



# Oleanolic acid suppresses ovalbumin-induced airway inflammation and Th2-mediated allergic asthma by modulating the transcription factors T-bet, GATA-3, ROR $\gamma$ t and Foxp3 in asthmatic mice

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## ABSTRACT

The natural product oleanolic acid is commonly found in a variety of medicinal plants. It is a triterpenoid compound known for its anti-inflammatory effects as well as various other pharmacological properties. The aim of the current study was to use a mouse model of allergic asthma to investigate whether oleanolic acid has anti-asthmatic effects, and if so, to determine the mechanism of these effects. Oleanolic acid suppressed eosinophil infiltration, allergic airway inflammation, and Penh, which occurred by suppressing the production of IL-5, IL-13, IL-17, and ovalbumin-specific IgE through the upregulation of T-bet and Foxp3, and the downregulation of GATA-3 and ROR $\gamma$ t. The therapeutic effect of oleanolic acid was due to suppression of Th2 cytokines (IL-5 and IL-13), B cell-dependent production of OVA-specific IgE, and Gr-1 expression through the T-bet, GATA-3, retinoic acid-related orphan receptor  $\gamma$ t (ROR $\gamma$ t) and forkhead box p3 (Foxp3) transcription pathways. It is therefore reasonable to suggest that the anti-inflammatory and anti-asthmatic effects of oleanolic acid may be exerted through inhibition of the GATA-3 and ROR $\gamma$ t pathways.

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## 1. Introduction

Allergic asthma is a chronic inflammatory disease of the airway wall that leads to bronchial hyper-responsiveness (also known as airway hyper-responsiveness [AHR]) and airway obstruction caused by inflammation, mucus hyper-production, and airway wall remodelling [1]. Allergic asthma is associated with the Th2-type immune response because Th2 cytokines are known to induce many features of the disease [2]. Furthermore, airway eosinophilia has long been linked to allergic asthma, and Th2 cytokines such as IL-4, IL-5, and IL-13 have been shown to cause eosinophilic inflammation in the airway [3]. The immune response to inhaled allergens is characterized by an aberrant Th2 lymphocyte response that can produce the features of asthma through production of IL-4, IL-5, and IL-13 [4]. Activation of allergen-induced Th2 cells in the lungs leads to eosinophilic lung inflammation, oedema, goblet cell hyperplasia with increased mucus secretion, and recurring bronchospasm [5]. In particular, IL-4, IL-5, and IL-13 are all associated with inflammatory changes in the airway via the activation of eosinophils and the production of immunoglobulin E (IgE) by B cells [6]. These three Th2 cell-derived cytokines play critical roles in orchestrating and amplifying allergic inflammation in asthma.

The transcription factor T-bet drives Th1 differentiation, while the transcription factor GATA-3 drives Th2 differentiation [7]. Compared to its expression level in control subjects, T-bet production is downregulated in severely asthmatic patients with signs of airway remodelling [8]. T-bet may therefore be an attractive target for the development of anti-asthmatic drugs. GATA-3, on the other hand, has been shown to be an important factor in mediating allergic airway inflammation in vivo [9]. Furthermore, local targeting of GATA-3 expression in the lungs leads to suppression of established airway inflammation, AHR, and IL-4 production in experimental asthma [10]. Effective immunotherapeutic strategies would aim to either downregulate Th2 cytokines or to downregulate their receptors or the transcription factors that control their production. Naturally occurring, thymus-derived, CD4<sup>+</sup>, CD25<sup>+</sup>, and Foxp3<sup>+</sup> T regulatory cells (Tregs) and inducible Tregs are known to inhibit the development of allergy via several mechanisms. These mechanisms include suppression of other effector cells (Th1, Th2, and Th17), suppression of eosinophils, induction of antibody isotype change from IgE to IgG4, suppression of inflammatory dendritic cells (DC), and suppression of inflammatory cell migration to tissues [11].

The natural product oleanolic acid is a biologically active pentacyclic triterpenoid compound that has been isolated from various edible and medicinal plants [12]. Oleanolic acid exerts antioxidant effects through Nrf2-mediated increase in the expression of antioxidant enzymes such as catalase and thioredoxin peroxidase and enhanced biosynthesis of the antioxidant glutathione [13,14]. In addition, there are anticancer

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[15], hepatoprotective [13], anti-inflammatory [16,17], and anti-asthmatic effects of hydroxyl pentacyclic triterpene acids, including oleanolic acid from *Forsythia viridissima* [18].

Various reports have described the biological mechanisms of these effects. The anti-inflammatory effects of oleanolic acid most likely result from targeting NF- $\kappa$ B; however, the exact mechanism underlying this effect remains unknown [19]. Kawahara et al. reported an inhibitory effect of oleanolic acid on LPS-mediated inflammation and release of high mobility group box 1 (HMGB1) in RAW264.7 cells [20]. Oleanolic acid also downregulates the cell surface expression of the receptor of HMGB1, thereby inhibiting HMGB1-dependent pro-inflammatory responses by opposing the activation of NF- $\kappa$ B and production of tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) [21]. Due to its similar chemical formula, oleanolic acid acetate modulates atopic dermatitis and allergic contact dermatitis through the inhibition of Th1, Th2, and Th17 cytokine gene expression, and the downregulation of NF- $\kappa$ B and MAPKs [22].

Despite its known anti-inflammatory effects, the mechanisms of action and molecular functions of oleanolic acid are not yet fully elucidated. Moreover, there are no in vivo studies on the anti-asthmatic or the anti-inflammatory effects of oleanolic acid in a mouse model of allergic asthma.

The aim of this study was to use a mouse model of allergic asthma to evaluate the pharmacological activity of oleanolic acid on Foxp3, GATA-3 and T-bet expression, as well as the production of Th1, Th2, and Th17 cytokines. We also examined its effects on the development of allergic airway inflammation, and Penh in the asthma model mice.

## 2. Materials and methods

### 2.1. Oleanolic acid and other chemicals

Oleanolic acid was purchased from Sigma (Sigma-Aldrich Korea, Korea). The purity of the oleanolic acid was determined to be above 97% by HPLC analysis (data not shown). HPLC-grade reagents, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). All other chemicals were of analytical grade.

### 2.2. Animals

Five-week-old female BALB/c mice were obtained from Daehan Biolink Co. LTD. (Eumsung, Republic of Korea). Our study was approved by the Committee for Animal Welfare at the Institution of Daejeon University. All animal procedures were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Republic of Korea).

### 2.3. Ovalbumin sensitization and inhalation

Ovalbumin (OVA) preparation and delivery were performed as previously described with modifications [23]. OVA (500  $\mu$ g/ml) in PBS was mixed with equal volumes of 10% (w/v) aluminium potassium sulphate (alum; Sigma) in distilled water, incubated for 60 min at RT after adjustment to pH 6.5 using 10 N NaOH, and centrifuged at 750  $\times$ g for 5 min. The OVA/alum pellet was resuspended to the original volume in distilled water. All mice were immunized on 3 different days (first day of the second, third, and fourth weeks before inhalational exposure) by intraperitoneal (i.p.) injections of 0.2 ml alum-precipitated antigen containing 100  $\mu$ g OVA (Sigma-Aldrich Korea, Korea) bound to 4 mg aluminium hydroxide (Sigma-Aldrich Korea) in PBS. Seven days after the second sensitization by intratracheal injection with 250  $\mu$ g OVA (on day 21) on the back of the tongue, mice were exposed to aerosolized OVA for 30 min/day, 3 days/week for 5 weeks (at a flow rate of 250 l/min, 1% OVA in normal saline for the first 4 weeks and 2% OVA in normal saline for the final week). Oleanolic acid (2 and 20 mg/kg, Sigma-Aldrich Korea) and cyclosporine A (CsA, 10 mg/kg)

were orally administered 3 times a week for the last 5 weeks. One day after the last OVA exposure (2% OVA inhalation), Penh was determined and samples (bronchoalveolar lavage fluid, lung cells, and serum) were collected for molecular analyses.

### 2.4. Bronchoalveolar lavage fluid (BALF)

Immediately following Penh assessment, mice were anaesthetized with an i.p. injection of 10% chloral hydrate (0.2 ml/20 g body weight) in PBS. The trachea was cannulated and BAL performed by washing the airway lamina. Briefly, cells in the lungs were recovered by flushing 1 ml BALF (1 mM EDTA, 10% foetal bovine serum [FBS], phosphate-buffered saline [PBS]) into the lungs via the trachea. Total cell counts were determined and 100  $\mu$ l of fluid was cytospun onto glass slides using a Cytospin centrifuge (Cellspin, Hanil, Korea) (400  $\times$ g, 4 min). Differential cell counts were performed after staining with a Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, Florida, USA). The BALF supernatant was stored at  $-25^{\circ}\text{C}$ .

### 2.5. Digestion of pulmonary tissue and cell preparation

Single cell suspensions from the lung tissues and BALF were isolated by mechanical disruption in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 50  $\mu$ M 2-mercaptoethanol, 20 mM HEPES, and 2% heat-inactivated FBS (GIBCO). Briefly, the lungs were removed from the thoracic cavity. After mincing using sterile scalpels, the tissue was incubated in PBS containing 1 mg/ml collagenase IV and 2 mg/ml dispase for 40 min at  $37^{\circ}\text{C}$  in a sterile polypropylene tube. After incubation, the lung tissue was vigorously pipetted up and down to dissolve remaining tissue clumps and then filtered using a 70- $\mu$ m cell-strainer (Falcon, Le Pont de Claix, France). Cells were counted manually using a hemocytometer chamber (Fisher). Between 2 and  $4 \times 10^3$  cells were spun onto glass slides (Cytospin centrifuge) (400  $\times$ g, 4 min). Differential counts were performed according to standard morphologic criteria.

### 2.6. Determination of enhanced pause (Penh)

Penh in mice was estimated using a previously described method with modifications [23]. A Buxco system (Biosystem XA; Buxco Electronics Inc., Troy, CT, USA) was used to evaluate the extent of airway constriction mice as described previously.

Penh is equal to  $\text{Pause} \times \text{PEF} / \text{PIF}$ , where  $\text{Pause} = (\text{Te} - \text{Tr}) / \text{Tr}$  (PIF, peak inspiratory flow; PEF: peak expiratory flow; Te, expiratory time; Tr, relaxation time). In this experiment, mice were aerosolized with OVA for 30 min/day, 3 days/week for 5 weeks. At 24 h after the final inhalation, mice were given aerosolized normal saline, followed by 3.125, 6.25, 12.5, 25, or 50 mg/ml methacholine (Sigma). Airway reactivity was then monitored for 30 min. Differences in Penh values between groups were evaluated using an unpaired Student's *t*-test.

### 2.7. Hematoxylin–eosin (H&E), Masson–trichrome (M–T), and Periodic acid–Schiff (PAS) staining

BALB/c mice were injected, inhaled, and sprayed with OVA for 5 weeks (3 times/week) to induce asthma. Two experimental groups were treated with different concentrations of oleanolic acid for the latter 5 weeks (5 times/week). At the end of the experiment, the lungs were removed and subjected to histological analysis using a modified protocol [23].

Briefly, the lung tissue was embedded in paraffin, cut into 3  $\mu$ m sections, stained with H&E solution and M–T solution. The tissue was subsequently mounted and covered with Dako-mounting medium (Dako Cytomation; Carpinteria, CA, USA) and a coverslip. The degree of airway inflammatory cell infiltration was scored using a double-blind screen by 2 independent observers. The degree of peribronchiolar

and perivascular inflammation was evaluated by a subjective scale of 0–2 as described previously, with modifications [23]. Periodic acid–Schiff (PAS) staining was performed to identify mucus secretion in the lung tissue. Frozen sections (30  $\mu$ m) of each tissue sample were prepared. Each sample section was mounted on a gelatin-coated slide, stained with PAS reagents, dehydrated, and covered with permount. PAS-positive goblet cells were counted manually and normalized to the length of the bronchial epithelial perimeter on the basal side, and expressed as the number of PAS-positive cells per mm of basement membrane.

## 2.8. Antibodies and flow cytometry

All antibodies (anti-CD3, CD4, CD8, CD69, CD11b, Gr-1, T-bet, and GATA-3) for flow cytometry were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA, USA). Cells from the lung tissues and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01%  $\text{NaN}_3$ ) for 10 min on ice, and analysed by 2-colour flow cytometry on a FACSCalibur device using CellQuest software (BD Biosciences, Mountain View, CA, USA).

## 2.9. Enzyme-linked immunosorbent assay (ELISA)

Interleukin (IL-5, IL-13, etc.) production in BALF and anti-OVA IgE levels in serum ( $N = 6$ ) 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). OVA-specific IL-5, IL-13, and IL-17 production was detected in spleen cells suspended in RPMI 1640 medium supplemented with 2 mM L-glutamine and 5% FBS. The spleen cells were cultured for 48 h at  $1 \times 10^5$  cells/well in 96-well culture plates (Corning, Cambridge, MA) with or without 1  $\mu$ g/ml OVA in a humidified atmosphere of 5%  $\text{CO}_2$  in air at 37 °C. The culture supernatants were collected and assayed by ELISA for OVA-induced IL-5, IL-13, and IL-17 antibodies. All data represent the mean and standard deviation from at least 3 separate experiments and were compared by analysis of variance (ANOVA).

## 2.10. Quantitative real-time PCR

To study the anti-asthmatic effects of oleanolic acid on cytokine gene expression in the lung tissue, quantitative real-time PCR was performed after quantitative normalization to  $\beta$ -actin. Briefly, total cellular RNA was extracted from the lungs by the phenol-chloroform method (RNAzol<sup>®</sup>; Tel-Test, USA) according to the manufacturer's instructions. cDNA was synthesized from 3  $\mu$ g of total RNA using a ReverTraAce-a-cDNA Synthesis kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Real-time quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, USA) using the following TaqMan probes.

The TaqMan probes for Foxp3 (Mm, 00475156, FAM dye-labelled, ABI), IL-17 (Mm, 00439619, FAM dye-labelled, ABI) and GAPDH (pn, 4352339E, VIC dye-labelled, ABI) were selected using Assays-on-Demand Gene Expression Products (ABI). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ABI) gene expression was used as an endogenous control.

The following PCR parameters 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 amount of SYBR Green was measured at the end of each cycle. The cycle number at which the emission intensity of the sample rose above baseline was referred to as the RQ (relative quantity) and was proportional to the target concentration. Real-time PCR was performed in duplicate and analysed according to the Applied Biosystems 7500 Fast Real-Time PCR system manual (threshold: 0.05, baseline: 6–15 cycles). To generate standard curves for pro-inflammatory cytokine and  $\beta$ -actin, serially diluted cDNA (1/1–1/16) was prepared and real-time PCR was

performed as described. RQ evaluation by RT-PCR was determined and expressed for various samples.

## 2.11. Lung tissue preparation for immunofluorescence

For immunofluorescence staining, the animals were intraperitoneally anaesthetized with ketamine (40 mg/kg), xylazine (2 mg/kg), and acepromazine (0.4 mg/kg). The thorax was opened and transcardially perfused with 100 ml of saline, followed by 100 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The lungs were then carefully removed and fixed for 4 h in the same fixative at 4 °C before infiltration with 30% sucrose solution for 24–48 h at 4 °C until they sank. Each lung was sliced into small blocks. The blocks were then mounted in Tissue-Tek OCT compound (Sakura Finetech Co., Tokyo, Japan) and rapidly frozen. The blocks were stored at –80 °C until sliced into 5- $\mu$ m sections using a Cryostat Microtome (Thermo Electron Corp., Cambridge, UK).

## 2.12. Immunofluorescence and nucleus staining in lung tissues

Lung tissue staining was performed as previously described with some modifications [24,25]. Monoclonal antibodies against T-bet (Santa Cruz Biotechnology, CA, USA), GATA-3 (Santa Cruz Biotechnology, CA, USA), Foxp3 (Abcam, Cambridge, UK), and ROR $\gamma$ t (BD Biosciences, Mountain View, CA, USA) were used to detect expression of these transcription factors. Briefly, the frozen blocks were randomly chosen and sliced (5  $\mu$ m) with a Cryostat Microtome (Thermo Electron Corp., Cambridge, UK). The frozen sections were then affixed to poly-L-lysine-coated slide glass. The slides were washed for 10 min in 0.1 M phosphate buffer (PB) at room temperature. Subsequently, the slides were washed for 10 min with two changes of 0.1 M phosphate buffered saline (PBS). The slides were then incubated in a permeabilization buffer of 0.5% Triton X-100, 0.2  $\mu$ g EDTA, and 1% normal donkey serum in PBS for 30 min. The slides were rinsed for 10 min twice in phosphate buffered saline containing 0.1% Tween 20 (PBS-T) and then blocked in 10% normal donkey serum in PBS-T for 1 h and 40 min to reduce non-specific staining. The slides were then incubated with the primary antibodies, diluted appropriately in a blocking solution, at room temperature overnight. The following primary antibodies were used: rabbit anti-mouse T-bet antibody (1:300, Santa Cruz Biotechnology, CA, USA), rabbit anti-mouse GATA-3 antibody (1:300, Santa Cruz Biotechnology, CA, USA), rabbit anti-mouse Foxp3 antibody (1:1000, Abcam, Cambridge, USA), and anti-mouse ROR $\gamma$ t antibody (1:50, BD Biosciences, USA). The slides were rinsed for 10 min with two changes of PBS-T, and incubated in fluorescein isothiocyanate-(FITC) conjugated donkey anti-rabbit IgG in the blocking solution for 1 h and 20 min. For nuclear staining, DAPI (4',6-diamidino-2-phenylindol) was used.

## 2.13. Image recording

Photomicrographic images were acquired on an Olympus BX-51 advanced fluorescence and DIC microscope (Olympus America Inc., USA) fitted with a CoolSNAP CCD camera (Photometrics, USA) using MetaVeu<sup>™</sup> Research Imaging Software (version 5.0, Molecular Devices Corp., USA). The images were imported into Adobe Photoshop CS5.1 Extended (version 12.1) and were adjusted for brightness and contrast to optimize photographic representation of images observed under the microscope. There was no further digital modification of the images.

## 2.14. RNA preparation and RT-PCR in EL-4T cells

EL-4T cells were plated at a density of  $5 \times 10^5$  cells/ml in 6-well plates, then stimulated with phorbol 12-myristate 13-acetate (1 ng/ml) and 4-tert-octylphenol (OP, 5  $\mu$ M) for 1 h, followed by incubation with oleanolic acid 6 h. Total cellular RNA was isolated using TRIzol reagent, according to the manufacturer's instructions. RNA was

reverse-transcribed into cDNA, and then PCR amplification of the cDNA was performed using RT-PCR PreMix (Bioneer Corporation, Korea). PCR analyses were performed to detect T-bet, GATA 3, IL-5, and IL-13 gene expression using  $\beta$ -actin as an internal standard. The sequences of PCR primers were as follows: IL-5, sense 5'-GAGCACAGTGGTGAAGAGAC-3', antisense 5'-ATGACAGGTTTTGGAATAGCATTT-3'; IL-13, sense 5'-TTCAAATGAGATTGTGGGAAAAT-3', antisense 5'-ACCGATACAGTACAGTACAGTA-3'; T-bet, sense 5'-AACCAGTATCCTGTTCCAGC-3', antisense 5'-TGTCGCCACTGGAAGGATAG-3', GATA-3, sense 5'-CTCCTTTTGGCTCTCCTTTTTC-3', antisense 5'-AAGAGATGAGGACTGGAGTG-3', and  $\beta$ -actin, sense 5'-TCATGAAGTGTGACGTTGACATCCGT-3', antisense 5'-CTAGAAGCATTTCGGTGCACGATG-3'. The PCR reactions were run for 35 cycles at 95 °C (30 s), 58 °C (30 s), 72 °C (30 s) in a PCR Thermal Cycler (DNA Engine Dyad, Bio Rad, Hercules, CA, USA). After the amplification, the reverse transcription-polymerase chain reaction (RT-PCR) products were separated in 1% (w/v) agarose gel and visualized by ethidium bromide staining and LED transillumination.

### 2.15. Immunoblotting analysis and detection of cytokines by ELISA

EL-4T cells were plated at a density of  $5 \times 10^5$  cells/ml in 6-well plates and then incubated for 12 h. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) and 4-tert-octyphenol (OP, 5  $\mu$ M) for 1 h. Oleanolic acid was added to each plate at concentrations of 0, 10, or 50  $\mu$ M. After 24 h of incubation, the culture supernatants were collected and stored at  $-80$  °C for measurement of cytokine levels. The amount of each cytokine in the supernatants was determined using ELISA kits (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Cytoplasmic and nuclear extracts were separated using a Nuclear/Cytosol Fractionation Kit (BioVision Incorporated, CA, USA) according to the manufacturer's instructions. The nuclear lysates were separated using 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and the electrophoresed proteins were transferred to nitrocellulose membranes. Nonspecific binding was inhibited by exposing the membranes to TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 5% fat-free milk for 1 h at room temperature. The membranes were incubated overnight with specific antibodies against T-bet, GATA-3 and C23 (Santa Cruz Biotechnology, CA, USA) at 4 °C. Immunoreactive bands were detected by incubating the membranes with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence reagents. The bands were scanned and analysed using Image J software.

### 2.16. Isolation CD4<sup>+</sup> T cells

As previously described [23], splenocytes were isolated from naive BALB/c mice. Cells were enriched for CD4<sup>+</sup> cell populations by staining with anti-CD4 (BD PharMingen, California, USA). CD25-cells were isolated from this population by staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD25 mAb (BD PharMingen) followed by incubation with magnetic-activated cell sorting anti-FITC beads (Miltenyi Biotec, California, USA). CD4<sup>+</sup> T cells were selected on a (CS) column, and the flow-through was collected as CD4<sup>+</sup> T cells. Isolated cells were activated by overnight incubation on 24-well plates coated with 1  $\mu$ g/ml anti-CD3 (Serotec), 1  $\mu$ g/ml anti-CD28 (Serotec), and oleanolic acid (25, 12.5  $\mu$ M) added to RPMI medium supplemented with 1 unit/ml penicillin, 1  $\mu$ g/ml streptomycin, 20 ml l-glutamine, 50 mM-mercaptoethanol, and 5% FBS for 48 h after stimulation.

### 2.17. Intracellular staining for T-bet and GATA-3 in isolated CD4<sup>+</sup> T cells

For T-bet (FITC mouse anti-T-bet; BD PharMingen, USA) and GATA-3 (PE mouse anti-GATA3; BD PharMingen, USA) intracellular staining, isolated CD4<sup>+</sup> T cells were stimulated in culture medium containing rIL-4

(200 ng/ml; R&D Systems, Minneapolis, MN, USA), TGF- $\beta$  (10 ng/ml; R&D Systems, Minneapolis, MN, USA), and monensin (GolgiStop, 1 ml/ml, BD Biosciences, Minneapolis, MN, USA) in a cell incubator with 10% CO<sub>2</sub> at 37 °C for 4 days. After staining surface markers, the cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences, Minneapolis, MN, USA) according to the manufacturer's instructions.

### 2.18. Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA) or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (SPSS version 14.0 analysis software). The difference between the normal group and the control group (OVA plus vehicle) was evident, and statistical significance is not indicated in the figures and tables to emphasize the statistical differences between the experimental groups and the control group. Results (presented as mean  $\pm$  standard error of the mean) were considered statistically significant at *P* values <0.05 (\*), <0.01 (\*\*), or <0.001 (\*\*\*).

## 3. Results

### 3.1. Inhibitory effect of oleanolic acid and CsA on airway hyperresponsiveness (AHR)

AHR was assessed as the relative increase of Penh in response to increasing doses of methacholine. We measured Penh to determine whether treatment with oleanolic acid and CsA during allergen sensitization and allergen challenge influenced the physiologic outcome of OVA-induced airway inflammation. In OVA-sensitized and challenged mice, the dose-response curve of Penh was increased compared with that of control mice (Fig. 1B). Both oleanolic acid and CsA treatments were equally efficient at reducing the Penh response to methacholine, as determined by whole-body plethysmography (Fig. 1B).

As shown in Fig. 1B, compared to that in animals sensitized with OVA (Control group), Penh conversion to methacholine was less in oleanolic acid-treated mice (2, 20 mg/kg, *p* < 0.01) and CsA-treated mice (10 mg/kg, *p* < 0.01). These results indicate that oleanolic acid treatment reduces OVA-induced AHR.

### 3.2. Histological analysis of lung sections

Histological analyses of the mouse lung tissue revealed that the OVA group developed inflammatory changes, including peribronchial and perivascular infiltrates composed of numerous inflammatory cells, including eosinophils, neutrophils, and lymphocytes (Fig. 1C). However, both the oleanolic acid-treated group and the CsA-treated group showed marked reductions in the infiltration of inflammatory cells in the peribronchiolar and perivascular regions.

As shown in Fig. 1C, the magnitude of peribronchiolar and perivascular inflammation in mice treated with oleanolic acid was significantly lower in comparison to that in the OVA group. We also observed many histological changes in the resulting fibrotic tissue. Eosinophil infiltration was mainly observed in the peribronchial regions of the lungs. In contrast, histological sections from oleanolic acid- and CsA-treated mice indicated reduced airway inflammation in the lung tissue (Fig. 1C).

To determine the level of mucus secretion in the airway, PAS-positive and PAS-negative epithelial cells in individual bronchioles were analysed. Goblet cell hyperplasia and mucus hyperproduction were evaluated by PAS staining and quantification of PAS-positive cells. The mean numbers of PAS-positive cells were significantly higher in OVA-challenged control mice than in saline-challenged normal mice. Administration of oleanolic acid (2 or 20 mg/kg) to mice repetitively challenged with OVA resulted in significantly lower relative number of PAS-stained goblet cells, as well as PAS staining in airway epithelium,

than that in untreated mice (Fig. 1C). The increased total lung inflammation, cell infiltration, and mucus secretion were significantly reduced following administration of oleanolic acid. These results suggest that oleanolic acid inhibits antigen-induced inflammation in the lungs, including the influx of eosinophils and mucus production.

### 3.3. Inhibitory effect of oleanolic acid and CsA on airway eosinophil accumulation and influx of inflammatory cells into the lungs and BALF

The total number of lung cells obtained from the PBS-challenged group was  $1.13 \pm 0.25 \times 10^6$ , and few eosinophils were detected in this group. On the other hand, the total number of lung cells ( $6.65 \pm 0.85 \times 10^6$ ) and BALF cells obtained from the OVA-challenged mice were significantly higher than that obtained from the PBS-challenged group.

The total number of lung cells was significantly lower in the oleanolic acid-treated (2 or 20 mg/kg) and CsA-treated (10 mg/kg)

mice than that in control mice, and oleanolic acid (2 or 20 mg/kg) reduced the absolute number of eosinophils in BALF, as well as the number of neutrophils and eosinophils in PBMC (Fig. 2A). Compared with that in the PBS-challenged control group ( $2.0 \pm 0.55 \times 10^6$  cells), the total cell numbers in the BAL fluids were significantly higher after the last OVA challenge (Fig. 2C). OVA sensitization and challenge induced marked increase in total cell counts within the BALF ( $69.0 \pm 15.00 \times 10^4$  cells). However, in the oleanolic acid-treated group ( $33.5 \pm 2.50 \times 10^4$  cells), these numbers were significantly higher than those in the OVA-exposed group. This increase in the total cell counts was also associated with reduction in lung eosinophilia.

### 3.4. Effect of oleanolic acid and CsA on the absolute number of immune cell subtypes in murine OVA-induced asthma lung and BALF

To evaluate the effect of oleanolic acid and CsA on the number of immune cell subtypes, the lungs and BALF cells were stained with specific

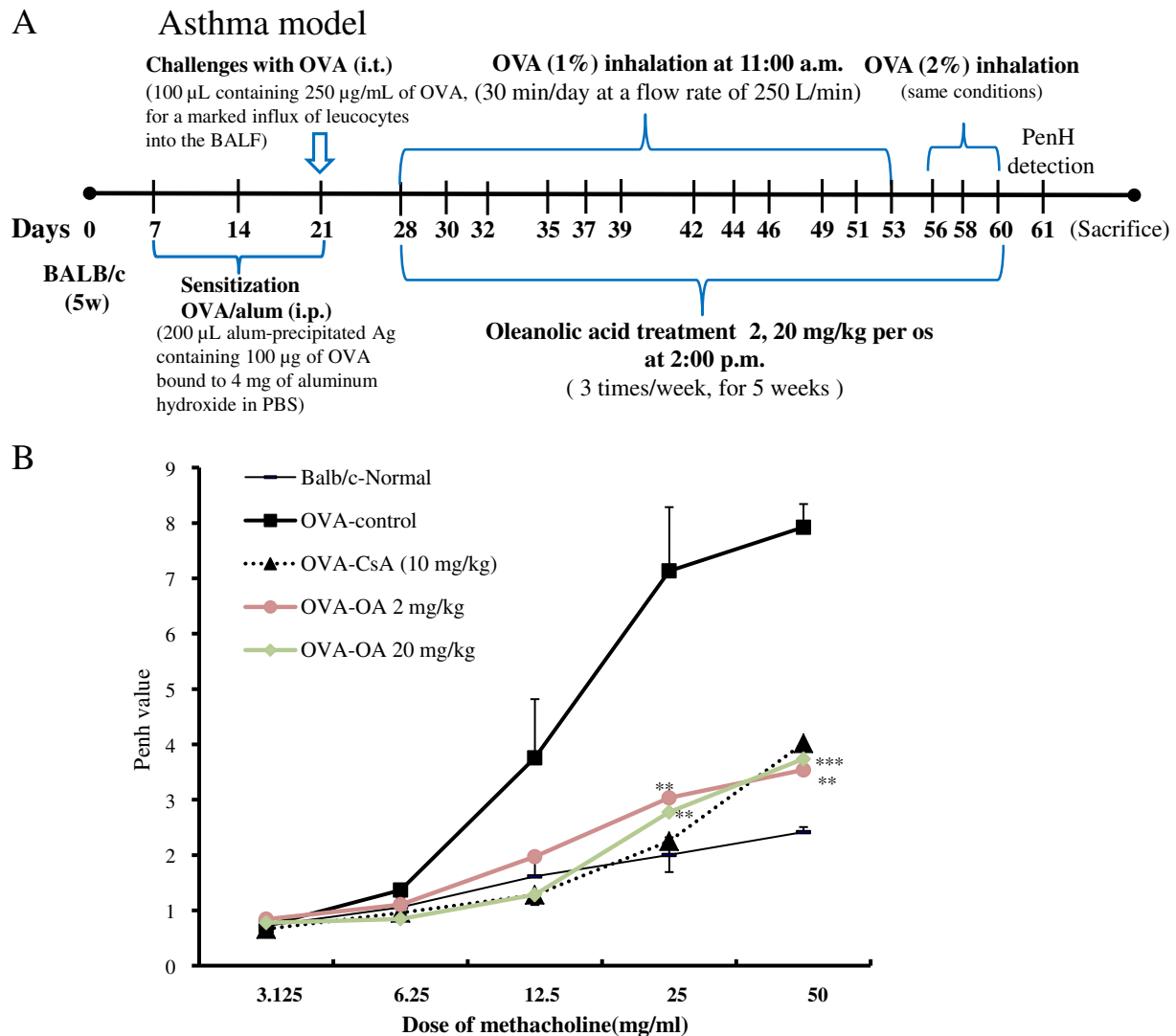


**Fig. 1.** Effect of oleanolic acid on airway hyperresponsiveness in OVA-challenged mice (A & B) and on histologic markers of airway inflammation (H&E, M-T, and PAS staining) in the lung tissue in the OVA-induced mouse model of allergic asthma. Mice were sensitized and challenged by OVA. Airway responsiveness to aerosolized methacholine was measured with a Buxco box. Mice were placed in the main chamber and nebulized first with PBS, then with increasing doses (3.125 to 50 mg/ml) of methacholine for 3 min per nebulization. Data represent the means  $\pm$  SEM of 5 independent experiments. \*\* $p < 0.01$  and \*\*\* $p < 0.01$  for control group vs. oleanolic acid-treated group comparisons (B). At the end of the experiment, the lungs were fixed and histologic analysis was performed. The lung sections of normal and asthmatic mice treated with OVA (control), oleanolic acid (2 mg/kg), or oleanolic acid (20 mg/kg) (C). H&E: Hematoxylin–eosin stain, M–T: Masson trichrome stain, PAS: Periodic acid–Schiff Stain, N: Normal BALB/c mice, CT (control): OVA inhalation + vehicle, OVA + oleanolic acid (2 or 20 mg/kg).

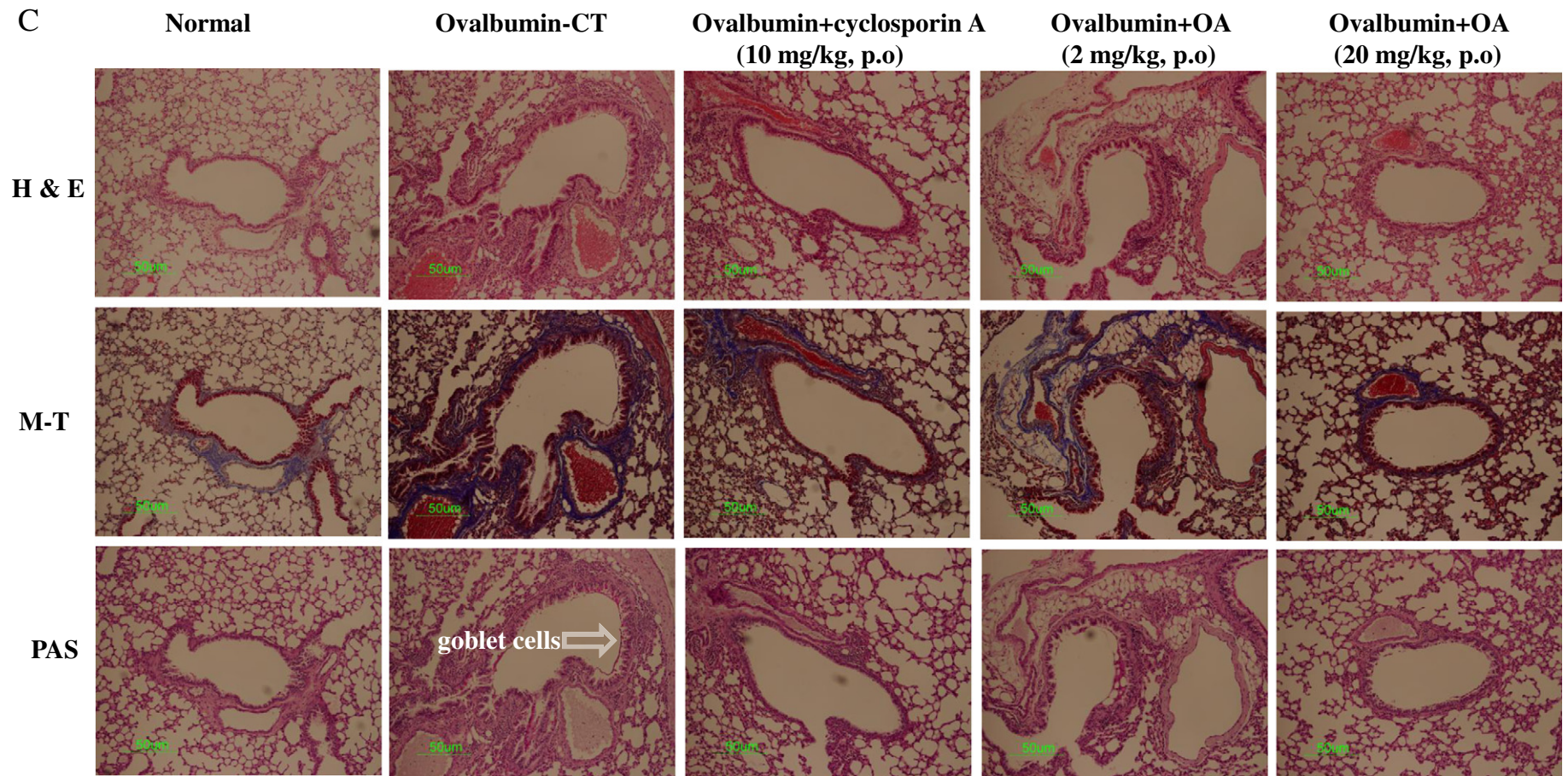


Fig. 1 (continued).

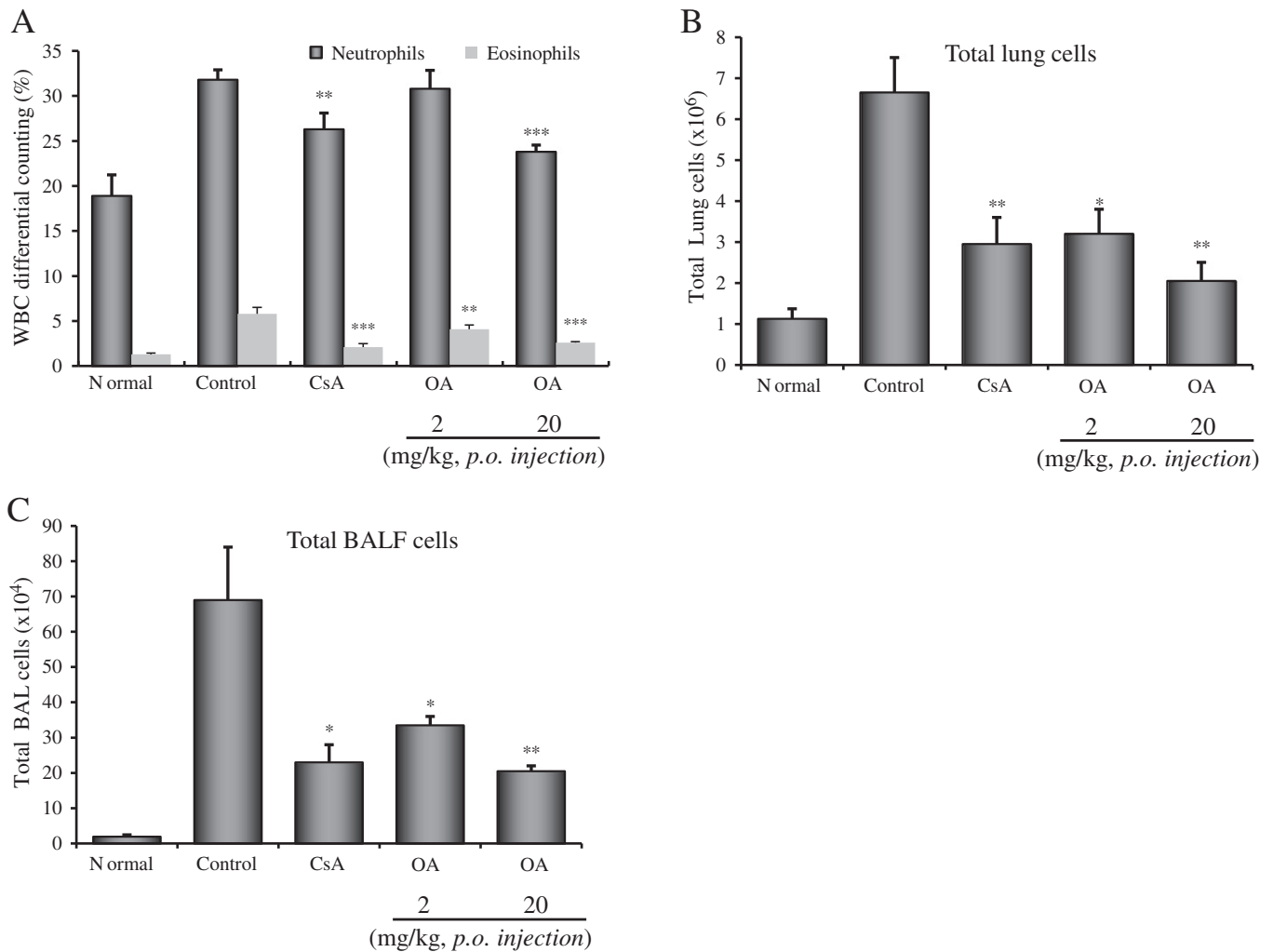


**Fig. 2.** The effects of oleanolic acid on total lung cells, total BALF cells, and eosinophils in PBMC. The lungs and BALF were harvested 24 h after the last OVA challenge. Total inflammatory cell numbers in the lungs and BALF were counted. Results are expressed as means  $\pm$  SEM (N = 6). Statistical significance between control and treatment groups was assessed by ANOVA or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (\**p* < 0.05, \*\**p* < 0.01, and \*\*\**p* < 0.001). N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA + CsA (10 mg/kg), OVA + oleanolic acid (2 or 20 mg/kg).

antibodies against CD3, CD4, CD8, CD69, CD11b, and Gr-1 and were subsequently analysed by flow cytometry. The numbers of CD3<sup>+</sup>/CD4<sup>+</sup>, CD3<sup>+</sup>/CD8<sup>+</sup>, CD11b<sup>+</sup>/Gr-1<sup>+</sup>, and CD3<sup>+</sup>/CD69 double-positive cells in the lungs and BALF of OVA-challenged mice were higher than that in the saline-treated group. In general, the cell count values were significantly lower in oleanolic acid- and CsA-treated mice than in OVA-challenged mice (Table 1).

Oleanolic acid and CsA both affected inflammatory leukocyte subsets in the lungs and BALF, as shown by the changes in the numbers of Gr-1<sup>+</sup>/CD11b<sup>+</sup> granulocytes, CD3<sup>+</sup>/CD69 early-activated T cells, and CD3<sup>+</sup>/CD4<sup>+</sup> T cells in this mouse model of asthma. Furthermore, there were deficits in Gr-1<sup>+</sup>/CD11b<sup>+</sup> granulocytes, accompanied by decrease in the number of eosinophils in PBMC, total lung cells, and total BALF cells (Fig. 2A, B, C).

**Table 1**

FACS analysis of immune cell subtypes in the lungs and BALF.

Absolute numbers of immune cell subtypes in the lungs were counted (described in Section 2.8 of Materials and Methods). The lungs and BALF cells were stained with anti-CD3, -CD4, -CD8, -CD69, -CD11b, -Gr-1 mAb, etc. Each point represents the mean  $\pm$  SEM values for 6 mice. Statistical significance between control and treatment groups was assessed by ANOVA or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001).

| Cell phenotypes in the lungs, bronchoalveolar lavage fluid   |       | Normal BALB/c   | Ovalbumin-induced asthma mice (absolute no.) |                                      |                                      |                                      |
|--------------------------------------------------------------|-------|-----------------|----------------------------------------------|--------------------------------------|--------------------------------------|--------------------------------------|
|                                                              |       |                 | Control                                      | Cyclosporin A                        | OA (2 mg/kg)                         | OA (20 mg/kg)                        |
| CD3 <sup>+</sup> /CD4 <sup>+</sup> ( $\times 10^6$ cells)    | Lungs | 3.99 $\pm$ 0.84 | 16.50 $\pm$ 2.7                              | <b>7.44 <math>\pm</math> 2.35*</b>   | <b>9.61 <math>\pm</math> 0.80*</b>   | <b>7.19 <math>\pm</math> 0.84*</b>   |
| CD3 <sup>+</sup> /CD8 <sup>+</sup> ( $\times 10^6$ cells)    |       | 1.53 $\pm$ 0.31 | 6.41 $\pm$ 0.49                              | <b>2.79 <math>\pm</math> 0.56***</b> | <b>3.62 <math>\pm</math> 0.11***</b> | <b>2.29 <math>\pm</math> 0.36***</b> |
| Gr-1 <sup>+</sup> /CD11b <sup>+</sup> ( $\times 10^5$ cells) |       | 0.62 $\pm$ 0.15 | 13.48 $\pm$ 2.12                             | <b>1.79 <math>\pm</math> 0.04***</b> | <b>4.80 <math>\pm</math> 0.25**</b>  | <b>2.25 <math>\pm</math> 1.07**</b>  |
| CD3 <sup>+</sup> /CD69 <sup>+</sup> ( $\times 10^5$ cells)   |       | 0.47 $\pm$ 0.01 | 8.57 $\pm$ 2.60                              | <b>2.41 <math>\pm</math> 0.73*</b>   | 5.24 $\pm$ 0.38                      | <b>3.07 <math>\pm</math> 0.69*</b>   |
| CD3 <sup>+</sup> /CD4 <sup>+</sup> ( $\times 10^4$ cells)    |       | 0.04 $\pm$ 0.01 | 24.99 $\pm$ 7.01                             | <b>6.53 <math>\pm</math> 0.78*</b>   | 9.18 $\pm$ 2.52                      | <b>5.75 <math>\pm</math> 0.76*</b>   |
| CD3 <sup>+</sup> /CD8 <sup>+</sup> ( $\times 10^4$ cells)    | BALF  | 0.02 $\pm$ 0.00 | 2.88 $\pm$ 0.56                              | <b>0.80 <math>\pm</math> 0.08**</b>  | <b>1.30 <math>\pm</math> 0.04*</b>   | <b>0.82 <math>\pm</math> 0.36*</b>   |
| CD3 <sup>+</sup> /CD69 <sup>+</sup> ( $\times 10^4$ cells)   |       | 0.03 $\pm$ 0.01 | 28.39 $\pm$ 3.28                             | <b>3.49 <math>\pm</math> 0.49***</b> | <b>11.63 <math>\pm</math> 1.15**</b> | <b>6.14 <math>\pm</math> 0.92***</b> |

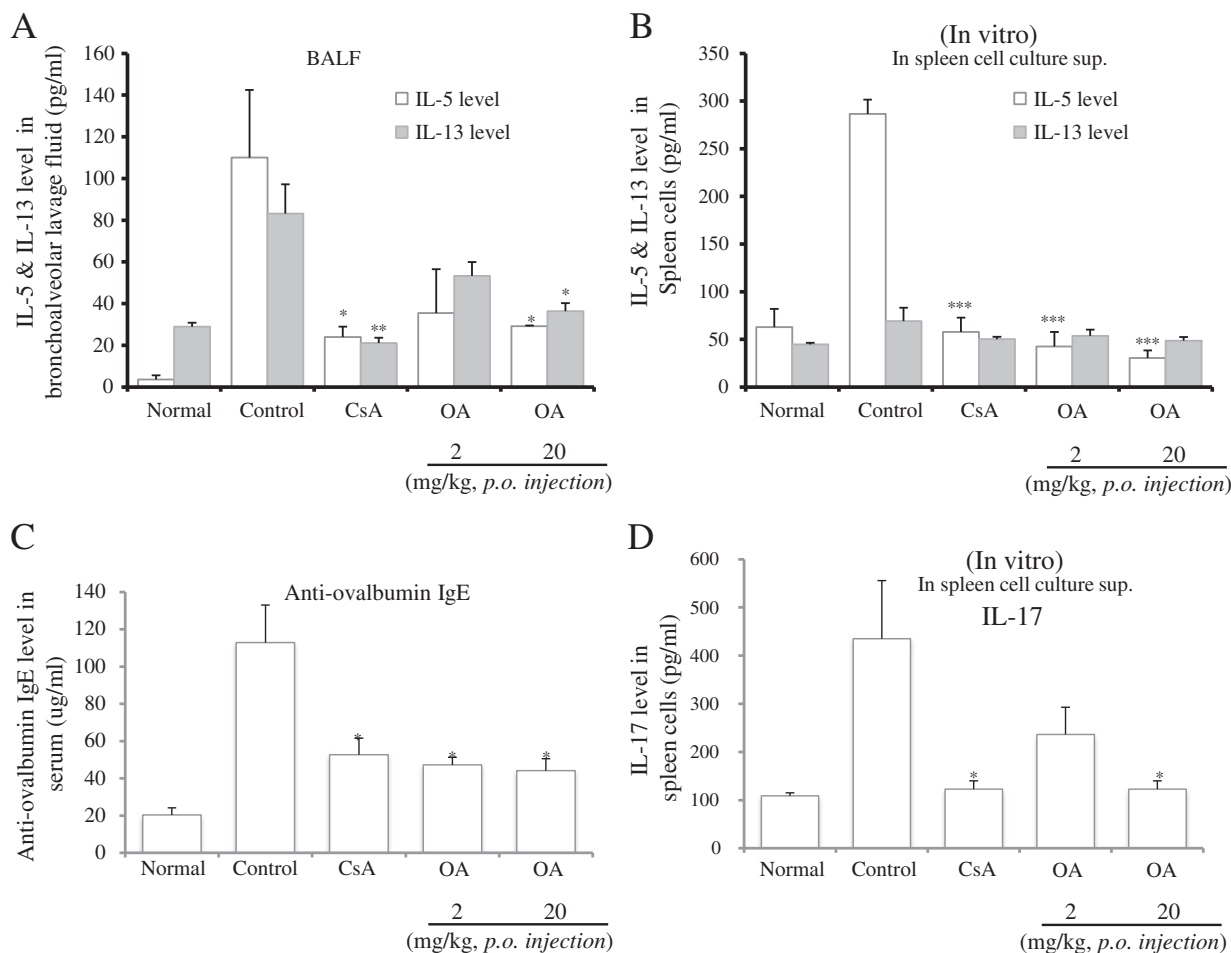


**Fig. 3.** Effect of oleanolic acid on Th2 cytokines (IL-5, IL-13) in BALF and OVA-specific IgE in serum and immunomodulatory effects of oleanolic acid on Th2 (IL-5, IL-13) and IL-17 cytokine production in spleen cells. Results are expressed means  $\pm$  SEM (N = 6). Statistical significance between control and treatment groups was assessed by ANOVA or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (\**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001). N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA + CsA (10 mg/kg), OVA + oleanolic acid (2 or 20 mg/kg).

### 3.5. Inhibition of Th2 cytokines (in vivo and in vitro) and OVA-specific IgE production in BALF and serum

To determine whether oleanolic acid and CsA influenced Th2 cytokine secretion in BALF, the levels of IL-4, IL-5, and IL-13 were measured by ELISA after the final challenge.

As shown in Fig. 3A and B, IL-5 and IL-13 levels were significantly reduced in oleanolic acid-treated (20 mg/kg) mice, CsA-treated (10 mg/kg) mice, and in spleen cell culture supernatants. An important characteristic of the allergic asthma model is the production of OVA-specific IgE. Therefore, levels of anti-OVA IgE in serum from the OVA-challenged mice and the PBS- and oleanolic acid-treated groups were measured.

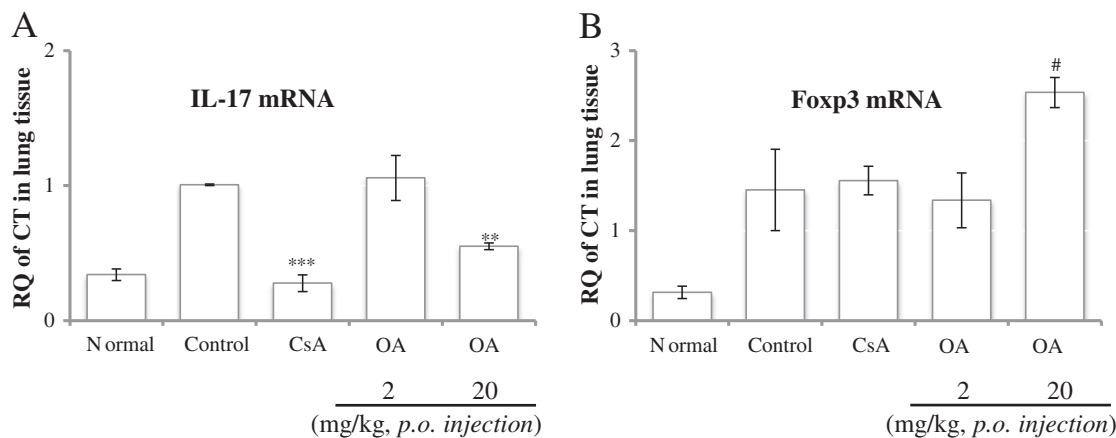


**Fig. 4.** Effects of oleanolic acid and CsA on IL-17, Foxp3 mRNA expression in the lung tissue of an OVA-induced murine model of asthma. BALB/c mice were administered OVA via injection, inhalation, or spraying for asthma induction, as described in Section 2.3 of the Materials and Methods. Three experimental groups were treated with different concentrations of oleanolic acid and CsA for 5 weeks (3 times a week). At the end of the experiment, the lungs were removed and analysed by real-time PCR. N Normal BALB/c mice, CT: OVA inhalation (control), OVA + CsA (10 mg/kg), OVA + oleanolic acid (2 or 20 mg/kg). The results are expressed as relative quantity (RQ).

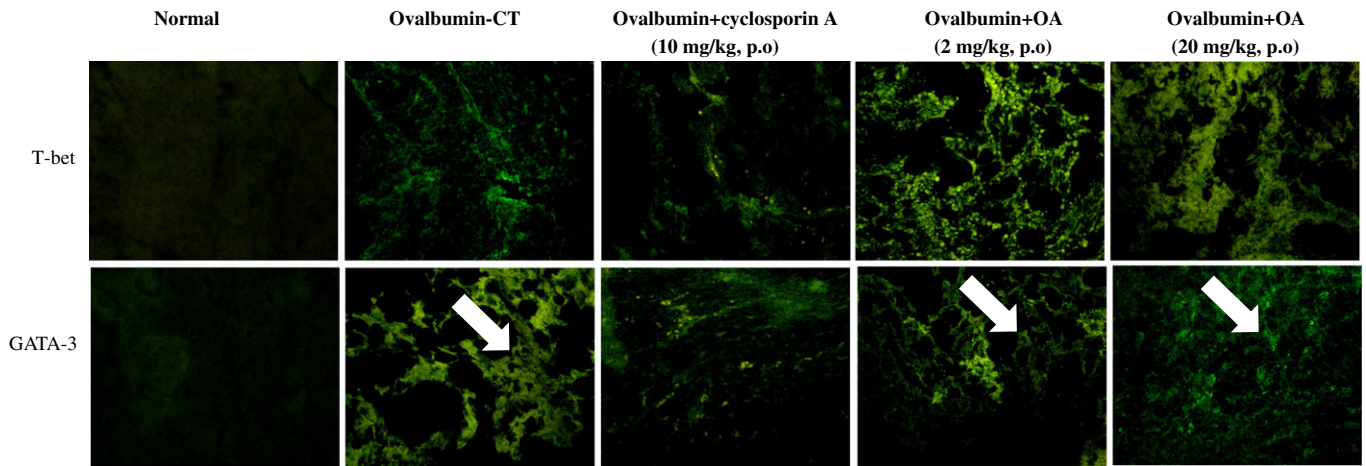


**Fig. 5.** The lung tissue immunofluorescence staining for T-bet and GATA-3 in vivo. Immunofluorescence staining of T-bet and GATA-3 in the lung tissues of mice with OVA-induced allergic asthma. N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA + CsA (10 mg/kg), OVA + oleanolic acid (2 or 20 mg/kg) (Immunofluorescence staining original magnification  $\times 200$ ).

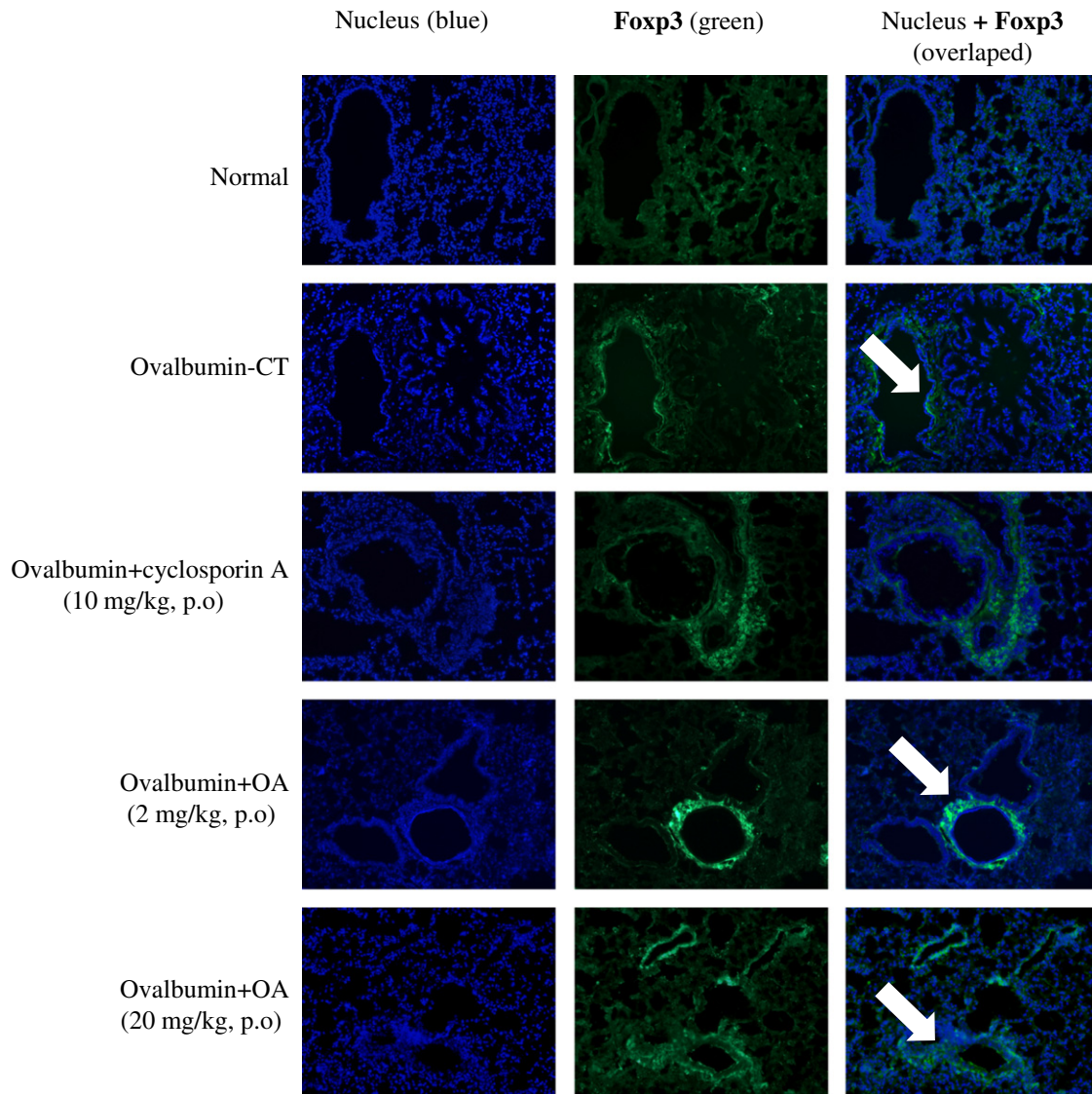


**Fig. 6.** The lung tissue immunofluorescence staining for Foxp3 (green) and DNA (blue) in vivo. Vertical line from left to right, in order: Images of DNA (blue), Foxp3 (green), DNA + Foxp3 (overlapped). Horizontal line from top to bottom, in order: N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA + CsA (10 mg/kg), OVA + oleanolic acid (2 or 20 mg/kg). Original magnification  $\times 200$ .

OVA-specific IgE levels in serum from OVA-induced asthmatic mice were significantly higher than that in serum from normal mice (PBS only), and oleanolic acid-treated (2 or 20 mg/kg) and CsA-treated (10 mg/kg) mice had significantly reduced OVA-specific IgE levels (Fig. 3C). Moreover, determination of IL-17 and IFN- $\gamma$  levels in culture supernatants by ELISA revealed that oleanolic acid (20 mg/kg) and CsA inhibited IL-17 production (Fig. 3D) but did not affect Th1 cytokine (IFN- $\gamma$ ) production in splenocytes (data not shown).

### 3.6. Detection of IL-17 and Foxp3 mRNA in lung tissue

IL-17 has revealed an important role for Th17 cells in allergic inflammation. Expression of IL-17 mRNA is upregulated in the airways in mouse asthma models, and IL-17 family members have been suggested to be potential therapeutic targets for severe asthma [4]. In addition, the importance of Foxp3 expression in immune regulation is supported by the fact that Foxp3<sup>+</sup> Tregs actively restrain the inflammatory response, suppress development of autoimmune diseases, and dampen a wide spectrum of immune responses [11].

As shown in Fig. 4, both IL-17 and FoxP3 mRNA was detected in the lung tissue of mice treated with PBS and OVA plus oleanolic acid (2 or

20 mg/kg), as well as in CsA-treated mice (10 mg/kg). The PCR products of IL-17 amplified from lung cell RNA preparations were fewer for the oleanolic acid-treated group than for the control mice (OVA alone), although the PCR products of Foxp3 increased in the oleanolic acid-treated group. These results were accompanied by changes in Foxp3 immunofluorescence staining in the lung tissue (Fig. 6), BAL cytokine levels (IL-5, IL-13), and IL-17 production in splenocytes (Fig. 3D).

### 3.7. Immunofluorescence staining of T-bet, GATA-3, Foxp3, and ROR $\gamma$ t in asthmatic mice

T-bet, GATA-3, Foxp3, and ROR $\gamma$ t staining in the asthma model mouse pulmonary tissue was altered compared in the control group, especially in the cell nucleus. After treatment with OVA plus CsA and OVA plus oleanolic acid (2 or 20 mg/kg), GATA-3 expression decreased significantly (Fig. 5). T-bet staining in pulmonary tissue in the OVA plus oleanolic acid-treated groups (2 or 20 mg/kg) was significantly stronger than that in the OVA alone control group, particularly in the cell nucleus (Fig. 5).

As shown in Fig. 6, immunofluorescence staining detected the Foxp3 transcription factor in the nucleus of the lung compartments of the OVA

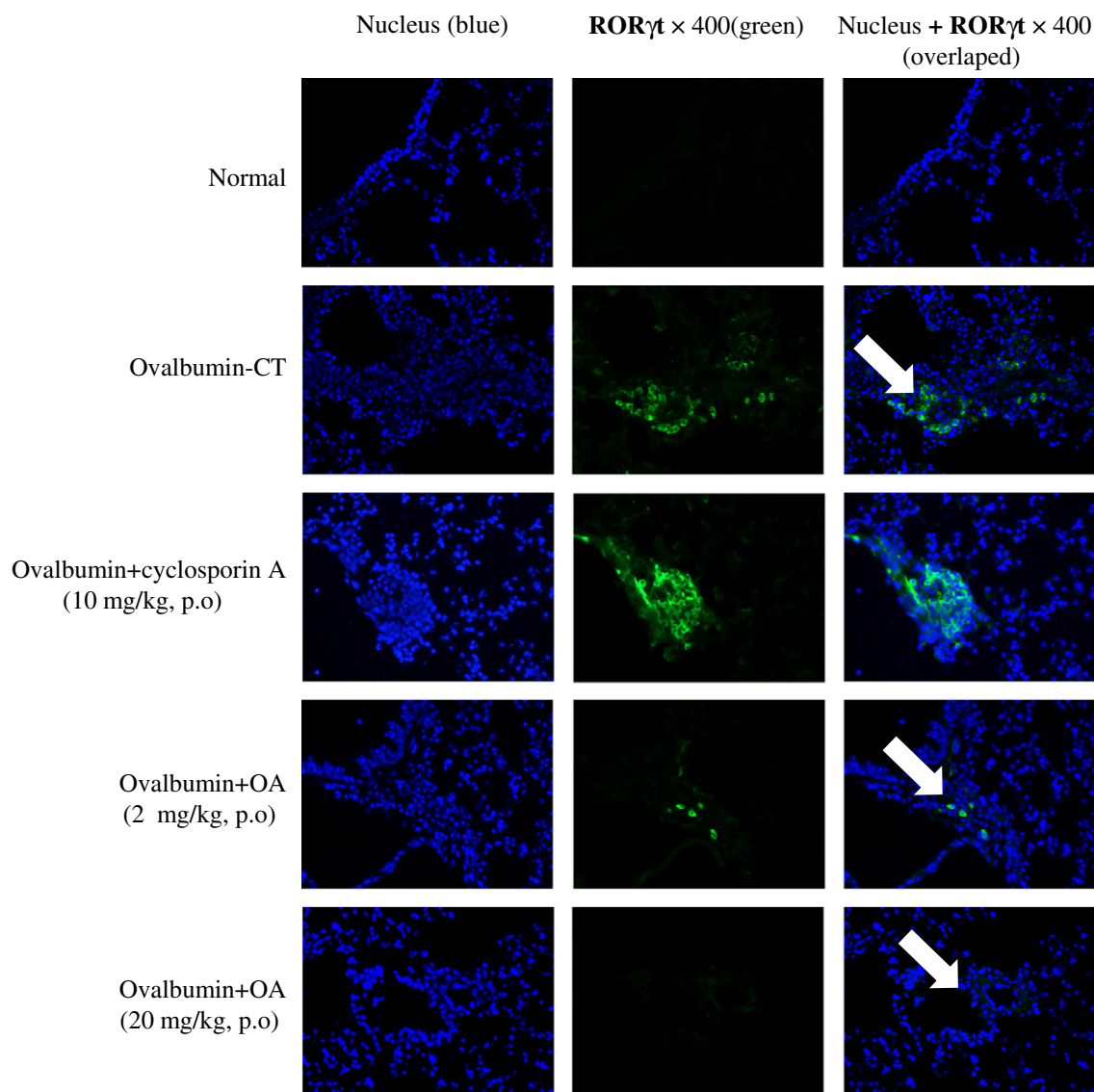


**Fig. 7.** The Lung tissue immunofluorescence staining for ROR $\gamma$ t (green) and DNA (blue) in vivo. Vertical line from left to right, in order: Images of DNA (blue), ROR $\gamma$ t (green), DNA + ROR $\gamma$ t (overlapped). Horizontal line from top to bottom, in order: N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA + CsA (10 mg/kg), OVA + oleanolic acid (2 or 20 mg/kg). Original magnification  $\times$  400.

alone group and the OVA plus oleanolic acid-treated groups (2 or 20 mg/kg).

Foxp3 staining in the lung tissues showed clear distinction between the lung tissue with bright Foxp3 nuclear staining and that with only faint positivity (Fig. 6). Foxp3 staining in pulmonary tissue was mildly greater in the OVA control groups than that in the normal group, whereas in the oleanolic acid-treated groups (2 or 20 mg/kg), it was significantly stronger than that in the OVA control group (Fig. 6). In addition, ROR $\gamma$ t staining in pulmonary tissue was mildly greater in the OVA control groups than in the normal group, whereas in the oleanolic acid-treated groups (2 or 20 mg/kg), the staining was significantly less than that in the OVA control group (Fig. 7).

### 3.8. Effect of oleanolic acid on IL-5, IL-13, T-bet, and GATA-3 mRNA expression and protein levels

To determine whether the observed effects of oleanolic acid on IL-5, IL-13, T-bet, and GATA-3 mRNA expression *in vivo* are related to a direct modulation of airway inflammation, we probed for expression of each of these mRNAs in EL4 cells *in vitro* (Fig. 8A). RT-PCR analysis revealed that expression of IL-5 and GATA-3 mRNA significantly decreased in oleanolic acid-treated cells vs. control cells (Fig. 8A). T-bet and GATA-3 protein expression were also assessed by western blotting of the nuclear fractions of EL-4T cells. Western blot analysis revealed that T-bet and GATA-3 protein levels in EL-4T cells were significantly

lower at 12 h after oleanolic treatment than the levels detected after treatment with only PMA or OP (Fig. 8B). Consistent with the results obtained from the RT-PCR analysis, ELISA results revealed that levels of IL-5 and IL-13 in EL-4T cell supernatants were also significantly lower at 12 h after oleanolic acid treatment (50  $\mu$ M) than that in either PMA or OP treatment alone (Fig. 8C).

### 3.9. T-bet and GATA3 expression in isolated CD4<sup>+</sup> T cells

As shown in Fig. 9, the levels of CD4<sup>+</sup>/T-bet<sup>+</sup> and CD4<sup>+</sup>/GATA-3<sup>+</sup> T cells increased in the control group (23.1% and 38.3%, respectively), stimulated with anti-CD3, anti-CD28, IL-4, and TGF- $\beta$  in comparison to unstimulated normal CD4<sup>+</sup> T cells (19.5% and 28.9%, respectively). Following oleanolic acid treatment (12.5 or 25  $\mu$ M), the proportion of CD4<sup>+</sup>/T-bet<sup>+</sup> T cells was slightly increased (30.3% and 27.4%, respectively) in comparison to the stimulated control group, but the proportion of CD4<sup>+</sup>/GATA-3<sup>+</sup> T cells was slightly smaller (30.5% and 34.6%, respectively) than that in the stimulated control group.

## 4. Discussion

Allergic asthma is an immune-mediated inflammatory disorder characterized by AHR and eosinophilic airway inflammation. Th2 cells are predominant in the airways in asthma, and Th2 cytokines such as IL-4, IL-5, and IL-13 play a pivotal role in its pathophysiology [26].

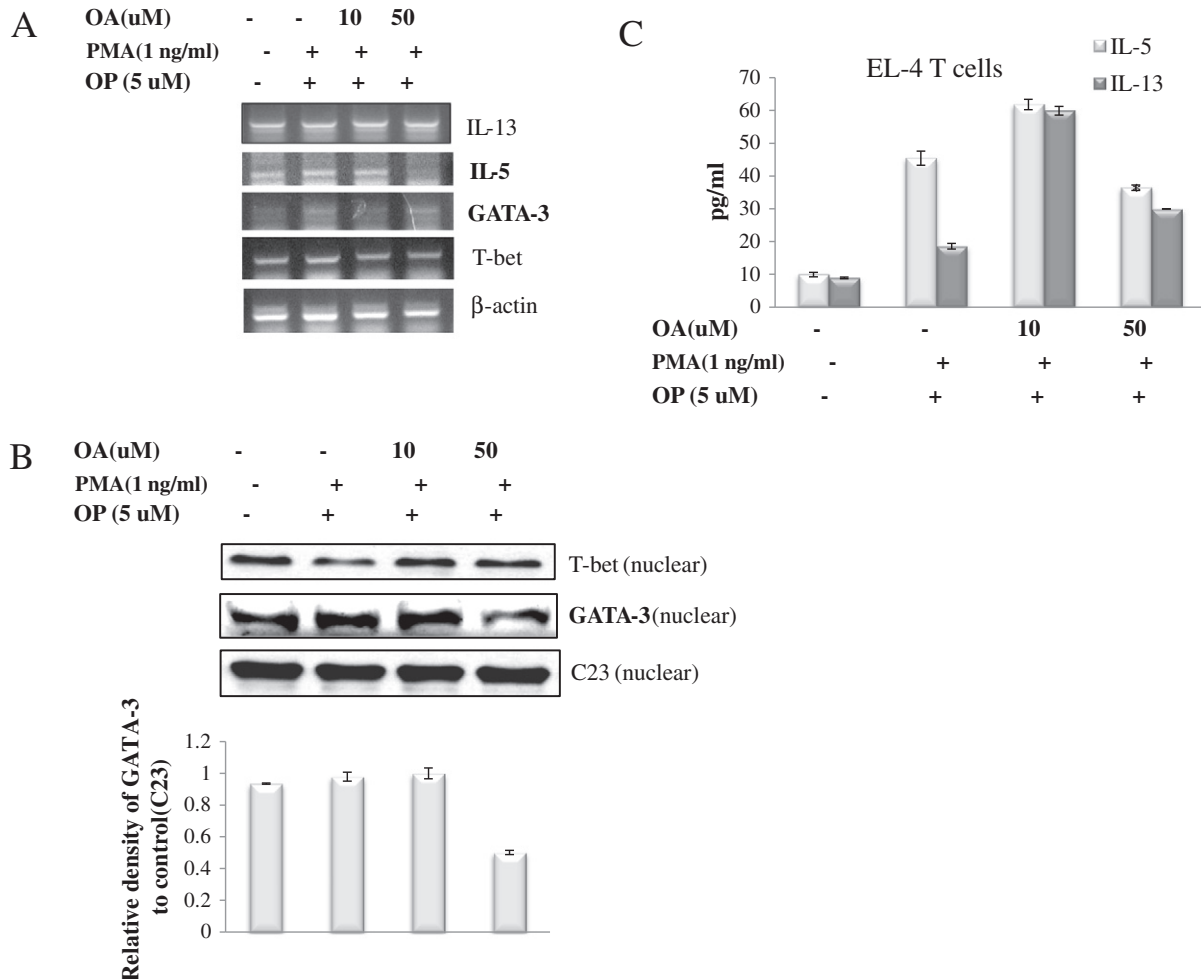


**Fig. 8.** Effects of oleanolic acid on PMA- and OP-induced mRNA expression of T-bet, GATA-3, IL-5 and IL-13; protein expression of T-bet and GATA-3; and levels of IL-5 and IL-13 in the supernatants of EL-4T cells. (A) Relative expression of T-bet, GATA-3, IL-5, and IL-13 mRNA. (B) Levels of T-bet, GATA-3, and C23 proteins were assessed by western blotting. Nuclear cell lysates were analysed using specific antibodies. Histograms represent quantifications of protein expression by Image J software. N represents control. (C) Levels of IL-5 and IL-13 were quantified in the supernatants of EL-4T cells by enzyme immunoassays. Data are expressed as mean  $\pm$  SEM \* $p$  < 0.05 vs. control.

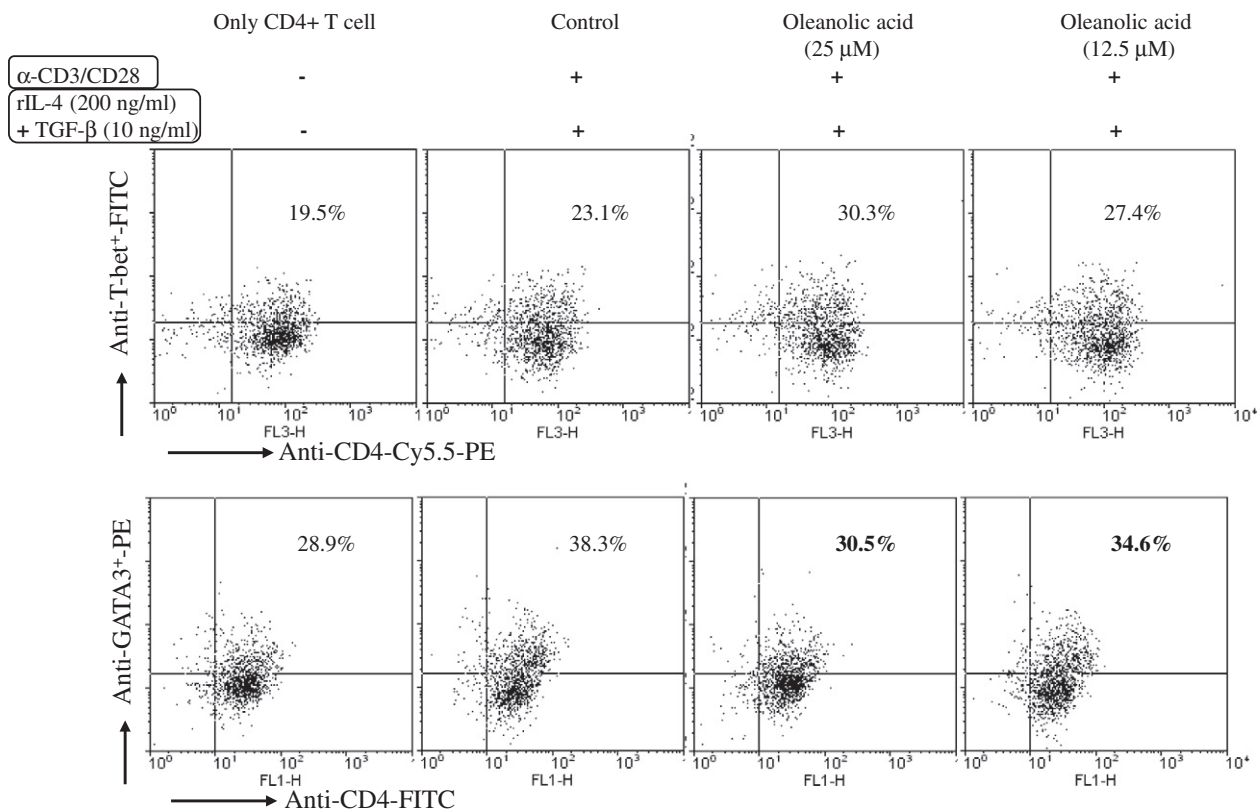


**Fig. 9.** Dot plot patterns of intracellular staining. X-axis: FITC, Cy5.5-PE intensity of CD4. Y-axis: PE intensity of GATA-3 and FITC intensity of T-bet. Isolated CD4<sup>+</sup> T cells were cultured without (normal group) or with (stimulated group) anti-CD3, anti-CD28, rIL-4, TGF-β, and oleanolic acid (12.5 or 20 μM) added to RPMI medium and stained with FITC, Cy5.5-PE-conjugated mAb to CD4, PE-conjugated mAb to GATA-3, or FITC-conjugated mAb to T-bet.

Allergic asthma is a complex syndrome broadly defined by inflammation of the airways associated with Penh and mucus hypersecretion [27].

Th2 cytokines have been implicated in many aspects of the allergic response, including IgE synthesis and eosinophilic inflammation and AHR [28]. It has been suggested that eosinophil recruitment in allergic reactions is regulated by Th2 lymphocytes, and cytokines that are synthesized by Th2 lymphocytes, such as IL-4, IL-5, and IL-13, have been investigated as potential therapeutic targets [29].

Oleanolic acid is a triterpenoid compound known for its various pharmacological and anti-inflammatory properties, and is commonly found in a variety of medicinal plants. It has numerous pharmacological activities, including anti-inflammatory, anti-cancer, antioxidant, and hepatoprotective effects. Derivatives of the natural triterpene oleanolic acid are known to suppress IL-17 and other pro-inflammatory mediators through Nrf2-dependent induction of anti-oxidant genes [30]. Oleanolic acid acetate inhibits the expression of cytokines and chemokines through the downregulation of NF-κB and MAPKs [22]. Additionally, it has been shown that oleanolic acid substantially suppresses STAT1 and STAT3 phosphorylation in adipocytes [31]. Despite these various studies of the pharmacological effects of oleanolic acid, the mechanism underlying its anti-asthmatic effect remains elusive. Furthermore, the precise nature of its influence on asthma has not been investigated.

Another therapeutic compound, CsA, has been shown to provide a clinical benefit in patients with chronic severe steroid-dependent asthma [32]. The effects of CsA on eosinophilic inflammation have been demonstrated using various asthma models [33,34]. CsA has been shown to inhibit the effects of single-allergen-induced allergic inflammation, such as eosinophilic and lymphocytic infiltration and mRNA expression of IL-4 and IL-5. In the present study, we used CsA as a positive control.

Recruitment of eosinophils to the airways is a well-known characteristic of asthma, and the degree of eosinophilia correlates with disease severity. Eosinophils are often considered to play a major role in inducing AHR. In this study, both oleanolic acid and CsA prevented the development of Penh (Fig. 1B), lung inflammation (Fig. 1C), airway eosinophilia (Fig. 2, Table 1), and decreased Th2 cytokine levels (IL-5 and IL-13) (Fig. 3A, B) in BALF and splenocytes. These results demonstrate that both oleanolic acid and CsA can inhibit allergic responses in the lungs in an OVA-induced asthma model.

Asthma produces immune abnormalities in a wide variety of cell populations. Studies in mice have demonstrated the attenuation of lung responsiveness in experimental asthma following the depletion of CD4<sup>+</sup> T cells. CD4<sup>+</sup> T cells are also known to play a key role in the induction of increased responsiveness of the airways to bronchoconstricting agents, a cardinal feature of asthma, and the recruitment of activated CD4<sup>+</sup> T cells to the airway wall is the primary determinant for the development of AHR [35].

Experimental models of allergic airway inflammation have shown that CD8<sup>+</sup> T cells, or Tc2 cells, can exacerbate inflammation by secreting type 2 cytokines such as IL-5 and IL-13 [36]. The mechanisms of these cell-mediated effects are thought to involve CD8<sup>+</sup> T cell-derived cytokines. However, no other mechanisms have been proposed to explain the regulatory effect of CD8<sup>+</sup> T cells during allergic airway inflammation [37]. In addition, CD69-deficient mice display a markedly attenuated airway inflammation. The administration of anti-CD69 antibody inhibits the induction of antigen-induced airway inflammation and hyperresponsiveness. CD69 plays a crucial role in the pathogenesis of arthritis and allergic airway inflammation, suggesting that CD69 may be a possible therapeutic target for both arthritis and asthma [38]. Thus, another goal in asthma research is the evaluation of the roles of specific cell subpopulations. In the present study, immunophenotyping

by flow cytometry showed similar patterns of total lymphocyte numbers in BALF and the lungs.

In the current study, the presence of elevated numbers of activated CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as well as CD11b<sup>+</sup>/Gr-1<sup>+</sup> and CD3<sup>+</sup>/CD69<sup>+</sup> double-positive T cells in the lungs and BALF of OVA-challenged mice was positively correlated with susceptibility to airway inflammation and AHR. Oleanolic acid and CsA effects on leukocyte subsets in the lungs and BALF were marked by changes in the numbers of CD3<sup>+</sup>/CD4<sup>+</sup>, CD3<sup>+</sup>/CD8<sup>+</sup>, CD11b<sup>+</sup>/Gr-1<sup>+</sup>, and CD3<sup>+</sup>/CD69<sup>+</sup> double positive cells (Table 1). The decrease in CD11b<sup>+</sup>/Gr-1<sup>+</sup> cells was accompanied by concurrent reduction in the eosinophil numbers in PBMC and BALF cells (Fig. 2A, C). Eosinophils express Gr-1, and therefore eosinophil populations may constitute a substantial portion of the CD11b<sup>+</sup>/Gr-1<sup>+</sup> population. Our results (Table 1) showed that Gr-1<sup>+</sup> cells increased with OVA challenge but significantly decreased with oleanolic acid and CsA treatment in mice. We observed significant correlations between IL-5 levels and Gr-1<sup>+</sup> expression in eosinophils. We therefore propose that oleanolic acid prevents airway inflammation and Penh by down regulating IL-5 production and reducing eosinophilia. Oleanolic acid and CsA also inhibited B cell-dependent production of OVA-specific IgE in serum (Fig. 3C).

The balance of Th1/Th2 cells is thought to be determined by the expression ratio of T-bet/GATA-3. Two transcription factors, T-bet and GATA-3, are determinants of Th cell differentiation. T-bet is a Th1-specific transcription factor thought to initiate Th1 development, whereas GATA-3 plays a pivotal role in the development of the Th2 phenotype [39]. Moreover, the Th1/Th2 paradigm was recently shifted to the Th1/Th2/Th17/regulatory T (Treg) hypothesis, a multi-lineage commitment from the same Th precursor cells [40]. After treatment with OVA plus CsA or OVA plus oleanolic acid (2 or 20 mg/kg), GATA-3 expression was significantly decreased (Fig. 5). The results of the in vitro experiments shown in Figs. 8 & 9 reveal similar patterns. In particular, oleanolic acid suppressed PMA- and OP-induced GATA-3 expression in EL-4T cells and suppressed CD4<sup>+</sup>/GATA-3<sup>+</sup> T cells slightly in comparison to the stimulated control group.

Both IL-17 receptor-deficient and IL-17A-deficient mice exhibit reduced recruitment of eosinophils into the airways and decreased levels of Th2 cytokines in the BALF [41]. Th17 cells, defined by their production of IL-17A/F, play important roles in autoimmune and inflammatory diseases [42]. Th17 cells are controlled by several transcription factors such as ROR $\gamma$ t, IRF4, BATF, FoxP3, T-bet, and PPAR $\gamma$  [43]. Our results show that oleanolic acid inhibits production of IL-17 (Figs. 3D, 4A) through ROR $\gamma$ t (Fig. 7), which plays critical roles in the differentiation of Th2 and Th17 cells. Similarly, the observation that administration of oleanolic acid significantly reduces IL-17 may also have implications for other immunoregulatory events. Furthermore, delayed specific antibody production following transplantation is most likely due to the ability of oleanolic acid to block both IL-4 and IL-17 synthesis [44]. The production of IL-17A in both serum and spinal cord of animals with EAE has been shown to be up-regulated, and oleanolic acid treatment is also known to suppress IL-17A production [45].

Foxp3<sup>+</sup> Treg cells are known to inhibit eosinophil recruitment, mucus hyperproduction, and Th2 cytokine release (IL-4, IL-5, and IL-13) [46]. There may thus be a crucial balance between Foxp3<sup>+</sup> Treg cells and Th17 cells, mediated by the acute phase protein IL-6 [47]. Foxp3 functions as a transcriptional repressor for various transcription factors such as the nuclear factor of activated T cells (NFAT), NF- $\kappa$ B, and Runx1 [48], which may form a mechanistic component of Foxp3-mediated suppression of immune responses. In Th17 cell differentiation, ROR $\gamma$ t has been identified as a master regulator of Th17 cell differentiation. As shown in Fig. 7, oleanolic acid suppressed ROR $\gamma$  expression in the lung tissues. We propose that induction of Foxp3 is the mechanism underlying suppression of IL-17 production (Figs. 4, 6, 7). Therefore, development of immunomodulatory molecules capable of targeting the main T cell-specific transcription factors T-bet,

GATA-3, and Foxp3 would be beneficial for treatment of allergic diseases.

Our findings shows that airway exposure to allergen in sensitized mice expands all effector T cells in the lungs, including T-bet<sup>+</sup>, GATA-3<sup>+</sup>, and Foxp3<sup>+</sup> cells. Among these, the GATA-3<sup>+</sup> effector Th2 cell is most prominently increased after OVA exposure, followed to a lesser extent by the T-bet<sup>+</sup> Th1 and Foxp3<sup>+</sup> Treg cells. Our study shows that Th2 cytokines and GATA-3 are prominent therapeutic factors in allergic inflammation, while concomitantly verifying the activity of Th1, Th17, and Treg cells. Our findings also indicate that oleanolic acid upregulates Th1 cell transcription factors. However, significant downregulation of the expression of GATA-3, IL-5, or IL-13 was observed following oleanolic acid treatment. Since GATA-3 is the principal Th2 transcription factor, and IL-5 and IL-13 are two of the most commonly recognized factors driving Th2 differentiation, our results suggest that oleanolic acid seems to have a greater effect on the Th2 pathway in T cell transcription. Because Foxp3 Treg cells suppress Th17 cells, it is possible that the therapeutic effect of oleanolic acid via inactivation of Th2 and Th17 cells is synergistically mediated by the GATA-3 and Foxp3/IL-17 pathways. Thus, the results of this study suggest oleanolic acid could be used as a new therapeutic compound for respiratory diseases, including allergic asthma, although further studies are required.

Asthma is an inflammatory disease of the lungs characterized by increased infiltration of leukocytes, especially eosinophils, into the airways and reduced respiratory function. This inflammation leads to increased Th2 cytokines, IL-17, IgE production, airway oedema, AHR, and mucus production. This inflammation is associated with the infiltration of eosinophils, neutrophils, and lymphocytes into the bronchial lumen and the lung tissues. These cellular infiltrates release various chemical mediators that can cause AHR. Recruitment of these inflammatory cells from the blood to the site of inflammation is regarded as a critical event in the development and persistence of airway inflammation [49].

It is widely accepted that differentiation, maturation, and activation of eosinophils are regulated by Th2 cytokines, especially by IL-5. In addition to Th2 cells, the potential involvement of Th17 cells in the development of the clinical features of this disease has also been the focus of intense investigation. In addition, IL-17 is a potent inducer of the migration and activation of inflammatory cells that perpetuate chronic airway inflammation. IL-17 levels are known to correlate with the severity of airway hypersensitivity in asthma. The present data suggest that GATA-3 gene expression was more responsive than T-bet under conditions favouring either Th1 or Th2. Thus, it appears that GATA-3 expression is a more important controller of Th1/Th2 differentiation than T-bet, consistent with reports that GATA-3 is a critical regulator of both Th1 and Th2 cell development. GATA-3 directly controls IL-5 gene expression through binding to transactivating elements in the IL-5 promoter. The findings of the present study suggest that inhibition of GATA-3 expression is more important than increased expression of T-bet for the production of IFN- $\gamma$ . Suppression of Th17 is achieved via Foxp3, and therefore Foxp3-expressing Tregs may contribute to the antiasthmatic effect of oleanolic acid [50].

Oleanolic acid may also ameliorate the imbalance of the Th1/Th2-associated transcription factors T-bet, GATA-3, ROR $\gamma$ t and Foxp3 in the pulmonary tissue of asthmatic mice. Furthermore, oleanolic acid could also inhibit the activation of Foxp3 protein in asthmatic mouse pulmonary tissue. Our data are consistent with the known functions of the respective transcription factors, namely, the downregulation of GATA-3-dependent expression of IL-5 and the upregulation of T-bet-dependent production of IFN- $\gamma$ . Oleanolic acid appears to have a strong suppressive effect on the Th2 cells during T cell activation through the GATA-3/Foxp3 pathway and a mild suppressive effect on Th17 activation through the ROR $\gamma$ t pathway.

Although the precise mechanism of action needs further investigation, this is the first report showing that oleanolic acid significantly inhibits airway inflammation and hyperresponsiveness in a mouse

model of allergic asthma. Therefore, we propose that oleanolic acid could be used to treat pathological inflammatory airway disorders, including allergic asthma. The results presented here demonstrate that oleanolic acid and CsA have profound inhibitory effects on airway inflammation, and that this effect is caused by suppression of Th2 cytokines (IL-5, IL-13), B cell-dependent production of OVA-specific IgE, and Gr-1 expression through the GATA-3, ROR $\gamma$ t and Foxp3 transcription pathways. It is reasonable to suggest that the anti-inflammatory and anti-asthmatic activity of oleanolic acid may be caused by inhibition of the GATA-3 and ROR $\gamma$ t pathways.

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