. Statistical analysis

Data are presented as the mean $\pm$ standard deviation. Group comparison was performed using one- or two-way analysis of variance (ANOVA). Post-hoc analysis was performed with Duncan's multiple comparison test. p-values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. Inhibitory effects of EEVP on HDM-induced airway inflammation

The HDM-induced asthmatic mouse model is a well-known model for the pathophysiological study of airway inflammation. This model shows enhanced methacholine responsiveness, increased serum HDM-specific IgE levels, and increased leukocyte recruitment to the airways [25]. In this study, we investigated the effect of EEVP on airway hyper-responsiveness by evaluating its effect on the methacholine-induced increase in Penh value, representing the change in airway reactivity. The Penh value of the HDM-control group increased in a dose-dependent manner upon methacholine administration compared to that of the normal control mice (Fig. 2B). However, the EEVP (50, 100, and 150 mg/kg) groups showed significantly decreased Penh values compared to the HDM-control group. Of note, the Penh value of the EEVP (150 mg/kg) group was similar to that of the dexamethasone group with 25 mg/mL methacholine. It was reported that increased Penh value is associated with an increase in IgE production due to an increase of infiltrated inflammatory cell proportion in the allergic region [26]. In addition, serum IgE level is directly correlated with the allergic asthma and inflammatory cytokine levels such as IL-4 [27,28]. As shown in Fig. 2C, HDM-induced IgE levels were markedly higher in the

HDM-control group than those in the normal group, but comparatively lower in the EEVP (150 mg/kg) group. To investigate the effect of EEVP on HDM-induced airway inflammation, we further examined immune cell infiltration by investigating the total change in cell numbers in the lung and BAL after the HDM challenge. Similar to a previous study which used the HDM mouse model, HDM exposure significantly increased the total number of cells in the lung and BAL (Fig. 2D). However, administration of a high concentration of EEVP (150 mg/kg) markedly attenuated HDM-mediated inflammatory cell recruitment, whereas low or middle doses of EEVP (50 and 100 mg/kg) had a much less effect. It has been suggested that changes in Penh values are related to ventilatory time and changes in pulmonary resistance at least in allergy experiments using mice and small animals [29]. Therefore, these results suggested that pretreatment of EEVP could prevent inflammatory cell infiltration related to systemic IgE induction and, at least in part, an aberrant lung dysfunction in the HDM-induced asthma mouse model.

3.2. Inhibitory effects of EEVP on HDM-induced histological change of airway inflammation

Increased numbers of inflammatory cells in the lung and BAL are correlated with the severity of airway remodeling in the HDM model [30,31]. To verify the pharmacological effects of EEVP on airway

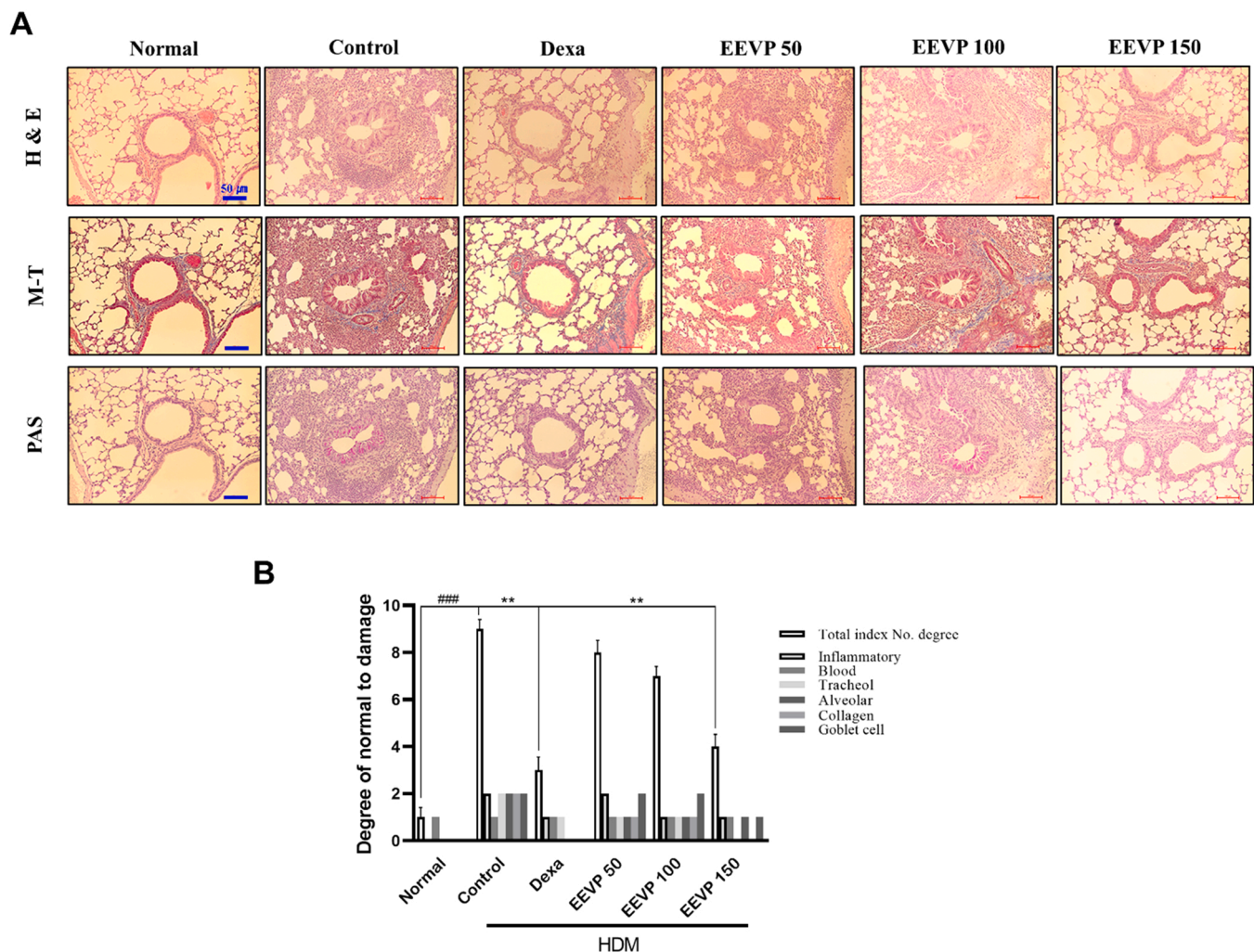


Fig. 3. Inhibitory effect of EEVP on the lung tissue of HDM-sensitized mice. (A) Representative images of hematoxylin and eosin (H&E), Masson trichrome (M-T), and periodic acid-Schiff (PAS) staining in each group. (B) Quantitative analyses of the degree of lung tissue damage. Normal, normal mice; Control, HDM-sensitized mice; HDM-Dexa, HDM-sensitized dexamethasone (3 mg/kg)-treated mice; HDM-EEVP 50, HDM-sensitized EEVP (50 mg/kg)-treated mice; HDM-EEVP 100, HDM-sensitized EEVP (100 mg/kg)-treated mice; HDM-EEVP 150, HDM-sensitized EEVP (150 mg/kg)-treated mice.

remodeling due to pathogenic inflammation, H&E, M-T, and PAS staining were performed on lung tissues from normal and HDM-challenged mice. HDM challenge induced an increase in inflammatory cell (neutrophils and eosinophils) deposition and activation of macrophages in the inflammatory airway (Fig. 3A). Collagen deposition caused due to damaged alveolar epithelial cells, goblet cell hyperplasia, and increased inflammatory and blood cell infiltration to the airway were significantly observed in the HDM challenge group compared with those in the normal group (Fig. 3B). However, dexamethasone and 150 mg/kg EEVP-treated mice showed reduced pathophysiological changes induced by HDM in the airway. Therefore, these results suggest

that EEVP might have a pharmacological effect in alleviating the pathophysiological severity of airway remodeling, such as fibrosis, goblet cell hyperplasia, and mucus hypersecretion, in HDM-induced airway inflammation.

3.3. Inhibitory effects of EEVP on inflammatory cell numbers in the lung and BAL

The increase in leukocytosis and macrophage proportion in the HDM-induced asthma model is primarily due to the increase in neutrophil, lymphocyte, and eosinophil proportions [25]. In addition,

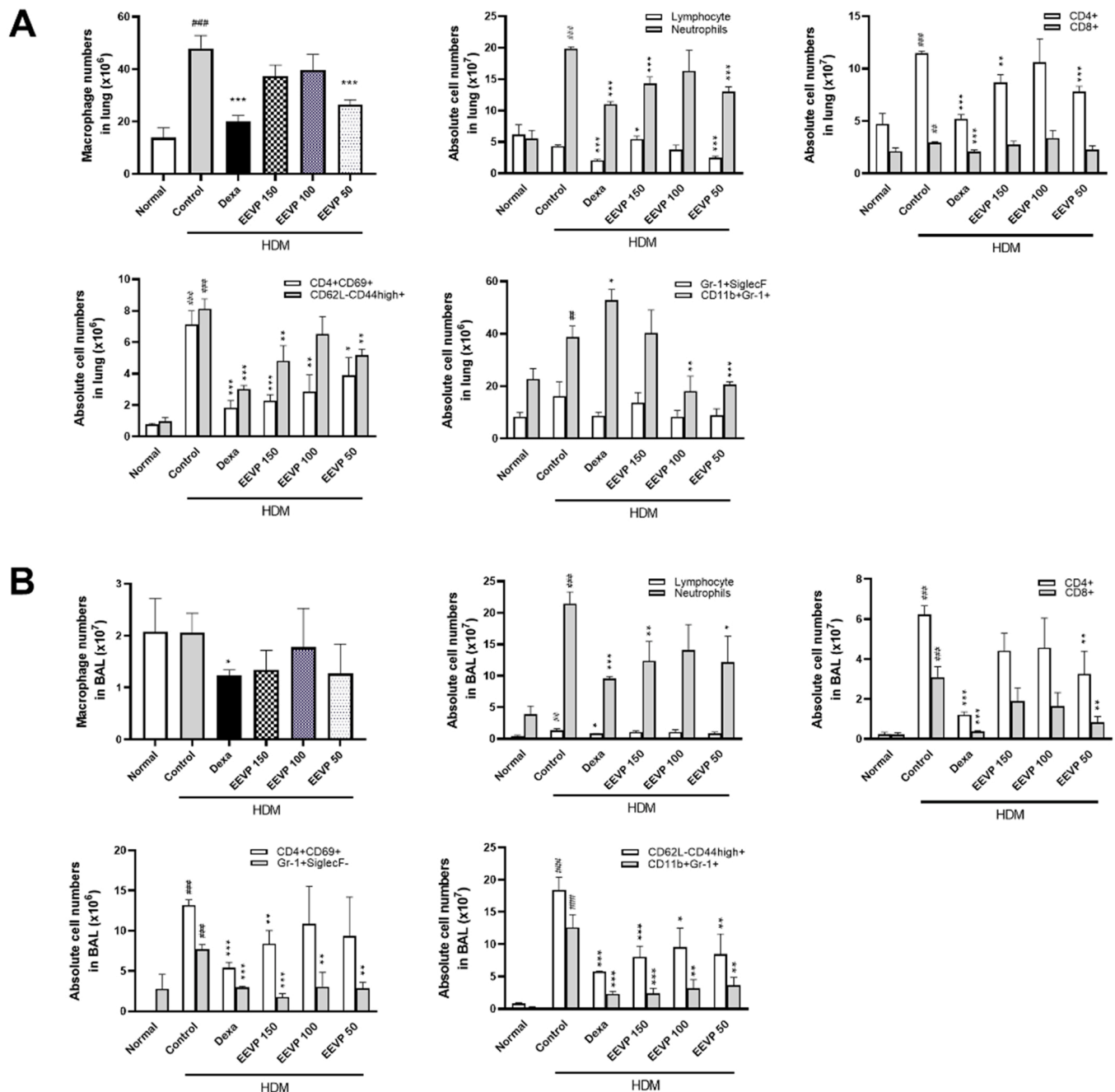


Fig. 4. FACS analysis of immune cell subtypes in lung and BAL of HDM-induced mice. Macrophage, lymphocyte, neutrophils, $CD4^+$, $CD8^+$, $CD4^+CD69^+$, $CD62L^-CD44^{high+}$, and $Gr-1^+SiglecF^-$ $CD11b^+Gr-1^+$ cell numbers in (A) lung or (B) BAL. Normal, normal mice; Control, HDM-sensitized mice; HDM-Dexa, HDM-sensitized dexamethasone (3 mg/kg)-treated mice; HDM-EEVP 50, HDM-sensitized EEVP (50 mg/kg)-treated mice; HDM-EEVP 100, HDM-sensitized EEVP (100 mg/kg)-treated mice; HDM-EEVP 150, HDM-sensitized EEVP (150 mg/kg)-treated mice. Data are expressed as means $\pm$ SEM. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs normal group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs HDM-sensitized control group.

eosinophils are considered one of the pathological cells to exacerbate airway inflammation in asthma in response to Th2 cytokines [32]. To clarify the change in inflammatory cell population infiltrating the lung and BAL, we analyzed the immune cell subtypes (lymphocytes, neutrophils, macrophages, and eosinophils) using Fluorescence-activated cell sorting (FACS) analysis. As shown in Fig. 4, the HDM challenge significantly increased the proportion of immune cells, including lymphocytes, neutrophils, macrophages, B cells, T cells, and their subtypes cells in the lung and BAL. Dexamethasone and 150 mg/kg EEVP significantly inhibited the HDM-induced increase in neutrophil proportions, including CD4⁺, CD8⁺, CD4⁺CD69⁺, and CD62L-CD44^{high} cells, in the lung and BAL. Among them, Gr-1⁺SiglecF⁺ cell proportions in BAL were significantly decreased in dexamethasone (2.9×10^6 cells) and 150 mg/kg EEVP treatment (1.76×10^6 cells) compared with HDM-induced control (7.7×10^6 cells). In addition, CD11b⁺Gr-1⁺ cell proportions in BAL and lung were significantly decreased in dexamethasone and 150 mg/kg EEVP treatment compared with HDM-induced control. Gr-1⁺SiglecF⁺ cells are an eosinophil subpopulation found within the allergen-challenged lung [33]. It is also suggested that eosinophils comprise a substantial portion of the CD11b⁺Gr-1⁺ cells constituted with the neutrophil and eosinophil populations [34]. Recruitment and activation of eosinophils amplify allergic inflammation by producing Th2 cytokines that mediate T cell recruitment, proliferation, and polarization [35]. Thus, these results suggested that the inhibitory effect of EEVP on HDM-induced airway inflammation could result from the reduction in the number of infiltrating eosinophils mediating airway inflammation in BAL and lung.

3.4. Inhibitory effects of EEVP on inflammatory cytokine levels in BALF and splenocyte culture medium

Allergen challenge after sensitization generates and maintains local and systemic Th2 memory response in lung and spleen of allergic asthma model [30,36]. Th2-derived cytokines play a significant role in the

development of airway inflammation in asthma [37,38]. It has reported that Th2 cytokine concentration in BAL in HDM-sensitized mice are very low or even nadir levels [39,40]. But, in vitro re-stimulation of splenocyte isolated from HDM-exposed mice generates significant amount of Th2-associated cytokine and some of Th1-associated cytokine [41], suggesting in vitro splenocyte culture as a tool for airway inflammatory study. As EEVP inhibition on eosinophil infiltration involved in Th2 cell response, we next evaluated the effect of EEVP on the levels of Th1 cytokines (CXCL-1, CXCL-2, IL-17, and TNF- α) in the BALF and the production of Th2 cytokines (IL-4, IL-5, and IL-10) in in vitro medium of splenocytes isolated from mice. As shown in Fig. 5A, the levels of Th1 cytokines in BALF were significantly increased upon HDM challenge, which decreased upon dexamethasone and EEVP administration. Although Th2 cytokine mostly initiates asthma inflammation, Th1 and Th2 cells together affect the exacerbation of the inflammation in chronic asthmatic response, suggesting suppression of Th1 cytokine secretion also is important for suppression of Th2 immunity. As Th1-response is involved in the differentiation and proliferation of Th2 cells, the inhibition of Th1 cytokines by dexamethasone or EEVP might attenuate the inflammation in asthma model. Interestingly, EEVP attenuated the production of Th2 cytokines (IL-4 and IL-5) but increased that of IL-10 in splenocytes compared with that in the HDM-challenged group (Fig. 5B). In addition, EEVP induced an increase in IFN- γ expression, which limits Th2 cell activation (Fig. 4B). IL-4, IL-5, and IL-13 production by activated Th2 cells are closely related to IgE production, airway infiltration, eosinophil activation and mucus secretion in airway inflammation [37, 42]. In contrast, IL-17 and TNF- α synergistically induce endothelial expression of the neutrophilic chemokines (CXCL1, CXCL2, and CXCL5), and endothelial activation leads to leukocyte influx into inflammatory sites [43]. Therefore, EEVP-mediated inhibition of Th1/Th2 cytokine production might attenuate inflammatory cell recruitment and activation in the inflammatory region initiating or developing airway inflammation.

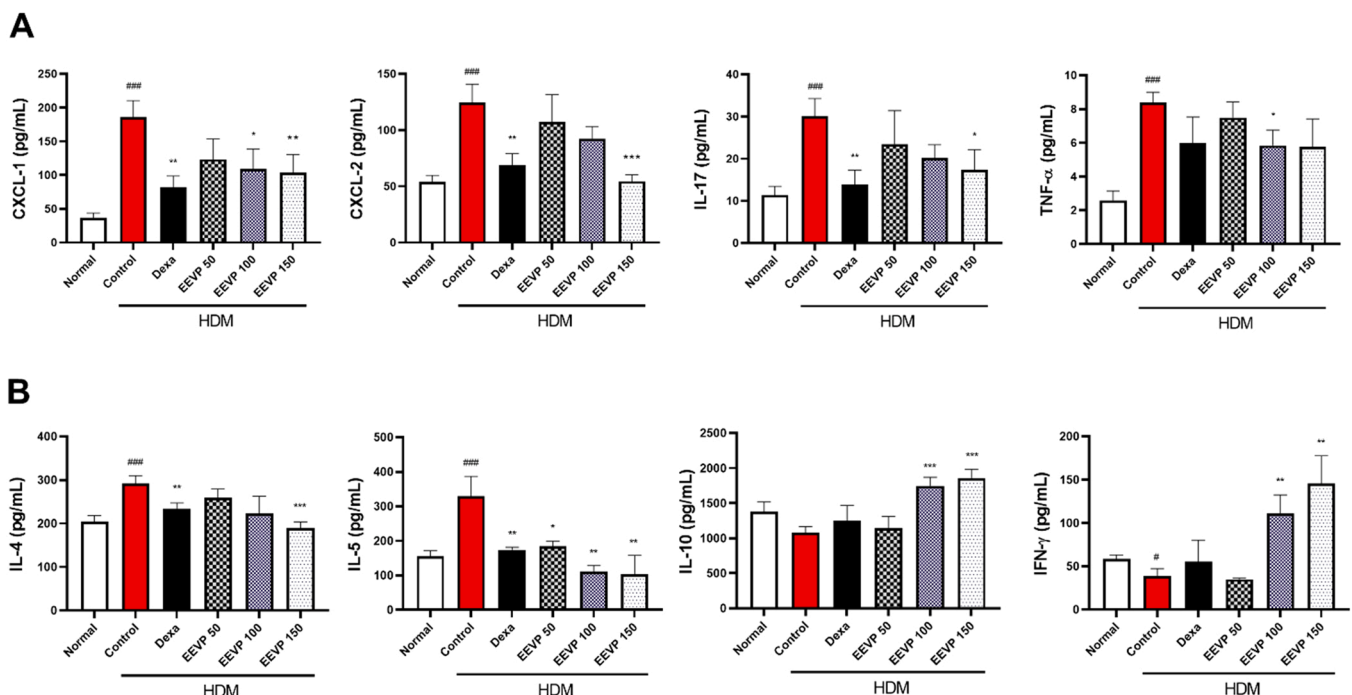


Fig. 5. Effects of EEVP on inflammatory cytokine levels in BALF and splenocyte culture media. BALF was collected 18 h after the last HDM induction. (A) CXCL-1, CXCL-2, IL-17, and TNF- α level in BALF in HDM-sensitized mice. (B) IL-4, IL-5, IL-10, and IFN- γ level in splenocyte culture media in HDM-sensitized mice. Normal, normal mice; Control, HDM-sensitized mice; HDM-Dexa, HDM-sensitized dexamethasone (3 mg/kg)-treated mice; HDM-EEVP 50, HDM-sensitized EEVP (50 mg/kg)-treated mice; HDM-EEVP 100, HDM-sensitized EEVP (100 mg/kg)-treated mice; HDM-EEVP 150, HDM-sensitized EEVP (150 mg/kg)-treated mice. ###p < 0.001 vs normal group; *p < 0.05, **p < 0.01, and ***p < 0.001 vs HDM-sensitized control group.

3.5. Inhibitory effects of EEVP on the expression of airway inflammatory factors in the lung

Regarding EEVP-mediated inhibition of Th1 and Th2 response in the

airway, we further investigated the effect of EEVP on the expression of genes related to airway inflammation, including Th2 cytokines (*Il-4*, *Il-5*, and *Il-13*), chemokines (*Tarc*), airway hyperreactivity (*Trpv1* and *Trpa1*), airway mucus (*Muc5ac*), or inflammatory factors (*Cox-2*, *Tnf- α* ,

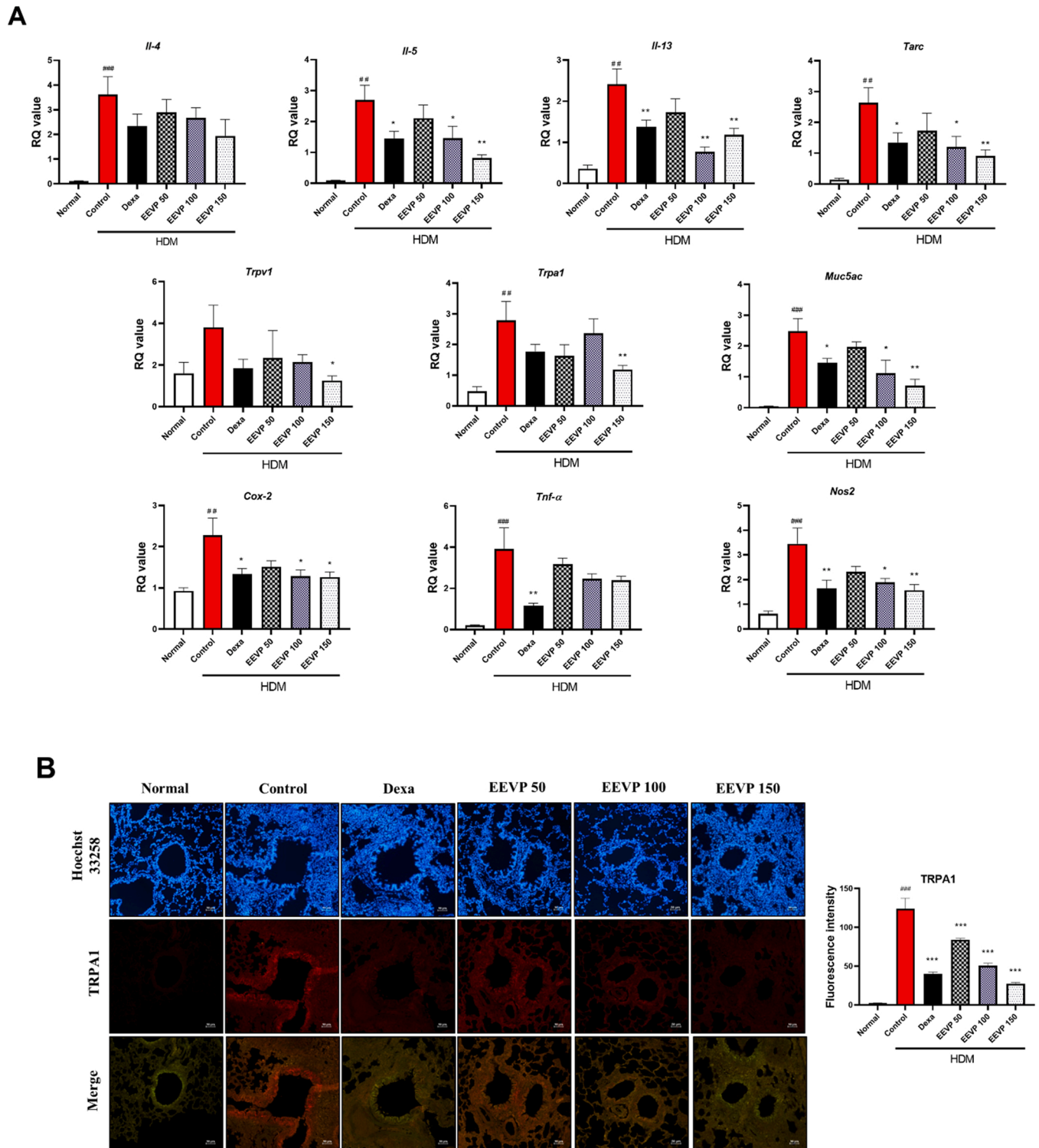


Fig. 6. Effects of EEVP on airway inflammation-related factors in the lung of HDM-sensitized mice. (A) After the last HDM induction, lung tissues were isolated and analyzed for expression levels of Th2 cytokines (*Il-4*, *Il-5*, and *Il-13*), chemokine (*Tarc*), airway hyperreactivity (*Trpv1* and *Trpa1*), airway mucus (*Muc5ac*), or inflammatory factors (*Cox-2*, *Tnf- α* , and *Nos2*) using qRT-PCR. (B) Fluorescent staining and signal quantification of TRPA1 in the lung tissue. Normal, normal mice; Control, HDM-sensitized mice; HDM-Dexa, HDM-sensitized dexamethasone (3 mg/kg)-treated mice; HDM-EEVP 50, HDM-sensitized EEVP (50 mg/kg)-treated mice; HDM-EEVP 100, HDM-sensitized EEVP (100 mg/kg)-treated mice; HDM-EEVP 150, HDM-sensitized EEVP (150 mg/kg)-treated mice. ##p < 0.01 and ###p < 0.001 vs normal group; *p < 0.05, **p < 0.01, and ***p < 0.001 vs HDM-sensitized control group.

and *Nos2*), in the lung tissue of mice. Consistent with EEVP-mediated inhibition of cytokine levels in BALF and splenocyte culture media, 150 mg/kg EEVP significantly decreased the expression levels of mRNAs, similar to dexamethasone treatment (Fig. 6A), although *Il-4* and *Tnf- α* levels were downregulated (not significant). Additionally, we further confirmed EEVP-mediated inhibition of TRPA1 levels in the lung tissue using immunofluorescence (Fig. 6B). Cytokine or gene expression levels in the inflammatory site revealed the inflammatory degree and inflammatory environment at the relevant time point. Therefore, the results of this study suggested that the pharmacological potential of EEVP in alleviating airway inflammation could result from the down-regulation of genes related to Th2 cell activation, airway mucus hypersecretion, or inflammation in allergic asthma.

3.6. Inhibitory effects of EEVP on STAT-3 and STAT-6 phosphorylation

Activated inflammatory cells interact with bronchial epithelial cells to secrete several chemokines that contribute to bronchial tissue damage and abnormal remodeling of pulmonary structure in allergic asthma [44, 45]. We found that EEVP attenuated the level of inflammatory cytokine (IL-1 β) and the epithelial cell adhesion molecule (Ep-CAM) related to inflammatory tissue damage (data not shown). In addition, EEVP

inhibition on the increase of pro-inflammatory factors and in animal model provides credibility to in vitro study for more detailed insight into molecular action mechanism. Therefore, we next evaluated the direct effect of EEVP on IL-4/13-stimulated chemokine level generated from primary bronchial/tracheal epithelial cells. There can also be significant discrepancies in the effective dose between in vivo study and in vitro study, suggesting an examination of an active concentration of EEVP having pharmacological activity without toxicity on the primary cells. As shown in Fig. 7A and B, EEVP significantly suppressed IL-4 and IL-13-induced monocyte chemotactic protein (MCP)-1 level in a dose-dependent manner, although it induced a little cytotoxicity at high concentration (300 μ g/mL). Furthermore, we also found that EEVP significantly, but partially, inhibits IL-4 and IL-13-induced STAT-3 as well as STAT-6 phosphorylation (Fig. 7C). Previously, it has reported that MCP-1 gene expression and production are significantly increased in IL-4/13-stimulated BEAS-2B cells via Janus kinase-2-mediated STAT-6 or STAT-1 signaling pathway [46]. Increased expression and concentration of MCP-1 in BALF of asthmatic patients suggest that a bronchial-specific elevation of the MCP-1 level represents a localized bronchial inflammation [47]. In addition, STAT-6 or STAT-3 acts as the primary transcription factor for IL-4 and IL-13 stimulation on airway epithelial or smooth muscle cells in asthmatic condition [44,48,49].

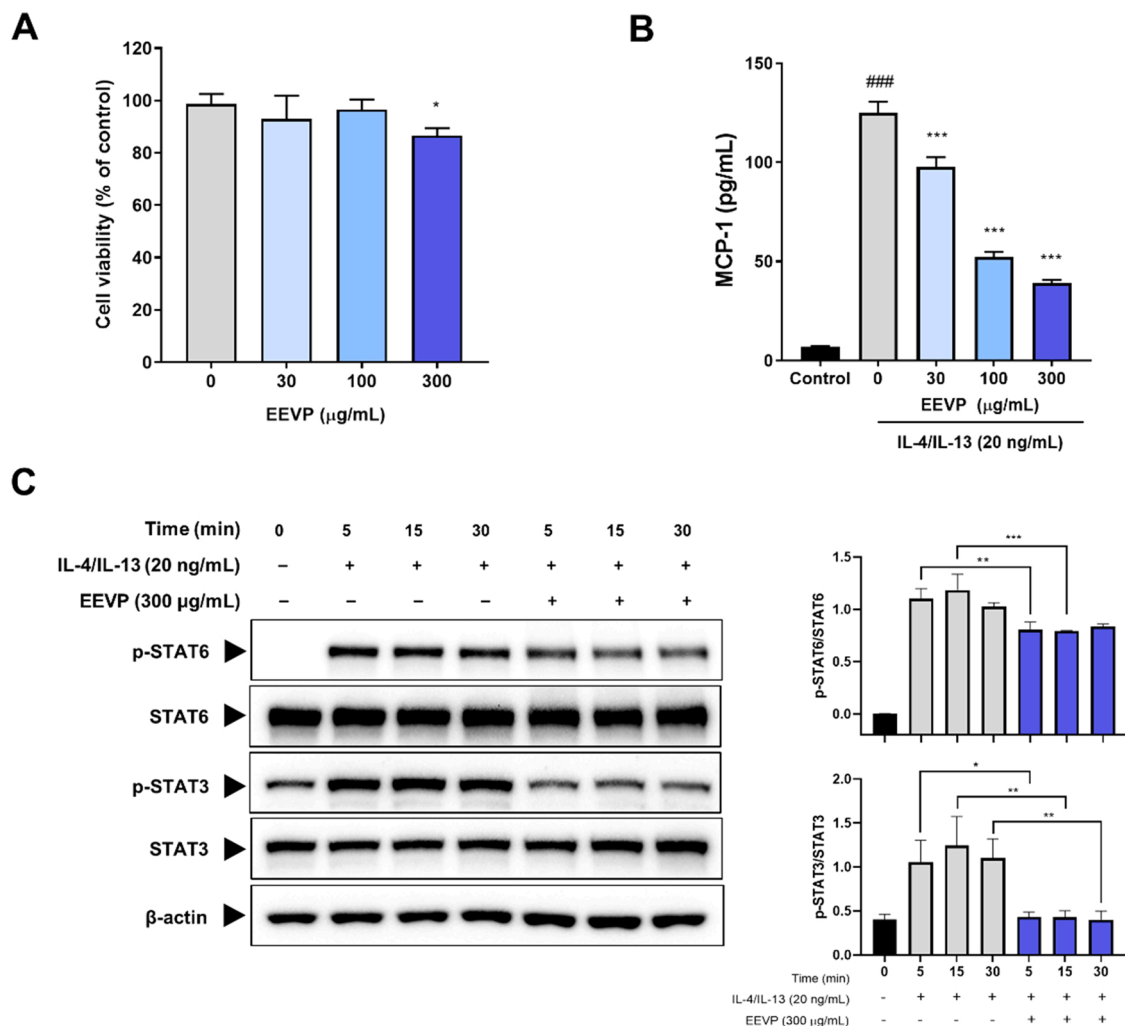


Fig. 7. Effects of EEVP on IL-4/13-induced monocyte chemotactic protein (MCP)-1 level and STAT phosphorylation. (A) Cell viability of primary bronchial/tracheal epithelial cells was determined after EEVP treatment for 24 h. (B) MCP-1 levels was measured via a bead-based immunoassay after IL-4, IL-13, and EEVP treatment for 48 h. (C) Cells pretreated with EEVP for 1 h were incubated with IL-4/IL-13 for 5, 15, and 30 min. Phosphorylation of STAT-3 and STAT-6 was evaluated using western blot analysis. Quantified western blot data shown as the means $\pm$ SEM of three separate experiments. ###p < 0.001 vs control; *p < 0.05, **p < 0.01, and ***p < 0.001.

Therefore, the results of this study suggested that EEVP inhibition of STAT signaling could result in the retardation of bronchial tissue damage and inflammation in HDM-challenged asthma. Regarding partial inhibition of EEVP on STAT activation, further research is warranted to reveal the more underlying molecular mechanism of EEVP regulating asthmatic inflammation.

4. Conclusions

In conclusion, the results of this study first showed the pharmacological effects of EEVP in alleviating HDM-induced airway inflammation. This effect might be attributed to EEVP-caused inhibition of the STAT-3/6-related signaling in bronchial/tracheal epithelial cells, and to EEVP inhibition of eosinophilic airway inflammation and T cell activation/polarization in the allergic lung. These results indicate the potential of EEVP to prevent airway inflammatory disease, suggesting a possible pharmacological application of EEVP for prophylactic agents to attenuate other allergic disorders of the skin or intestines.

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CRedit authorship contribution statement

T.K. and K.M.K. conceived the study and designed the experiments. K.-S.S. and K.M.K. wrote and revised the manuscript. H.-K. S, Y.-H.H, S.C, H.K.K, S. J, Y.H.K, B.-K.C, W.-K.Y, and S.-H.K performed the experiments. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that this study does not receive any commercial or financial supports related to a potential conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2022.113264](https://doi.org/10.1016/j.biopha.2022.113264).

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