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Anti-allergic effects of salvianolic acid A and tanshinone IIA from *Salvia miltiorrhiza* determined using *in vivo* and *in vitro* experiments

Jae-Yeong Heo, Dong-Soon Im*

College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea

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ABSTRACT

Salvia miltiorrhiza root has been used in Asian traditional medicine for the treatment of cardiovascular diseases, asthma, and other conditions. Salvianolic acid B from *S. miltiorrhiza* extracts has been shown to improve airway hyperresponsiveness. We investigated the effects of salvianolic acid A, tanshinone I, and tanshinone IIA from *S. miltiorrhiza* in allergic asthma by using rat RBL-2H3 mast cells and female Balb/c mice. Antigen-induced degranulation was assessed by measuring β-hexosaminidase activity *in vitro*. In addition, a murine ovalbumin-induced allergic asthma model was used to test the *in vivo* efficacy of salvianolic acid A and tanshinone IIA. Tanshinone I and tanshinone IIA inhibited antigen-induced degranulation of mast cells, but salvianolic acid A did not. Administration of salvianolic acid A and tanshinone IIA decreased the number of immune cells, particularly eosinophils in allergic asthma-induced mice. Histological studies showed that salvianolic acid A and tanshinone IIA reduced mucin production and inflammation in the lungs. Administration of salvianolic acid A and tanshinone IIA reduced the expression and secretion of Th2 cytokines (IL-4 and IL-13) in the bronchoalveolar lavage fluid and lung tissues of mice with ovalbumin-induced allergic asthma. These findings provide evidence that salvianolic acid A and tanshinone IIA may be potential anti-allergic therapeutics.

1. Introduction

Repeated exposure to environmental antigens induces inflammatory reactions in the airway, and inflammatory changes in the airway epithelium contribute to the pathophysiologic features of allergic asthma [1]. Increased infiltration of immune cells, such as, eosinophils, macrophages, and lymphocytes, and increased levels of cytokines, such as, IL-4, IL-5, IL-9, and IL-13, and also structural changes in the airway mucous membrane are characteristics of asthma [2,3]. Although pro-inflammatory cytokines and anti-inflammatory drugs have been discovered and developed, current therapeutics for allergic asthma are limited [4,5].

Salvia miltiorrhiza root, also referred to as Danshen in Chinese, has traditionally been used in China and other Asian countries for the treatment of cardiovascular diseases, asthma, and other conditions [6–9]. The components isolated and purified from *S. miltiorrhiza* include hydrophilic constituents, such as salvianolic acid A and B, danshensu, and protocatechuic aldehyde; and lipophilic constituents, such as tanshinone I, tanshinone IIA and IIB, cryptotanshinone, and dihydrotanshinone (Fig. 1) [6,10]. Pharmacological mechanism of tanshinone IIA and cryptotanshinone in platelet anti-aggregating effects have been studied [11]. Recently, salvianolic acid B was shown to improve airway hyperresponsiveness [12]. However, the *in vivo* anti-allergic efficacy of other constituents has not been evaluated yet. Hence, we aimed to investigate the effects of salvianolic acid A, tanshinone I, and tanshinone IIA in allergic asthma by using rat RBL-2H3 mast cells and female Balb/c mice.

2. Materials and methods

2.1. Materials

Salvianolic acid (Cas No.: 96574-01-5, ≥96% HPLC, molecular weight 494.45, Pubchem ID 329825115), tanshinone I (Cas No.: 568-73-0, ≥98% HPLC, molecular weight 276.29, Pubchem ID 329826820), tanshinone IIA (Cas No.: 568-72-9, ≥97% HPLC,

Abbreviations: OVA, ovalbumin; BALF, bronchoalveolar lavage fluid; PAS, periodic acid-Schiff; IL-4, interleukin-4; IL-13, interleukin-13; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; DNP, dinitrophenyl; DNP-hAb, human dinitrophenyl albumin; HRP, horseradish peroxidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

* Corresponding author at: Laboratory of Pharmacology, College of Pharmacy, Pusan National University, 63 Beon-gil 2, Busandaehag-ro, Geumjeong-gu, Busan 46241, Republic of Korea.

E-mail address: imds@pusan.ac.kr (D.-S. Im).

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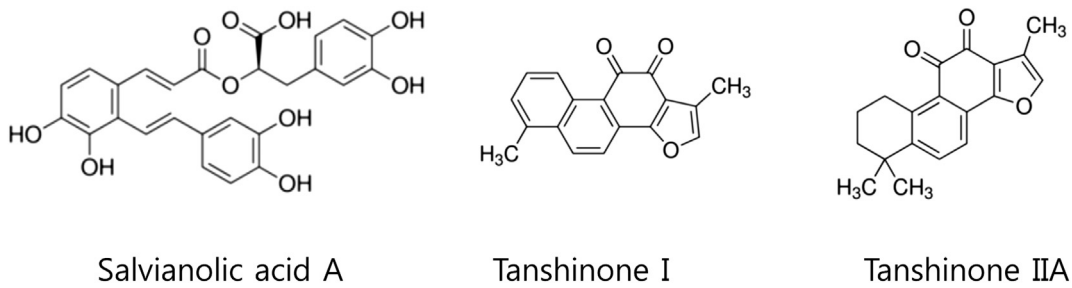

Fig. 1. Structures of salvianolic acid A, tanshinone I, and tanshinone IIA.

molecular weight 294.34, Pubchem ID 329826804) and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Rat RBL-2H3 mast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RBL-2H3 cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) containing 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin, 50 μg/mL streptomycin, and 10% (v/v) heat-inactivated fetal bovine serum at 37 °C in a 5% CO_2-humidified incubator [13].

2.3. Animals

Five-week-old female Balb/c mice were purchased from Daehan Biolink (DBL; Seoul, Korea) and housed in the laboratory animal facility at Pusan National University. They were provided food and water *ad libitum*. The protocol used in this study was reviewed and approved by the Pusan National University Institutional Animal Care Committee (PNU–IACUC) with respect to ethical issues and scientific care (Approval Number, PNU-2018-298).

2.4. Assessment of degranulation

Degranulation was evaluated by measuring β-hexosaminidase release from mast cells [14]. Briefly, RBL-2H3 cells were sensitized by incubation with monoclonal anti-dinitrophenyl mouse immunoglobulin E overnight. Before stimulation, the cells were washed twice with PIPES buffer (pH 7.2) containing 25 mM PIPES, 0.05 mM NaOH, 110 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$ to remove DNP-IgE, and 0.1% bovine serum albumin (BSA). Cells were then incubated in PIPES buffer containing different concentrations of salvianolic acid A, tanshinone I, or tanshinone IIA at 37 °C for 30 min. The cells were then incubated for additional 15 min at 37 °C with human dinitrophenyl albumin to induce degranulation. Twenty-five microliters of the medium were then transferred to a 96-well microplate and incubated for 110 min with 5 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide in 0.1 M citrate buffer (pH 4.5). The reaction was terminated by addition of 0.05 M sodium carbonate buffer (pH 10; 0.05 M Na_2CO_3/0.05 M $NaHCO_3$). The absorbance (optical density; OD) of the final product was measured at 405 nm using a microplate reader.

2.5. Induction of asthma in Balb/c mice and administration of salvianolic acid A and tanshinone IIA

Six-week-old Balb/c mice (22 g) were divided into eight groups ($n = 5$) as follows: phosphate-buffered saline (PBS)-injected control group, ovalbumin (OVA)-injected asthma group, OVA-injected plus salvianolic acid A-treated group (5 or 10 mg/kg); and PBS-injected control group, ovalbumin (OVA)-injected asthma group, and OVA-injected plus tanshinone IIA-treated group (5 or 10 mg/kg). Experiments were conducted as two sets for salvianolic acid A and tanshinone IIA. Salvianolic acid A is water soluble; and therefore, easily dissolved in PBS. However, tanshinone IIA is water-insoluble; therefore, it was dissolved as a β-cyclodextrane conjugate. Hence, we used both, PBS and β-cyclodextrane solutions, as vehicles for each set. Asthma was induced by intraperitoneal injection of OVA (50 μg; A5503, Sigma-Aldrich) and aluminum hydroxide (1 mg; 239,186, Sigma-Aldrich) on days 0 and 14. Mice were exposed to nebulized OVA for 30 min for three consecutive days from day 28 [15]. Each constituent was administrated intraperitoneally 30 min before OVA challenge. On day 32, bronchoalveolar lavage fluid (BALF) was collected from the lungs, and immune cells in BALF were stained and counted.

On day 32, lungs were excised and fixed with 10% formalin solution. Next, they were cryoprotected using 30% sucrose solution in PBS (pH 7.4) overnight at 4 °C. Tissues were embedded in O.C.T. compound and sectioned at 6 μm thickness. Sections were then thaw-mounted onto Superfrost® microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at −80 °C until further use.

2.6. Histological examination of the lungs and cell counting in BALF

Lung sections from mice of different experimental groups were prepared and examined. In brief, the left lungs were fixed in 10% formalin and dehydrate 30% sucrose solution and embedded in O.C.T. compound. Sections of 6 μm thickness were stained with periodic acid-Schiff (PAS) to identify mucus-secreting cells (goblet cells) in the airways, and hematoxylin and Eosin (H&E) staining was performed to confirm eosinophil infiltration. Lung tissues from all five mice in each group were examined. For PAS staining, O.C.T. compound was removed from the sections; the sections were hydrated, and placed in periodic acid solution at room temperature for 5 min. After rinsing with water, sections were stained with Schiff's regent (ab150680, Abcam) for 15 min, rinsed with running tap water, and stained with hematoxylin for 90 s. The sections were then rinsed, dehydrated, and cover-slipped. For H&E staining, O.C.T. compound was removed from the sections; the sections were hydrated, and were stained with hematoxylin regent for 30 s. After rinsing with running tap water, sections were stained again with eosin regent for 10 s, followed by rinsing, dehydration, and mounting the sections on the cover-slipped.

Lung inflammation was scored by a treatment-blind observer. Degree of lung inflammation was evaluated using a subjective scale of 0–3, as previously described [16,17]. Total lung inflammation was defined as the average lung inflammation score. Five lung sections per mouse were evaluated, and the average inflammation score was calculated [18]. Mucin-secreting cells in the airways were stained with PAS and counted in the large-caliber preterminal bronchi from at least two lung sections per mouse. The length of the basal lamina of bronchi was measured using ImageJ software (National Institute of Health).

Cells in BALF were made to adhere to a glass slide using a Cellspin® centrifuge (5 min, 500 rpm; Hanil Electric) and fixed in methanol for 30 s. Subsequently, the cells were processed with May-Grünwald solution (32,856, Sigma-Aldrich) for 8 min followed by Giemsa solution (32,884, Sigma-Aldrich) for 12 min. Slides were then rinsed and covered.

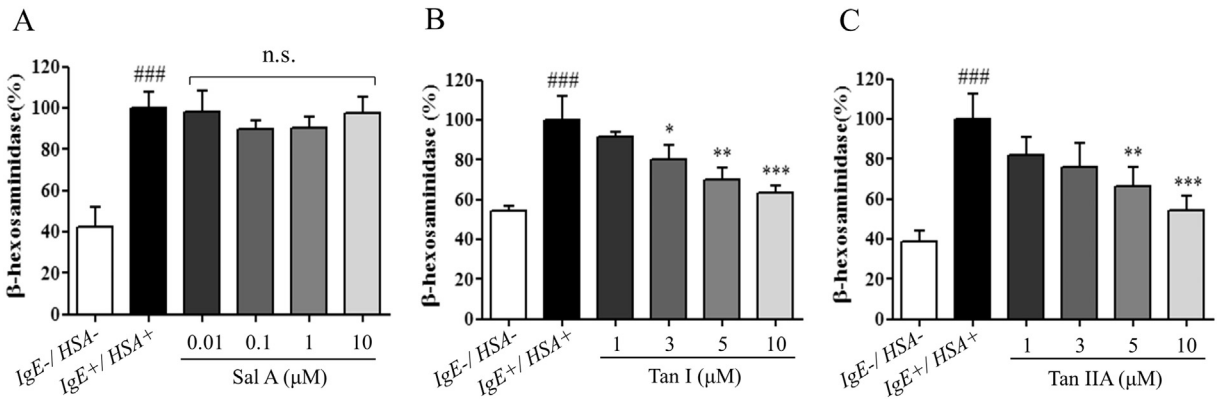

Fig. 2. Effects of salvianolic acid A, tanshinone I, and tanshinone IIA on antigen-induced β-hexosaminidase release in RBL-2H3 mast cells
RBL-2H3 cells were sensitized with anti-DNP IgE for 18 h, followed by a challenge with DNP human serum albumin (HSA). Salvianolic acid A, tanshinone I, or tanshinone IIA was administered at the indicated concentrations 30 min prior to antigen challenge. The samples without IgE and HSA represent basal degranulation from the cells and samples with IgE and HSA served as positive control of antigen-induced degranulation. The results are presented as means ± SEs of values from three independent experiments. The released enzyme of control cells stimulated with IgE/HSA without inhibitors was 22% of total cellular enzyme. $^{###}p < 0.001$ *vs.* the HSA-treated group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the OVA-treated group.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Expression levels of asthmatic markers from type 2 T-helper (Th2) cells and Th1 cells were analyzed by RT-PCR. Total RNA was isolated from all five mice in each group using TRIzol™ reagent (Invitrogen, Waltham, MA, USA), and was used for cDNA synthesis. The synthesized cDNA, primers for each gene, and Promega GoTaq® DNA polymerase (Promega Corporation, Madison, WI, USA) were used to amplify the specific genes. Specific primers for IL-4 (sense 5′-CTA GTT GTC ATC CTG CTC TTC TTT-3′, antisense 5′-CTT TAG GCT TTC CAG GAA GTC TTT-3′), IL-13 (sense 5′-CAG CAT GGT ATG GAG TGT GG-3′, antisense 5′-TGG GCT ACT TCG ATT TTG GT-3′) were used to amplify gene fragments. PCR was performed as follows: over 30 amplification cycles (denaturation at 95 °C for 30 s, annealing at 49 °C for 30 s, and elongation at 72 °C for 30 s) in SimpliAmp Thermal Cycler PCR machine (Thermo Fisher Scientific Singapore). Specific primers for IL-5 (sense 5′-AGA ATC AAA CTG TCC GTG GG-3′, antisense 5′-GTC TCT CCT CGC CAC ACT TC-3′), IL-2 (sense 5′-AAC CTG AAA CTC CCC AGG AT-3′, antisense 5′-TCC ACC ACA GTT GCT GAC TC-3′), IFN-γ (sense 5′-ACT GGC AAA AGG ATG GTG AC-3′, antisense 5′-TGA GCT CAT TGA ATG CTT GG-3′) were used and annealing was performed at 53 °C. Specific primers for GAPDH (sense 5′-TTC ACC ACC ATG GAG AAG GC-3′, antisense 5′-GGC ATG GAC TGT GGT CAT GA-3′) were used and annealing was performed at 60 °C (27 amplification cycles). Aliquots of 5 μL were electrophoresed on 1.2% agarose gel and stained with StaySafe™ nucleic acid gel stain (Real Biotech Corporation, Taipei, Taiwan) [19].

2.8. Measurement of cytokine levels

Interleukin (IL)-4 and IL-13 levels in BALF isolated from five mice per group were measured by using enzyme-linked immunosorbent assay (ELISA) kits. Briefly, 96-well plates (NUNC, Penfield, NY, USA) were coated overnight at 4 °C with a capture antibody specific for IL-4 (cat. 14-7041-68, eBioscience, San Diego, CA, USA) or IL-13 (cat. 14-7043-68, eBioscience). Biotinylated detection antibody specific for IL-4 (cat. 33-7042-68C, eBioscience) or IL-13 (cat. 33-7135-68B, eBioscience) was used. Avidin-horseradish peroxidase (HRP) was used, and the absorbance was measured at 450 nm [14].

2.9. Statistical analysis

Results are expressed as means ± standard errors (SEs). Differences among groups were tested for statistical significance using analysis of variance (ANOVA), followed by Turkey's *post hoc* test. p Values < 0.05 indicated statistical significance. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Tanshinone I and tanshinone IIA, but not salvianolic acid a inhibited antigen-induced degranulation in RBL-2H3 mast cells

Mast cells play an important role in the initiation and development of allergic responses. Fc epsilon RI (FcεRI) on the cell membranes of mast cells traps circulating IgEs. This antigen exposure induces cross-linking of the cell-bound IgE, resulting in degranulation [20]. Degranulation releases histamine, leukotrienes, and prostaglandins from antigen-exposed mast cells and is a key step in the induction of allergic responses. Rat basophilic RBL-2H3 leukemia cells, the tumor analog of mast cells with high FcεRI surface expression, were used to determine whether constituents from *S. miltiorrhiza* root (Dansen) could inhibit antigen-induced degranulation. Degranulation was assessed by measuring β-hexosaminidase activity in the medium after antigen exposure, as previously described in materials and methods. Tanshinone I and tanshinone IIA inhibited antigen-induced release of β-hexosaminidase in a concentration-dependent manner (Fig. 2). Tanshinone I and tanshinone IIA inhibited degranulation at concentrations higher than 5 and 3 μM, respectively (Fig. 2). Salvianolic acid A was however, not effective in inducing the degranulation in RBL-2H3 cells (Fig. 2).

3.2. Salvianolic acid A and tanshinone IIA inhibited eosinophil accumulation in BALF of OVA-induced mice

Next, OVA-treated female Balb/c mice with allergic asthma were employed as a model to confirm the inhibitory effects of three constituents of *S. miltiorrhiza*. We noted that, tanshinone I-injected mice died within two days of injection. Except the tanshinone I-injected group, cell number and distribution of cell populations in BALF were analyzed. The total cell number in BALF increased to 313.8% in the OVA-induced asthma mice compared to those in the PBS-treated control group (Fig. 3-B). On the other hand, salvianolic acid A 5 mg/kg and 10 mg/kg inhibited OVA-induced increase in total cell number by 37.8 and 43.0%, respectively (Fig. 3-B). The total cell number in BALF increased to 175.6% in the OVA-induced asthma group compared to that in the PBS-treated control group for a set of tanshinone IIA experiment (Fig. 3-D). Tanshinone IIA 5 mg/kg and 10 mg/kg inhibited OVA-induced increase in total cell number by 22.5 and 50.6%, respectively

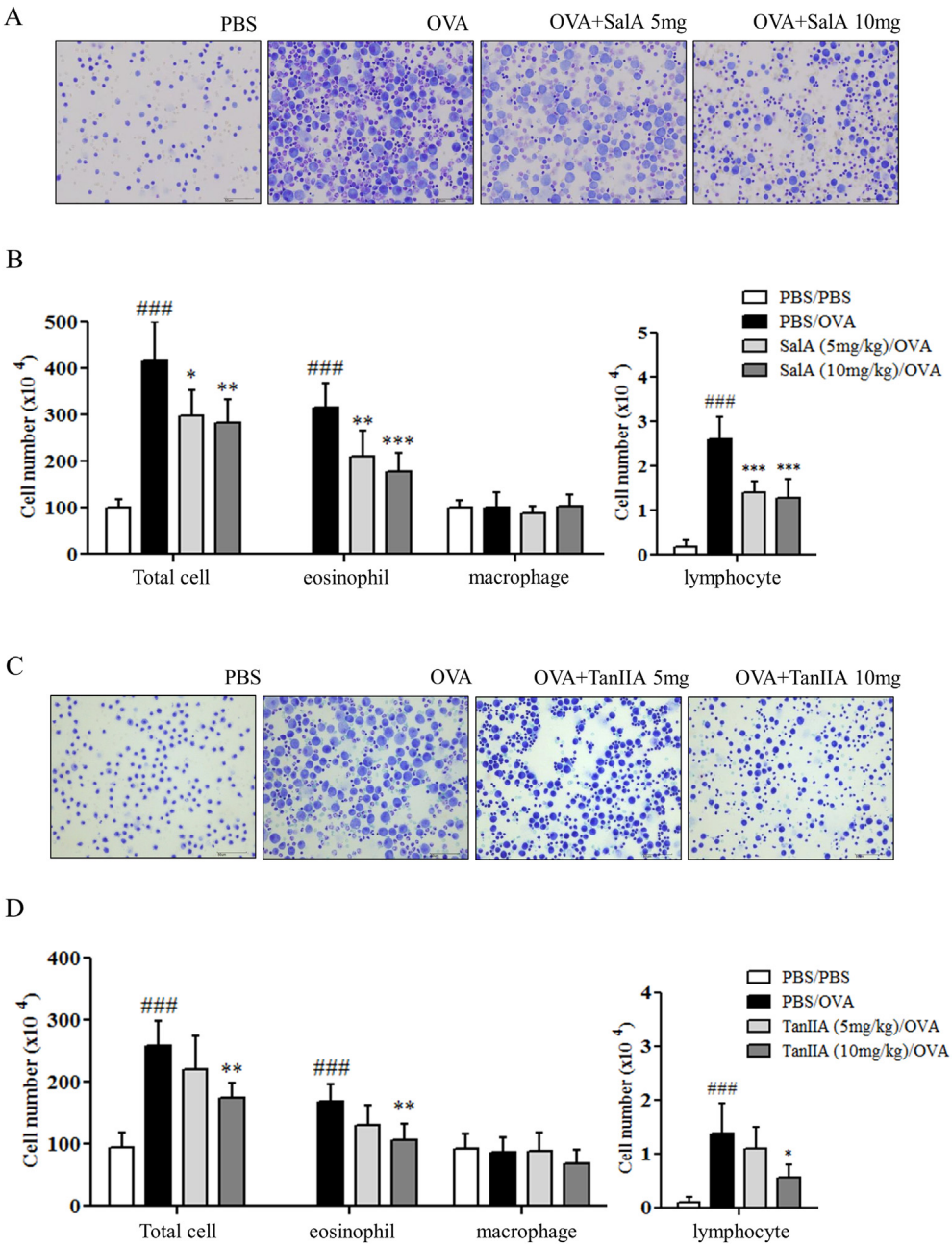

Fig. 3. Effects of salvianolic acid A and tanshinone IIA on immune cell accumulation in BALF of OVA-induced asthmatic mice.
(A and C) Mice ($n = 5$ per group) were sensitized with OVA twice by intraperitoneal injection on days 0 and 14, and later challenged with nebulized OVA on days 28, 29, and 30. Salvianolic acid A or tanshinone II A was intraperitoneally administered at the dose of 5 and 10 mg/kg, 30 min before OVA challenge. Cells from BALF of these mice were stained using May-Grünwald stain and counted. (B) Total cell counts in BALF of PBS-treated control mice, OVA-induced asthmatic mice (OVA), and salvianolic acid A (5 mg/kg and 10 mg/kg) administrated OVA groups; and eosinophils, macrophages, and lymphocytes counts from the BALF samples obtained from these four groups. (D) Total cell counts in BALF of PBS-treated control mice, OVA-induced asthmatic mice (OVA), and OVA-induced and tanshinone IIA-treated (5 mg/kg and 10 mg/kg) groups; and eosinophils, macrophages, and lymphocytes counts from the BALF samples obtained from these four groups. The results are presented the mean ± SE cell count values ($n = 5$). $^{###}p < 0.001$ *vs.* the PBS-treated group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the OVA-treated group.

(Fig. 3-D). Further analyses of cell populations from the BALF supported this observation (Fig. 3-B and D); OVA-induced increase in eosinophil numbers in BALF of the asthmatic mice was significantly inhibited by salvianolic acid and tanshinone IIA 10 mg/kg by 43.3 and 37.5%, respectively (Fig. 3-B and D). Lymphocyte counts were noted to be lesser than eosinophil counts in these mice. OVA administration resulted in a significant increase in lymphocyte counts, which was reduced upon salvianolic acid A or tanshinone IIA treatment (Fig. 3-B and D). However, BALF macrophage population did not significantly increase in the OVA-induced group and was not altered by salvianolic acid A or tanshinone IIA treatment (Fig. 3-B and D).

3.3. Salvianolic acid A and tanshinone IIA inhibited inflammation and mucin secretion in the lungs of OVA-induced asthmatic mice

Histological examination of lung samples was performed two days after final antigen challenge. Fig. 4-A and B shows representative H&*E*-stained lung sections from mice in the PBS-treated, OVA-induced, OVA-induced and salvianolic acid A-treated, and OVA-induced and tanshinone IIA-treated mice groups. Eosinophils appear as small, navy blue dots, as indicated by the green arrows. In the PBS control group, eosinophils were rarely observed (Fig. 4-A and B); however, in the OVA-induced group, eosinophils densely surrounded the bronchioles (Fig. 4-A and B). Treatment with salvianolic acid A or tanshinone IIA reduced eosinophil infiltration in the lungs of OVA-induced mice (Fig. 4-A and B). Moreover, lung inflammation was semi-quantitatively evaluated using a subjective scale of 0–3, as previously described [16,17,21]. The average inflammation score in the OVA-treated group was approximately 2, whereas treatment with salvianolic acid A or tanshinone IIA significantly reduced the inflammation score (Fig. 4-C and D).

PAS was used to stain mucins or mucous glycoproteins, which are produced by goblet cells. Secreted or stored mucins stain purple after PAS staining. As shown in Fig. 5-A and B, dark violet-stained cells surrounded the bronchioles in the OVA-treated group, as indicated by

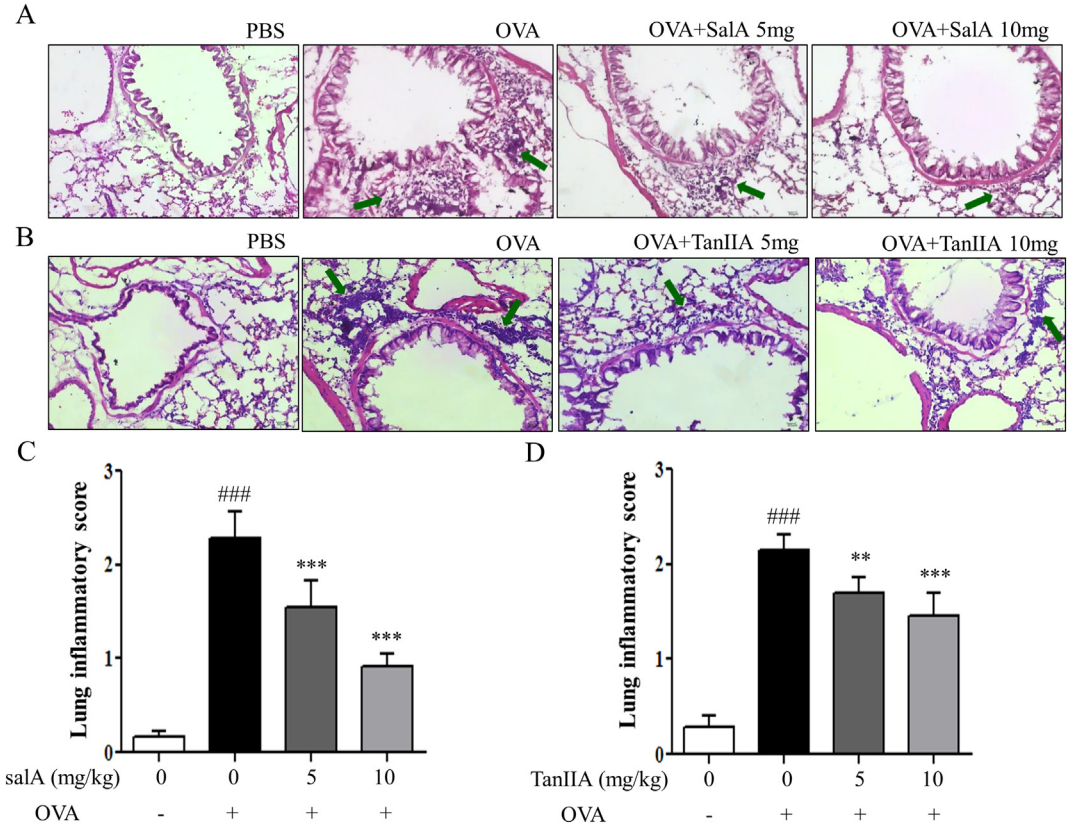

Fig. 4. Protective effects of salvianolic acid A and tanshinone IIA against airway inflammation.
(A and B) Panels show hematoxylin & eosin (H&E)-stained sections of lung tissues from the PBS group, OVA group, and salvianolic acid A (5 mg/kg and 10 mg/kg) or tanshinone IIA-treated (5 mg/kg and 10 mg/kg) OVA groups. Sections from mice in the OVA-induced and salvianolic acid A or tanshinone IIA-treated OVA-induced groups showed an accumulation of eosinophils (small navy blue dots) around the bronchioles, whereas eosinophils were rarely observed in the PBS group. Moreover, eosinophils densely accumulated around the bronchioles in the OVA group (green arrows); however, eosinophil accumulation was less obvious in the 5 and 10 mg/kg salvianolic acid A-treated or 5 and 10 mg/kg tanshinone IIA-treated OVA groups, than in the OVA group, suggesting that salvianolic acid A or tanshinone IIA treatment reduced airway inflammation. (C and D) Lung inflammation was semi-quantitatively evaluated; histological findings were scored as described in the materials and methods section, and by Tournoy et al. (2000) and Kwak et al. (2003). Graphs represent the mean ± SE values ($n = 5$). $^{###}p < 0.001$ *vs.* the PBS-treated group, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the OVA-treated group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the red arrows. However, sections from mice in the OVA-induced and salvianolic acid A-treated or OVA-induced and tanshinone IIA-treated groups showed fewer mucin stained cells, compared to the OVA-treated group, which indicated inhibition of mucin production by salvianolic acid A and tanshinone IIA treatment upon OVA induction (Fig. 5-A and B). In addition, mucin production was semi-quantitatively analyzed by counting PAS-positive cells per mm of the bronchioles (Fig. 5-C and D) [22]. PAS staining was rarely observed in the vehicle group; however, approximately 110 PAS-positive cells/mm were detected in the OVA-treated group. Salvianolic acid A or tanshinone IIA significantly reduced PAS-positive cells in the OVA-treated asthmatic mice (Fig. 5-C and D).

3.4. Salvianolic acid A and tanshinone IIA inhibited the production of Th2 cytokines in lung tissues

Th2 cytokines play a key role in the pathogenesis of asthma [2,3]. In particular, IL-4, IL-5, and IL-13 induce eosinophil recruitment and activation, mucus hypersecretion by epithelial cells, goblet cell metaplasia, and proliferation of smooth muscle cells [2,3]. Therefore, we measured the mRNA levels of IL-4, IL-5, and IL-13 in BALF-associated cells and lung tissues. The mRNA levels of IL-4, IL-5, and IL-13 increased by 1215.6, 2989.1 and 4034.3%, respectively, in the BALF of mice from the OVA group; however, these elevations in the cytokine transcript levels were inhibited by salvianolic acid A (Fig. 6-A). Salvianolic acid A 5 mg/kg and 10 mg/kg inhibited the mRNA expression of IL-4 by 42.2 and 65.0%; of IL-5 by 16.0 and 62.9%; and of IL-13 by 28.1 and 53.9%, respectively (Fig. 6-A). The mRNA levels of IL-4, IL-5, and IL-13 increased by 783.7, 3031.5 and 2940.9%, respectively, in the BALF of mice from the OVA group for a set of tanshinone IIA experiment; however, these elevations were inhibited by tanshinone IIA (Fig. 6-C). Tanshinone IIA 5 mg/kg and 10 mg/kg inhibited the mRNA expression of IL-4 by 24.7 and 75.3%; of IL-5 by 16.1 and 68.1%; and of IL-13 by 48.9 and 61.4%, respectively (Fig. 6-C). Furthermore, salvianolic acid A or tanshinone IIA inhibited mRNA expression of Th1 cytokines, such IL-2 and INF-γ (Fig. 6-B and D).

Moreover, as shown in Fig. 7-A and C, the mRNA levels of IL-4, IL-5, and IL-13 increased by 1358.5, 3637.5 and 2128.4%, respectively, in the lung tissues of mice from the OVA group; however, salvianolic acid A inhibited the mRNA expression of IL-4 by 43.2 and 68.3%; of IL-5 by 29.8 and 63.6%; and of IL-13 by 41.7 and 59.9%, respectively (Fig. 7-A). The mRNA levels of IL-4, IL-5, and IL-13 increased by 910.9, 1855.7 and 3600.9%, respectively, in the lung tissues of mice from the OVA-treated group; however, tanshinone IIA treatment inhibited the mRNA expression of IL-4 by 67.8 and 52.8%; of IL-5 by 52.2 and 60.8%; and of IL-13 by 57.2 and 58.0%, respectively (Fig. 7-C). Again, salvianolic acid A and tanshinone IIA inhibited mRNA expression of Th1 cytokines, such IL-2 and INF-γ in the lung tissues (Fig. 7-B and D).

To confirm the effect of salvianolic acid A or tanshinone IIA treatment on the expression of Th2 cytokines, the levels of IL-4 and IL-13 in

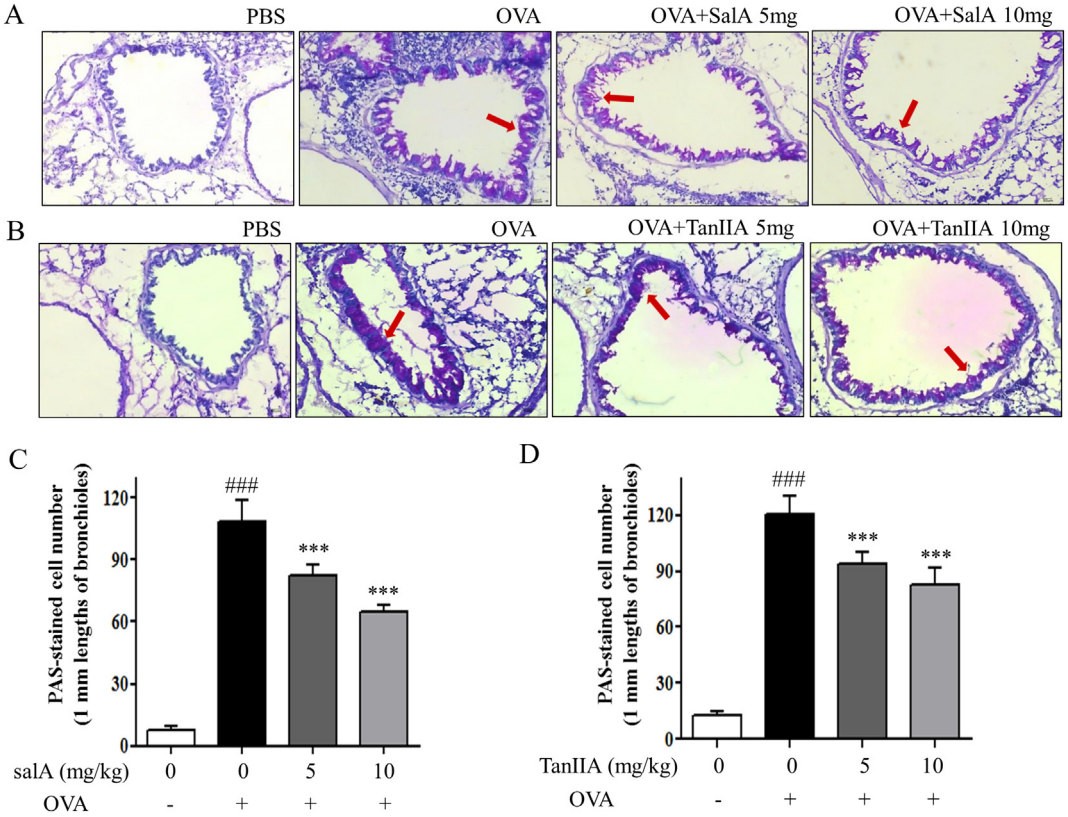

Fig. 5. Protective effects of salvianolic acid A and tanshinone IIA against mucin production.
(A and B) Panels show PAS-stained sections of lung tissues from the PBS group, OVA group, and salvianolic acid A (5 mg/kg and 10 mg/kg) or tanshinone IIA-treated (5 mg/kg and 10 mg/kg) OVA groups. Post PAS staining, mucin is stained in purple color. In OVA-induced group, darker and thicker purple color is observed surrounding the bronchioles compare to PBS-treated group. (C and D) Mucous production was evaluated by counting the number of PAS-positive cells (red arrows) per mm of bronchiole ($n = 5$ per group). Bar = 50 μm. $^{###}p < 0.001$ *vs.* the PBS-treated group, $^{***}p < 0.001$ *vs.* the OVA-treated group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

BALF were measured by ELISA. IL-4 and IL-13 levels increased in the OVA-induced group, compared to the PBS control group (Fig. 8). OVA-induced increase in IL-4 and IL-13 levels was significantly inhibited by salvianolic acid A or tanshinone IIA administration (Fig. 8).

4. Discussion

In the present study, we investigated effects of three constituents of *Salvia miltiorrhiza* root (Dansen), salvianolic acid A, tanshinone I, and tanshinone IIA in the lungs of OVA-induced asthmatic mice. Our study indicates significant anti-allergic effects of constituents from *S. miltiorrhiza* which have not been fully investigated otherwise, except salvianolic acid B [12]. Although the relaxant effect of tanshinone IIA sulphonate on mouse tracheal smooth muscle was reported [23], *in vitro* and *in vivo* efficacy tests of three constituents have been studied for the first time in this study. Recently, daily treatment with *S. miltiorrhiza* ethanolic or water extract showed significant reduction in OVA-induced airway inflammatory cell infiltration, Th1/Th2 cytokine amounts, and goblet cells hyperplasia [24]. Furthermore, chromatography showed that levels of salvianic acid A and caffeic acid are higher in water extract than ethanol extract, while levels of salvianolic acid B and tanshinone IIA are higher in ethanol extract than water extract [24]. In the RBL-2H3 cell-based tests, we found that tanshinone I and tanshinone IIA inhibit mast cell degranulation. However, in *in vivo* tests, salvianolic acid A and tanshinone IIA showed significant efficacy in inhibiting allergic features in mice. Because tanshinone I was lethal for the mice, it was omitted from the animal experiments.

Because tanshinone IIA showed inhibitory effect on mast cell degranulation, this may contribute to the *in vivo* efficacy of tanshinone IIA. On the other hand, salvianolic acid A did not show an inhibitory effect on the degranulation *in vitro*. Nevertheless, it significantly suppressed allergic asthma *in vivo*. Salvianolic acid A significantly suppressed immune cell accumulation in the BALF of OVA-induced mice at the dose of 5 mg/kg, but tanshinone IIA showed significant effects at the dose of 10 mg/kg. In a previous study, salvianolic acid B was effective at 50 mg/kg but not at 10 mg/kg, which was used as effective doses for salvianolic acid A and tanshinone IIA in the present study [12]. Therefore, salvianolic acid A may be the most potent constituent for anti-asthma therapy. Recently, suppressive effects of tanshinone IIA on FcεRI-mediated mast cell activation and anaphylaxis were reported to be mediated through activation of the inhibitory Sirt1-LKB1-AMPK pathway [25].

Although both salvianolic acid A and tanshinone IIA exhibited similar efficacy in reducing lung inflammation and mucin production, salvianolic acid A showed a better reduction in lung inflammation at 10 mg/kg than tanshinone IIA. Similarity in efficacy of both these *S. miltiorrhiza* constituents was further supported by a decrease in cytokine production in BALF-associated cells and lung tissues of the OVA-induced mice. Both constituents showed inhibitory effects on Th1 cytokines in addition to Th2 cytokines, implying that anti-allergic activity of both salvianolic Acid A and tanshinone IIA stems from anti-inflammatory effects. In previous studies, salvianolic acid A treatment showed a downregulation of NF-κB p65 expression and reduced the inflammation response mediated by interleukin-1β and tumor necrosis factor-α [26–28]. Also tanshinone IIA alleviated inflammatory response in LPS-stimulated RAW264.7 cells, and ameliorated inflammatory microenvironment of colon cancer cells *via* repression of microRNA-155 [29,30]. Therefore, in line with these studies we hypothesize that anti-

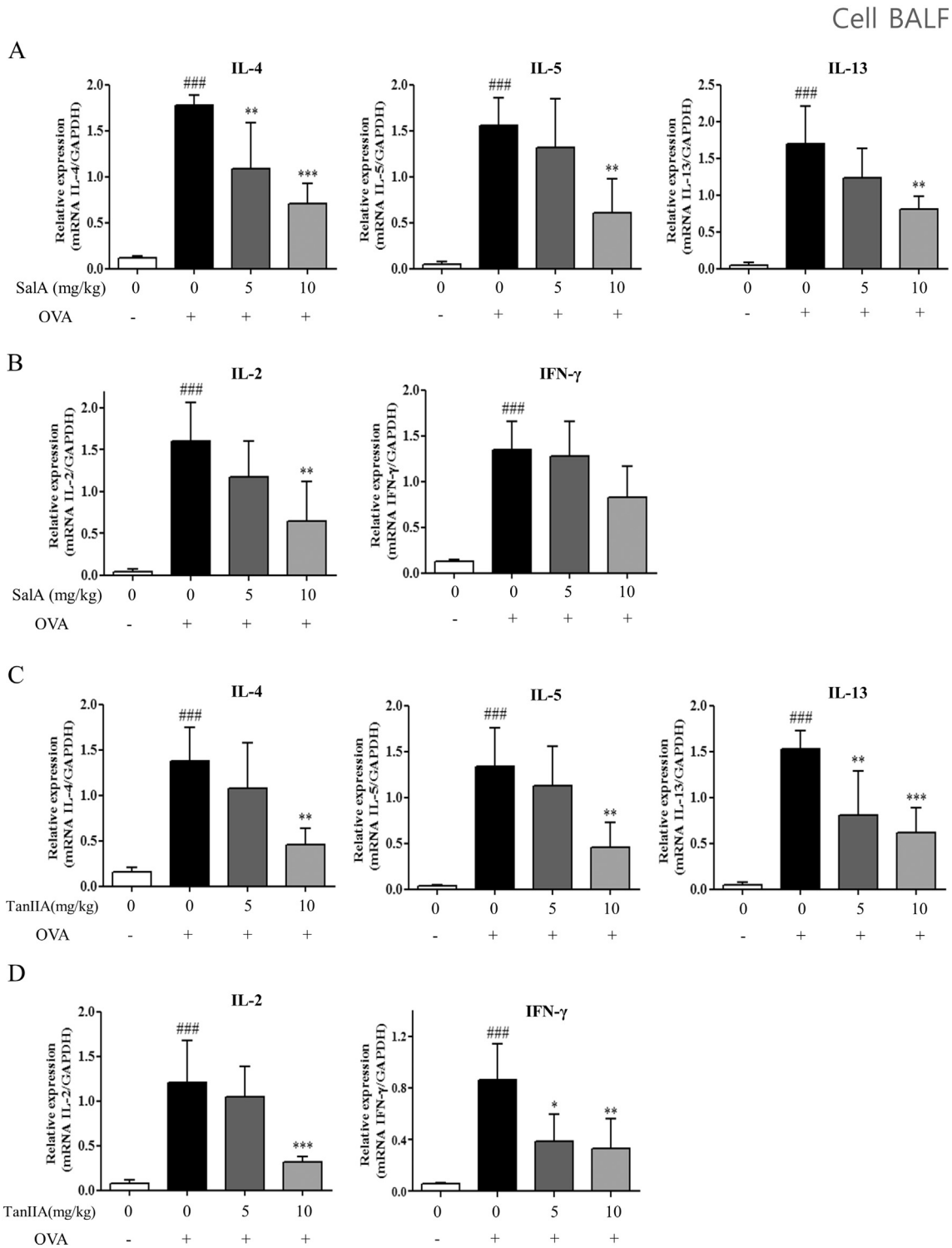

Fig. 6. Salvianolic acid A and tanshinone IIA treatment inhibited Th2 and Th1 cytokine expression at the mRNA level in BALF-associated cells.
(A and C) Analysis of mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) from the BALF cells of OVA-induced and salvianolic acid (A) or tanshinone IIA (C)-treated mice groups. (B and D) Analysis of mRNA expression of Th1 cytokines (IL-2 and IFN-γ) from the BALF cells of OVA-induced and salvianolic acid A (B) or tanshinone IIA (D)-treated mice groups. Relative mRNA levels of cytokines were quantified as ratios to GAPDH transcript levels. Each lane represents one of five different mice from the group. Values represent the mean ± SE ($n = 5$). $^{###}p < 0.001$ *vs.* the PBS-treated group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the OVA-treated group.

inflammatory effects of both constituents may contribute to the anti-allergic effects in the present study.

In summary, we report that tanshinone I and tanshinone IIA have inhibitory effects on mast cell degranulation, and also that salvianolic acid A and tanshinone IIA have anti-allergic activity in OVA-induced mouse asthma model, providing evidence for the therapeutic potential of these constituents from *S. miltiorrhiza*.

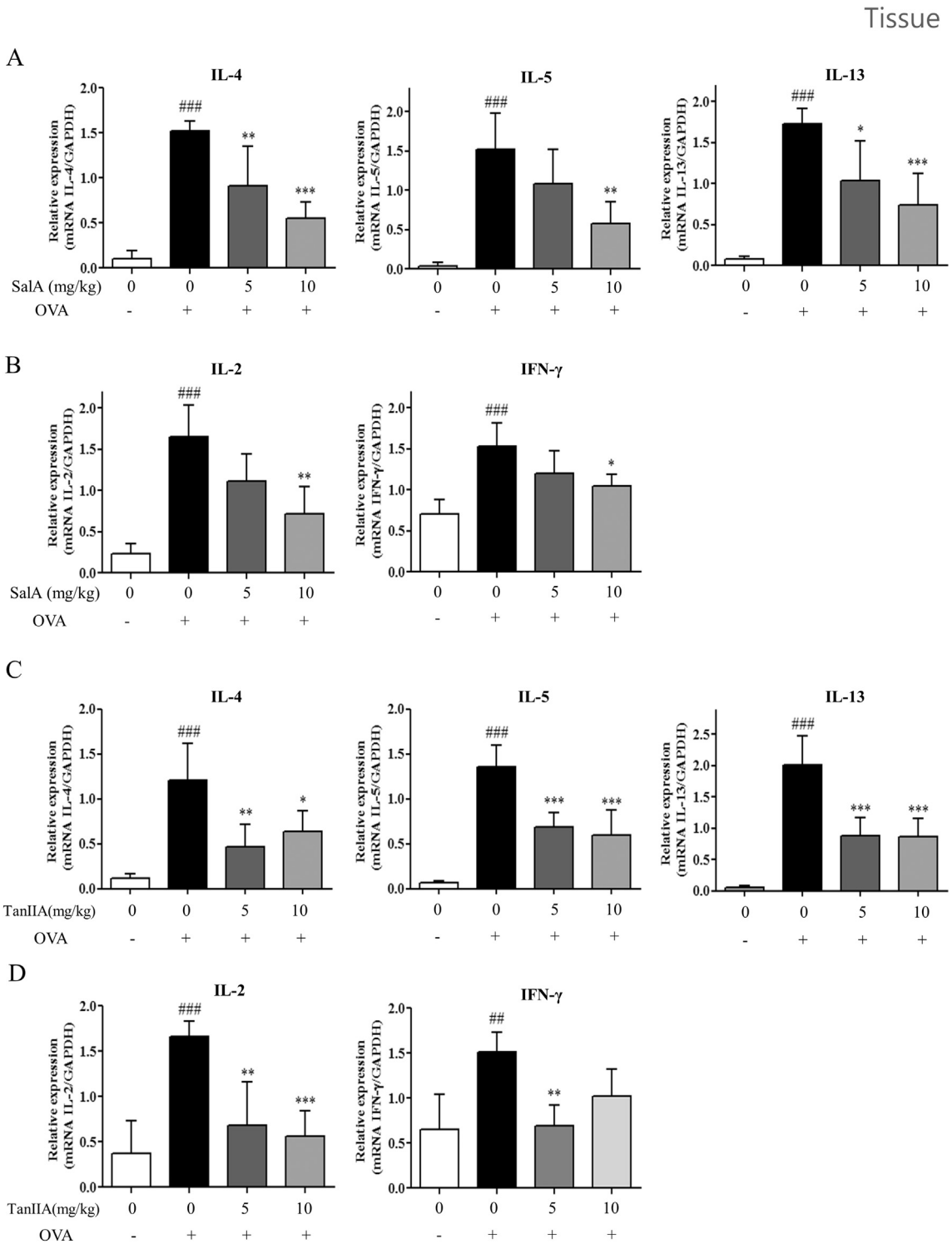

Fig. 7. Salvianolic acid A or tanshinone IIA treatment inhibited Th2 and Th1 cytokine expression at the mRNA level in the lung tissues of OVA-induced asthmatic mice.

(A and C) Analysis of mRNA expression of Th2 cytokines (IL-4, IL-5, and IL-13) from lungs of salvianolic acid A(A) or tanshinone IIA (C)-treated asthmatic mice. (B and D) Analysis of mRNA expression of Th1 cytokines (IL-2 and IFN-γ) from lungs of salvianolic acid A (B) or tanshinone IIA (D)-treated asthmatic mice. Relative mRNA levels of cytokines were quantified as ratios to GAPDH transcript levels. Each lane represents one of the five different mice from the group. Values represent the mean ± SE ($n = 5$). $^{###}p < 0.001$ *vs.* the PBS-treated group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the OVA-treated group.

Statement of contribution

JY Heo and DS Im designed the experiments. JY Heo performed the experiments and analyzed the data. JY Heo and DS Im wrote manuscript.

Conflict of interest

Authors declare there is no conflict of interest.

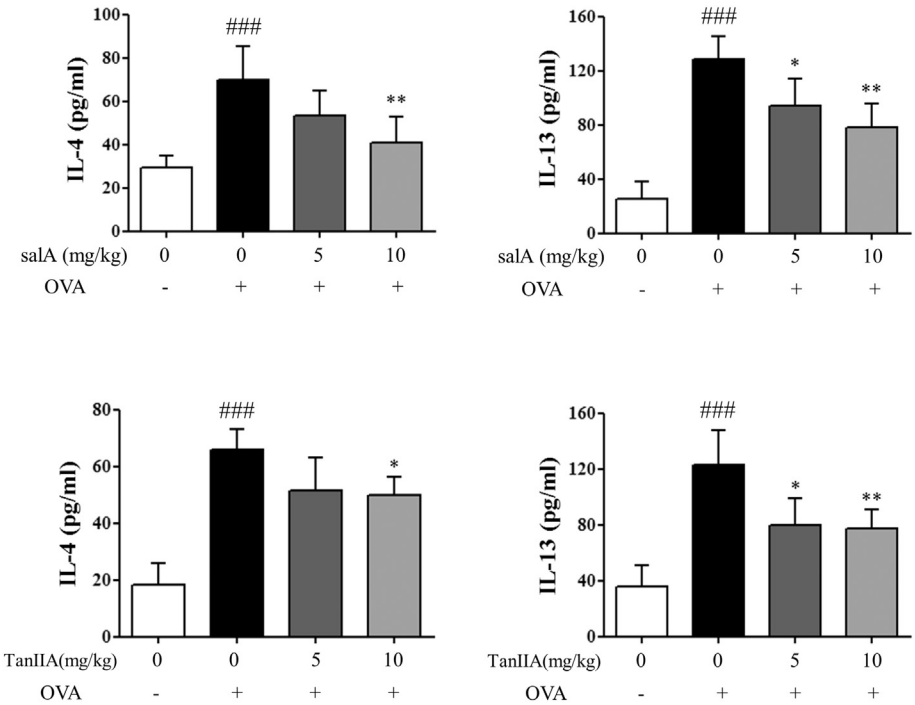

Fig. 8. Effects of salvianolic acid A or tanshinone IIA treatment on IL-4 and IL-13 protein levels in BALF. ELISA was used to measure IL-4 and IL-13 protein levels in BALF samples from PBS-treated group, OVA-induced group, and salvianolic acid A or tanshinone IIA-treated OVA group ($n = 5$ per group). The results represent the mean ± SE of protein levels ($n = 5$). $^{###}p < 0.001$ *vs.* the PBS-treated group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *vs.* the OVA-treated group.

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