



Pulmonary, gastrointestinal and urogenital pharmacology

Ursolic acid, a potential PPAR γ agonist, suppresses ovalbumin-induced airway inflammation and Penh by down-regulating IL-5, IL-13, and IL-17 in a mouse model of allergic asthmaSeung-Hyung Kim^{a,1}, Jung-Hee Hong^b, Young-Cheol Lee^{b,*,1}^a Institute of Traditional Medicine & Bioscience, Daejeon University, Daejeon 300-716, Republic of Korea^b Department of Herbology, College of Oriental Medicine, Sangji University, Wonju 220-702, Republic of Korea

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ABSTRACT

Allergic asthma is a chronic airway disorder characterized by airway hyperresponsiveness to allergens, chronic airway inflammation, airway edema, increased mucus secretion, excess production of Th2 cytokines, and eosinophil accumulation in the lungs. Ursolic acid is known for its pharmacological effects, such as its anti-tumor, anti-inflammatory and antimicrobial activities. To investigate the anti-asthmatic effects and mechanism of ursolic acid, we studied the development of pulmonary eosinophilic inflammation and enhanced pause (Penh) in a mouse model of allergic asthma.

In this study, BALB/c mice were systemically sensitized to ovalbumin followed by intratracheal, intraperitoneal, and aerosol allergen challenges. We investigated the effect of ursolic acid and Cyclosporin A (CsA) on Penh, pulmonary eosinophilic infiltration, various immune cell phenotypes, Th2 cytokines, IL-17 production, and ovalbumin specific IgE production in a mouse model of asthma.

In BALB/c mice, ursolic acid had 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 by blocking the GATA-3 and STAT6 pathways.

Our data suggest the therapeutic mechanism of ursolic acid in asthma is based on reductions of Th2 cytokines (IL-5 and IL-13), ovalbumin-specific IgE production, and eosinophil infiltration via the Th2-GATA-3, STAT6, and IL-17-NF- κ B pathways.

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1. Introduction

The pathogenesis of asthma is the result of chronic allergic inflammation accompanied by progressive airway dysfunction and is characterized by wheezing, cough, breathlessness, and airway inflammation. The cytokines produced by Th2 cells are critical to the pathogenesis of allergic asthma. Memory Th2 cells within the lungs are activated and up-regulate IL-4, IL-5, and IL-13 within 1 h of aeroallergen challenge (Mojtabavi et al., 2002). The rapid response initiates a cascade, which recruits inflammatory cells into the lungs, induces airway hyperresponsiveness, and evokes goblet cell mucus hypersecretion. Inhibiting the effector arm of memory Th2 cells would effectively prevent the cascade. Inhibition of Th2 effector cytokines would serve to treat disease (Epstein, 2006). In asthma, activation of allergen-induced Th2 cells in the lungs leads to eosinophilic lung inflammation, edema,

goblet cell hyperplasia with increased mucus secretion, and recurring bronchospasm (Bousquet et al., 2000). Eosinophilic inflammation is the hallmark of airway inflammation in asthma (Umetsu et al., 2002). The cytokines produced by Th2 cells are critical to the pathogenesis of allergic asthma. In particular, IL-4, IL-5, and IL-13, which are produced by Th2 cells, are related to inflammatory changes in the airway via activation of eosinophils and production of immunoglobulin E (IgE) by B cells (Sanderson et al., 1985). Whereas IL-4 is important for allergic sensitization and IgE production, and IL-5 is crucial for eosinophil survival, IL-13 has pleiotropic effects in the lungs, including a central role in the development of airway hyperresponsiveness and tissue remodeling (Finkelman et al., 2010). Moreover, there is a strong interaction between eosinophils and Th2 cells in the asthmatic airway and Th2 cell-derived cytokines, namely IL-4, IL-5, and IL-13, play critical roles in orchestrating and amplifying allergic inflammation in asthma (Nakajima et al., 2010).

Ursolic acid is a triterpenoid compound found in plants used in the human diet and in medicinal herbs, in the form of the free acid or as aglycones of triterpenoid saponins (Furtado et al., 2008). Ursolic acid possesses hepatoprotective (Liu, 1995),

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antiallergic (Banno et al., 2004), anti-ulcer (Ovesná et al., 2004), cardioprotective (Senthil et al., 2007), anti-inflammatory, and analgesic (Vasconcelos et al., 2006) activities. Ursolic acid has also been used in the treatment of kidney diseases (Yoshimura et al., 2003) and hypertension (Somova et al., 2004). Recently, ursolic acid has been reported to have anti-arthritic (Ahmad et al., 2006), anti-diabetic (Zhang et al., 2006), and immunomodulatory activities (Jang et al., 2009).

Several biological mechanisms of action have been proposed; ursolic acid inhibits nuclear factor- κ B activation induced by carcinogenic agents through suppression of I κ B α kinase and p65 phosphorylation, and correlates with down-regulation of cyclooxygenase 2, matrix metalloproteinase 9, and cyclin D1 (Shishodia et al., 2003; Zeng et al., 2011). Ursolic acid also suppresses MMP-2 and MMP-9 activity in cancer cell lines such as B16F10 and HT-1080 (Cha et al., 2011). Th17 cells have recently emerged as a major player in inflammatory and autoimmune diseases via production of the pro-inflammatory cytokines interleukin-17 (IL-17), IL-17F, and IL-22. Differentiation of Th17 cells and the associated cytokine production is directly controlled by ROR γ t. Xu et al. (2011) reported that ursolic acid suppresses IL-17 production by selectively antagonizing the function of ROR γ t and may be a valuable drug candidate or leading compound for development of therapies for Th17-mediated inflammatory diseases such as asthma. However, no in vivo studies on the anti-asthmatic and anti-inflammatory activity of ursolic acid have been reported.

The aim of this study was to evaluate the pharmacological activity of ursolic acid on Th1, Th2, and Th17 cytokines, the development of allergic airway inflammation, and Penh. Therefore, we investigated the anti-inflammatory, anti-asthmatic, and possible mechanisms of ursolic acid activity in a mouse model of allergic asthma.

2. Materials and methods

2.1. Identification of ursolic acid by HPLC analysis

2.1.1. Chemicals and reagents

Ursolic acid was purchased from Sigma (Sigma-Aldrich Korea, Korea). The purity of each compound was determined to be above 98% by HPLC analysis. HPLC-grade reagents, acetonitrile, and water were obtained from J.T. Baker (Phillipsburg, NJ, USA). Other chemicals were of analytical grade.

2.1.2. Chromatographic system and conditions

Analysis was performed using a Waters HPLC system equipped with a Waters 600 pump, a Waters 996 PDA detector, an Empower system controller, and an HPLC column (Optima Pak C18 column, 4.6 $\times$ 250 mm i.d., 5 μ m particle size). The mobile phase consisted of water and acetonitrile (10:90 for ursolic acid). The column temperature was maintained 40 °C. The analysis was performed at a flow rate of 1.0 ml/min with PDA detection (200 nm for ursolic acid analysis). The injection volume was 20 μ L.

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 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

As described with modifications (Kim et al., 2011), ovalbumin (500 μ g/ml) in PBS was mixed with equal volumes of 10% (w/v) aluminum potassium sulfate (alum; Sigma) in distilled water, then incubated for 60 min at RT after adjustment to pH 6.5 using 10 N NaOH, and centrifuged at 750g for 5 min. The ovalbumin/alum pellet was resuspended to the original volume in distilled water. All mice were immunized on 3 different days (first day of 2, 3, 4 weeks before inhalational exposure) by intraperitoneal (i.p.) injections of 0.2 ml alum-precipitated antigen containing 100 μ g ovalbumin (Sigma-Aldrich Korea, Korea) bound to 4 mg aluminum hydroxide (Sigma-Aldrich Korea) in PBS. Seven days after the second sensitization by intratracheal injection with 250 μ g ovalbumin (on day 21) on the back of the tongue, mice were exposed to aerosolized ovalbumin for 30 min/day, 3 days/week for 5 weeks (at a flow rate of 250 L/min, 1% ovalbumin in normal saline for the first 4 weeks and 2% ovalbumin in normal saline for the final week). Ursolic acid (2 and 20 mg/kg, Sigma-Aldrich Korea) and CsA (10 mg/kg) were orally administered 3 times a week for the last 5 weeks. One day after the last ovalbumin exposures (2% ovalbumin inhalation), Penh was determined and samples (bronchoalveolar lavage fluid, lung cells, and serum) were collected for molecular analyses.

2.4. Bronchoalveolar lavage fluid

Immediately following Penh assessment, mice were sacrificed with an i.p. injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and BAL obtained by washing the airway lamina. Briefly, cells in the lungs were recovered by flushing 1 ml bronchoalveolar lavage fluid (1 mM EDTA, 10% fetal bovine serum [FBS], phosphate-buffered saline [PBS]) into the lungs via the trachea. Total cell counts were determined and 100 μ L fluid was cytospun onto glass slides using a Cytospin centrifuge (Cellspin, Hanil, Korea) (400g, 4 min). Differential cell counts were performed after staining with a Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, FL, USA). The bronchoalveolar lavage fluid supernatant was stored at –25 °C.

2.5. Digestion of pulmonary tissue and cell preparations

Single cell suspensions from lung tissues and bronchoalveolar lavage fluid 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 (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 °C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down to dissolve remaining tissue clumps and then filtered using a 70- μ m cell-strainer (Falcon, Le Pont de Claix, France). Cells were counted manually using a hemocytometer chamber (Fisher). Between 2 and 4 $\times$ 10³ cells were spun onto glass slides (Cytospin centrifuge) (400g, 4 min). Differential counts were performed according to standard morphologic criteria.

2.6. Determination enhanced pause (Penh)

Penh in mice was estimated using a previously described method with modifications (Kim et al., 2011). A Buxco system (Biosystem XA; Buxco Electronics Inc., Troy, CT) 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 $\text{Pause} = (T_e - T_r)/T_r$ (PIF is the peak inspiratory flow; PEF is the peak expiratory flow; T_e is the expiratory time; T_r is the relaxation time). In this experiment, mice were aerosolized with ovalbumin 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, 50 mg/ml methacholine (Sigma). Airway reactivity was then monitored for 30 min. Differences in Penh values between groups were evaluated by unpaired Student's *t*-test.

2.7. Hematoxylin–eosin (H&E), Masson–trichrome (M–T), periodic acid–Schiff (PAS) staining and bronchoalveolar lavage fluid cytospin

BALB/c mice were injected, inhaled, and sprayed with ovalbumin for 5 weeks (3 times a week) to induce asthma. Two experimental groups were treated with different concentrations of ursolic acid for the latter 5 weeks (5 times/week). At the end of the experiment, the lungs were removed and analyzed histologically using a modified protocol (Kim et al., 2011).

Briefly, the lung tissue was embedded in paraffin, cut into 3- μ m sections, stained with H&E solution, M–T solution. The tissue was subsequently mounted and covered with Dako-mounting medium (Dako cytometry; Denmark Carpinteria CA) and a coverslip. The degree of airway inflammatory cell infiltration was scored in a double-blind screen by two independent observers. The degree of peribronchiole and perivascular inflammation was evaluated by a subjective scale of 0–2 as described previously, with modifications (Kim et al., 2011). Periodic acid–Schiff (PAS) stain was performed to identify mucus secretion in lung tissue. Frozen sections (30 μ m) of each tissue sample were made. 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 (such as anti-CD3, CD4, CD8, CCR3, CD69, B220, CD23, CD11b, Gr-1) for flow cytometry were purchased from Becton Dickinson (BD) PharMingen (San Diego, CA). Cells from lung tissues and bronchoalveolar lavage fluid 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 on a FACSCalibur using CellQuest software (BD Biosciences, Mountain View, CA).

2.9. Enzyme-linked immunosorbent assay (ELISA)

Interleukin (IL-4, IL-5, IL-13 etc.) and IFN- γ production in bronchoalveolar lavage fluid and anti-ovalbumin IgE in serum ($n=5$) was measured by ELISA according to the manufacturer's instructions with a monoclonal antibody-based mouse interleukin ELISA kit (R&D system) ovalbumin-specific IL-5, IL-13, IL-4, and IFN- γ 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 1×10^5 cells/well in 96-well culture plates (Corning, Cambridge, MA) with or without 1 μ g/ml ovalbumin in a humidified atmosphere of 5% CO_2 in air at 37 °C. The culture supernatants were collected and assayed by ELISA for ovalbumin-induced IL-5, IL-13, IFN- γ , and IL-4 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 ursolic acid on cytokine gene expression from lung tissue, quantitative real-time PCR was performed after quantitative normalization to β -actin. Briefly, total cellular RNA was extracted from the lung by the phenol–chloroform method (RNAsol^B; Tel-Test, USA) according to the manufacturer's instructions. cDNA was synthesized from 3 μ g 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) with the following primers.

Mouse IL-17, 5'-TCTCATCCAGCAAGAGATCC-3' and 5'-AGTTTGGGACCCCTTTACAC-3'; Peroxisome proliferator-activated receptor γ (PPAR γ), 5'-ATGCCATTCTGGCCCACTT-3' and 5'-CCCTTGCATCCTTCAAGCATG-3'; CCR3, 5'-CCCGAAGTGTGACTTTTGCT-3' and 5'-CCTCTGGATAGCGAGACTG-3'; β -actin, 5'-TGGAACTCTGTGTCCATGAAAC-3' and 5'-GTCACAGTCAGCTGTATAGGG-3'.

Pro-inflammatory cytokine gene expression was analyzed with SYBR Green PCR MasterMix (ABI) and 200 nM primers, using β -actin as the internal standard.

The TaqMan probes for Foxp3 (pn,4331182, FAM dye-labeled, ABI) and GAPDH (pn,4352339E, VIC dye-labeled, 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 relative quantity (RQ) and was proportional to the target concentration. Real-time PCR was performed in duplicate and analyzed 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 β -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. PPAR γ RNA preparation and RT-PCR

EL-4 T cells were purchased from the Korea Cell Bank (Seoul, Korea) and maintained in RPMI-1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10% FBS (Lonza) and 100 mg/ml penicillin–streptomycin (Lonza) at 37 °C in 5% CO_2 humidified air. EL-4T cells (5×10^5 cells/ml) were treated with PMA (50 ng/ml) and ionomycin (1 μ M) for 12 h in the absence or presence of ursolic acid. Total cellular RNA was isolated using TRIzol reagent, according to the manufacturer's instructions. RNA (7 μ g) was reverse-transcribed from each sample using Bioneer RT PreMix. PCR analyses were performed on aliquots of the cDNA preparations to detect PPAR γ gene expression (using β -actin as an internal standard) using a thermal cycler (DNA Engine Dyad). Reactions were carried out in 20 μ L containing 1 U Top DNA polymerase, 250 μ M dNTP, reaction buffer, and 10 pmol primers. After initial denaturation for 5 min at 95 °C, 34 amplification cycles were performed for PPAR γ (30 s of 95 °C denaturation, 30 s of 58 °C annealing, and 30 s of 72 °C extension), and β -actin (30 s of 95 °C denaturation, 30 s of 58 °C annealing, and 30 s of 72 °C extension). PCR primers are listed below and were purchased from Bioneer: sense strand PPAR γ , 5'-ATGCCATTCTGGCCCACTT-3'; antisense strand PPAR γ , 5'-CCCTTGCATCCTTCAAGCATG-3'; β -actin, 5'-TCATGAAGTGTGACGTTGACATCCGT-3'; antisense strand β -actin, 5'-CCTAGAAGCATTTGCGGTGCACGATG-3'. After amplification, portions each PCR product

were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining and UV irradiation.

2.12. Immunoblotting analysis for PPAR γ and STAT6

RAW 264.7 cells were plated at 5×10^5 cells/ml in 6-well plates, then incubated for 12 h. The cells were treated with ursolic acid in 1 μ g/ml LPS or 10 ng/ml IFN- γ . Ursolic acid was added to each plate at 20 μ M. After 24 h incubation, the cells were rinsed twice in ice-cold PBS and 50 μ L cell lysis buffer was added to each sample. The proteins were denatured in sodium dodecyl sulfate (SDS); the samples were then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. Nonspecific binding was inhibited by exposure of the cell membranes to TBST (50 mM Tris-HCl, pH 7.5, 150 mM NaCl 0.1% Tween-20) containing 5% nonfat milk for 1 h at room temperature. The membranes were incubated overnight with specific antibodies against PPAR γ and STAT6 at 4 °C. Immunoreactive bands were detected by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies and enhanced chemiluminescence reagents.

2.13. Isolation CD4 $^+$ T cells

As previously described (Roh et al., 2008), splenocytes were isolated from naive Balb/c mice. Cells were enriched for CD4 $^+$ 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 $^+$ T cells were selected on a (CS) column, and the flow-through was collected as CD4 $^+$ T cells. Isolated cells were activated by overnight incubation on 24-well plates coated with 1 μ g/ml anti-CD3 (Serotec.), 1 μ g/ml anti-CD28 (Serotec), and ursolic acid (20, 10 μ g/ml) added to RPMI medium supplemented with 1 unit/ml penicillin, 1 μ g/ml streptomycin, 20 ml L-glutamine, 50 mM-mercaptoethanol, and 5% FBS for 48 h after stimulation.

2.14. Intracellular staining for GATA-3

For GATA-3 (PE mouse anti-GATA3; BD PharMingen) intracellular staining, isolated CD4 $^+$ T cells were stimulated in culture medium containing rIL-4 (200 ng/ml; R&D Systems), TGF-beta (10 ng/ml; R&D Systems), and monensin (GolgiStop, 1 ml/ml, BD Biosciences) in a cell incubator with 10% CO $_2$ at 37 °C for 4 h. After staining surface markers, cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions.

2.15. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (SPSS version 14.0 statistic software). The difference between the normal group and the control group (ovalbumin plus vehicle) was clearly distinguished; for this reason, statistical significance is not indicated in the figures and tables to put an emphasis on 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. HPLC analysis of ursolic acid

Retention time of ursolic acid was about 8.3 min. Fig. 1A shows chromatograms of the reference compound with detection of eluent at 200 nm. The component peak was identified as ursolic acid.

3.2. Inhibitory effect of ursolic acid and CsA on Penh

We measured Penh to confirm that treatment with ursolic acid and CsA during allergen sensitization and allergen challenge influenced the physiologic outcome of ovalbumin-induced airway inflammation. In a mouse model of allergic asthma, we compared the effects of ursolic acid, delivered by means of nebulization only or in association with oral administration. Both ursolic acid and CsA treatments were equally efficient at reducing Penh to methacholine, as measured on the basis of whole-body plethysmography (Fig. 1C).

Penh was measured using a Buxco system on day 1 after final inhalation and samples were immediately collected. Methacholine treatment is useful to exhibit the distinct effect of drugs on Penh value. In ovalbumin-sensitized and ovalbumin-challenged mice, the dose-response curve of the Penh value was shifted to the left in comparison to normal mice (Fig. 1C). As shown in Fig. 1C, relative to animals sensitized with ovalbumin (Control group), Penh conversion to methacholine was reduced in ursolic acid-treated mice (2, 20 mg/kg, *p* < 0.01) and CsA-treated mice (10 mg/kg, *p* < 0.01).

3.3. Histological analysis of lung sections

Histological studies of the mouse lungs showed that the ovalbumin group developed inflammatory changes typical in this model, including peribronchial and perivascular infiltrates composed of inflammatory cells such as eosinophils, neutrophils, and lymphocytes (Figs. 1D and 2A, B, D). However, the ursolic acid-treated group and CsA treated group demonstrated significantly reduced airway infiltration of inflammatory cells.

As shown in Fig. 1D, the inflammation degree of peribronchiole and perivascular regions in mice treated with UA decreased significantly in comparison to the ovalbumin group. We also observed many histological changes in the resulting fibrotic tissue. The histopathological investigation of ovalbumin-challenged and UA-treated mice showed inflammatory changes in comparison to saline-challenged normal mice. In this study, infiltration of leukocytes in histologic sections of lungs from ovalbumin-challenged control mice and lung tissue sections from ovalbumin-challenged mice showed distinct inflammatory infiltration and erosion in peribronchial and perivascular areas. The peribronchial and perivascular inflammatory infiltrate consisted of eosinophils and mast cells, admixed with lymphocytes. Eosinophil infiltration was mainly observed in the peribronchial regions of the lung. In contrast, histological sections from ursolic acid and CsA treated mice indicated reduced airway inflammation in lung tissue (Fig. 1D).

To investigate the level of mucus expression in the airway, PAS-positive and PAS-negative epithelial cells in individual bronchioles were analyzed. The degree of goblet cell hyperplasia and mucus hyperproduction was evaluated by PAS staining and quantification of PAS-stained cells. The ovalbumin-challenged control mice significantly increased the mean numbers of PAS-positive cells in comparison to saline-challenged normal mice.

In particular, there was a greater reduction in the mean number of PAS-stained goblet cells in the ursolic acid-treated (2, 20 mg/kg) asthma mice than in ovalbumin-sensitized/challenged mice (Fig. 1D).

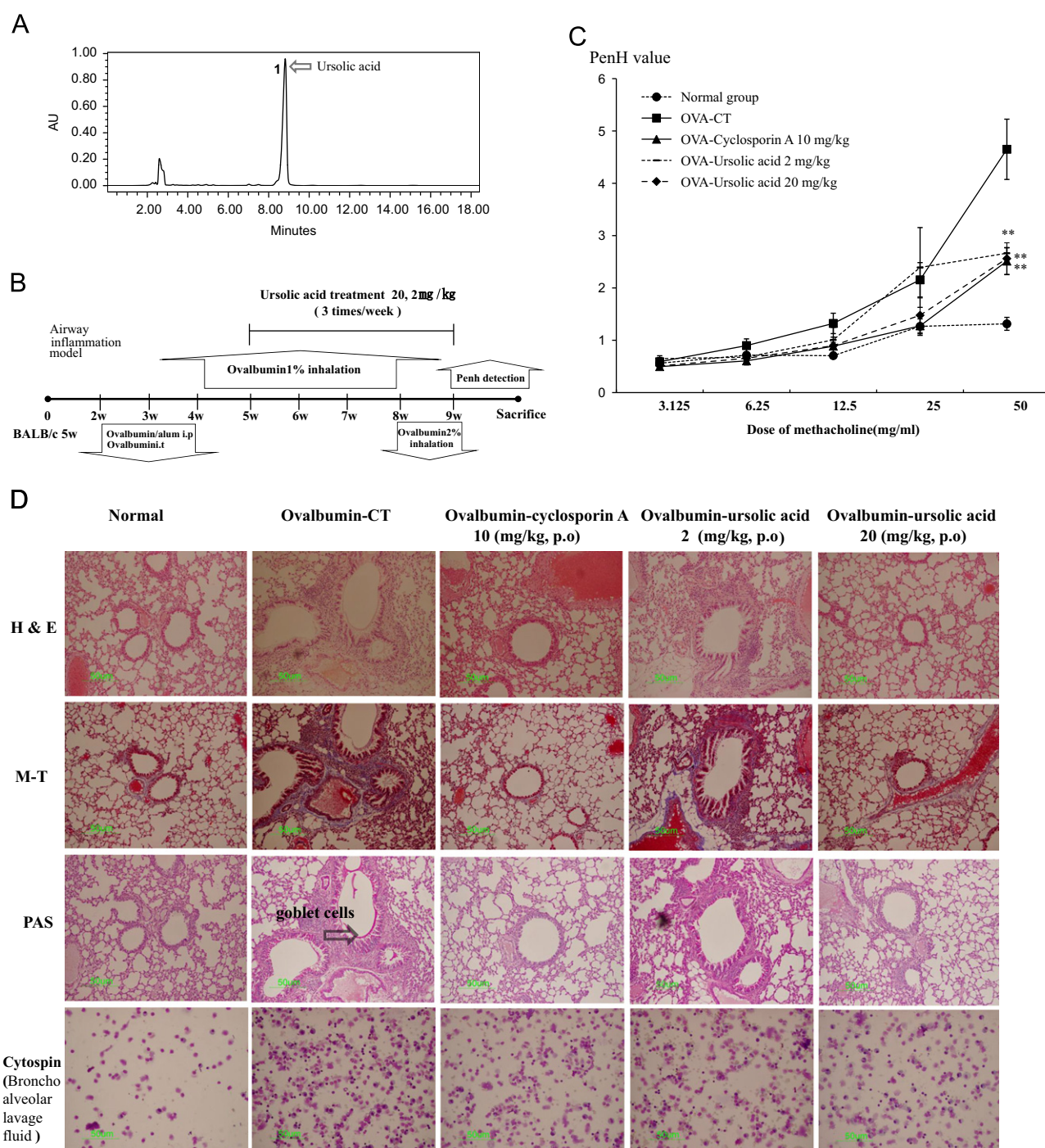


Fig. 1. HPLC chromatogram of standard ursolic acid (A), effect of ursolic acid on airway hyperresponsiveness in ovalbumin-challenged mice (B and C) and effect of ursolic acid on histologic examination of airway inflammation (H&E, M-T, and PAS staining) in lung tissue and bronchoalveolar lavage fluid cytospin in the ovalbumin-induced mouse model of allergic asthma (D). Ursolic acid was subjected to HPLC analysis with an Optima Pak C18 (4.6 × 250 mm) column and eluted with water and acetonitrile (10:90) at 1.0 ml/min (A). Mice were sensitized and challenged by ovalbumin. 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 (3.125–50 mg/ml) of methacholine for 3 min per nebulization. Data represent the means ± SEM of 5 independent experiments. * $p < 0.05$, ** $p < 0.01$ for control group versus Ursolic acid-treated groups (C). At the end of the experiment, lungs were fixed and histologic analysis was performed. Lung sections of normal, asthmatic mice treated with vehicle (control), ursolic acid (2 mg/kg), and ursolic acid (20 mg/kg) (D). H&E: Hematoxylin–eosin stain, M-T: Masson trichrome stain, PAS: Periodic acid–Schiff Stain, N: Normal BALB/c mice, CT (control): Ovalbumin inhalation+vehicle, ovalbumin+ursolic acid (2, 20 mg/kg)

3.4. Inhibitory effect of ursolic acid and CsA on airway eosinophil accumulation and influx of inflammatory cells into lung and bronchoalveolar lavage fluid

The number of total lung cells obtained from the PBS saline-challenged group was $2.19 \pm 0.39 \times 10^7$ cells, indicating that few

eosinophils were detected in this group. On the other hand, the number of total lung cells ($5.58 \pm 0.3 \times 10^7$) and eosinophils in the bronchoalveolar lavage fluid cytospin of the ovalbumin-challenged was significantly higher than that in the PBS saline-challenged group.

The total number of lung cells was significantly reduced in ursolic acid-treated (2, 20 mg/kg) and CsA-treated (10 mg/kg)

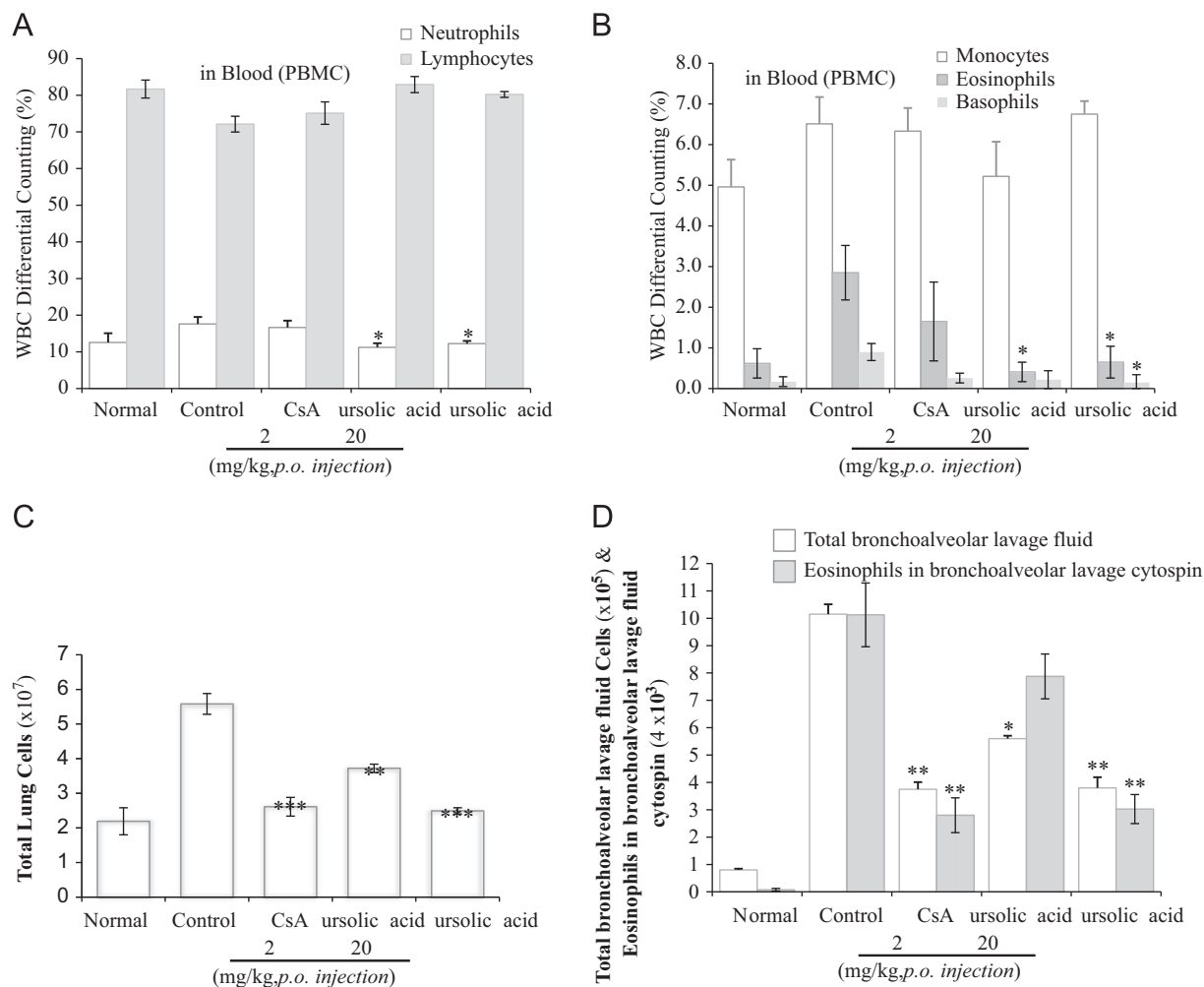


Fig. 2. Effects of ursolic acid on total lung cells, total bronchoalveolar lavage fluid cells, eosinophils in bronchoalveolar lavage fluid and hematology in PBMC. Lung and bronchoalveolar lavage fluid were harvested 24 h after the last ovalbumin challenge. Total inflammatory cell numbers in lung and bronchoalveolar lavage fluid were counted, and lymphocyte classification was performed on a minimum of 200 cells. Results are expressed as means $\pm$ SEM ($N=5$). 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: Ovalbumin inhalation + vehicle, CsA: Ovalbumin + CsA (10 mg/kg), Ovalbumin + ursolic acid (2, 20 mg/kg).

mice versus control mice, and ursolic acid (2, 20 mg/kg) also reduced the absolute number of eosinophils in bronchoalveolar lavage fluid and neutrophils and eosinophils in PBMC (Fig. 2).

3.5. Inhibitory effect of ursolic acid and CsA on absolute number of immune cell subtypes in murine ovalbumin-induced asthma lung and bronchoalveolar lavage fluid

To evaluate the effect of ursolic acid and CsA on immune cell subtypes, we performed flow cytometry. The numbers of CD3, CD4, CD8, CCR3, CD69, B220, CD23, CD11b, Gr-1 positive cells of ovalbumin-challenged mice in the lungs and bronchoalveolar lavage fluid were increased in comparison to the saline-treated group; generally, values in ursolic acid and CsA treated mice were significantly lower than in ovalbumin-challenged mice (Fig. 4).

Ursolic acid and CsA affected leukocyte subsets in lungs and bronchoalveolar lavage fluid, as shown by changes in Gr-1⁺/CD11b⁺ granulocytes and CD3⁺/CCR3⁺ eosinophils in a mouse model of asthma and by the deficits in CD3⁺/CCR3⁺ eosinophils accompanied by decreased eosinophils in bronchoalveolar lavage fluid cytospin (Fig. 2D).

3.6. Inhibition of Th2 cytokines (in vivo and in vitro) and ovalbumin-specific IgE production in bronchoalveolar lavage fluid and serum

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

As shown in Fig. 4A and D, IL-5 and IL-13 levels were significantly reduced in ursolic acid-treated (20 mg/kg) mice, CsA-treated (10 mg/kg) mice and in spleen cell culture supernatants. An important component of the allergic asthma model is the production of ovalbumin-specific IgE. Therefore, levels of anti-ovalbumin IgE were measured in serum from ovalbumin-challenged mice, and in the PBS and ursolic acid-treated groups. Ovalbumin-specific IgE levels in serum from ovalbumin-induced asthmatic mice were significantly higher than in normal mice (PBS only), and ursolic acid-treated (20 mg/kg) and CsA-treated (10 mg/kg) mice had significantly reduced ovalbumin-specific IgE (Fig. 4C). IL-4 and IFN- γ in culture supernatants were measured by ELISA; ursolic acid (20 mg/kg) and CsA inhibited Th2 cytokine (IL-4) production and did not affect Th1 cytokine (IFN- γ) in splenocytes (Fig. 4D).

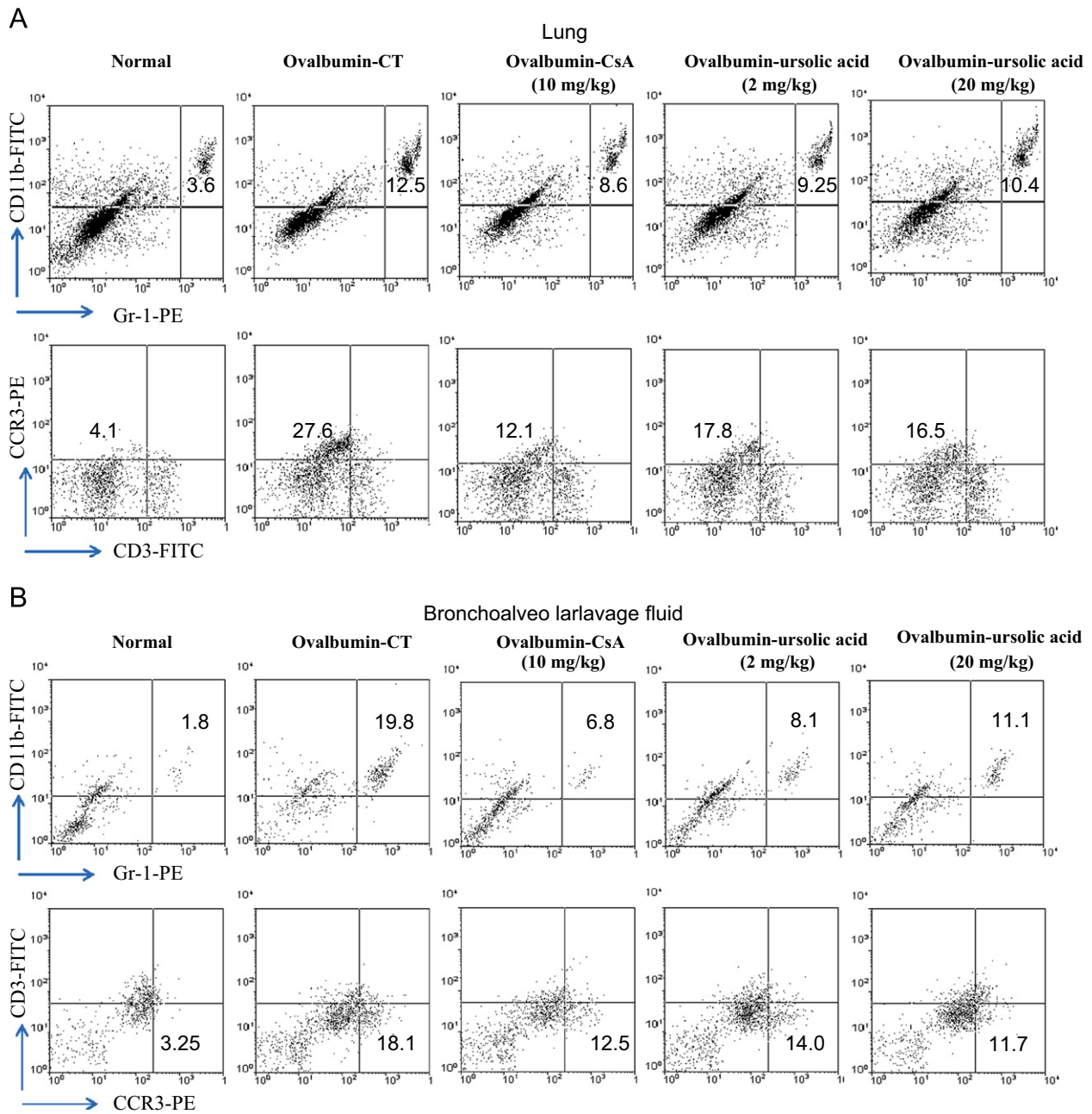


Fig. 3. Dot plot of representative flow cytometry profiles on the gated $CD3^{-}/CCR3^{+}$ and $Gr-1^{+}/CD11b^{+}$ (A, B). Results are expressed as mean $\pm$ SEM ($N=5$). Statistical significance between control and treatment groups was assessed by ANOVA or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). N: Normal BALB/c mice, CT: Ovalbumin inhalation+vehicle, CsA: Ovalbumin+CsA (10 mg/kg), Ovalbumin+ursolic acid (2, 20 mg/kg).

3.7. Detection of CCR3, IL-17, and $PPAR\gamma$ mRNA by SYBR green and FoxP3 mRNA in lung tissue

CCR3 is highly expressed on eosinophils; moreover, expression of both molecules is increased in asthmatics. Inhibiting pulmonary eosinophilia by blocking the CCR3 receptor with small molecule antagonists may reduce inflammation and airway responsiveness in asthma (Smit et al., 2006). Careful studies of $CD4^{+}IL-17^{+}$ T cells and IL-17 have revealed an important role for Th17 cells in allergic inflammation. Expression of IL-17 mRNA is up-regulated in the airways of a mouse model of asthma. IL-17 family members may be an interesting therapeutic target for severe asthma (Lloyd and Hessel, 2010). $PPAR\gamma$ possesses anti-inflammatory activity that may be mediated by suppression of pro-inflammatory cytokines and T-cell proliferation. $PPAR\gamma$

ligands suppress airway eosinophil adhesion and have proven therapeutic effects in animal models of inflammatory diseases such as asthma (Hirasawa et al., 2008; Lee et al., 2006). $CD4^{+}CD25^{+}FoxP3^{+}$ T regulatory cells (Tregs) actively restrain the inflammatory response, suppress development of autoimmune diseases, and dampen a wide spectrum of immune responses (Davidson et al., 2007).

As shown in Fig. 5, CCR3, IL-17, $PPAR\gamma$, and FoxP3 mRNAs were detected in lung tissue treated with PBS, ovalbumin plus ursolic acid-treated groups (2, 20 mg/kg) and CsA-treated group (10 mg/kg).

PCR products of CCR3 and IL-17 amplified from lung cell RNA preparations were reduced in ursolic acid-treated vs. control mice (ovalbumin-induced asthma model group), but PCR products of $PPAR\gamma$ and FoxP3 increased in the ursolic acid-treated group.

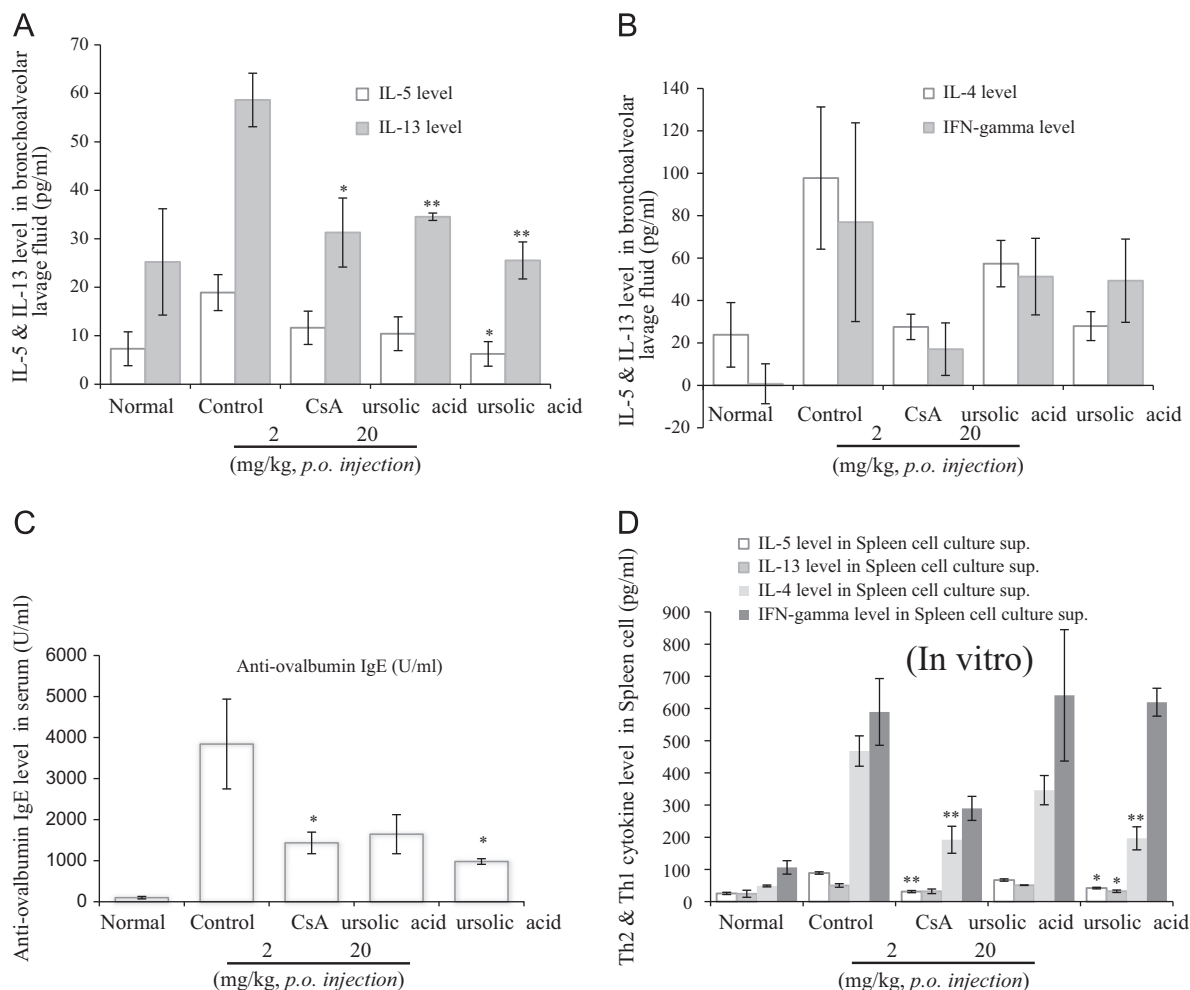


Fig. 4. Effect of ursolic acid on Th2 cytokines (IL-5, IL-13) in bronchoalveolar lavage fluid and ovalbumin-specific IgE in serum, and immunomodulatory effects of ursolic acid on ovalbumin-specific Th1/Th2 cytokine production in spleen cells. Results are expressed means $\pm$ SEM ($N=5$). Statistical 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: Ovalbumin inhalation+vehicle, CsA: Ovalbumin+CsA (10 mg/kg), Ovalbumin+ursolic acid (2, 20 mg/kg).

These results were accompanied by changes in eosinophil influx ($CD3^-/CCR3^+$) (Table 1) and BAL cytokines (IL-5, IL-13).

3.8. PPAR γ expression in EL4 T cells and RAW 264.7 macrophages, and STAT6 in RAW 264.7 macrophages

Recently, several *in vitro* studies have indicated that ursolic acid inhibits NF κ B activation through suppression of I κ B α kinase and p65 phosphorylation (Shishodia et al., 2003; Zeng et al., 2011) and selectively inhibits ROR γ t function, greatly reducing IL-17 expression in developing and differentiated Th17 cells (Xu et al., 2011), significantly reducing MMP-2 and MMP-9 enzymatic activity (Cha et al., 2011), and inhibiting the STAT3 activation pathway (Pathak et al., 2007).

To confirm that the observed effects of ursolic acid on PPAR γ expression *in vivo* are related to a direct modulation of airway inflammation, PPAR expression was analyzed in EL4 and RAW 264.7 cells *in vitro*. PPAR γ expression was assessed in 2 different cell lines by 3 independent techniques. Transcripts of PPAR γ (Fig. 6A) were detected by RT-PCR in EL4 cells and confirmed by western blotting (Fig. 6B).

RT-PCR analysis revealed that expression of PPAR γ mRNA increased 2-fold in ursolic acid-treated vs. vehicle-treated control cells (Fig. 6A).

PPAR γ expression was also investigated by western blotting of the nuclear and cytosolic fractions of RAW264.7 cells. As shown in Fig. 6A, PPAR γ and STAT6 were detected in the nucleus of LPS- and IFN- γ -treated cells. Treatment with ursolic acid markedly increased PPAR γ protein expression and inhibited STAT6. Thus, ursolic acid prevents airway inflammation and Th2 cytokine production in bronchoalveolar lavage fluid by blocking the STAT6 transcription factor in the nuclei of LPS- and IFN- γ -stimulated RAW 264.7 cells.

3.9. Ursolic acid and GATA3 expression in CD4 $^+$ T cells

As shown in Fig. 7, the levels of CD4 $^+$ /GATA3 $^+$ CD4 $^+$ T cells significantly increased in the control group (19.7%) stimulated with anti-CD3, anti-CD28, IL-4, and TGF- β in comparison to unstimulated normal CD4 $^+$ T cells (1.63%). Following ursolic acid 20, 10 μ g/ml treatment, the number of CD4 $^+$ /GATA3 $^+$ T cells decreased (3.85%, 3.61%) in comparison to the stimulated control group.

4. Discussion

Allergic asthma is a complex syndrome and chronic inflammatory disease broadly defined by inflammation of the airways associated with Penh and mucus hypersecretion (Busse and Lemanske, 2001). The airways develop predominantly eosinophilic

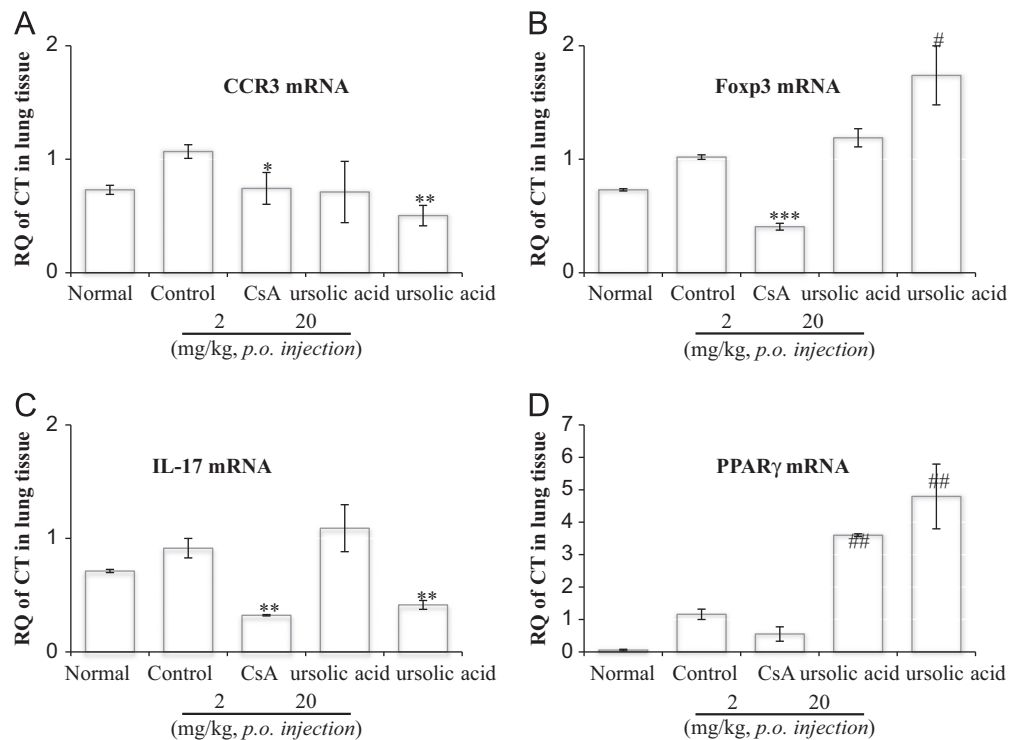


Fig. 5. Effects of ursolic acid and CsA on CCR3, Foxp3, IL-17, and PPAR γ mRNA expression in lung tissue of an ovalbumin-induced murine model of asthma. BALB/c mice were injected, inhaled, and sprayed with ovalbumin for 9 weeks (3 times a week) for asthma induction. Three experimental groups were treated with different concentrations of ursolic acid and CsA for 5 weeks (3 times a week). At the end of the experiment, lungs were removed and analyzed by real-time PCR. WT: Normal BALB/c mice, CT: Ovalbumin inhalation (control), Ovalbumin+CsA (10 mg/kg), Ovalbumin+ursolic acid (2, 20 mg/kg). The results are expressed as the RQ (relative quantity).

Table 1

Quantification by FACS analysis of immune cell subtypes in lung, bronchoalveolar lavage fluid, and MLN.

Cell phenotypes in lung, bronchoalveolar lavage fluid and MLN		Normal Balb/c	Ovalbumin-induced asthma mice (absolute no.)			
			Control	Cyclosporin A	Ursolic acid (2 mg/kg)	Ursolic acid (20 mg/kg)
CD3 ⁺ /CD4 ⁺ ($\times 10^5$ cells)	Lung	2.60 $\pm$ 0.77	9.60 $\pm$ 0.18	3.60 $\pm$ 0.44^c	5.40 $\pm$ 1.36^a	3.20 $\pm$ 0.32^c
CD3 ⁺ /CD8 ⁺ ($\times 10^5$ cells)		1.70 $\pm$ 0.43	4.10 $\pm$ 1.14	1.90 $\pm$ 0.09	2.90 $\pm$ 0.26	1.70 $\pm$ 0.19
Gr-1 ⁺ /CD11b ⁺ ($\times 10^6$ cells)		0.60 $\pm$ 0.01	7.20 $\pm$ 0.55	2.30 $\pm$ 0.30^c	3.40 $\pm$ 0.06^b	2.70 $\pm$ 0.18^c
CD3 ⁺ /CCR3 ⁺ ($\times 10^5$ cells)		0.70 $\pm$ 0.02	13.50 $\pm$ 1.05	3.30 $\pm$ 0.46^c	5.90 $\pm$ 0.48^b	3.90 $\pm$ 0.05^c
CD3 ⁺ /CD69 ⁺ ($\times 10^5$ cells)		0.70 $\pm$ 0.06	7.30 $\pm$ 0.23	2.70 $\pm$ 0.20^c	3.50 $\pm$ 0.31^c	2.70 $\pm$ 0.15^c
B220 ⁺ /CD23 ⁺ ($\times 10^5$ cells)		0.50 $\pm$ 0.09	7.10 $\pm$ 0.77	1.20 $\pm$ 0.18^b	3.90 $\pm$ 0.31^a	1.40 $\pm$ 0.02^b
CD3 ⁺ /CD4 ⁺ ($\times 10^4$ cells)	Broncho alveolar lavage fluid	0.50 $\pm$ 0.29	39.40 $\pm$ 14.04	8.20 $\pm$ 0.11^a	15.10 $\pm$ 1.29	7.50 $\pm$ 3.35 ^a
CD3 ⁺ /CCR3 ⁺ ($\times 10^4$ cells)		0.40 $\pm$ 0.29	19.80 $\pm$ 8.75	4.30 $\pm$ 0.72	8.30 $\pm$ 1.03	4.70 $\pm$ 0.14
Gr-1 ⁺ /CD11b ($\times 10^4$ cells)		0.20 $\pm$ 0.09	17.20 $\pm$ 5.11	2.60 $\pm$ 0.23^a	7.00 $\pm$ 2.81	3.60 $\pm$ 0.98^a
CD3 ⁺ /CD4 ⁺ ($\times 10^5$ cells)	Mediastinal lymph node (MLN)	3.10 $\pm$ 1.19	20.50 $\pm$ 3.26	7.70 $\pm$ 1.82^b	14.20 $\pm$ 4.55	12.10 $\pm$ 0.97^a
CD3 ⁺ /CD8 ⁺ ($\times 10^5$ cells)		1.20 $\pm$ 0.39	8.40 $\pm$ 1.08	4.80 $\pm$ 0.44^a	7.30 $\pm$ 2.43	6.30 $\pm$ 1.27
CD3 ⁺ /CD69 ⁺ ($\times 10^5$ cells)		0.60 $\pm$ 0.14	10.00 $\pm$ 3.29	1.60 $\pm$ 0.50^a	4.90 $\pm$ 0.33	3.60 $\pm$ 0.13
Gr-1 ⁺ /CD11b ⁺ ($\times 10^5$ cells)		0.30 $\pm$ 0.15	4.20 $\pm$ 1.15	1.30 $\pm$ 0.39^a	1.80 $\pm$ 0.49^a	1.80 $\pm$ 0.32^a
B220 ⁺ /CD23 ⁺ ($\times 10^5$ cells)		0.20 $\pm$ 0.04	6.30 $\pm$ 2.31	1.00 $\pm$ 0.16^a	3.20 $\pm$ 0.96	1.40 $\pm$ 0.18^a

Absolute numbers of immune cell subtypes in lung were counted (described in Section 2). Lung, bronchoalveolar lavage fluid, and MLN cells were stained with anti-CD3, CD4, CD8, CCR3, CD69, B220, CD23, CD11b, Gr-1 mAb, etc. Each point represents the mean $\pm$ SEM of 5 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.

^a *p* < 0.05.

^b *p* < 0.01.

^c *p* < 0.001.

inflammation with prominent edema and mucus production in response to antigen inhalation (Medoff et al., 2008). Th2 cells and their signature cytokines IL-4, IL-5, and IL-13 have key pathogenic roles in asthma (Tomkinson et al., 2001). Murine models of allergic pulmonary inflammation and airway hyperresponsiveness have elegantly demonstrated that Th2 cells and the cytokines they secrete are essential for the development of eosinophilia and airway hyperresponsiveness (Wills-Karp et al., 1998). Eosinophilic

inflammation is regulated by activated T lymphocytes in the airways that secrete the Th2 cytokine IL-5. This cytokine is a central mediator of eosinophilic inflammation through effects on the proliferation, differentiation, and activation of eosinophils (Yamaguchi et al., 1988).

Ursolic acid exists widely in food products (vegetable oils) and in more than 120 plant species (Somova et al., 2003), many of which are used in traditional medicine. It has several

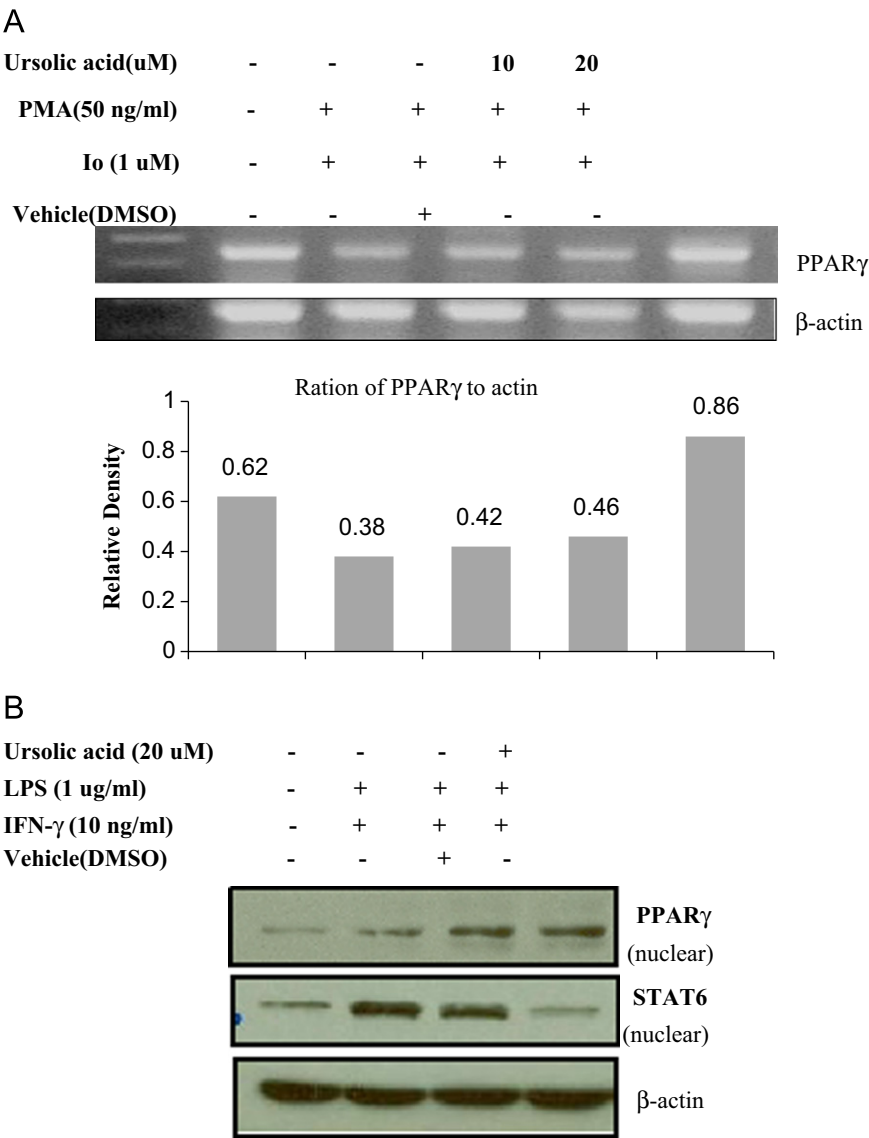


Fig. 6. Effects of ursolic acid on PMA or ionomycin-induced PPAR γ mRNA expression in EL-4 cells (A). Expression of LPS or IFN- γ -induced PPAR γ and STAT6 expression in RAW 264.7 cells by ursolic acid (B). EL-4 T cells (5×10^5 cells/ml) were treated with PMA (50 ng/ml) and ionomycin (1 μ M) for 12 h in the absence or presence of ursolic acid. Total cellular RNA was isolated using TRIzol reagent, according to the manufacturer's instructions (A). PPAR γ and STAT6 were induced in RAW 264.7 cells by LPS and IFN- γ treatment for 24 h. Proteins in total cell lysates were resolved on 7% SDS-PAGE for PPAR γ and STAT6. β -actin served as a loading control (B).

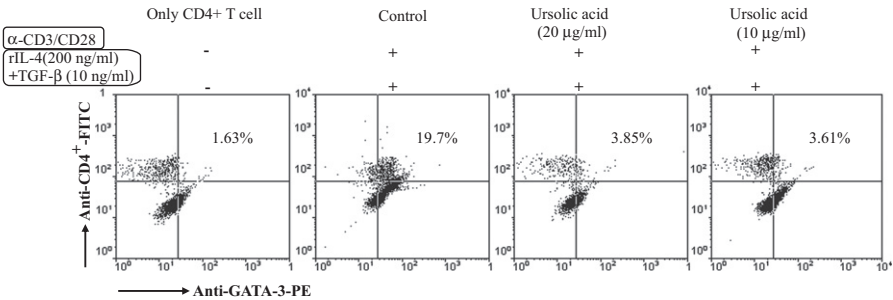


Fig. 7. Dot plot of intracellular staining. X-axis: PE intensity of GATA-3. Y-axis: FITC intensity of CD4. Isolated CD4 $^+$ T cells were cultured without (normal group) or with (stimulated group) anti-CD3, anti-CD28, rIL-4, TGF- β , and ursolic acid (20, 10 μ g/ml) added to RPMI medium and stained with FITC-conjugated mAb to CD4 and PE-conjugated mAb to GATA-3.

pharmacological uses, including anti-inflammatory (Fan et al., 2004) and analgesic (Costa et al., 2003). Moreover, ursolic acid provide hepatoprotective (Liu, 1995), antiallergic (Banno et al., 2004), anti-ulcer (Ovesná et al., 2004), cardioprotective (Senthil

et al., 2007), anti-inflammatory, analgesic (Vasconcelos et al., 2006), anti-hyperlipidemic (Ahmad et al., 2006), anti-diabetic (Zhang et al., 2006), and immunomodulatory (Jang et al., 2009) activities. ursolic acid has also been used to treat kidney diseases

(Yoshimura et al., 2003), hypertension (Somova et al., 2004), tumorigenesis (Huang et al., 1994), tumor promotion (Tokuda et al., 1986), and angiogenesis (Sohn et al., 1995).

Several of the effects of ursolic acid are mediated by suppression of lipoxygenase, COX-2, MMP-9, and iNOS (Cha et al., 1996; Suh et al., 1998), all of which are regulated by NF- κ B. Ursolic acid inhibits NF- κ B activation by carcinogenic agents through suppression of I κ B α kinase and p65 phosphorylation (Shishodia et al., 2003; Zeng et al., 2011). Ursolic acid also significantly suppresses MMP-2 and MMP-9 activity in cancer cell lines such as B16F10 and HT-1080 (Cha et al., 2011).

However, the mechanism underlying the biological efficacy of ursolic acid remains elusive, and its influence on asthma has not been addressed.

CsA has been shown to provide a clinical benefit in patients with chronic severe steroid-dependent asthma (Nizankowska et al., 1995). The effects of CsA and corticosteroids on eosinophilic inflammation have been demonstrated using various asthma models (Nagai et al., 1995; De, Bie et al. (1996); Eum et al., 1996). CsA has been shown to inhibit single-allergen induced allergic inflammation such as eosinophilic and lymphocytic infiltration and mRNA expression for 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. These cells are often considered to play a major role in inducing airway hyperresponsiveness. Ursolic acid and CsA prevented the development of Penh (Fig. 1C), lung inflammation (Fig. 1D), airway eosinophilia (Figs. 2 and 3; Table 1), and decreased Th2 cytokine levels (IL-5 and IL-13) (Fig. 4A) in bronchoalveolar lavage fluid. These results demonstrate ursolic acid and CsA inhibition of lung allergic responses in the ovalbumin-induced asthma model.

Asthma produces immune abnormalities in a wide variety of cell populations. Thus, another goal in asthma research is the evaluation of specific cell subpopulations. Immunophenotyping by flow cytometry showed similar patterns of total lymphocyte numbers in bronchoalveolar lavage fluid and lung.

Ursolic acid and CsA effects on leukocyte subsets in the lungs and bronchoalveolar lavage fluid were marked by changes in the numbers of Gr-1⁺/CD11b⁺ granulocytes and CD3⁺/CCR3⁺ eosinophils in a mouse model of asthma (Table 1 and Fig. 3), and the decrease in CD3⁺/CCR3⁺ eosinophils was accompanied by concurrent eosinophil reductions in bronchoalveolar lavage fluid cytospin (Fig. 2D). Ursolic acid and CsA also inhibited B cell-dependent production of ovalbumin-specific IgE in serum (Fig. 4C).

CC chemokine receptor 3 (CCR3) is expressed on eosinophils, MC, basophils, and a subset of Th2 lymphocytes (Kitauro et al., 1996). Eosinophils express the chemokine receptor CCR3 and are likely recruited via production of the STAT6-dependent chemokines CCL11 and CCL24 (in mice and humans) (Lilly et al., 2001).

The number of CCR3⁺ cells is increased in bronchial biopsies in asthma (Ying et al., 1999) and CCR3 is considered an important potential therapeutic target in asthma and other allergic diseases (De, Lucca (2006)). Inhibition of pulmonary eosinophilia by blocking the CCR3 receptor with antagonists may reduce inflammation and airway responsiveness in asthma. This approach has been investigated by several research groups.

Moreover, eosinophils express Gr-1; therefore, eosinophil populations may constitute a substantial portion of the CD11b⁺Gr-1⁺ population. Our results (Table 1 and Fig. 3) showed that Gr-1⁺ cells increased with ovalbumin challenge but significantly decreased with ursolic acid and CsA treatment in mice. We observed significant correlations between IL-5 levels and CCR3 expression on eosinophils. We hypothesized that ursolic acid prevents Penh by down regulating IL-5 production and reducing eosinophilia.

The balance of Th1/Th2 cells is thought to be determined by the expression ratio of T-bet/GATA-3 and is important for inducing autoimmune and allergic responses (Kiwamoto et al., 2006). However, 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 (Weaver et al., 2007). Th17 cells have emerged as a major player in inflammatory and autoimmune diseases via production of pro-inflammatory cytokines interleukin-17 (IL-17), IL-17F, and IL-22. Th17 cells are responsible for host defense by producing pro-inflammatory cytokines, most notably IL-17 (Bauquet et al., 2009). Differentiation of Th17 cells and the associated cytokine production is directly controlled by ROR γ t. Xu et al. (2011) reported that ursolic acid suppresses IL-17 production by selectively antagonizing the function of ROR γ t. PPAR γ is expressed by CD4⁺ T cells and regulates their differentiation into at least 4 functionally distinct subsets referred to as T helper (Th) 1, Th2, Th17 (Hontecillas and Bassaganya-Riera, 2007). In monocytes and macrophages, PPAR γ agonists inhibit expression of pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 (Ricote et al., 1998). Moreover, it has been implicated as a negative regulator of Inflammation. This negative regulation of pro-inflammatory genes by PPAR γ is termed trans-repression and occurs in a ligand-dependent manner by antagonizing the activities of other transcription factors, such as NF- κ B and AP-1 (Ricote and Glass, 2007). The observation that PPAR γ is expressed in T cells and may modulate T-cell cytokine production prompted us to examine its role in a murine model of a Th2-mediated disease, allergic asthma.

Th17 cells are controlled by several transcription factors such as ROR γ t, IRF4, BATF, FoxP3, T-bet, and PPAR γ (Hwang, 2010). Our studies have shown that ursolic acid, a potential PPAR- γ agonist, inhibits production of IL-5 and IL-17 (Fig. 5), which play critical roles in the differentiation of Th2 and Th17 cells, respectively.

Various transcription factors such as c-maf, GATA-3, NFATc1, NIP45, JunB, and STAT6 induce or augment Th2 cytokine production, although only c-maf and GATA-3 are expressed selectively in Th2 cells (Glimcher and Murphy, 2000). IL-25 (IL-17E) induces Th2 responses including IL-4, IL-5, and IL-13 expression, increased serum IgE, airway eosinophil influx, mucus production, and epithelial cell hyperplasia (Fort et al., 2001). Monoclonal antibody neutralization of IL-25 reverses Th2-associated pathology in the lungs through a signal transducer and activator of transcription 6 (STAT6) and Th2 cell-dependent mechanism. Studies have shown that IL-4 and/or IL-13 induce TGF- β production through activation of the STAT6 pathway. Regulatory T cells inhibit eosinophil recruitment, mucus hyperproduction, and Th2 cytokine release (IL-4, IL-5, and IL-13) (Nemeth et al., 2010). Additional studies have shown that STAT6-deficient mice were protected from Th2-mediated bronchial inflammation and airway hyperresponsiveness (Akimoto et al., 1998). STAT6 is an important factor for the development of airway hyperresponsiveness in asthma. Regulation of chemokine production is dependent on the STAT6-mediated pathway (Zhang et al., 2000), a pathway primarily activated by Th2 cells. In particular, GATA-3 promotes expression of several Th2 cytokines, including IL-4, IL-5, and IL-13 (Asnagli and Murphy, 2001), and GATA-3 expression is regulated by STAT6 in Th2 cells (Awasthi et al., 2008). We aimed to understand the impact of cellular signals on PPAR γ activity in EL-4 and macrophages. We have demonstrated an inverse relationship between PPAR γ and STAT6 in RAW264.7 macrophages (Fig. 6B).

Nuclear factor-kappaB (NF- κ B) is a major family of transcription factors activated during the inflammatory response in asthma. Because NF- κ B has many functions in the cell and inflammatory cascade, it has been implicated as a mediator of many immune suppressive agents including CsA. IL-17 activates NF- κ B and mitogen-activated protein kinase (MAPK) pathways

(Maitra et al., 2007). MAPK activates similar inflammatory genes and is activated in patients with severe asthma (Bhavsar et al., 2008). An antisense oligonucleotide that blocks p38 MAPK demonstrated efficacy in a murine asthma model (Duan et al., 2005). p38 MAPK plays a key role in activation of GATA-Binding protein (GATA) 3, a transcription factor that regulates Th2 cell differentiation and expression of Th2 cytokines (Maneechotesuwan et al., 2007).

There might be a balance between Foxp3+ Treg cells and Th17 cells, and this critical balance is mediated by the acute phase protein IL-6 (Awasthi et al., 2008). Foxp3 functions as a transcriptional repressor for various transcription factors such as nuclear factor of activated T cells (NFAT), NF- κ B, and Runx1 (Bettelli et al., 2005), which could be a part of the mechanisms for Foxp3-mediated suppression of immune responses. We propose that induction of Foxp3 is the mechanism for suppression of IL-17 (Fig. 5B and C).

Ursolic acid inhibits NF- κ B activation through suppression of I κ B α kinase and p65 phosphorylation (Shishodia et al., 2003; Zeng et al., 2011). Ursolic acid exerts anti-inflammatory and analgesic effects by suppressing COX-2 and iNOS expression through down-regulation of NF- κ B binding activity (Cha et al., 1996; Suh et al., 1998). Numerous papers have suggested that MAPKs participate in the regulation of NF- κ B transcriptional activity. It is reasonable to assume our results might be caused by up-regulation of PPAR γ and inhibition of GATA-3 (Fig. 7) and STAT6 through NF- κ B pathway.

Because Th17 cells activate NF- κ B pathways, it is plausible that the therapeutic effect of ursolic acid via inactivation of Th2 and Th17 cells is mediated by the synergistic effect of the IL-13-STAT6 and IL-17-NF- κ B pathways. The results of this study suggest the possibility of using ursolic acid as a new therapeutic material for respiratory diseases including allergic asthma, although further studies are required. Although the precise mechanism of action needs further investigation, this is the first report that ursolic acid significantly inhibits airway inflammation and hyperresponsiveness in a mouse model of allergic asthma. Therefore, we propose that ursolic acid could be used to treat pathologic inflammatory airway disorders such as allergic asthma.

Although the results are not sufficient to describe the precise mechanisms, our data demonstrate that ursolic acid and CsA have profound inhibitory effects on airway inflammation in a mouse model of asthma, and this effect was caused by suppression of Th2 cytokines (IL-5), B cell-dependent production of ovalbumin-specific IgE, and eosinophil CCR3 expression. It is reasonable to assume the anti-inflammatory and anti-asthmatic activity of ursolic acid might be caused by inhibition the GATA-3 and STAT6 pathways.

5. Conclusion

Ursolic acid treatment increased expression of PPAR γ and reduced expression of GATA-3 and STAT6, leading to decreased antigen-induced Penh, lung inflammation, eosinophilia, and cytokine production as well as serum levels of antigen-specific IgE. Thus, ursolic acid may be an antagonist of Th2 (IL-5, IL-13) and Th17 cytokines (IL-17) and have a therapeutic effect on allergic asthma.

Acknowledgments

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