



18 β -Glycyrrhetic acid, the major bioactive component of Glycyrrhizae Radix, attenuates airway inflammation by modulating Th2 cytokines, GATA-3, STAT6, and Foxp3 transcription factors in an asthmatic mouse model

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ARTICLE INFO

Keywords:

18 β -glycyrrhetic acid
Asthma
IL-5
IL-13
GATA-3
Foxp3

ABSTRACT

18 β -Glycyrrhetic acid (18Gly), the major bioactive component of Glycyrrhizae Radix, possesses anti-ulcerative, anti-inflammatory, and other pharmacological properties. Although 18Gly is associated with immunoregulatory functions of allergic diseases, the pathophysiological mechanisms of 18Gly action in allergic inflammatory lung disease have not been examined. Moreover, there are no *in vivo* studies on the anti-asthmatic effects of 18Gly in allergic asthma. We investigated its effect and mechanism of action in airway inflammation in a BALB/c mouse model of allergic asthma.

Interestingly, 18Gly strongly suppressed airway hyperresponsiveness, accumulation of inflammatory cells, and levels of T helper type 2 (Th2) cytokines (interleukin (IL)-5 and IL-13) in bronchoalveolar lavage fluid (BALF). It also attenuated lung IL-5, IL-13, and IL-4 expression, but it upregulated peroxisome proliferator-activated receptor gamma (PPAR γ) mRNA expression in lungs. Moreover, it exerted immunomodulatory effects by suppressing Th2 cytokines (IL-5, IL-13) production through upregulation of forkhead box p3 (Foxp3), and downregulation of signal transducer and activator of transcription (STAT6), GATA-binding protein 3 (GATA-3), and retinoic acid-related orphan receptor γ t (ROR γ t) expression. These results suggest that the anti-asthmatic activity of 18Gly may occur by the suppression of IL-5, IL-13, and OVA-specific Immunoglobulin E (IgE) production through inhibition of the ROR γ t, STAT6, GATA-3 pathways and upregulation of the Foxp3 transcription pathway. Also, 18Gly treatment was protective against the oxidative stress by inducing significant decrease of reactive oxygen species (ROS) generation in MH-S alveolar macrophage cells. Our results suggest that 18Gly can improve allergic asthma and can be a novel therapeutic component for the treatment of allergic asthma.

1. Introduction

Allergic asthma is an inflammatory disorder of the airways associated with pathological structural changes such as hyperplasia of mucus glands and myofibroblasts, subepithelial fibrosis (Elias et al., 1999), and characterized by airflow obstruction, airway inflammation, T helper type 2 (Th2) cytokine production, airway hyperresponsiveness (AHR), and infiltration of a variety of inflammatory cells, including eosinophils and T-lymphocytes (Busse and Lemanske, 2001).

Eosinophils have since long been associated with Th2 responses in allergic asthma, and Th2 cytokines such as IL-5, and IL-13 have been shown to cause eosinophilic inflammation of the airways (Pope et al., 2001). In particular, IL-4 and IL-13 are known to promote isotype

switching to IgE (Fish et al., 2005). Furthermore, IL-4, IL-5, and IL-13 are important in the development and persistence of eosinophilic, allergic airway inflammation (Barnes, 2008).

It is well known that GATA-binding protein 3 (GATA-3) is important for the differentiation of uncommitted naive T cells into Th2 cells, and it also regulates the secretion of Th2-type cytokines such as IL-4, IL-5, and IL-13. 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 cells (Chakir et al., 2003). T-bet-deficient mice undergo airway remodelling and show increased expression of GATA-3 and induction of Th2-type cytokines (IL-4, IL-5, IL-13), confirming that T-bet is an important regulator of GATA-3 (Finotto et al., 2002).

The important role of signal transducer and activator of transcrip-

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tion (STAT) 6 in asthma is demonstrated by the inability of STAT6-deficient mice to develop asthma. STAT6 interacts directly with GATA-3 to activate Th2 cytokine promoters and enhancers (Ting et al., 1996). Targeted expression of STAT6 in epithelial cells is sufficient for IL-13-induced AHR and mucus production (Kuperman et al., 2002).

Forkhead box p3 (Foxp3)⁺ T regulatory cells (Tregs) have suppressive effects on other T cells and may have an important role in regulating Th2 cell function in asthma (Larché, 2007). Foxp3⁺ Tregs can suppress Th2 maturation (Xu et al., 2003), possibly by inhibiting GATA-3 expression (Mantel et al., 2007). T-bet, GATA-3, STAT6, and Foxp3 orchestrate the allergic inflammatory responses in asthma, and these transcription factors may therefore be attractive targets for the development of anti-asthmatic drugs.

Glycyrrhizae Radix (general name Licorice) is the root of three Glycyrrhiza species, i.e., *Glycyrrhiza uralensis* Fischer, *Glycyrrhiza glabra* Linné, and *Glycyrrhiza inflata* Batalin used clinically in ancient Egyptian, Greek and China (Sun and Pan, 2006). For popular use, it has been cultivated in many countries, such as China, Korea, Japan and Europe. It has been used in medicine for thousands of years. According to the traditional use, Glycyrrhizae Radix is primarily effective for asthma with coughing, excessive phlegm, fatigue and for relieving drug toxicity. It is a widely used herbal medicine native to southern Europe and parts of Asia and has beneficial applications in the medical sector (Wang et al., 2013). Recent studies showed that Glycyrrhizae Radix has anti-inflammatory, antioxidant, and antiallergic effects (Li et al., 2015; Singh et al., 2016; Dong et al., 2014; Shin et al., 2007).

18 β -glycyrrhetic acid (18Gly), the major bioactive component of Glycyrrhizae Radix, possesses anti-inflammatory (Gumprich et al., 2005) and other pharmacological properties including antiproliferative activity in Jurkat T cells (Fu et al., 2013) and anti-invasive effects via inhibition of PI3K/Akt-dependent NF- κ B activity (Jayasooriya et al., 2014).

Glycyrrhizin (glycyrrhizic acid), a major active component of Glycyrrhizae Radix, is a triterpene glycoside which consists of one molecule of 18Gly. Glycyrrhizin alleviates asthmatic features in mice and has anti-inflammatory effects (Ram et al., 2006). 18Gly, an aglycone of glycyrrhizin, inhibits passive cutaneous anaphylaxis (Park et al., 2004). Additionally, 18Gly reduces inflammation and articular damage in collagen-induced arthritis (Puchner et al., 2012).

Both glycyrrhizic acid and 18Gly reduce inflammatory cytokines (TNF- α , IL-1, IL-6) production via PI3K/Akt pathway activation (Kao et al., 2010). Glycyrrhizic acid also attenuates the development of carrageenan-induced lung injury (Menegazzi et al., 2008). Since 18Gly can be transformed from glycyrrhizic acid, we hypothesized that 18Gly can inhibit airway and lung inflammation.

Despite its numerous pharmacological activities including anti-inflammatory effects, the molecular mechanisms of action of 18Gly are not yet fully elucidated. There are a few reports describing the biological activity of 18Gly, and further investigations are needed. Moreover, there are no *in vivo* studies on the anti-asthmatic effects of 18Gly and the underlying mechanisms remain unclear. We hypothesized that 18Gly might be able to improve the inflammatory response in a mouse model of allergic asthma. Therefore, we used a mouse model of allergic asthma to evaluate the pharmacological activity of 18Gly.

2. Materials and methods

2.1. 18Gly and other reagents

18Gly, ovalbumin (OVA), aluminium hydroxide, and methacholine were purchased from Sigma. The purity of the 18gly 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 reagents were of analytical grade and were obtained from Sigma unless indicated otherwise.

2.2. Animals

Five-week-old female BALB/c mice, weighing 20–25 g, were purchased from Daehan Biolink Co. Ltd. (Eumsung, Republic of Korea) and maintained in specific pathogen-free conditions at our animal breeding facility. Our animal 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 Daejeon University, Republic of Korea.

2.3. OVA sensitisation, inhalation, and enhanced pause (Penh) measurement

OVA preparation, delivery, and Penh measurements were performed as previously described, with certain modifications (Kim et al., 2011). 18Gly (2 and 20 mg/kg), cyclosporine A (CsA, 10 mg/kg), and rosiglitazone (Rosi., 2 mg/kg) were orally administered 3 times a week for 5 weeks. One day after the last OVA exposure, Penh values were determined and samples (BALF, lung cells, and serum) were collected for molecular analyses. The protocols for sensitisation, inhalation challenge, and Penh measurement have previously been described in detail Fig. 1A (Kim et al., 2014b).

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).

2.4. Bronchoalveolar lavage fluid (BALF)

Bronchoalveolar lavage (BAL) is a medical procedure which is called “bronchoalveolar washing” and the fluid is squirted into a small part of the lung and then collected for examination as follows. Immediately after Penh measurement, mice were anesthetized with an i.p. injection of 10% chloral hydrate (0.2 mL/20 g body weight) in phosphate-buffered saline (PBS). The trachea was cannulated and BALF were collected by washing the airway lamina. Briefly, cells in the lungs were recovered by flushing 1 mL BAL (1 mM ethylenediaminetetraacetic acid (EDTA), 10% foetal bovine serum (FBS), PBS) into the lungs via the trachea. Total cell counts were determined and 100 μ L of fluid were cytospun onto glass slides using a cytospin centrifuge (400 $\times$ g, 4 min). Differential cell counts were performed after staining with a Diff-Quik Stain Set (Baxter Healthcare Corp., Miami, FL, USA). The rest of the BALF supernatant was stored at -25°C for further experiments.

2.5. Digestion of pulmonary tissue and cell preparation

Single cell suspensions from lung tissues and BALF were isolated by mechanical disruption in Roswell Park Memorial Institute (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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 2% heat-inactivated FBS. Briefly, the extracted right lungs were minced using sterile scalpels followed by incubation in PBS containing 1 mg/mL collagenase IV and 2 mg/mL dispase for 40 min at 37°C in a sterile polypropylene tube. After incubation, lung tissue was vigorously pipetted up and down, and then filtered using a 70- μ m cell-strainer. The remaining lobe of the left lung was stored for RNA extraction (upper lobe) and histological analyses (bottom lobe).

Between 2 and 4×10^3 cells were spun onto glass slides (Cytospin centrifuge, 400 $\times$ g, 4 min). Cells were counted manually using a hemocytometer chamber. Differential counts were performed according to standard morphological criteria.

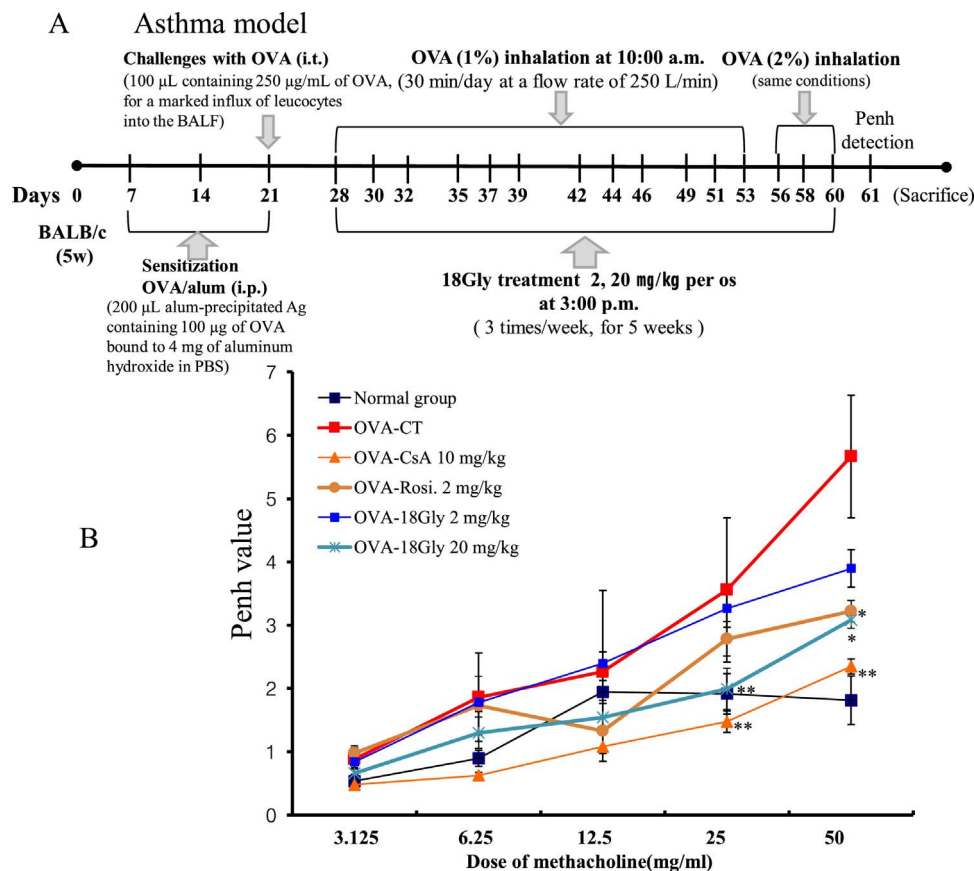


Fig. 1. Scheme of OVA sensitization and challenge protocol (A). The effects of 18Gly on AHR in OVA-challenged mice were measured using non-invasive whole-body plethysmography (B). Effects of 18Gly on histological markers of airway inflammation (H & E, M-T, and PAS staining) in lung tissue in the OVA-induced mouse model of allergic asthma (C). Airway responsiveness to aerosolised methacholine was measured with a Buxco box. Mice were placed in the main chamber and nebulised first with PBS, then with increasing doses (3.125–50 mg/mL) of methacholine for 3 min per nebulization. Data represent the mean $\pm$ SEM of 6 independent experiments. N = 6. * P < 0.05, ** P < 0.01, and *** P < 0.001 for the experimental groups versus the OVA control group comparisons. N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18- β -glycyrrhetic acid (2 or 20 mg/kg).

2.6. Hematoxylin-eosin (H & E), masson-trichrome (M-T), and periodic acid-Schiff (PAS) staining

One day after the last OVA exposure, mice were sacrificed and the lungs were harvested and subjected to histological analysis using a previously described protocol, with some modifications (Kim et al., 2011).

Briefly, the lung tissues were fixed with formalin, embedded in paraffin, and cut into 3- μ m sections. Infiltration of inflammatory cells into lung tissues was assessed by microscopic investigation of sections stained with H & E.

Other sections were stained with either PAS to quantify airway goblet cells and mucus secretion, or with M-T to determine collagen deposition in the lungs.

Frozen sections of each tissue sample were prepared. The tissue was subsequently mounted and covered with Dako-mounting medium (Dako Cytomation; Carpinteria, CA, USA) and a coverslip. All slides were examined in a blinded manner, and the degree of airway inflammatory cell infiltration and its distribution were 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 (Kim et al., 2011).

2.7. Antibodies and fluorescence-activated cell sorting (FACS) analysis

All antibodies (anti-cluster of differentiation (CD)3, CD4, CD8, CD11b, Gr-1, B220, and CD23) for flow cytometry were purchased

from Becton Dickinson (BD) PharMingen (San Diego, CA, USA). Briefly, cells (1×10^6) were washed with flow cytometry staining buffer. Then, cells from lung tissues and BALF were stained with the indicated antibodies in staining buffer (PBS containing 1% FBS and 0.01% NaN_3) for 10 min on ice, and analysed by 2-colour flow cytometry using a FACS Calibur device using CellQuest software (BD Biosciences, Mountain View, CA, USA).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Interleukins (IL-5, IL-13, etc.) levels in BALF and anti-OVA IgE levels in serum 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-induced interferon gamma ($\text{IFN-}\gamma$), IL-4, IL-5, and IL-13 production were 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, USA) with or without 1 μ g/mL OVA in a humidified atmosphere with 5% CO_2 at 37 $^\circ\text{C}$. The culture supernatants were collected and assayed by ELISA for OVA-induced $\text{IFN-}\gamma$, IL-4, IL-5, and IL-13. All data represent the mean and standard deviation from at least 3 separate experiments and were compared by analysis of variance (ANOVA).

2.9. Quantitative real-time PCR (RT-qPCR) in vivo

Total cellular RNA was extracted from the lung by the phenol-

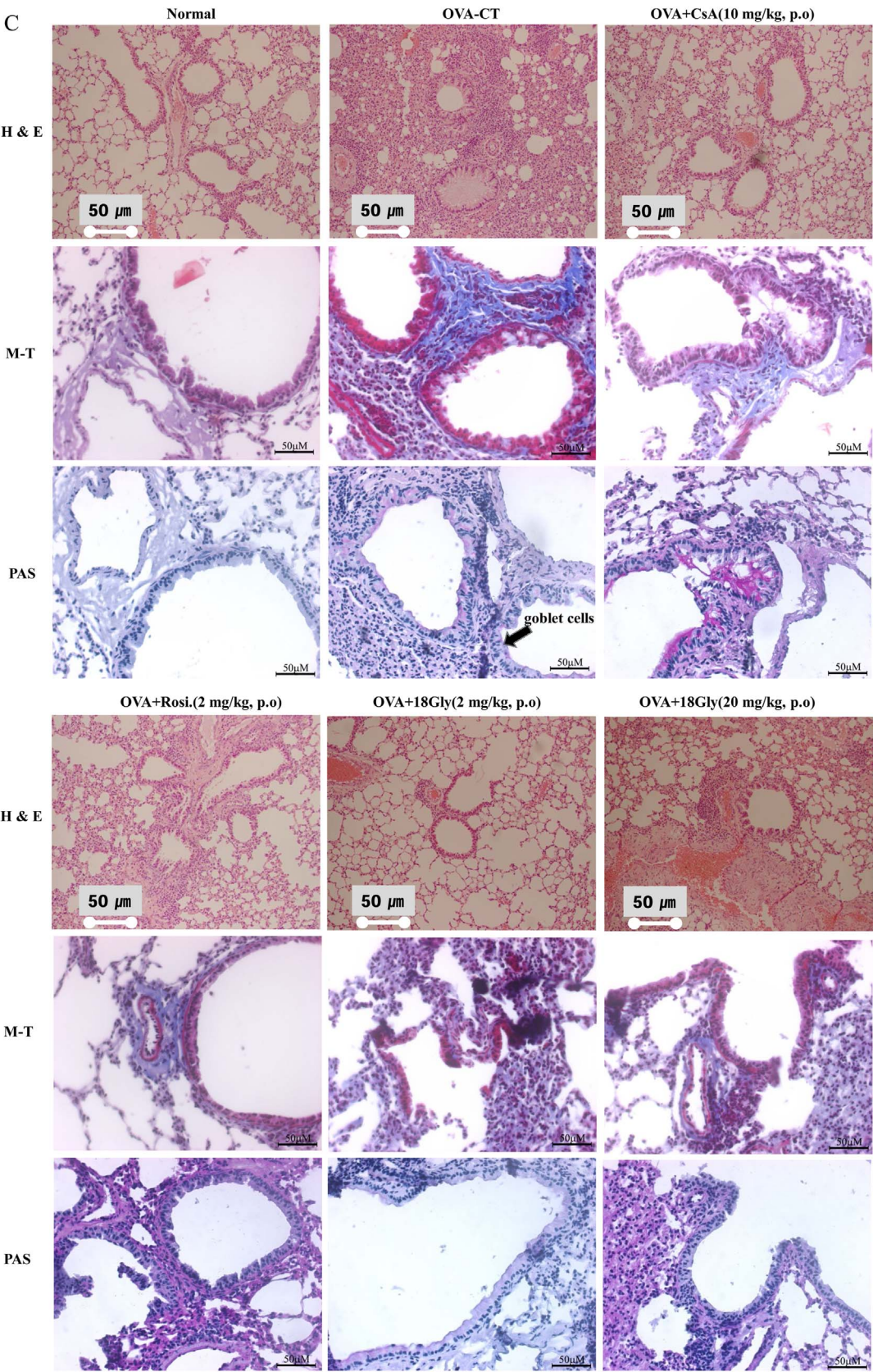


Fig. 1. (continued)

chloroform method (RNAsol^B) and cDNA was synthesised from 3 µg of total RNA using a ReverTraAce-a-cDNA Synthesis kit (Toyobo) according to the manufacturer's instructions. RT-qPCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, USA) using the following TaqMan probes and primers.

The TaqMan probes for PPAR γ (Mm, 00440940, FAM dye-labelled, ABI), IFN- γ (Mm, 00801778, FAM dye-labelled, ABI), and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (pn, 4352339E, VIC dye-labelled, ABI) were selected using Assays-on-Demand Gene Expression Products (ABI). GAPDH (ABI) gene expression was used as an endogenous control. The primers for mouse IL-5, 5'-AGCACAGTGGTGAAAGAGACCTT-3' and 5'-TCCAATGCATAGCTGGTGATT-3'; IL-13, 5'-CAGTTGCAATGCCATCCACA-3' and 5'-AGCCACATCCGAGGCCTTT-3'; IL-4, 5'-GGATGTAACGACAGCCCTCT-3' and 5' –GTGTTCTTGTGCGTAAG-3' were used for analysis with SYBR Green PCR Mastermix (ABI) using β -actin as the internal standard. 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, 1 min at 60 °C, and 1 min at 72 °C. The cycle number at which the emission intensity of the sample rose above the baseline was referred to as the RQ (relative quantity) and was proportional to the target concentration. RT-qPCR was performed in duplicates and analysed according to the system manual (threshold: 0.05, baseline: 6–15 cycles).

2.10. Preparation of lung tissue for immunofluorescence

For immunofluorescence staining, the animals were intraperitoneally anesthetized 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 Finetechnical Co., Tokyo, Japan) and rapidly frozen. The blocks were stored at –80 °C until sliced into 5-µm sections using a Cryostat Microtome (Thermo Electron Corp., Cambridge, UK).

2.11. Immunofluorescence and nucleus staining in lung tissues

Lung tissue staining was performed as previously described with some modifications (Li et al., 2009). Briefly, the sections of the frozen tissue were 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 PBS. The slides were then incubated in a permeabilization buffer of 0.5% Triton X-100, 0.2 µg 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 40 min to reduce non-specific staining. The slides were then incubated with primary antibodies, diluted appropriately in a blocking solution, at room temperature, overnight. The following monoclonal 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), rabbit anti-mouse Foxp3 antibody (1:1000, Abcam, Cambridge, USA), and anti-mouse ROR γ t, pSTAT6 antibodies (1:50, BD Biosciences). 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.

2.12. Image acquisition

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 MetaVeuTM 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.

2.13. Cell culture

MH-S mouse alveolar macrophage cells (KCLB 40071, Korean Cell Line Bank, Seoul, South Korea) were cultured in RPMI supplemented with 10% foetal bovine serum, and 100 IU/ml penicillin–100 µg/mL streptomycin at 37 °C in a 5% CO₂ humidified air incubator.

Cells were plated at a density of 2.5×10^4 cells/ml in 96-well plates. After 2 h 18Gly, Rosi. and CsA treatment, MH-S alveolar macrophage cells were exposed to lipopolysaccharide (LPS; 500 ng/mL) for 24 h. Then, cells were prepared to be used in the following test.

2.14. Intracellular reactive oxygen species (ROS) level measurement

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) Cellular ROS Detection Assay Kit (ab113851, Abcam, Cambridge, MA, USA) according to the protocol provided by the manufacturer. After incubation, a 100 µL suspension of MH-S cells was washed in DCFDA solution and stained with DCFDA (Sigma-Aldrich Korea, Seoul, Korea) in DCFDA solution containing DCFDA at a final concentration of 25 µM MH-S cells were incubated at 37 °C for 45 min in the dark and washed two times in DCFDA solution. The DCFDA-loaded MH-S cells were resuspended in 100 µL DCFDA solution and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm on a microplate reader (SpectraMax M2; Molecular Devices, Inc., Sunnyvale, CA). The intracellular ROS levels were expressed as relative fluorescence unit values.

2.15. Cytotoxicity

Cells were seeded into 96-well plates at a density of 2.5×10^5 cells/ml and incubated for overnight. Then the cells were exposed to various concentrations of 18 β -Glycyrrhetic acid for 24 h, applied either alone or together with LPS. The cell survival fractions were determined by the water-soluble tetrazolium test (WST, EZ-CYTOX assay Kit, Daeillab, Korea) according to the manufacturer's instruction. Absorbance was measured at 600 or 650 nm as the reference wavelength in a microplate reader (Epoch Micro-Volume Spectrophotometer System, BioTek, USA). The assays were conducted more than three times. The optical density (O.D.) was calculated as the difference between the reference wavelength and the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/O.D. of non-treated sample) $\times$ 100.

2.16. Statistical analysis

Data were analysed using ANOVA or unpaired Student's *t*-test followed by Dunnett's multiple comparison test (SPSS analysis software, version 14.0). 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 significance in the 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. Effects of 18Gly on AHR in response to methacholine stimulation

AHR to methacholine (doses of 3.125, 6.25, 12.5, 25, 50 mg/kg) was assessed one day after the final OVA challenge by non-invasive whole-body plethysmography. The responses of the normal mice to methacholine were weak as shown in Fig. 1B. However, methacholine administrations significantly increased the Penh values in OVA-induced mice compared with the normal group.

Both 18Gly (20 mg/kg) and Rosi. treatments significantly reduced the Penh values ($P < 0.01$). However, there was no significant difference between the AHR in 18Gly (2 mg/kg)-supplemented OVA-challenged mice and OVA-induced control mice. Treatment of mice with 18Gly (20 mg/kg) attenuated the increased AHR and showed similar effects as cyclosporine A (CsA) (Fig. 1B).

3.2. Histopathological analysis of lung tissues

To investigate the effects of 18Gly on the histological changes in the OVA-challenged asthma model, lung tissues were stained with H & E, M-T, and PAS staining solution. Lung tissues of the mice in the normal group (PBS only) exhibited normal parenchyma with little infiltration of inflammatory cells, a little collagen staining, and no mucus staining around the respiratory epithelium.

Typical histological features of asthma showing numerous infiltrating inflammatory cells composed of lymphocytes, eosinophils, and neutrophils around peribronchial and perivascular regions were ob-

served in OVA-induced control group (Fig. 1C). However, both 18Gly and Rosi. treatments reduced the degree of infiltration of inflammatory cells and airway inflammation in the peribronchiolar and perivascular region, and similar results were obtained upon CsA treatment.

Subepithelial fibrosis, collagen deposition, and mucus secretion within the bronchi of the lungs were also examined. To determine the level of goblet cell hyperplasia and mucus hypersecretion, PAS-positive epithelial cells in individual bronchioles were analysed. The mean numbers of PAS-positive cells were significantly higher in the control group than in the normal group. 18Gly (2 or 20 mg/kg) treatment resulted in a significantly lower number of PAS-stained goblet cells (Fig. 2B and C). The lungs of 18Gly and Rosi. treated OVA-induced mice exhibited the histological features of mild eosinophil infiltration, less airway epithelial cell hypertrophy, mild mucus production, and less collagen deposition as compared to the control mice (Fig. 1C). As expected, CsA significantly reduced peribronchial and perivascular lung inflammation.

3.3. 18Gly and rosi. suppress airway eosinophil accumulation and influx of inflammatory cells into lung and BALF

To investigate the anti-asthmatic effect of 18Gly in an *in vivo* mouse model, the total number of leukocytes and the number of eosinophils in the BALF and lung tissues were determined. As shown in Fig. 2, the total number of lung cells isolated from normal mice was $3.10 \pm 0.25 \times 10^6$ while control mice showed a significantly higher number, $12.60 \pm 2.05 \times 10^6$. However, 18Gly (20 mg/kg) and CsA inhibited the OVA-induced recruitment of total leukocytes into the lung

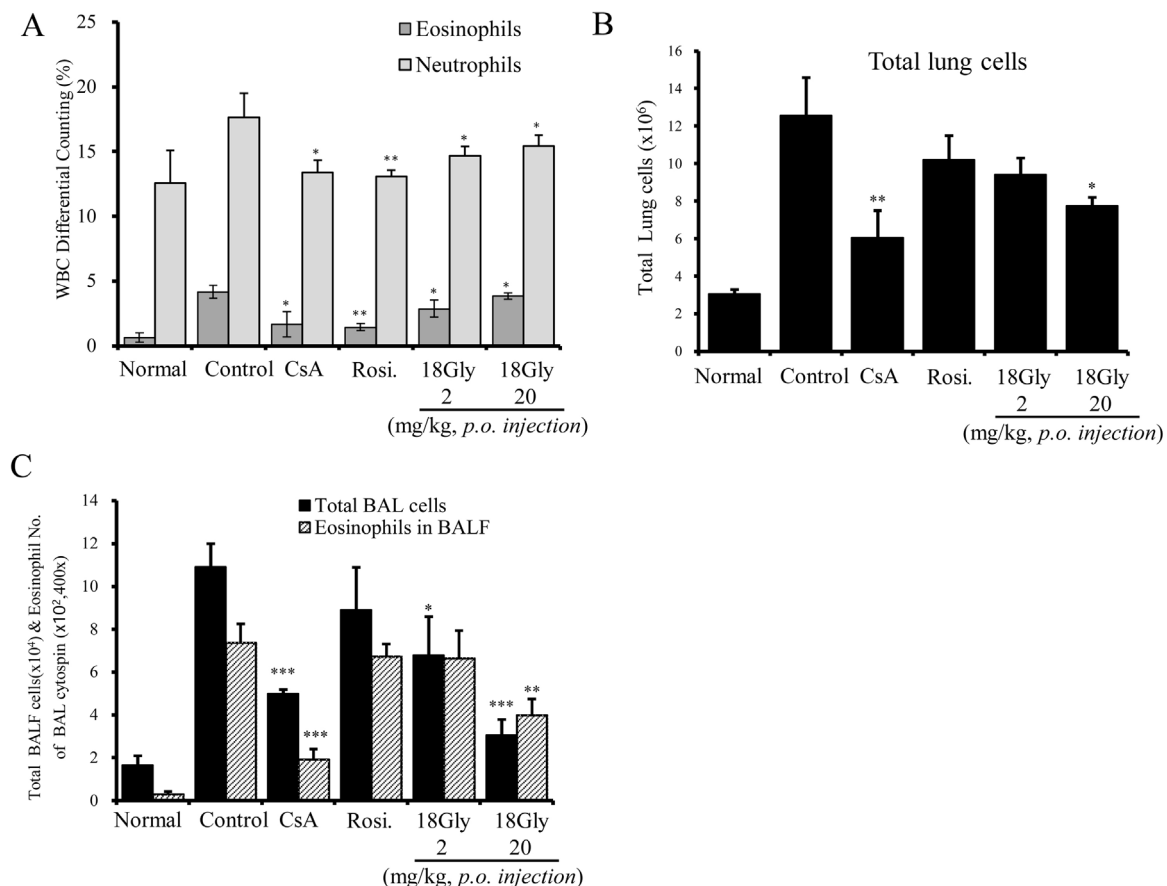


Fig. 2. The effects of 18Gly on total lung cells, total BALF cells, eosinophils, and neutrophils in PBMC. Results are expressed as mean $\pm$ SEM ($N = 6$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for the experimental groups versus the OVA control group comparisons. N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18 β -glycyrrhetic acid (2 or 20 mg/kg).

Table 1

Fluorescence-activated cell sorting analysis (FACS) of immune cell subtypes in lung and BALF. Each point represents the mean $\pm$ SEM values for 6 mice.

Cell phenotypes in Lung, BALF	Ovalbumin-induced asthma mice (Absolute No.)					
	Normal BALB/c	Control	Cyclosporin A	Rosi. (2 mg/kg)	18Gly (2 mg/kg)	18Gly (20 mg/kg)
CD3 ⁺ /CD4 ⁺ ($\times 10^6$ cells)	7.30 $\pm$ 0.52	44.40 $\pm$ 8.47	13.70 $\pm$ 1.85**	20.70 $\pm$ 3.30*	18.70 $\pm$ 0.91**	16.80 $\pm$ 0.15**
CD3 ⁺ /CD8 ⁺ ($\times 10^6$ cells)	1.90 $\pm$ 0.20	12.20 $\pm$ 1.94	4.50 $\pm$ 1.63**	9.30 $\pm$ 2.93	7.90 $\pm$ 0.71*	6.30 $\pm$ 0.02*
Gr-1 ⁺ /CD11b ⁺ ($\times 10^5$ cells)	5.40 $\pm$ 1.09	52.00 $\pm$ 15.58	14.40 $\pm$ 4.17*	22.10 $\pm$ 8.18	25.30 $\pm$ 2.99	17.60 $\pm$ 3.26*
B220 ⁺ /CD23 ⁺ ($\times 10^5$ cells)	2.10 $\pm$ 0.72	24.70 $\pm$ 2.27	7.70 $\pm$ 2.46***	12.60 $\pm$ 0.30***	14.60 $\pm$ 4.10*	8.30 $\pm$ 1.14*
CD3 ⁺ /CD4 ⁺ ($\times 10^5$ cells)	1.50 $\pm$ 0.53	25.70 $\pm$ 0.11	7.30 $\pm$ 0.09***	10.00 $\pm$ 1.05***	11.60 $\pm$ 2.50**	3.10 $\pm$ 0.47***
CD3 ⁺ /CD8 ⁺ ($\times 10^5$ cells)	0.2 $\pm$ 0.05	9.2 $\pm$ 3.41	0.70 $\pm$ 0.01**	5.90 $\pm$ 4.82	3.00 $\pm$ 0.07*	0.90 $\pm$ 0.12*
Gr-1 ⁺ /CD11b ⁺ ($\times 10^5$ cells)	1.00 $\pm$ 0.39	32.40 $\pm$ 7.58	6.40 $\pm$ 0.75**	16.90 $\pm$ 5.08*	13.30 $\pm$ 4.37*	3.80 $\pm$ 0.98**

*P < 0.05, **P < 0.01, and ***P < 0.001 for the experimental groups versus the OVA control group comparisons. N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18 β -glycyrrhetic acid (2 or 20 mg/kg).

tissues (Fig. 2B). However, there was no significant difference in the levels of these inflammatory cells among the OVA-sensitized and challenged mice, 18Gly-treated (2 mg/kg) mice, Rosi.-treated mice.

Significant infiltration of inflammatory cells (total leukocytes and eosinophils) to BALF was observed in the mice that had been challenged with OVA. 18Gly (2, 20 mg/kg) also reduced the absolute number of total BALF cells (Fig. 2C), as well as the percentage of eosinophils and neutrophils in peripheral blood mononuclear cells (PBMC) (Fig. 2A). Compared with $1.7 \pm 0.45 \times 10^4$ cells in normal group, the total cell numbers, $10.9 \pm 1.10 \times 10^4$, in the BALF were significantly higher in the control group. However, the 18Gly-treated mice (2 and 20 mg/kg) showed $6.8 \pm 1.80 \times 10^4$ and $3.1 \pm 0.75 \times 10^4$ cells, respectively. These numbers were significantly lower than those of the control group. CsA also significantly reduced inflammatory cells in BALF. In CsA-treated mice, total BALF cell number is consistent with the percentage of neutrophils and eosinophils in PBMC, and total number of lung cells. However, in Rosi.-treated mice, total BALF cell number does not correspond to the percentage of neutrophils and eosinophils in PBMC.

3.4. Inhibitory effect of 18Gly and Rosi. on the absolute number of immune cell subtypes in murine OVA-induced asthma lungs and BALF

To confirm whether the AHR, inflammatory changes, and histopathological changes demonstrated in mice subjected to various treatments were reflected in altered T cell subtypes distributions, lung and BALF cells were analysed by flow cytometry. The effects of 18Gly and Rosi. on T cell subtype counts were studied in normal as well as OVA-induced mice and other experimental groups (Table 1).

In OVA-induced control mice, the numbers of CD3⁺/CD4⁺, CD3⁺/CD8⁺, Gr-1⁺/CD11b⁺, and B220⁺/CD23⁺ double-positive cells in the lungs and BALF were higher than those in normal mice. The eosinophil population may constitute a substantial portion of the CD11b⁺/Gr-1⁺ population. 18Gly and Rosi. administration resulted in significant reduction in inflammatory leukocyte subsets in the lungs and BALF as shown by the changes in the numbers of Gr-1⁺/CD11b⁺ granulocytes. As expected, CsA administration resulted in significant reduction in T cell subtypes and Gr-1⁺/CD11b⁺ granulocytes.

Furthermore, there were deficits in Gr-1⁺/CD11b⁺ granulocytes, accompanied by a decrease in the number of eosinophils in PBMC, total lung cells, and total BALF cells (Fig. 2A–C, Table 1).

3.5. Inhibition of *th2* cytokines (in vivo and in vitro) in BALF

To investigate whether 18Gly, Rosi. and CsA influenced Th2 cytokines secretion in BALF, the levels of IL-4, IL-5, and IL-13 were measured by ELISA after the final OVA challenge. As shown in Fig. 3A, IL-5 and IL-13 levels were significantly reduced in 18Gly-treated (20 mg/kg), Rosi., and CsA-treated mice. No significant differences were observed in IFN- γ , and IL-4 levels in BALF between these groups

(data not shown). Moreover, determination of Th1 cytokine (IFN- γ) levels in spleen cell culture supernatants by ELISA revealed that 18Gly (2, 20 mg/kg) and Rosi., but not CsA, increased IFN- γ production (Fig. 3B).

3.6. Inhibitory effect of 18Gly on OVA-specific IgE production in serum

An important feature of the asthmatic model mice is the high level of OVA-specific IgE in serum. Therefore, levels of anti-OVA IgE in serum from OVA-induced mice and the PBS only, 18Gly, Rosi., and CsA-treated groups were measured.

OVA-specific IgE levels in the sera of OVA-induced asthmatic mice were significantly higher than those in sera from normal mice (PBS only). 18Gly, Rosi., and CsA significantly inhibited the increases in the levels of OVA-specific IgE (Fig. 3C). Specifically, 18Gly reduced the levels of OVA-specific IgE in a dose-dependent manner.

No significant differences were observed in OVA-specific IgE levels in BALF between the groups (data not shown).

3.7. Effects of 18Gly on IL-5, IL-13, IL-4, IFN- γ , and PPAR γ mRNA levels in lung tissue

As shown in Fig. 4, IL-5, IL-13, IL-4, IFN- γ , and PPAR γ mRNA were detected in lung tissue treated with PBS, ovalbumin plus 18Gly-treated groups (2, 20 mg/kg), Rosi. (2 mg/kg), and CsA-treated group (10 mg/kg).

IL-5, IL-13, IL-4 mRNA levels in lung cells were reduced in 18Gly-treated (20 mg/kg) mice, but PPAR γ and IFN- γ mRNA levels were increased in the 18Gly-treated (2, 20 mg/kg) group. These results were accompanied by changes in eosinophil influx (Gr-1⁺/CD11b⁺) (Fig. 2A and C, Table 1), BAL cytokines (IL-5, IL-13) (Fig. 3A), IFN- γ production in spleen cells (Fig. 3B), and OVA-specific IgE level in serum (Fig. 3C).

3.8. Immunofluorescence staining of T-bet, GATA-3, foxp3, ROR γ t, and pSTAT6 in asthmatic mice

The differentiation of Th1, Th2, Treg and Th17 cells is controlled by the transcriptional regulators T-bet, GATA-3 (and pSTAT6), Foxp3, and retinoic acid-related orphan receptor γ t (ROR γ t), respectively. To confirm the transcriptional expression of the above factors in lung tissues, we performed immunofluorescence staining of lung tissue samples from mice in each group.

T-bet, GATA-3, Foxp3, ROR γ t, and pSTAT6 levels in the asthma model mouse lung tissue were altered compared to the control group, especially in the cell nuclei. After treatment with OVA plus CsA and OVA plus 18Gly (2 or 20 mg/kg), GATA-3 expression decreased significantly in a dose-dependent manner (Fig. 5). However, T-bet staining in lung tissue in the OVA plus 18Gly-treated groups (2 or 20 mg/kg) was slightly decreased than that in the OVA alone control group, particularly in the cell nuclei (Fig. 5).

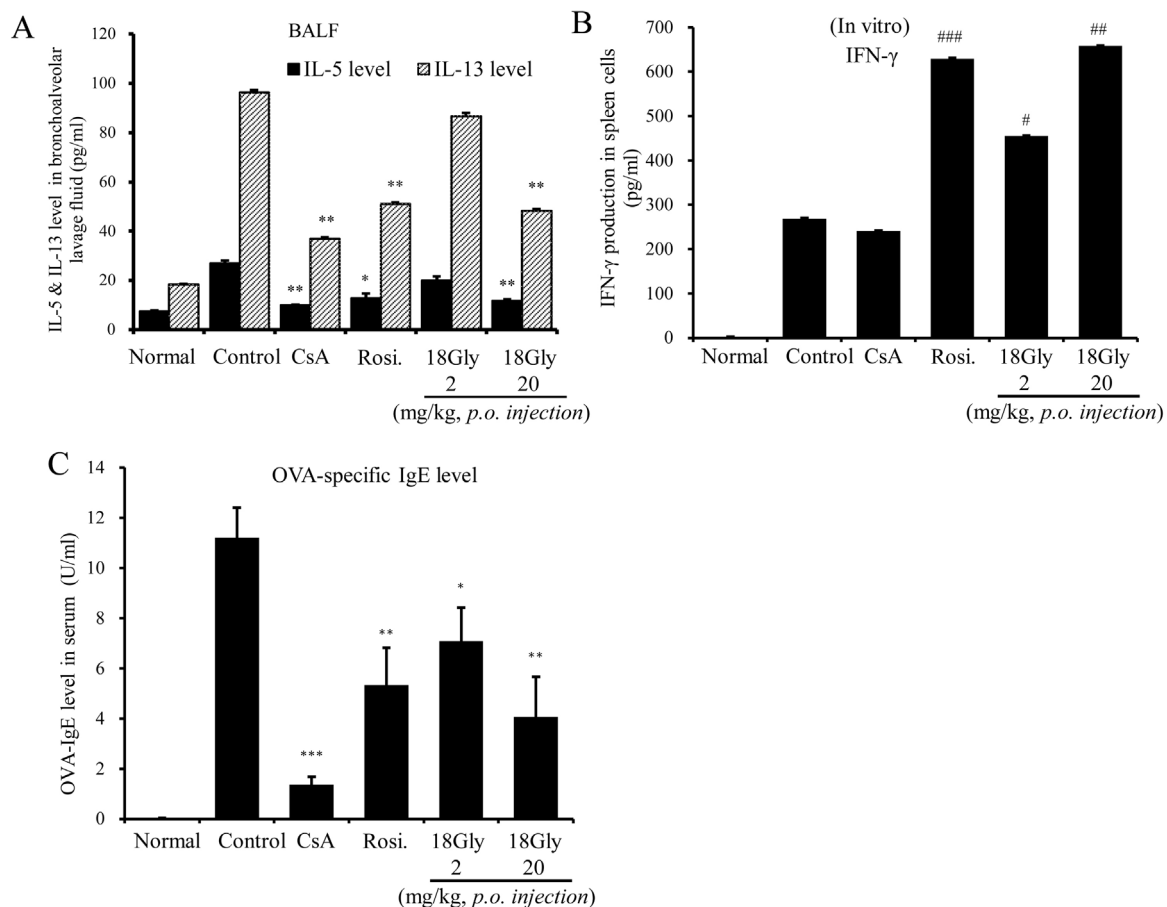


Fig. 3. The effects of 18Gly on Th2 cytokines (IL-5 and IL-13) in BALF, the levels of OVA-specific IgE in serum, and the immunomodulatory effects of 18Gly on Th1 (IFN- γ) cytokine production in spleen cells. Each point represents the mean $\pm$ SEM values for 6 mice. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for the experimental groups versus the OVA control group comparisons. # $P < 0.05$, ## $P < 0.01$ and ### $P < 0.001$ for significant increases between control and treatment groups (analysis of variance). N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18 β -glycyrrhetic acid (2 or 20 mg/kg).

Fig. 6 shows that the immunofluorescence intensities of Foxp3 in the asthmatic groups were lower than those in the normal group, but the expression of Foxp3 was significantly high in 18Gly-treated group (2 or 20 mg/kg) as compared to the mice group. CsA was much more effective against inhibition of GATA-3, while 18Gly was more effective against Foxp3 expression.

In addition, pSTAT6 and ROR γ t levels in the pulmonary tissue were higher in the OVA control groups than in the normal group; whereas in the 18Gly-treated groups (2 or 20 mg/kg), the levels were significantly lower than that in the OVA control group (Figs. 6 and 7). The changes in pSTAT6 levels were accompanied by changes in GATA-3 fluorescence intensities (Fig. 5). These results indicate that 18Gly controlled the differentiation of Th2 cells by inhibiting GATA-3 and pSTAT6 transcription factors.

3.9. Inhibitory effects of 18Gly, Rosi. and CsA on intracellular ROS levels and cytotoxicity in MH-S cells

Excessive production of ROS correlates with the degree of airway hyperresponsiveness, and overproduction of ROS results in bronchial hyperreactivity which is characteristic of asthma (Lee and Yang, 2012). We investigated the effects of 18Gly, Rosi. and CsA on the generation of ROS in LPS induced MH-S cells. The addition of 18Gly suppressed the LPS-induced production of ROS dose-dependently (Fig. 8A). Little cytotoxic effect was presented in 18Gly at above concentrations (Fig. 8B).

We found that medium alone did not significantly affect ROS

generation in MH-S alveolar macrophage cell lines (Fig. 8A). LPS alone significantly promoted ROS generation compared with the medium alone group (Fig. 8A). This ROS generation was significantly inhibited by 40 μ M, 20 μ M and 10 μ M 18Gly, Rosi. and CsA.

18Gly significantly reduced the ROS generation in a concentration-dependent manner, but Rosi. did not decrease dose-dependently ROS generation in MH-S cells. Further studies are needed to clarify these results of Rosi.

Compared with the control group, 40 μ M and 20 μ M 18Gly significantly decreased the intracellular ROS levels in MH-S alveolar macrophage cells ($P < 0.01$; Fig. 8A). Furthermore, 10 μ M 18Gly significantly inhibited the intracellular ROS levels in these cells ($P < 0.05$; Fig. 8A). The 5 μ M 18Gly group also showed this effect, but was weaker than the higher concentration group. These results showed that the higher concentrations (40 μ M, 20 μ M and 10 μ M) of 18Gly decreased the ROS generation induced by LPS better than the lower concentration (5 μ M) of 18Gly (Fig. 8A), indicating that the appropriate concentrations of 18Gly in MH-S protects against excessive ROS generation.

4. Discussion

Allergic asthma is known to be a Th2 cell-dependent, IgE-mediated disease characterized by airway inflammation, mucus secretion, smooth muscle hyperplasia, and tissue infiltration of eosinophils and other cell types (Tattersfield et al., 2002).

Numerous experimental models are used to study asthma. In this

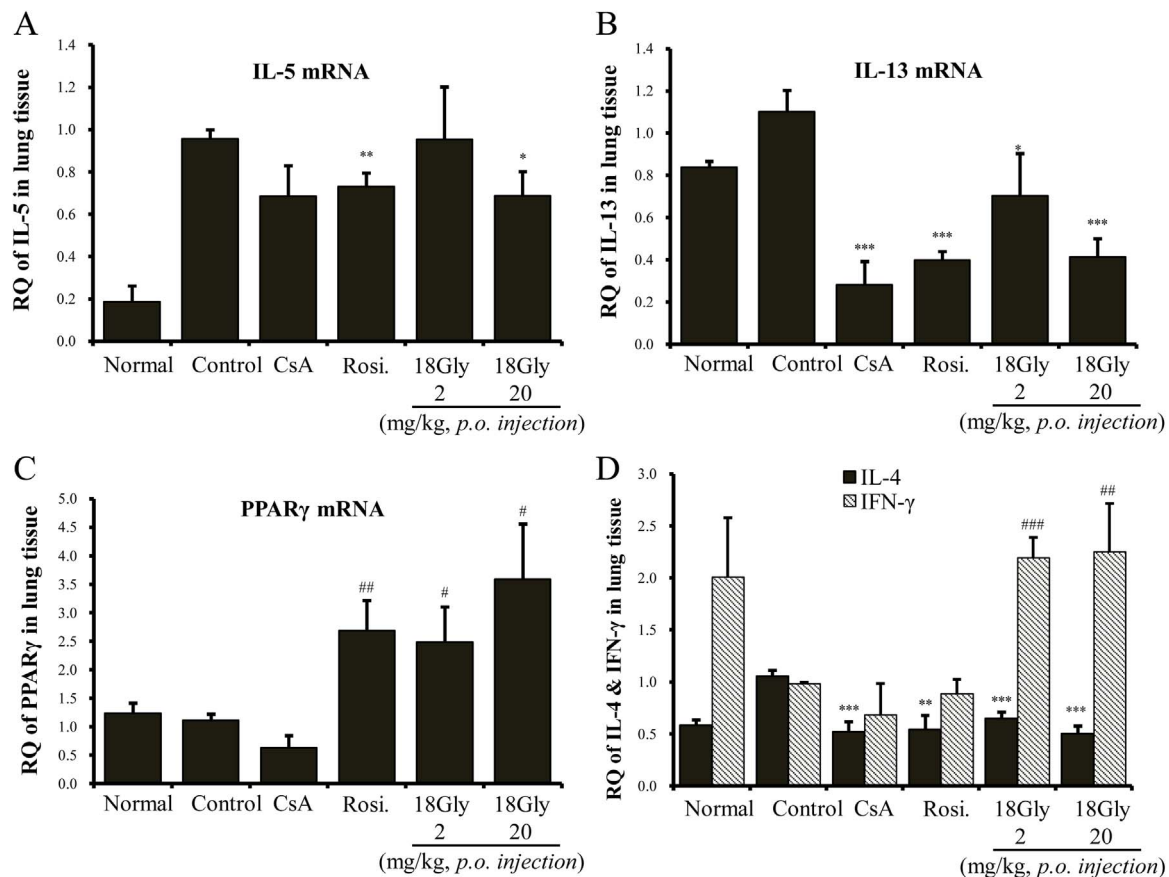


Fig. 4. Effects of 18Gly and CsA on IL-5, IL-13, PPAR γ , IL-4, and IFN- γ mRNA expression in lung tissue of an OVA-induced murine model of asthma. Samples were analysed by RT-qPCR. The results are expressed as RQ to control.

study, we chose to use a mouse model of allergic asthma with BALB/c mice, as described in Fig. 1A.

18Gly reduces inflammatory cytokines (TNF- α , IL-1, IL-6) production via the activation of the PI3 K/Akt pathway, and has an anti-inflammatory, anti-invasive effect via the inhibition of PI3 K/Akt-dependent nuclear factor- κ B (NF- κ B) activity and other pharmacological properties. Moreover, Kim et al. (Kim et al., 2013) showed that 18Gly significantly suppresses the expression of surface molecules CD80, CD86, major histocompatibility complex (MHC) class I and MHC class II as well as IL-12 in LPS-stimulated DCs, and impairs the induction of the Th1 immune response. These findings indicate that 18Gly can be a therapeutic candidate for the treatment of inflammatory diseases, including asthma. Therefore, we hypothesized that 18Gly can inhibit airway and lung inflammation.

In our results, both 18Gly and CsA prevented the development of Penh, eosinophil infiltration, mucus production, lung inflammation, and also decreased the Th2 cytokines IL-5, IL-13, and OVA-specific IgE in BALF and serum (Figs. 1–4 and Table 1). The mRNA levels of IL-5, IL-13, IL-4 in lung tissues were reduced in 18Gly-treated (20 mg/kg) mice, but those of PPAR γ and IFN- γ were increased in the 18Gly-treated (2, 20 mg/kg) group.

CsA has been shown to inhibit allergic inflammation, such as eosinophilic and lymphocytic infiltration and the expression of IL-4 and IL-5. Hence, we used CsA as a positive control.

One of the main strategies in the treatment of asthma is the evaluation of the roles of specific T cell subpopulations. CD4 $^{+}$ T cells are important for immune responses and they play a critical pathogenic role as drivers of allergic diseases, including asthma. CD8 $^{+}$ T cells, like CD4 $^{+}$ T cells, have been experimentally shown to produce IL-4, IL-5, and IL-13 (Kemp and Ronchese, 2001). CD8 $^{+}$ T cells can exacerbate inflammation by secreting Th2 cytokines such as IL-5 and IL-13

(Miyahara et al., 2004). Additionally, the mechanisms of these CD8 $^{+}$ T cell-mediated effects are thought to involve CD8 $^{+}$ T cell-derived cytokines, especially IFN- γ (Enomoto et al., 2012).

Eosinophils are one of the cell types known to express Gr-1; therefore, eosinophil population may constitute a substantial portion of the Gr-1 + CD11b $^{+}$ population (Hoffmann et al., 2007).

Analysis of CD23 expression on B220 $^{+}$ cells in the mice revealed a significant increase in the presence of B cells (Outram et al., 2008). The surface marker CD23, which is a low affinity IgE receptor (Fc ϵ RII), is found on the cell surface of B and T lymphocytes and eosinophils. The soluble fragment of this receptor (sCD23) appears to be identical to the IgE binding factor and to participate in the regulation of IgE synthesis (Conrad, 1990).

The elevated numbers of activated CD4 $^{+}$ and CD8 $^{+}$ T cells, as well as Gr-1 $^{+}$ /CD11b $^{+}$ and B220 $^{+}$ /CD23 $^{+}$ positive cells in lung and BALF of OVA-induced mice correlated with susceptibility to lung inflammation. Moreover, these cell numbers significantly decreased with 18Gly (20 mg/kg) and CsA treatment (Table 1). These results were accompanied by a concurrent reduction in the eosinophil numbers in PBMC and BALF cells (Fig. 2A and C).

A recent study has shown that the expression patterns of the Th2 and Th17 cells in the bronchial epithelial cells of asthmatics are reciprocal, which indicates a regulation of the Th2 and Th17 inflammatory pathways in asthma (Choy et al., 2015).

Although Th2 cells have been the most frequently evaluated T cells in asthma, there is evidence that Th1 cells are involved in the pathogenesis of asthma. Tregs are assumed to control not only pathogenic Th2 cells, but also Th1 cells. Moreover, these cells are regulated by the transcription factors T-bet and GATA-3 (Vale-Pereira et al., 2011).

Recently, ROR γ t has been shown to be indispensable for the

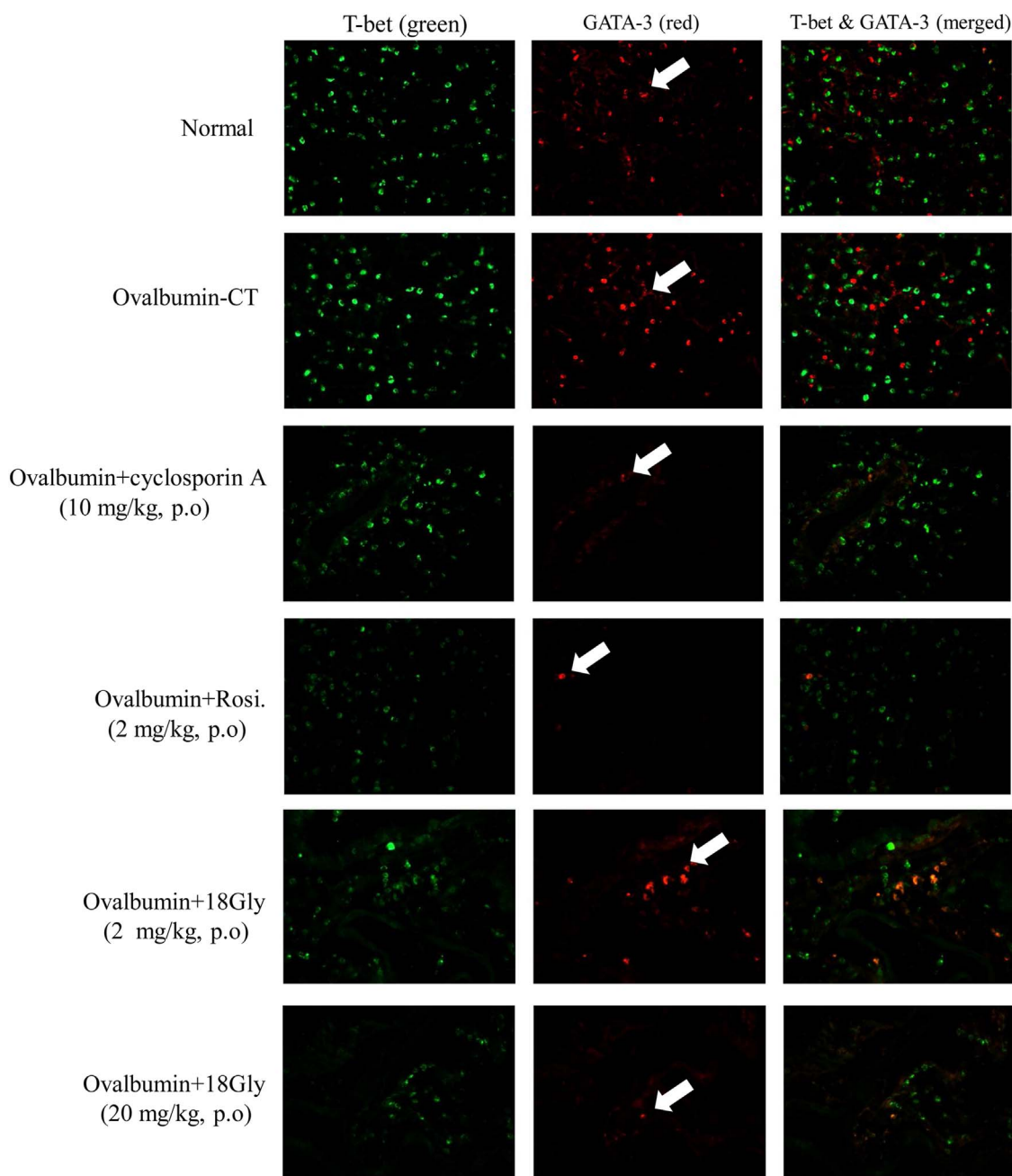


Fig. 5. Lung tissue immunofluorescence staining for T-bet and GATA-3 *in vivo*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Immunofluorescence staining of T-bet (green) and GATA-3 (red) in the lung tissues of mice with OVA-induced allergic asthma. N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18 β -glycyrrhetic acid (2 or 20 mg/kg). (Magnification $\times$ 200).

differentiation of Th17 cells (Ivanov et al., 2006), and was directly involved in IL-17A production, and Foxp3 inhibits IL-17A expression through interaction with ROR γ t (Ichiyama et al., 2008). It was reported that increased IL-17A expression synergized with IL-13 that was present during allergic airway inflammation, resulting in increased AHR (Lajoie et al., 2010). IL-17A increased airway mucus expression in mouse models of airway inflammation (Chen et al., 2003).

CD4 + Foxp3+ Treg cells play a crucial role in the regulation of asthma, and the Tregs prevent development of allergic diseases, including asthma, by suppressing Th2, Th17 cytokines production (Meiler et al., 2008).

It was reported that PPAR γ agonists such as rosiglitazone reverse the pathophysiological features of asthma and suppress IL-17 expression in the lungs. Inhibition of PPAR γ expression and IL-17 production

also markedly reduces airway inflammation, AHR, and the increased levels of Th2 cytokines and OVA-specific serum IgE (Lee et al., 2006). Moreover, PPAR γ ligands also inhibit antigen-induced AHR, lung inflammation, eosinophilia, Th2 cytokine production, and GATA-3 expression, as well as serum levels of antigen-specific IgE in different animal models of asthma (Belvisi et al., 2006).

PPAR γ expression was increased in the 18Gly-treated (2, 20 mg/kg) group, corresponding to the OVA-specific IgE, IL-5, and IL-13 levels (Figs. 3 A and C, 4). 18Gly (2 or 20 mg/kg) decreased GATA-3 expression significantly in a dose-dependent manner (Fig. 5). However, T-bet levels in the lung tissue in the 18Gly-treated groups (2 or 20 mg/kg) were slightly lower than those in the OVA alone control group, particularly in the cell nuclei (Fig. 5). The immunofluorescence intensities of Foxp3 in 18Gly-treated (2 or 20 mg/kg) mice were

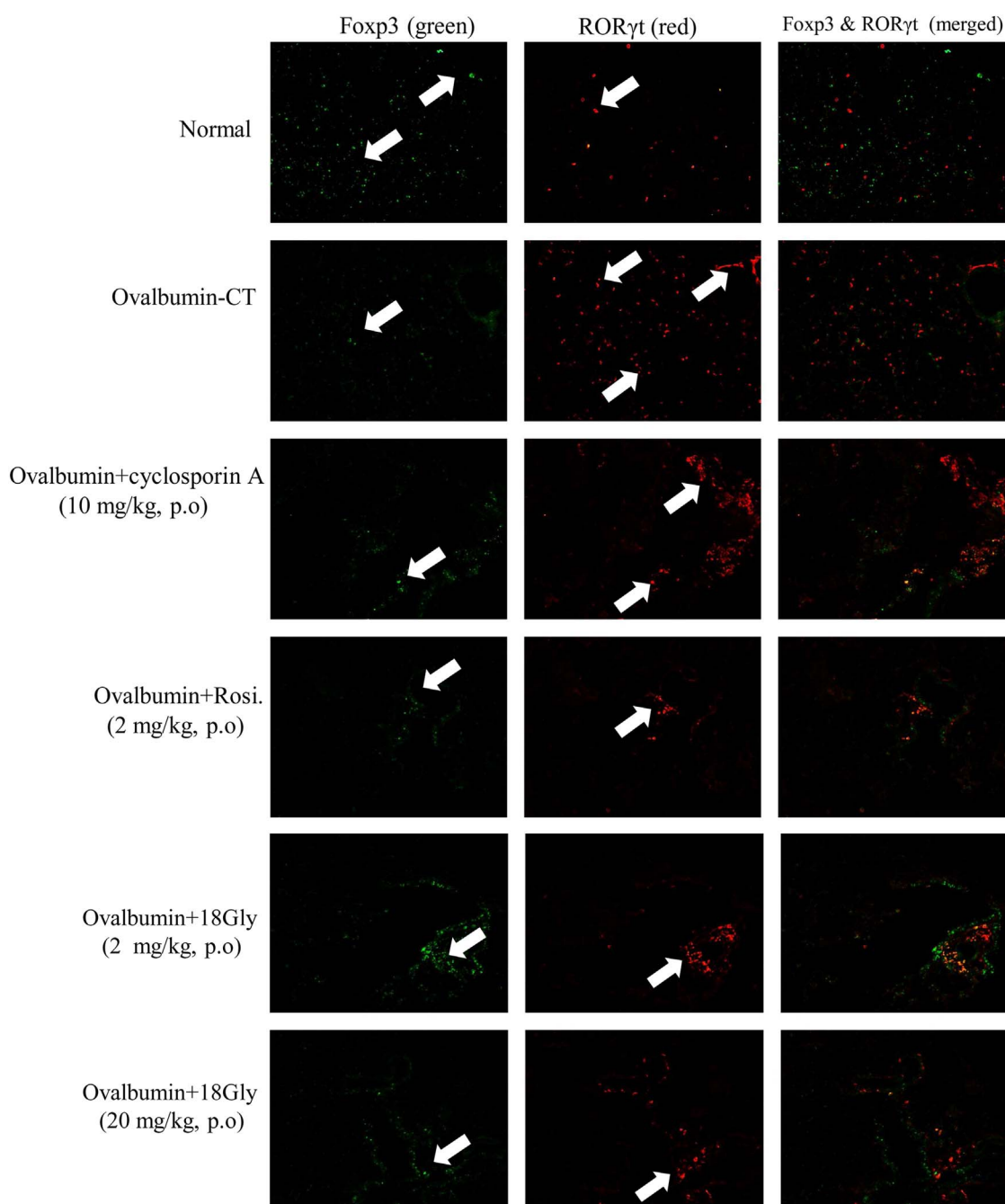


Fig. 6. Lung tissue immunofluorescence staining for Foxp3 and ROR γ t *in vivo*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) From left to right, in order: Images of Foxp3 (green), ROR γ t (red), Foxp3 plus ROR γ t (overlapped) stained tissues. From top to bottom, in order: N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18 β -glycyrrhetic acid (2 or 20 mg/kg). (Magnification $\times$ 200).

significantly higher as compared to the control mice (Fig. 6).

In addition, pSTAT6 and ROR γ t levels in pulmonary tissue were higher in the OVA control group than in the normal group; whereas in the 18Gly-treated groups (2 or 20 mg/kg), the levels were significantly lower than in the OVA control group (Figs. 6 and 7).

We propose that a decrease in ROR γ t is the mechanism underlying the upregulation of the transcription factor Foxp3. Since Foxp3+ Treg cells suppress Th17 cells, it is possible that the therapeutic effect of 18Gly via inactivation of Th2 (IL-5, IL-13) and ROR γ t is synergistic with the Foxp3 and GATA-3 pathways (Figs. 5 and 6).

We also observed significant correlations among IL-5, IL-13, and IFN- γ levels in BALF and lung tissues (Figs. 3 and 4).

We observed that 18Gly suppressed IL-5, IL-13, GATA-3 and pSTAT6 expression in BALF, and lung tissues, both at mRNA and protein levels (Figs. 3–5,7).

The critical role of the STAT6 pathway in IgE isotype switching in B cells is well established, and IL-13 can activate STAT6 and induce IgE production in mice (Finkelman et al., 1988; McKenzie et al., 1993). Both, STAT6 and NF- κ B activities are required for the induction of IgE production (Godava et al., 2013). Th2-mediated responses such as airway eosinophilia and mucus production were significantly reduced in STAT6 knockout mice when compared to the control group (Mathew et al., 2001). STAT6 also regulates the expression of GATA-3, which is responsible for the expression of the Th2 cytokines (Maier et al., 2012).

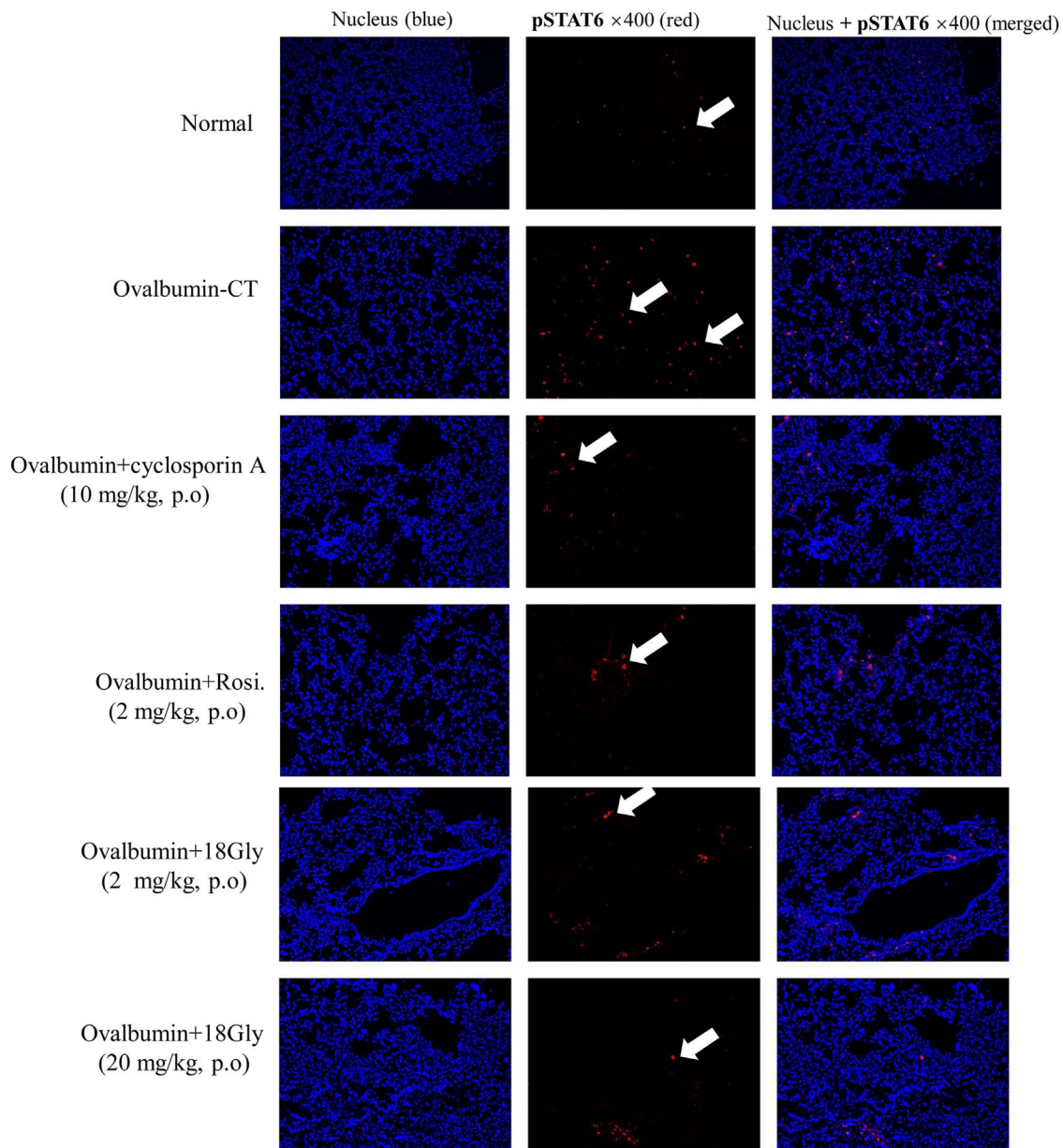


Fig. 7. Lung tissue immunofluorescence staining for pSTAT6 *in vivo*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) From left to right, in order: Images of nuclei (blue), pSTAT6 (red), nuclei + ROR γ t (overlapped) staining in tissues. From top to bottom, in order: N: Normal BALB/c mice, CT: OVA inhalation + vehicle, CsA: OVA inhalation + cyclosporine A, 10 mg/kg, Rosi.: OVA inhalation + rosiglitazone, 2 mg/kg, 18Gly: OVA inhalation + 18 β -glycyrrhetic acid (2 or 20 mg/kg). (Magnification $\times 200$). Original magnification $\times 400$.

However, GATA-3 can also promote transcription of Th2 cytokines independent of the STAT6 pathway via Notch signalling (Rayees et al., 2014).

18Gly (5 mg/kg) and CsA also inhibited B cell-dependent production of OVA-specific IgE in BALF (Fig. 3), and therefore we infer that 18Gly suppressed OVA-specific IgE production through downregulation of STAT6 and GATA-3 (Figs. 5 and 7).

Our results showed that Th2 cytokines, GATA-3, STAT6, ROR γ t, and Foxp3 play an important role in allergic inflammation. 18Gly may also regulate the imbalance of the Th1/Th2-associated transcription factors, T-bet, STAT6, GATA-3, ROR γ t, and Foxp3 in asthmatic mice. However, STAT6 and GATA-3 transcription factors for Th2 cells are significantly induced after OVA exposure, followed to a lesser extent by T-bet for Th1.

Thus, low levels of ROR γ t, STAT6, and GATA-3 expression are inversely correlated with high levels of Foxp3 expression by Tregs. One possible mechanism could be that Foxp3 directly inhibits ROR γ t,

STAT6, and GATA-3. However, the precise mechanism remains unclear because of the involvement of numerous transcription factors such as NF- κ B, activator protein-1 (AP-1), and nuclear factor of activated T-cells (NF-AT) besides these transcription factors. Further studies are needed to confirm these mechanisms.

Oxidative stress like reactive oxygen species (ROS) may initiate and augment inflammation, and may also result from inflammation. ROS are involved in airway smooth muscle contraction, decreased numbers and function of epithelial cilia, increased mucus production, altered release of inflammatory mediators, the influx of inflammatory cells and increased vascular permeability (Lee and Yang, 2012). Tregs may contribute to the defense against tissue damages induced by ROS (Kim et al., 2014a.). However, oxidative stress may contribute to the depletion of Treg cells in asthma model mice, and it can also promotes Th17 cell differentiation and related cytokines production (Yang et al., 2016). Airway oxidative inflammation by ROS may contribute to systemic inflammation through upregulation of Th17

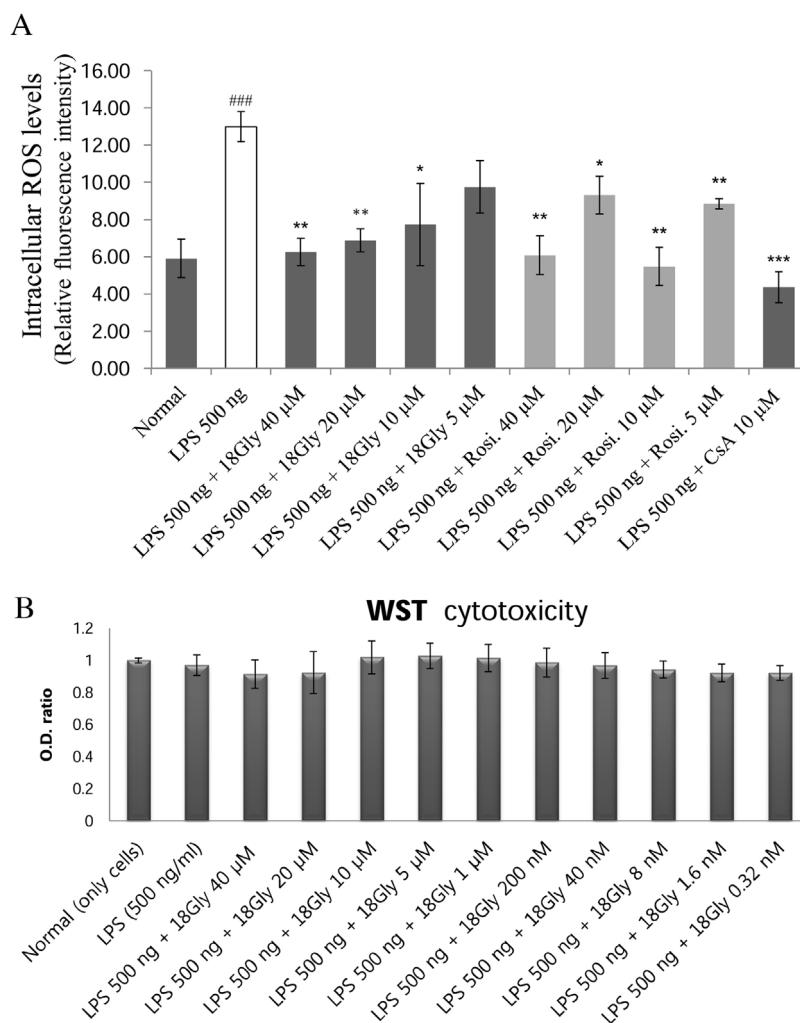


Fig. 8. Antioxidant effects of 18Gly, Rosi, and CsA on the intracellular ROS levels in MH-S alveolar macrophage cells (A). The cytotoxicity of 18Gly on MH-S alveolar macrophage cells (B). MH-S alveolar macrophage cells were incubated with 18Gly (40, 20, 10 and 5 μ M), Rosi. (40, 20, 10 and 5 μ M) and CsA (10 μ M) for 24 h. MH-S cells were pre-treated with 18Gly for 2 h and incubated with LPS for 24 h. The cells were then incubated with 25 μ M DCFDA for 45 min, washed thoroughly, and intracellular ROS level immediately analysed by Cellular Reactive Oxygen Species Detection Assay Kit (ab113851, Abcam, Cambridge, MA, USA). The generation of fluorescent oxidized DCF, shown as the means $\pm$ SE optical density (O.D.) measurements from triplicate cultures. The cytotoxicity of 18Gly on MH-S cells at different concentrations was evaluated by a WST assay (EZ-CYTOX assay Kit) described materials and methods. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ for the experimental groups versus LPS treated control. ### $P < 0.05$ for the LPS treated control versus untreated normal group.

immune responses (Al-Harbi et al., 2016). ROR γ t expression in lung tissue was reduced in 18Gly-treated (20 mg/kg) mice, but Foxp3 expression was increased in the 18Gly-treated (2, 20 mg/kg) group (Fig. 6). It is possible that 18Gly can inhibit Th17 cell expansion via inhibiting ROR γ t.

Recent investigations (Abd El-Twab et al., 2016; Mahmoud and Al Dera, 2015; Oztanir et al., 2014; Agarwal et al., 2005) have demonstrated that 18Gly reduces the oxidative stress and possesses strong antioxidant capabilities in addition to its anti-inflammatory properties.

In our result, the induction of ROS production was significantly lower for the 18Gly treatment than LPS treated control group (Fig. 8). These data indicate 18Gly treatment was protective against the oxidative stress by inducing significant decrease of ROS.

However, anti-oxidant effect and precise mechanisms of 18Gly cannot be explained by a simple decrease in intracellular ROS production. Although we have yet to test antioxidant effect of 18Gly in asthma model directly, our result showed that 18Gly may promote the differentiation of Treg cells via antioxidative stress effects. It can be argued that 18Gly may contribute the Treg/Th17 imbalance via antioxidative stress effects in asthma model.

The results demonstrated that 18Gly has inhibitory effects on airway inflammation, and that this effect is caused by suppression of

Th2 cytokines (IL-5, IL-13), and OVA-specific IgE production through the ROR γ t, STAT6, GATA-3, and Foxp3 transcription pathways.

Both CsA and 18Gly have effects on Penh, tissue eosinophil infiltration, airway inflammation, inhibition of Th2 cytokines (such as IL-13) and IgE in the lung, while CsA had a small or no effect on IL-5, to the Th1 cytokine (IFN- γ), PPAR γ in lung tissue. CsA was much more effective against inhibition of GATA-3, while 18Gly was more effective against Foxp3 expression. Similar results were shown in ROR γ t, pSTAT6 and cell phenotypes in lung and BALF. It is not clear why there should be a difference in the responsiveness to CsA and 18Gly among these factors. One possibility is that the differential effects of CsA on cytokine, transcription factors may determine the overall response.

CsA has been shown to provide a clinical benefit in patients with chronic severe steroid-dependent asthma. However, the oral medication of asthmatic patients with CsA is limited because of systemic side-effects (Nizankowska et al., 1995).

Herbal medicines and natural components play an important role both in preventive and curative treatments in traditional system of medicine, despite advances in modern western medicine. Although numerous modern medicines for the treatment of asthma have been identified, they suffer from various drawbacks, such as lack of efficacy,

excessive side effects and high cost. It is well known that herbal medicines and natural components from those are more effective with fewer side effects based on numerous reports.

Based on previous results (Kalani et al., 2013; Ram et al., 2006; Ukil et al., 2005; Mahmoud and Dera, 2015; Kalaiarasi and Pugalendi, 2009) which were conducted to show safety data, the effective and safety concentrations were used in our study.

Side effects including cardiac dysfunction, oedema and hypertension have been reported in high doses (400 mg/day) of glycyrrhizic acid (Størmer et al., 1993).

Consumed liquorice in doses of 50–200 g/day (corresponding to 75–540 mg/day glycyrrhethinic acid) can cause a significant rise in blood pressure (Sigurjonsdottir et al., 2001). However, further studies are needed to assess the systematic safety evaluation and clinical safety evaluation of 18Gly is needed for future direction

Therefore, it can be argued that 18Gly can be more effective candidate material with fewer side effects based on our results and numerous reports in this respect.

In conclusion, this is the first report showing that 18Gly significantly inhibits OVA-induced airway inflammation, eosinophil infiltration, and AHR in a mouse model of allergic asthma by suppression of Th2 cytokines (IL-5, IL-13) via STAT6, GATA-3, and Foxp3 transcription pathways. These data suggested that 18Gly might be a novel therapeutic compound for the treatment of inflammatory airway disorders, including allergic asthma.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology (2011-0006527) and in part funded by the Ministry of Science, ICT and Future Planning (2016R1A2B4A006269).

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