



Original research article

Chelidonine, a principal isoquinoline alkaloid of *Chelidonium majus*, attenuates eosinophilic airway inflammation by suppressing IL-4 and eotaxin-2 expression in asthmatic mice

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ABSTRACT

Background: Chelidonine, a major bioactive, isoquinoline alkaloid ingredient in *Chelidonium majus*, exhibits anti-inflammatory and other pharmacological properties. However, its molecular mechanisms in asthma remain unclear. In this work we investigated chelidonine's effect and mechanism in airway inflammation in a mouse model of allergic asthma.

Methods: The mice were sensitized to ovalbumin followed by aerosol allergen challenges and determination of chelidonine's effect on enhanced pause (Penh), pulmonary eosinophilic infiltration, eotaxin-2, interleukin-4 (IL-4), IL-13, OVA-specific IgE production, and several transcription factors.

Result: Chelidonine strongly suppressed airway eosinophilia, expression of eotaxin-2, IL-4, and IL-13 cytokine production in bronchoalveolar lavage fluid (BALF). It also attenuated lung IL-17, and eotaxin-2 mRNA expression levels. Moreover, it suppressed eotaxin-2 and IL-17 production in accordance with up- and downregulation of forkhead box p3 (Foxp3), and signal transducer and activator of transcription (STAT6) expression, respectively.

Conclusions: Chelidonine has profound inhibitory effects on airway inflammation and this effect is caused by suppression of IL-4, eotaxin-2, and OVA-specific IgE production through the STAT6 and Foxp3 pathways. So chelidonine can improve allergic asthma in mice and be a novel anti-asthma therapeutic.

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Introduction

Allergic asthma is a inflammatory disease of the airways characterized by airway inflammation, Th2 cytokine production, airway hyperresponsiveness (AHR), and infiltration of inflammatory cells, including eosinophils and T-lymphocytes [1]. Eosinophilic inflammation has been linked to allergic asthma, and Th2 cytokines such as interleukin IL-4, IL-5, and IL-13 [2]. In particular, IL-4 and IL-13 are associated with inflammatory changes in the airways via the production of immunoglobulin E (IgE) by B cells [3]. Furthermore, IL-4 is a primary signal influencing B lymphocytes to undergo switching from IgM to IgE, and they also induce eotaxin-2 mRNA and protein release [4,5].

Eotaxin-2 is a chemokine involved in the pathogenesis of asthma. STAT6 is a central downstream mediator of airway

reactivity. Targeted expression of STAT6 in epithelial cells is sufficient for IL-13-induced AHR and mucus production [6]. T box-containing protein (T-bet) is a Th1-specific transcription factor thought to initiate Th1 development, whereas GATA binding protein 3 (GATA-3) plays a pivotal role in the development of the Th2 cells [7]. Moreover, IL-4 and IL-13 were increased in the BALF of T-bet-deficient mice [8].

Foxp3⁺ T regulatory cells (Tregs) can suppress Th2 maturation [9], possibly by inhibiting IL-4 production [10]. T-bet, GATA-3, STAT6, and Foxp3 may therefore be attractive targets for the development of anti-asthmatic drugs.

Chelidonine is one of the principal alkaloids of *Chelidonium majus*, a plant with broad pharmacological activities. Both crude extracts of *C. majus* and its purified chelidonine exhibit broad biological properties, including anti-inflammatory, immunomodulatory, analgesic, antimicrobial, and antiviral ones [11–13].

Despite its known anti-inflammatory effects, the mechanisms of action and molecular functions of chelidonine are not yet fully elucidated. Only a few investigations on the biological activity of chelidonine are available. Moreover, there are no *in vivo* studies on

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the anti-asthmatic effects of chelidonine. We hypothesized that chelidonine might be able to improve the inflammatory response in an asthma mouse model. To test this, we used a mouse model of allergic asthma to evaluate the pharmacological activity of chelidonine.

Materials and methods

Chelidonine and other chemicals

Chelidonine and ovalbumin (OVA) were purchased from Sigma. HPLC-grade reagents, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were purchased from Sigma Chemical and of analytical grade.

Animals

Five-week-old female BALB/c mice, weighing 20–25 g, were obtained from Daehan Biolink Co. Ltd. (Eumsung, Republic of Korea). Our study was approved by the Committee for Animal Welfare at the Institution Daejeon University. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and the Institutional Animal Care and Use Committee of Daejeon University, Republic of Korea.

Ovalbumin sensitization, inhalation and Penh measurement

OVA sensitization, inhalation and Penh measurement were performed as previously described with modifications [14].

Chelidonine (5 and 1 mg/kg) and dexamethasone (0.5 mg/kg) were orally administered 3 times a week for the last 5 weeks. One day after the last OVA exposure, Penh was measured and samples (BALF, lung cells, and serum) were collected for molecular analyses (Fig. 1A).

BALF

Immediately after Penh assessment, mice were anesthetized with an intraperitoneal (*ip*) injection of 10% chloral hydrate (0.2 mL/20 g body weight) in PBS. The trachea was cannulated and BALF performed by washing the airway lamina. Lung cells were recovered by flushing 1 mL BAL (1 mM EDTA, 10% fetal bovine serum (FBS), PBS) into the lungs *via* the trachea. Total cell counts were determined and 100 μ L of fluid were cytopspun onto glass slides using a cytopspin centrifuge (400 $\times$ g, 4 min). Differential cell counts were performed after staining with a Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, FL, USA) to determine the percentage of eosinophils. Four hundred cells were counted per slide. The rest of BALF supernatant was stored at -25°C for further analyses.

Digestion of lung tissue and cell preparation

Cell suspensions from lung tissues and BALF were isolated by mechanical disruption in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, 50 μ M 2-mercaptoethanol, 20 mM HEPES, and 2% heat-inactivated FBS. Lung tissues were minced using sterile scalpels followed by

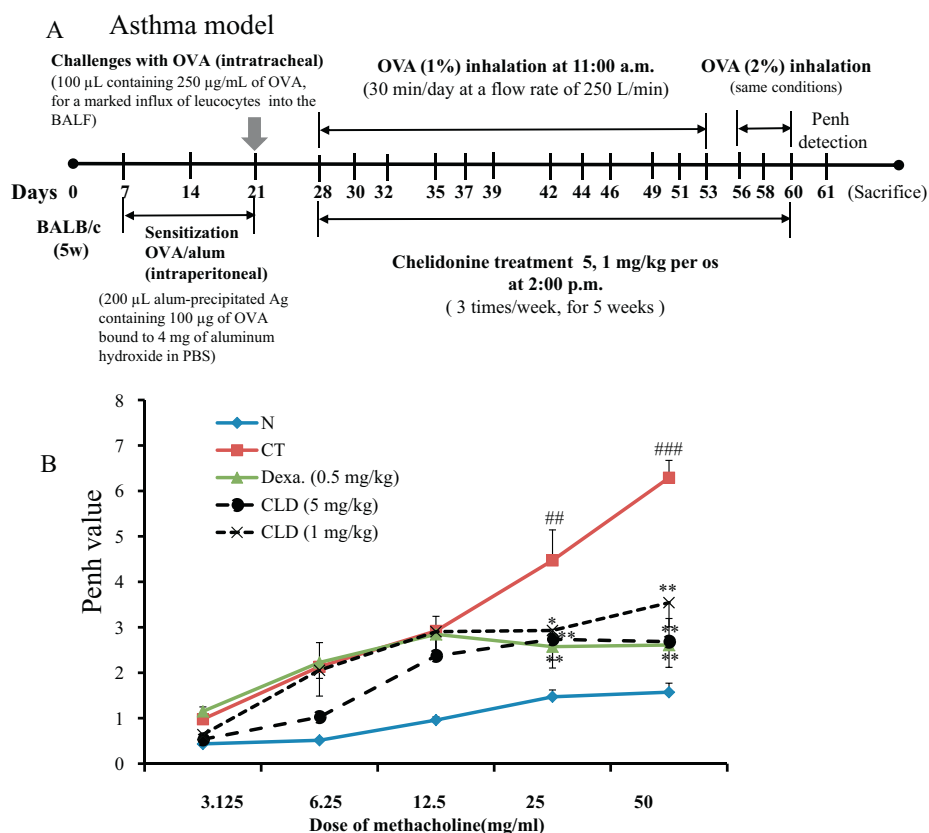


Fig. 1. Scheme of OVA sensitization and challenge protocol (A). Effect of chelidonine on airway hyperresponsiveness in OVA-challenged mice (B). Airway responsiveness to aerosolized methacholine was assessed with a Buxco box. Mice were placed in the main chamber and nebulized first with PBS, then with increasing doses (3.125–50 mg/mL) of methacholine for 3 min per nebulization. Data represent the means $\pm$ SEM of six independent experiments. $N = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the normal group versus the control group comparison, and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the OVA control group versus the experimental groups comparisons. N: Normal BALB/c mice, CT: OVA inhalation + vehicle, Dexa.: OVA inhalation + Dexamethasone, 0.5 mg/kg, CLD: OVA inhalation + chelidonine (5 or 1 mg/kg).

incubation in PBS containing 1 mg/mL collagenase IV and 2 mg/mL dispase for 40 min at 37 °C in a sterile polypropylene tube. After incubation, lung tissues were vigorously pipetted up and down to dissolve, then filtered using a 70 µm cell-strainer. Cells were counted manually using a hemocytometer chamber. Differential cell counts were performed according to standard morphologic criteria.

Hematoxylin–eosin (H&E), Masson-trichrome (M-T), periodic acid-Schiff (PAS), and Alcian blue periodic acid-Schiff (AB-PAS) staining

The right lobe of the lungs from each animal was harvested and subjected to histopathological analysis using a modified protocol previously described [14].

Harvested lung tissues were embedded in paraffin, cut into 3 µm sections, stained with H&E, M-T, PAS, and AB-PAS solution. Above tissue was subsequently mounted and covered with Dako-mounting medium (Dako Cytomation; Carpinteria, CA, USA) and a coverslip. PAS-positive goblet cells were counted manually and normalized to the length of the bronchial perimeter on the basal region, and expressed as the number of goblet cells per mm of basement membrane.

Antibodies and flow cytometry

All antibodies (anti-CD3, CD4, CD8, Gr-1, CD11b, CCR3, and Foxp3) for flow cytometry were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA, USA). Lung cells and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% Na₂S₂O₃) for 10 min on ice, and analyzed by 2-color flow cytometry on a FACSCalibur device using CellQuest software (BD Biosciences, Mountain View, CA, USA).

Enzyme-Linked Immunosorbent Assay (ELISA)

Interleukins (IL-4, IL-13, IL-17, etc.), eotaxin-2 and anti-OVA IgE levels in BALF (*N* = 6) were measured by ELISA using a monoclonal antibody-based mouse interleukin ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). All data represent the mean and standard error of mean from at least three separate experiments and were compared by analysis of variance (ANOVA).

Quantitative real-time PCR (qRT-PCR)

The total RNA was extracted from the lung by the phenol-chloroform method (RNAsol^B) and cDNA was synthesized from 3 µg of total RNA using a ReverTraAce-a-cDNA Synthesis kit (Toyobo) according to the manufacturer's instructions. qRT-PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system with the following mouse primers: mouse IL-17, 5'-TCTCATCCAGCAAGAGATCC-3' and 5'-AGTTTGGGACCCCTTACAC-3'; RORγt, 5'-CAGTGAGAGCCCAGAAGGAC-3' and 5'-TCATCCCATCCATTTTGGT-3'; eotaxin-2, 5'-CTGTGACCATCCCCTCATCT-3' and 5'-CTTATGGCCCTTCTGGTGA-3'; β-actin, 5'-TGGAACTCTGTGCTCCATGAAC-3' and 5'-GTCACAGTCAGCTGATAGGG-3'. The above gene expressions were analyzed with SYBR Green PCR Mastermix (ABI) using β-actin as the internal standard. The following PCR conditions were used: 2 min at 50 °C, 10 min at 94 °C, then 40 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C. The cycle number at which the emission intensity of the sample rose above baseline was referred to as the relative quantification (RQ) and was proportional to the target concentration. qRT-PCR was used in duplicate and analyzed according to the system manual (threshold: 0.05, baseline: 6–15 cycles).

Isolation of CD4⁺ T cells

Isolation of CD4⁺ T cells was performed as previously described [14]. CD4⁺ T cells were activated by overnight incubation on 24-well plates coated with 1 µg/mL anti-CD28 (Serotec, Oxford, U.K.), 1 µg/mL anti-CD3 (Serotec, Oxford, U.K.), and chelidonine (20, 10, and 5 µg/mL) added to RPMI medium supplemented with 1 µg/mL streptomycin, 1 U/mL penicillin, 50 mM-mercaptoethanol, 20 mL L-glutamine, and 5% FBS for 48 h after stimulation.

Intracellular staining for CD4⁺ and Foxp3 in isolated CD4⁺ T cells

For CD4 (FITC mouse anti-CD4; BD PharMingen) and Foxp3 (PE mouse anti-Foxp3; BD PharMingen) intracellular staining, the isolated CD4⁺ T cells were stimulated in culture medium containing anti-CD28 (1 µg/mL; Serotec, Oxford, U.K.), anti-CD3 (1 µg/mL; Serotec, Oxford, U.K.), TGF-β (5 ng/mL; R&D Systems, Minneapolis, MN, USA), and monensin (GolgiStop, 1 mL/mL, BD Biosciences, Mountain View, CA, USA) in a cell incubator with 10% CO₂ at 37 °C for 4 days. After staining with markers, they were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences, Minneapolis, USA) according to the manufacturer's instructions.

RNA preparation and qRT-PCR in EL-4 T and spleen cells

EL-4 T cells (5×10^5 cells/mL) were stimulated with 4-*tert*-octylphenol (OP, 5 µM) and phorbol 12-myristate 13-acetate (PMA, 1 ng/mL) for 1 h and spleen cells (1×10^6 cells/mL) were stimulated with concanavalin A (ConA) (2 µg/mL) for 1 h. They were followed by incubation with chelidonine for 6 h. The method was mentioned in qRT-PCR in this paper about total RNA and cDNA synthesis. qRT-PCR was performed using the Applied Biosystems StepOnePlus Real-Time PCR system. IL-17A, IL-17F, IL-4, IL-13, and eotaxin 2 were amplified using a SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA, USA). The sequences of PCR primers were as follows: IL-17A, sense 5'-TCCCTCTGTGATCTGGGAAG-3', antisense 5'-CTCGACCTGAAAGTGAAGG-3'; IL-17F, sense 5'-TTGCAGAAGGCTGGGAAGT-3', antisense 5'-GGGGTCTCGAGTGATGTTGTAAT-3'; IL-4, sense 5'-CTCATGGAGCTGCAGAGACTCTT-3', antisense 5'-CATTCATGGTGCAGCTTATCGA-3'; IL-13, sense 5'-GCTTGCCTTGGTGGTCTTGC-3', antisense 5'-CCATACCATGCTGCTGTGCAC-3'; eotaxin-2, 5'-CTGTGACCATCCCCTCATCT-3' and 5'-CTTATGGCCCTTCTGGTGA-3'; and β-actin, sense 5'-TCATGAAGTGTGACGTTGACATCCGT-3', antisense 5'-CTAAGCATTTGCGGTGCACGATG-3'. The following PCR conditions were used: 2 min at 50 °C, 10 min at 94 °C, then 40 cycles of 1 min at 94 °C and 1 min at 60 °C. The target genes were calibrated by comparative threshold (*C_T*) of β-actin. The *C_T* method ($\Delta\Delta C_T$ method) was used to determine the RQ of target nucleic acid in samples. qRT-PCR quality control was performed by verifying a single peak in the melting curve analysis according to the system's manual. The oligonucleotides were manufactured by Genotech Corp (Daejeon, Korea).

Immunoblotting analysis

EL-4 T cells (1×10^6 cells/mL) were stimulated with OP (5 M) and phorbol 12-myristate 13-acetate (PMA), 1 ng/mL for 1 h followed by 24 h incubation of chelidonine (20, 10, or 1 µM). The nuclear extracts were separated using a Nuclear/Cytosol Fractionation Kit (BioVision Incorporated) according to manufacturer's instructions. Immunoblotting analysis was performed according to the method of our previous study [15] with specific antibodies against pSTAT6, T-bet, GATA-3 (Santa Cruz, CA, USA), RORγt (BD Biosciences, MD, USA), Foxp3, nuclear factor-κB (NF-κB) p65, TATA

(Abcam, MA, USA) at 4 °C. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. The bands were scanned and we performed densitometry using Image J software.

Statistical analysis

Data were analyzed using unpaired Student's *t*-test followed by Dunnett's multiple comparison test or ANOVA (SPSS analysis software, version 14.0). The difference between the normal group and the control group (OVA plus vehicle) was significant. Results (presented as mean $\pm$ standard error of the mean) were considered statistically significant at *p* values <0.05 (*), <0.01 (**), or <0.001 (***) for the control group versus the experimental groups comparisons and *p* values <0.05 (#), <0.01 (##), or <0.001 (###) for the normal group versus the control group comparison.

Results

Chelidonine inhibits AHR in response to methacholine stimulation

AHR was detected as the relative increase of Penh in response to increasing doses of methacholine. Control group showed an increased airway resistance (represented as Penh) and the Penh values were significantly greater than those in normal group. Both dexamethasone (*p* < 0.01) and chelidonine (*p* < 0.01) treatments were equally efficient at reducing the Penh compared to that in control group as determined by whole-body plethysmography (Fig. 1B).

Histological analysis of pulmonary inflammation

Histological analyses of the lung and trachea revealed typical pathologic features of asthma in control group showing numerous infiltrating cells composed of eosinophils, and lymphocytes around peribronchial and perivascular regions (Fig. 2). However, both the chelidonine-treated group and the dexamethasone-treated group revealed marked reductions in the infiltration of inflammatory cells in the perivascular and peribronchiolar regions.

To determine the level of goblet cell hyperplasia, AB-PAS-positive and PAS-positive cells in individual bronchioles were analyzed. The mean numbers of AB-PAS and PAS-positive cells were significantly higher in control group than in normal group. Administration of chelidonine (5 or 1 mg/kg) resulted in a significantly lower relative number of AB-PAS and PAS-positive goblet cells than that in control group (Fig. 2B and C). Chelidonine and dexamethasone markedly attenuated OVA-induced eosinophil infiltration, goblet cell hyperplasia, and collagen deposition in lung tissues as compared with that in control mice (Fig. 2).

Chelidonine and dexamethasone suppress airway eosinophil accumulation and influx of inflammatory cells into lung and BALF

We examined the alternation of total cells in the BALF and lung tissues. As shown in Fig. 3, the total lung cells of normal was $1.44 \pm 0.57 \times 10^7$ while control showed significantly higher number, $2.85 \pm 0.34 \times 10^7$. However, these cells were significantly lower in the chelidonine-treated (5 mg/kg) and dexamethasone-treated mice than that in control. Chelidonine (5 mg/kg) also reduced the absolute number of total BALF cells, as well as the number of eosinophils in PBMC. Compared with $6.5 \pm 1.5 \times 10^4$ cells in normal group, the total cell numbers, $20.5 \pm 0.05 \times 10^4$, in the BALF were significantly higher in control group. However, the chelidonine-treated groups (5, 1 mg/kg), showed $5.0 \pm 1.0 \times 10^4$ and $15.0 \pm 4.0 \times 10^4$ cells respectively. These numbers were significantly lower than that in control group.

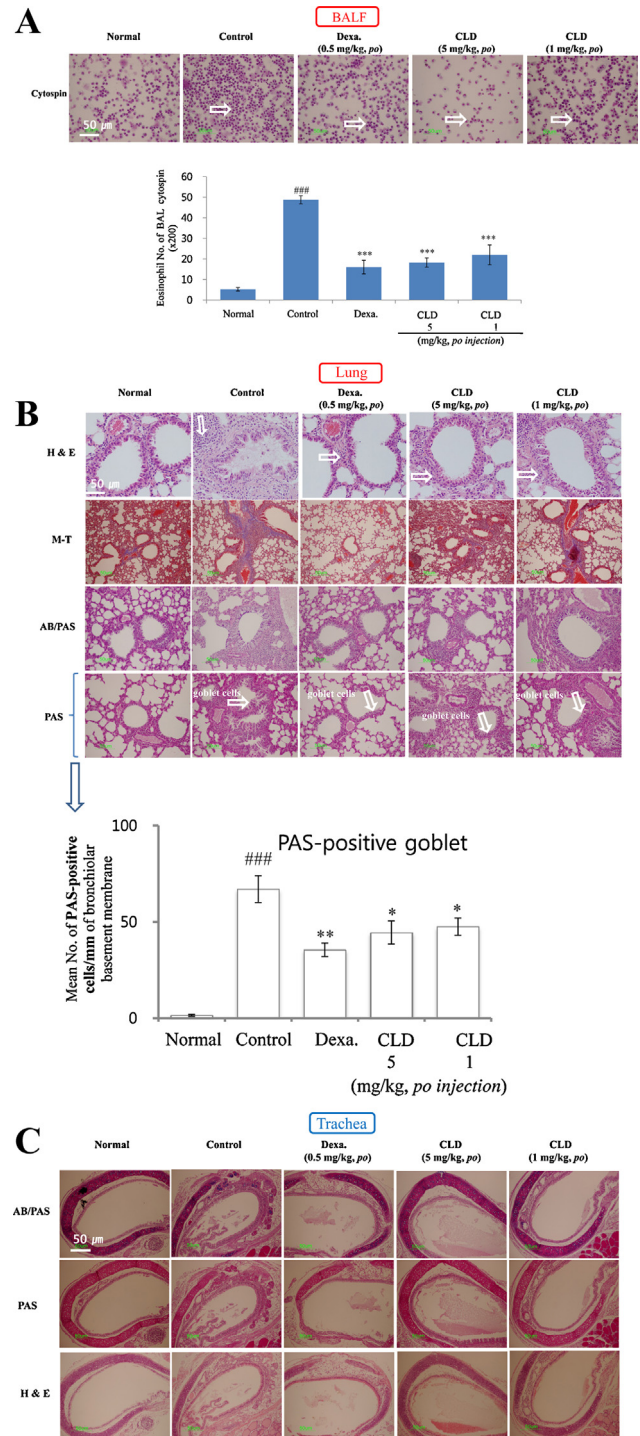


Fig. 2. Effect of chelidonine on histologic markers of airway inflammation (H&E, M-T, AB-PAS, and PAS staining) in lung tissue in the OVA-induced mouse model of allergic asthma. Photomicrograph of BALF cytospins (A). Lung and trachea sections (B and C). ###*p* < 0.001 for the normal group versus the control group comparison, and **p* < 0.05, ***p* < 0.01, and ****p* < 0.001 for the control group versus the experimental groups comparisons.

Inhibitory effect of chelidonine and dexamethasone on the absolute number of immune cell subtypes in murine OVA-induced asthma lungs and BALF

The numbers of CD3⁺/CD4⁺, CD3⁺/CD8⁺, Gr-1⁺/CD11b⁺, and CD3⁺/CCR3⁺ double-positive or single-positive cells in the lungs

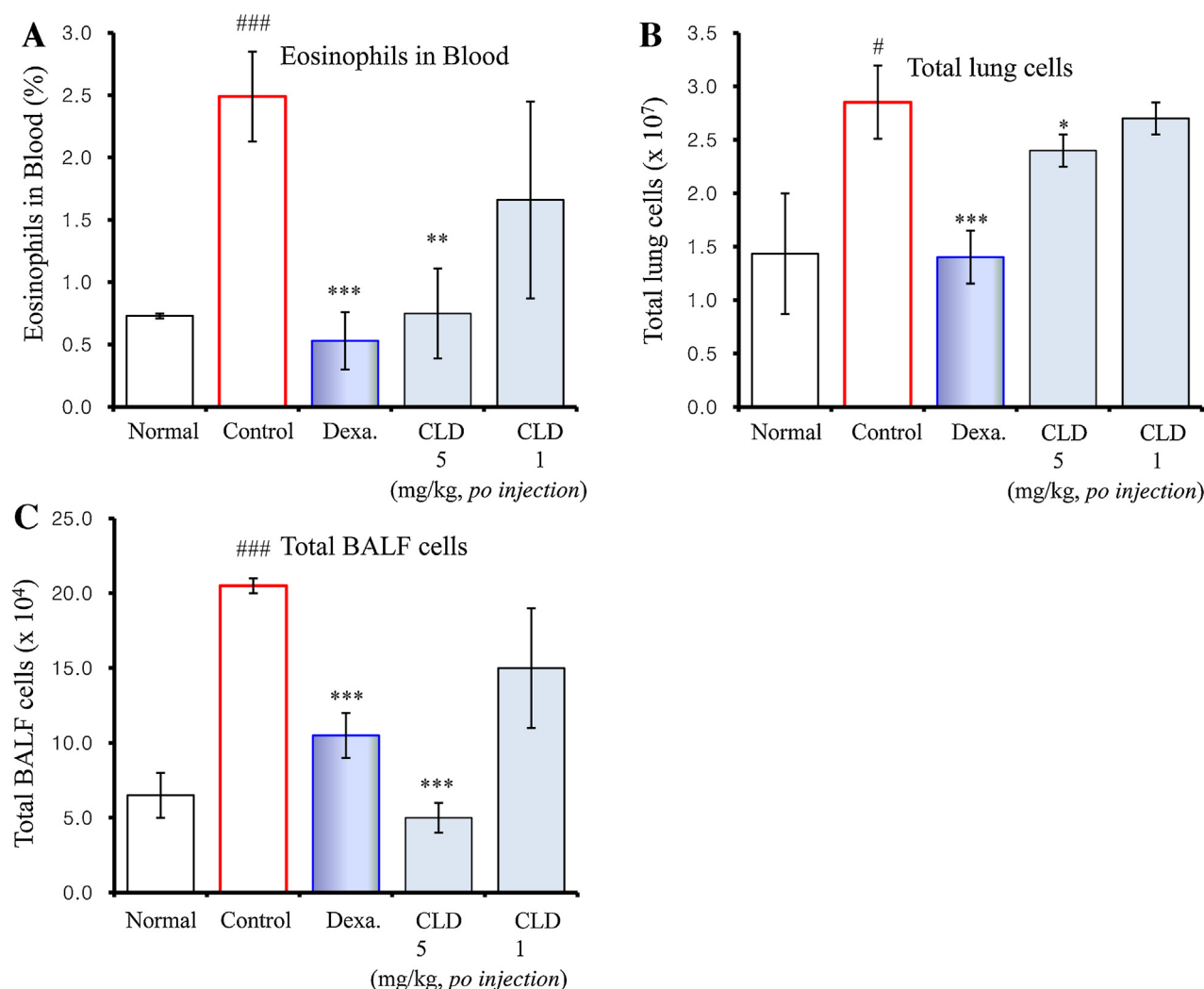


Fig. 3. The effects of chelidonine on total lung cells, total BALF cells, and eosinophils in blood. The BALF and lungs were harvested one day after the last OVA challenge. Total inflammatory cell numbers in the BALF and lungs were counted. Results are expressed as means $\pm$ SEM ($N = 6$). $^{\#}p < 0.05$, $^{###}p < 0.001$ for the normal group versus the control group comparison, and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the control group versus the experimental groups comparisons.

and BALF of control group were higher than those in normal group. The eosinophil numbers may constitute a substantial portion of the $CD11b^{+}/Gr-1^{+}$ population [16]. Chelidonine and dexamethasone both affected inflammatory leukocyte subsets in lungs and BALF as shown by the changes in the numbers of $CD3^{-}/CCR3^{+}$ and $Gr-1^{+}/CD11b^{+}$ granulocytes. There were deficits in $Gr-1^{+}/CD11b^{+}$ granulocytes, accompanied by a decrease in the

number of eosinophils in blood, and total BALF cells (Fig. 3A–C and Table 1).

Inhibition of IL-17, eotaxin-2, IL-4, and IL-13 in BALF

The levels of IL-17, eotaxin-2, IL-4, and IL-13 were measured by ELISA. As shown in Fig. 4, IL-17, eotaxin-2, and IL-4 levels were

Table 1
Fluorescence-activated cell sorting analysis (FACS) of immune cell subtypes in lung and BALF. Cells from lung tissues and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN_3) for 10 min on ice, and analyzed by 2-color flow cytometry. Each point represents the mean $\pm$ SEM values for 6 mice. $^{\#}p < 0.05$, $^{###}p < 0.001$ for the normal group versus the control group comparison, and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the control group versus the experimental groups comparisons.

Cell phenotypes in lung, BALF	Ovalbumin-induced asthma mice (absolute no.)				
	Normal	Control	Dexa.	CLD (5 mg/kg)	CLD (1 mg/kg)
Lung					
$CD3^{+}/CD4^{+}$ ($\times 10^6$ cells)	2.50 ± 1.03	$7.15 \pm 0.90^{###}$	$1.36 \pm 0.18^{***}$	$4.35 \pm 0.24^{*}$	$5.33 \pm 0.05^{*}$
$CD3^{+}/CD8^{+}$ ($\times 10^6$ cells)	1.19 ± 0.75	$1.75 \pm 0.14^{\#}$	$0.45 \pm 0.03^{***}$	$1.25 \pm 0.13^{*}$	$1.13 \pm 0.16^{*}$
$Gr-1^{+}/CD11b^{+}$ ($\times 10^5$ cells)	1.08 ± 0.48	$8.21 \pm 0.38^{###}$	$1.93 \pm 0.30^{***}$	$3.87 \pm 0.22^{***}$	5.58 ± 1.43
$CD3^{-}/CCR3^{+}$ ($\times 10^5$ cells)	1.71 ± 0.71	$9.93 \pm 1.45^{###}$	$2.45 \pm 0.93^{**}$	$4.51 \pm 0.13^{**}$	8.00 ± 0.70
BALF					
$CD3^{+}/CD4^{+}$ ($\times 10^3$ cells)	0.39 ± 0.13	$44.21 \pm 5.99^{###}$	$3.23 \pm 0.19^{***}$	$6.17 \pm 1.81^{***}$	24.66 ± 12.78
$Gr-1^{+}/CD11b^{+}$ ($\times 10^4$ cells)	0.20 ± 0.08	$4.41 \pm 0.13^{###}$	$1.35 \pm 0.14^{***}$	$0.69 \pm 0.13^{***}$	$2.66 \pm 0.82^{*}$
$CD3^{-}/CCR3^{+}$ ($\times 10^3$ cells)	4.50 ± 0.55	$66.02 \pm 2.18^{###}$	$10.49 \pm 4.39^{***}$	$10.80 \pm 4.08^{***}$	51.65 ± 18.65

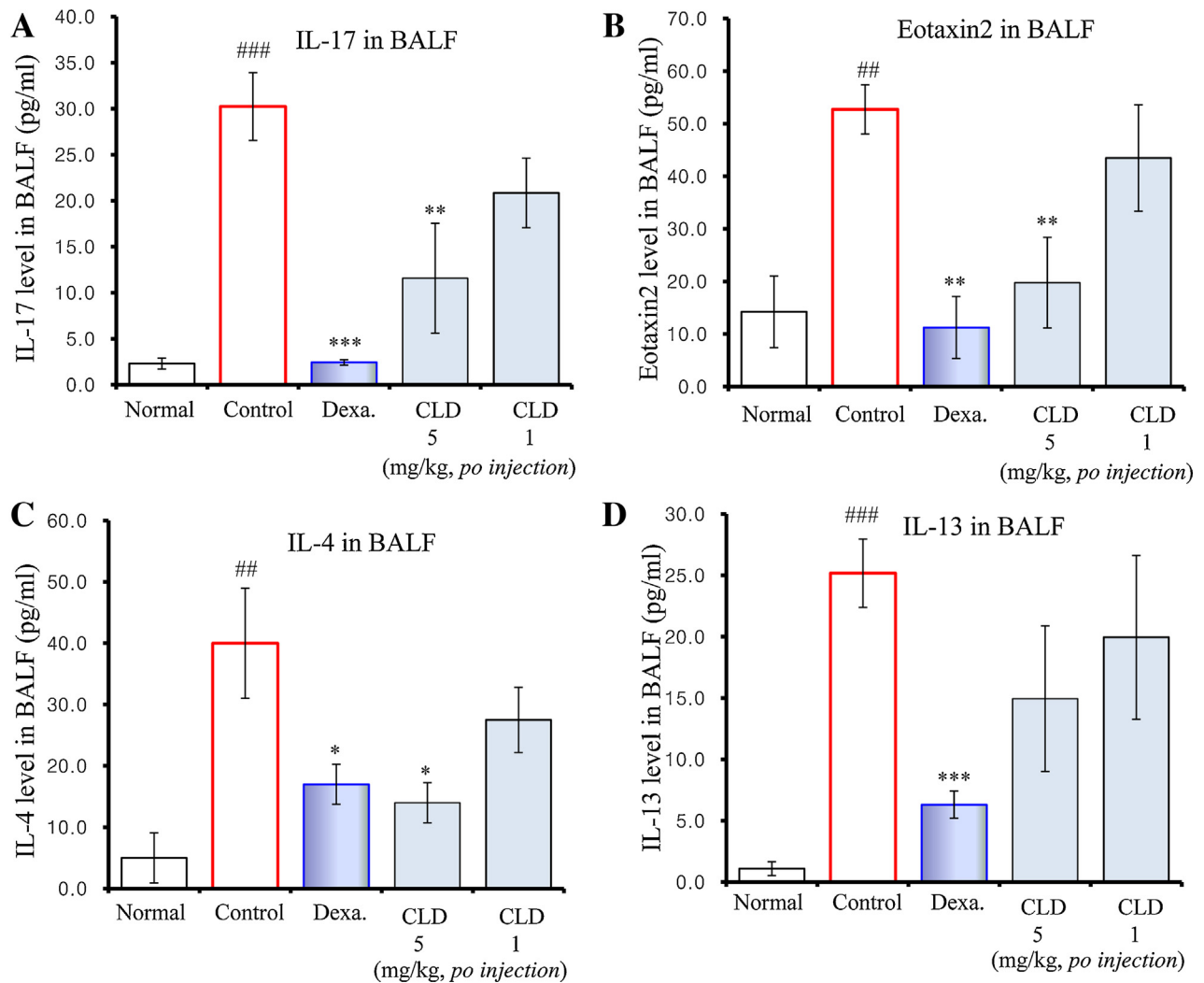


Fig. 4. Effect of chelidonine on IL-17, eotaxin-2, and Th2 cytokines (IL-4 and IL-13) in BALF. Interleukins (IL-4, IL-13, IL-17, etc.), eotaxin-2 in BALF ($N = 6$) were measured by ELISA using a monoclonal antibody-based mouse interleukin ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). $^{##}p < 0.05$, $^{###}p < 0.001$ for the normal group versus the control group comparison, and $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ for the control group versus the experimental groups comparisons.

significantly reduced in chelidonine-treated (5 mg/kg) and dexamethasone-treated mice. We also determined eotaxin-1, IFN- γ , and IL-5 levels in BALF, but no differences in their levels were observed between these groups (data not shown).

Effect of chelidonine on OVA-specific IgE production in BALF

OVA-challenged mice significantly increased the levels of OVA-specific IgE. The treatment with chelidonine significantly inhibited the increases in the levels of OVA-specific IgE at a dose of 5 mg/mL, whereas chelidonine at a low dose did not significantly inhibit the level of OVA-specific IgE (Fig. 5). We also determined OVA-specific IgE levels in serum, but no differences were observed between the groups (data not shown).

Chelidonine attenuates IL-17, ROR γ t, and eotaxin-2 mRNA levels in lung tissue

As shown in Fig. 6, the mRNA levels of IL-17, ROR γ t, and eotaxin-2 were fewer in the chelidonine-treated group than in control group. These results were accompanied by the changes in cytokine levels IL-17, IL-4, and eotaxin-2 production in BALF (Fig. 4).

Effect of chelidonine on the expression of Foxp3 in CD4 $^{+}$ T cells

TGF- β induces Foxp3 expression, which is further synergized with anti-CD28 and anti-CD3. To determine whether chelidonine can also express Foxp3 in response to these factors, we investigated the relative proportion of Foxp3 positive cells in the isolated CD4 $^{+}$ cells after treatment with or without anti-CD28 and anti-CD3 antibodies and TGF- β . As shown in Fig. 7, the levels of CD4 $^{+}$ /Foxp3 $^{+}$ double-positive cells (11.8%) in CD4 $^{+}$ T cells were significantly increased compared to that of only CD4 $^{+}$ T cells (2.08%). Following treatment with 20 or 10 μ g/mL chelidonine, the numbers of CD4 $^{+}$ /Foxp3 $^{+}$ T cells were increased to 12.7% and 14.1%, respectively.

Effect of chelidonine on mRNA expression of cytokines in vitro

To determine a possible effect of chelidonine on T cell responses, we evaluated the effects of chelidonine on IL-17A and IL-17F mRNA expression in PMA and OP-activated EL4T cells and IL-4, IL-13, IL-17A, and eotaxin-2 mRNA in ConA-activated splenocyte cultures *in vitro*. qRT-PCR analysis revealed that expression of IL-17A and eotaxin-2 mRNA significantly decreased in chelidonine-treated cells than control cells (Fig. 8). These results

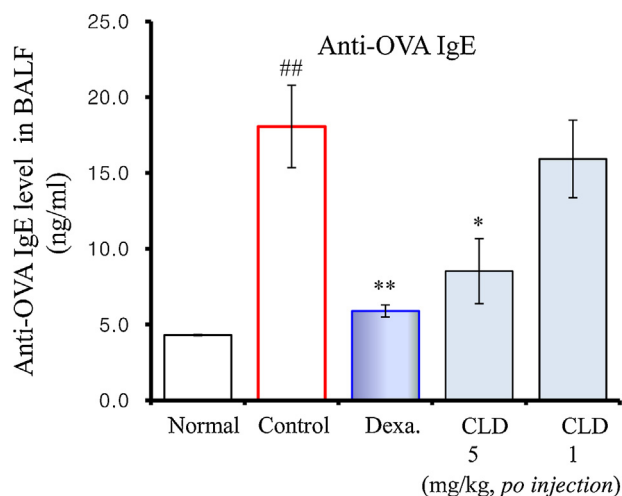


Fig. 5. Effects of chelidonine on the levels of OVA-specific IgE in BALF. OVA-specific IgE in BALF ($N = 6$) were measured by ELISA using a monoclonal antibody-based mouse interleukin ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). ## $p < 0.01$ for the normal group versus the control group comparison, and * $p < 0.05$, ** $p < 0.01$ for the control group versus the experimental groups comparisons.

were accompanied by a decrease in IL-17A and eotaxin-2 mRNA expression in BALF and lung tissues (Figs. 4 and 6).

Effect of chelidonine on the protein levels of transcription factors in EL4T cells

T-bet, GATA3, ROR γ t, Foxp3, p-STAT6, and p65 protein expression were also assessed by Western blotting of the nuclear fractions of EL-4 T cells. Western blot analysis revealed that nuclear expression of T-bet and Foxp3 protein levels were increased by chelidonine (20 μ M) stimulation. Chelidonine inhibited the nuclear expression of pSTAT6 and p65; however, the levels of the GATA3 and ROR γ t proteins were difficult to determine (Fig. 9). Chelidonine did not show a significant effect on STAT3 expression (data not shown). In accordance with the increase of Foxp3 in intracellular staining analysis (Fig. 7), chelidonine increased the nuclear expression of T-bet and Foxp3 protein levels in EL-4 T cells.

Discussion

Allergic asthma is a chronic inflammatory disease characterized by infiltration of eosinophils and other cell types in airway and tissue (lung, trachea). In this study, both chelidonine and dexamethasone prevented the development of Penh, eosinophil

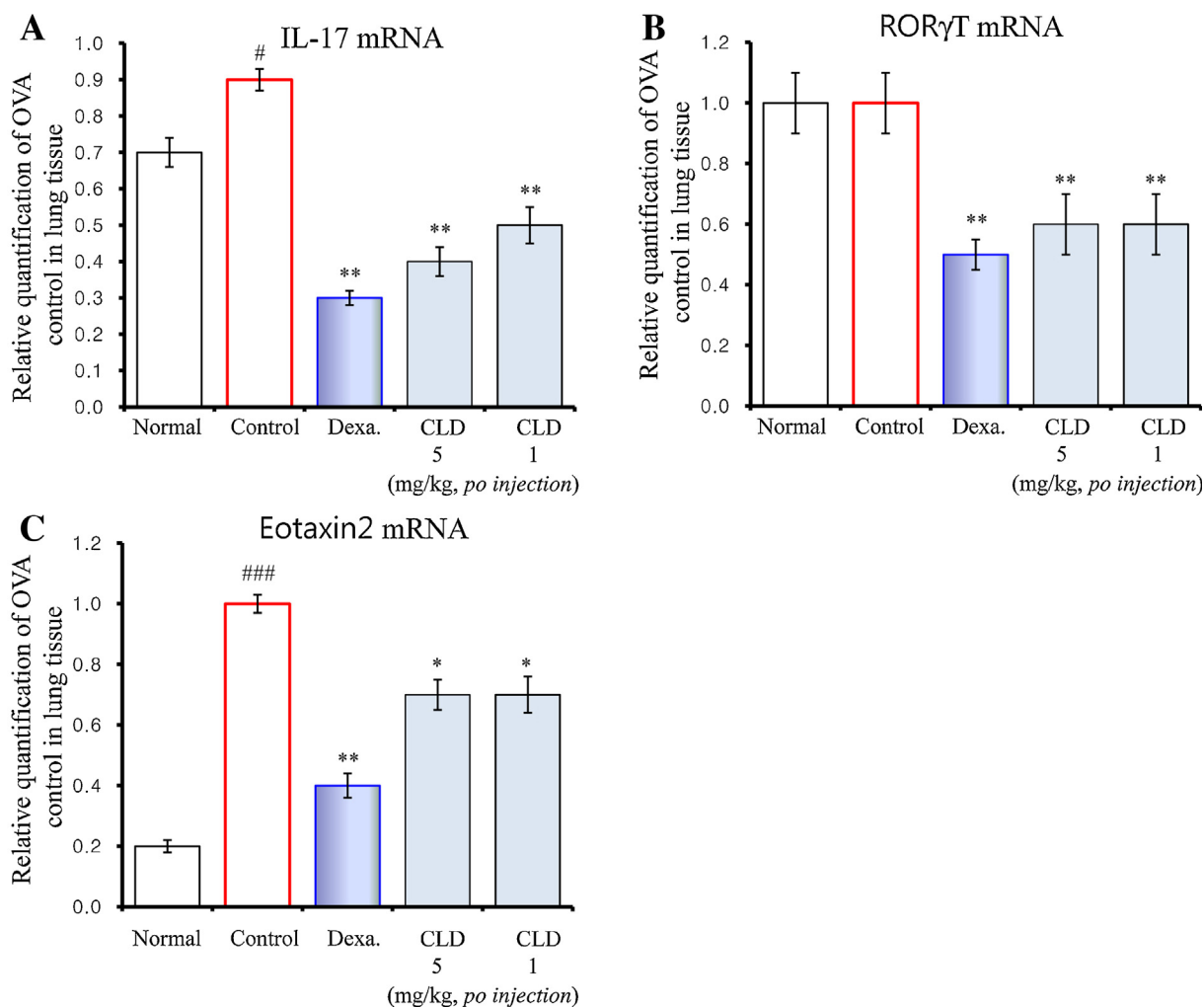


Fig. 6. Effects of chelidonine and dexamethasone on IL-17, ROR γ t, and eotaxin-2 mRNA expression in lung tissue of an OVA-induced murine model of asthma. Lungs were analyzed by qRT-PCR. The results are expressed as RQ to control. * $p < 0.05$, *** $p < 0.001$ for the normal group versus the control group comparison, and * $p < 0.05$, ** $p < 0.01$ for the control group versus the experimental groups comparisons.

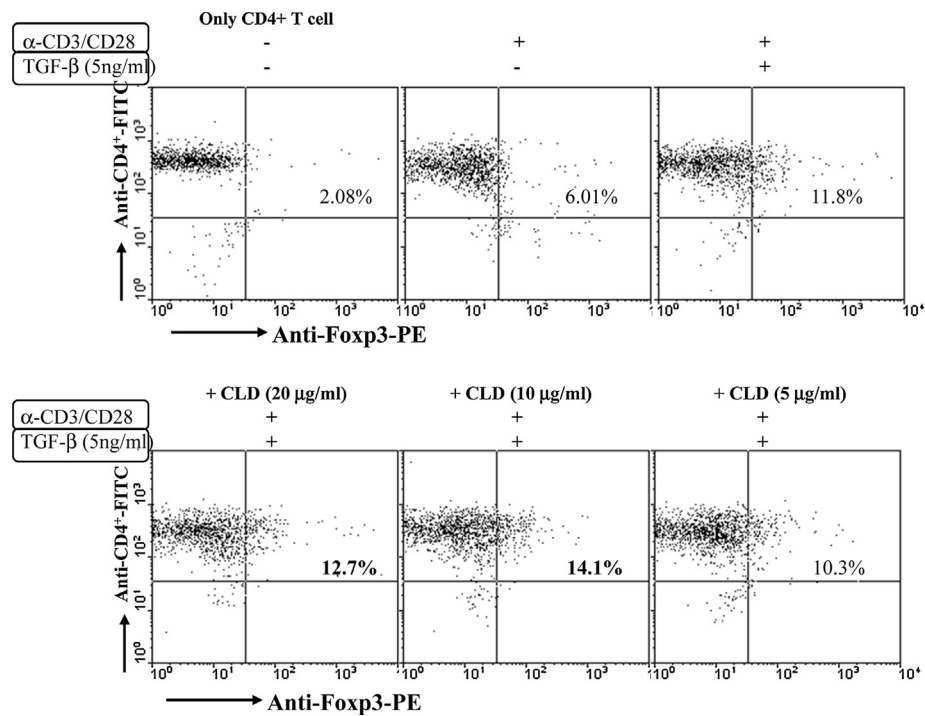


Fig. 7. Dot plot pattern of intracellular cytokine staining. X-axis: PE intensity of Foxp3. Y-axis: FITC intensity of CD4. Isolated CD4⁺ T cells were cultured without (normal group) or with (stimulated group) anti-CD3, anti-CD28, TGF- β , and CLD (20, 10, or 5 μ g/mL) added to RPMI medium and stained with PE-conjugated mAb to Foxp3, FITC-conjugated mAb to CD4. The intracellular stained cells were analyzed by the FACSCalibur device using CellQuest software.

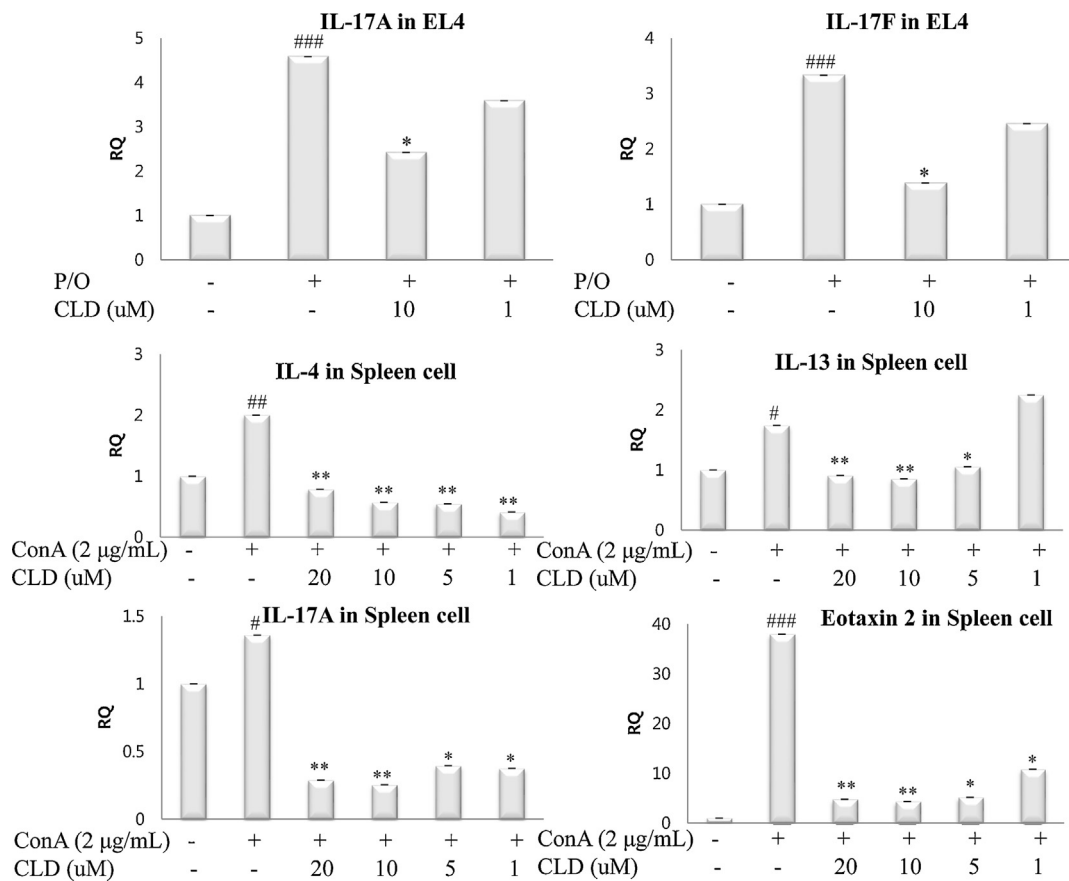


Fig. 8. Effects of chelidonine on PMA- and OP-induced mRNA expression of IL-17A and IL-17F in EL-4 T cells and ConA-induced mRNA expression of IL-4, IL-13, IL-17A, and eotaxin-2 in spleen. P/O indicates PMA, 1 ng/mL and OP, 5 μ M stimulation. The values are presented as the mean $\pm$ SEM ($N = 3$) for triplicate samples from each group. The results are expressed as RQ to normal. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ for the not treated group (P/O, ConA, and CLD) versus the control group (only P/O or ConA) comparison, and * $p < 0.05$, ** $p < 0.01$ for the control group versus the experimental groups comparisons.

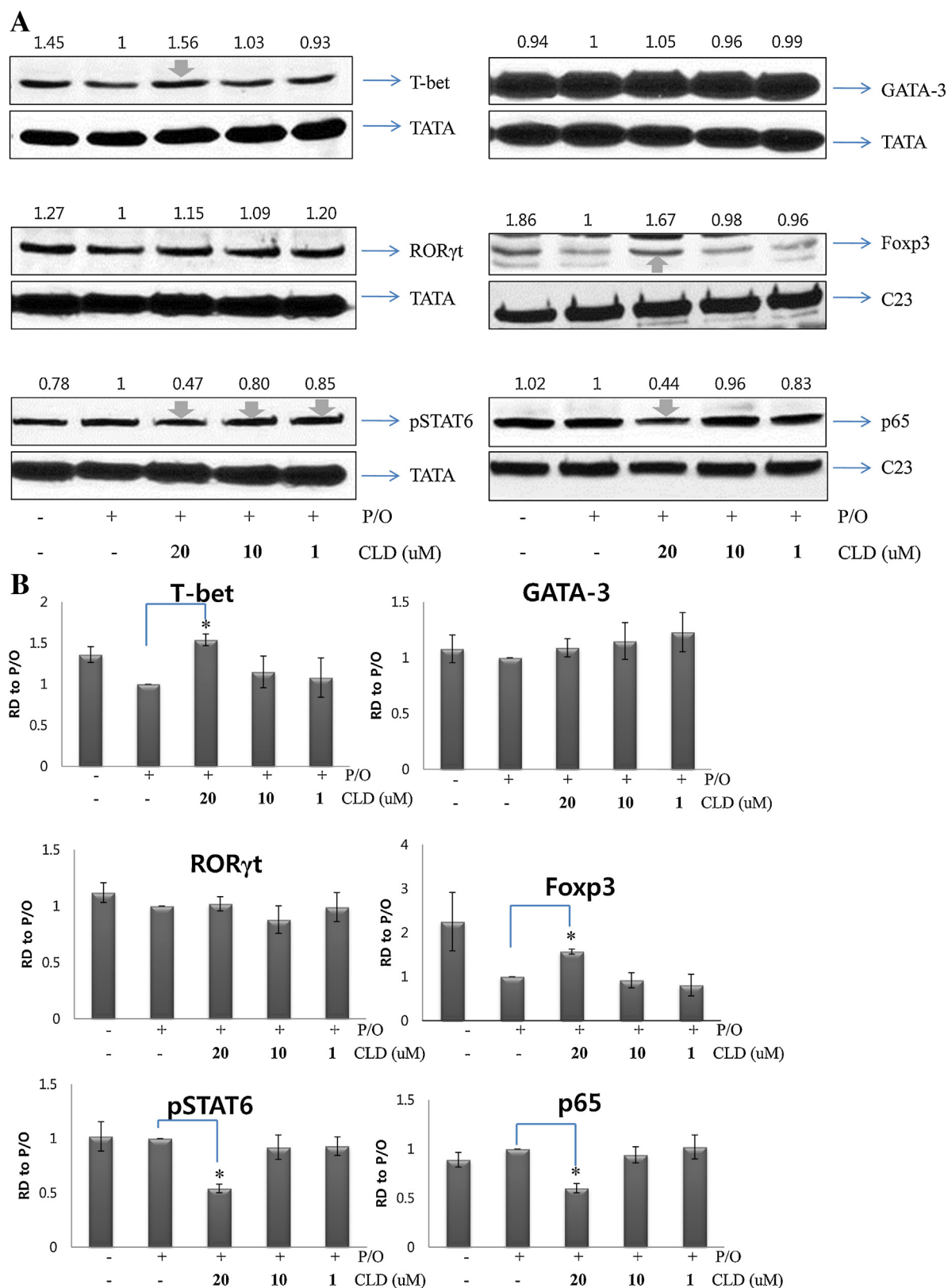


Fig. 9. Effect of chelidonine on the nuclear expression of T-bet, GATA3, RORγt, Foxp3, p-STAT6, and NF-κBp65 in EL4T cells by Western blot. The 60 μg of protein was loaded onto a gel electrophoresis. C23 or TATA were used as an internal control. The bands were scanned and a image of each protein was shown in (A) and every scanned images were analyzed density using Image J software (B). The density of each band was divided by the density of internal control, and we refer to this as the relative density (RD). The RD value in the cells stimulated by P/O without CLD treatment was set to 1.00 and the effect by CLD treatment was quantitated. P/O indicates the group that stimulated with PMA, 1 ng/mL and OP, 5 μM. The experiment was repeated at least three times independently. Data shows mean ± standard error. **p* < 0.05 for the control group versus the experimental groups comparisons.

infiltration, mucus production, lung inflammation, and a decrease in the Th2 cytokine levels IL-4, IL-17, and eotaxin-2 in BALF (Figs. 1–4 and Table 1).

Allergic airway inflammation have shown that CD8⁺ T cells can exacerbate inflammation by secreting Th2 cytokines [17]. The CCR3 receptor seems to be involved in the degranulation of eosinophils [18]. Th2 cells express CCR3 chemokine receptors, and eosinophils express specific mRNA for CCR3. Our results showed significant decrease of CD4⁺ and CD8⁺ T cells, Gr-1⁺/CD11b⁺ and CD3⁺/CCR3⁺ positive cells in chelidonine (5 mg/kg) and dexamethasone treated groups (Table 1). These results were accompanied by a concurrent reduction in the eosinophil numbers in PBMC and BALF cells (Figs. 2A and 3A).

Foxp3 functions as a transcriptional repressor for various transcription factors such as the nuclear factor of activated T cells (NFAT), NF- κ B [19]. CD4⁺Foxp3⁺ Treg cells play an important role in the regulation of asthma and the induction of Tregs has become one potential strategy for asthma therapy (Fig. 7).

As shown in Fig. 6, chelidonine did not suppressed ROR γ t expression in lung tissues. However, there is no significant increase in lung ROR γ t, between normal and control mice (Fig. 6B), which indicates that ROR γ t likely do not play a significant role in the development of airway inflammation in this model. Induction of IL-17 mRNA is the mechanism underlying suppression of other transcription factor not the ROR γ t.

Because Foxp3 Treg cells suppress Th17 cells, it is possible that the therapeutic effect of chelidonine via inactivation of Th2 (IL-4) and Th17 cells is mediated by the Foxp3 pathways (Figs. 6–9). We also observed significant correlations between IL-17 and eotaxin-2 levels in BALF and lung tissues (Figs. 4, 6, and 8). IL-17 creates a synergy effect with IL-4 and IL-13 to enhance Th2 cytokines and CCL11 (eotaxin), and a separate study showed that IL-17 neutralization inhibits Th2 disease [20,21]. Eotaxin-2 mRNA levels in the mouse lung can be increased by administration of IL-4 via a STAT-6-dependent mechanism [22]. In our study, similar patterns of results was shown that chelidonine suppressed IL-4 and STAT-6 expression in BALF, EL-4, and spleen cells (Figs. 4, 8, and 9). The critical role of IL-4 and STAT6 pathway in IgE isotype switching in B cells is well established [23,24]. Here, Western blot analysis revealed that chelidonine (20 μ M) treatment inhibited nuclear expression of pSTAT6 and NF- κ Bp65 (Fig. 9). Chelidonine (5 mg/kg) and dexamethasone also inhibited B cell-dependent production of OVA-specific IgE in BALF (Fig. 5), and therefore we suggest that chelidonine suppressed OVA-specific IgE production through downregulation of the expression of STAT6 and NF- κ Bp65 (Figs. 5 and 9).

Our study shows that IL-4, eotaxin-2, STAT6, and Foxp3 are prominent therapeutic factors in allergic inflammation, while concomitantly verifying the activity of T-bet, NF- κ Bp65, and STAT6. Chelidonine may also ameliorate the imbalance of the Th1/Th2-associated transcription factors, T-bet, STAT6, and Foxp3 in asthmatic mice, followed to a lesser extent by the GATA-3 and ROR γ t.

The results demonstrated that chelidonine has profound inhibitory effects on airway inflammation, and that this effect is caused by suppression of IL-4, IL-17, eotaxin-2, and OVA-specific IgE production through the STAT6, and Foxp3 transcription pathways. This is the first report showing that chelidonine significantly inhibits airway inflammation, eosinophil infiltration, and hyperresponsiveness in a mouse model of allergic asthma. Therefore, we propose that chelidonine could be used to treat pathologic inflammatory airway disorders, including allergic asthma.

Completing interests

The authors have no conflict of interest to declare.

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