



meso-Dihydroguaiaretic acid attenuates airway inflammation and mucus hypersecretion in an ovalbumin-induced murine model of asthma

Ji-Won Song^a, Chang-Seob Seo^b, Eun-Sang Cho^a, Tae-In Kim^c, Young-Suk Won^d, Hyo-Jung Kwon^a, Jong-Keun Son^{c,*}, Hwa-Young Son^{a,**}

^a Department of Veterinary Pathology, College of Veterinary Medicine, Chungnam National University, Daejeon, Republic of Korea

^b K-herb Research Center, Korea Institute of Oriental Medicine, Daejeon, Republic of Korea

^c College of Pharmacy, Yeungnam University, Gyeongsan, Republic of Korea

^d Laboratory Animal Resource Center, Korea Research Institute of Bioscience and Biotechnology, Chungbuk, Republic of Korea

ARTICLE INFO

Article history:

Received 28 May 2015

Received in revised form 9 December 2015

Accepted 22 December 2015

Available online 11 January 2016

Keywords:

Asthma

IgE

meso-Dihydroguaiaretic acid

NF- κ B

MAPKs

Th2 cytokines

ABSTRACT

meso-Dihydroguaiaretic acid (MDGA), which is a dibenzylbutane lignin isolated from the ethyl acetate fraction of *Saururus chinensis*, has various biological activities, including anti-oxidative, anti-inflammatory, anti-bacterial, and neuroprotective effects. However, no report has examined the potential anti-asthmatic activity of MDGA. In this study, we evaluated the protective effects of MDGA on asthmatic responses, particularly airway inflammation and mucus hypersecretion in an ovalbumin (OVA)-induced murine model of asthma. Intragastric administration of MDGA significantly lowered the productions of interleukin (IL)-4, IL-5, IL-13, tumor necrosis- α (TNF- α), eotaxin, monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and immunoglobulin (Ig)E in bronchoalveolar lavage fluid (BALF), plasma, or lung tissues. Histological studies showed that MDGA inhibited OVA-induced inflammatory cell infiltration and mucus production in the respiratory tract. Moreover, MDGA markedly attenuated the OVA-induced activations of nuclear factor kappa B (NF- κ B), extracellular-signal-regulated kinases 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (p38 MAPK). Together, these results suggest that MDGA effectively inhibits airway inflammation and mucus hypersecretion by downregulating the levels of T helper 2 (Th2) cytokines, chemokines, and adhesion molecules, and inhibiting the activations of NF- κ B and MAPKs.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Asthma is a chronic inflammatory disease characterized by reversible airway obstruction, airway hyperresponsiveness (AHR), mucus hypersecretion, and airway remodeling [1]. The pathogenesis of asthma is associated with the infiltration of inflammatory cells, including eosinophils, mast cells, macrophages, and T helper (Th) cells. It is generally accepted that Th2-mediated immune responses to allergens are responsible for the development of allergic asthma [1]. Under asthmatic condition, allergens processed by antigen-presenting cells activate Th2 cells, triggering the release of Th2 cytokines, including interleukin (IL)-4, IL-5, and IL-13 [2]. These cytokines exacerbate the allergic response by increasing inflammatory cell infiltration into the airway, triggering immunoglobulin (Ig)E switching, and promoting chemokine

release, which together lead to mucus hypersecretion in the bronchiolar epithelial region [3,4]. Therefore, inhibition of Th2 cell function and/or Th2 effector responses are logical therapeutic goals for treating Th2-associated asthma.

Nuclear factor kappa B (NF- κ B) is an important transcription factor involved in the expression of various proinflammatory genes, including cytokines [IL-1, IL-2, and tumor necrosis factor- α (TNF- α)], chemokines [IL-8, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractant protein-1 (MCP-1), and eotaxin], and adhesion molecules [intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM), and E-selectin] [5]. Increased activation of NF- κ B has been demonstrated in the lungs after allergen challenge, and in airway epithelial cells and macrophages from asthmatic patients [6]. Many studies have also reported that allergic asthma could be improved by regulating the activation of NF- κ B signaling [5,7].

Mitogen-activated protein kinases (MAPKs), which include serine and threonine kinases, play critical roles in the activation of inflammatory cells. The MAPKs comprise three major subgroups: extracellular signal-related kinase 1/2 (ERK1/2), p38, and c-Jun. N-terminal kinase 1/2 (JNK1/2) [8]. Among them, p38 MAPK is markedly activated in the lungs of asthmatic mice compared with those of normal mice.

* Correspondence to: J.-K. Son, College of Pharmacy, Yeungnam University, 280 Daehak-ro, Gyeongsan 712-749, Republic of Korea.

** Correspondence to: H.-Y. Son, Department of Veterinary Pathology, College of Veterinary Medicine, Chungnam National University, 99 Daehak-ro, Yuseong-gu, Daejeon 305-764, Republic of Korea.

E-mail addresses: jkson@yu.ac.kr (J.-K. Son), hyson@cnu.ac.kr (H.-Y. Son).

Moreover, blockade of p38 MAPK has been shown to have an anti-inflammatory effect in allergic asthma, indicating that the inhibition or regulation of p38 MAPK could be essential for minimizing asthmatic symptoms [9].

meso-Dihydroguaiaretic acid (MDGA) is a dibenzylbutane lignin isolated from *Saururus chinensis* [10]. Because it exhibits diverse pharmacological effects with no marked toxicity, *S. chinensis* has been used in Korean folk medicine to treat various disorders, including jaundice, pneumonia, edema, and fever [11]. Recently, several studies showed that MDGA exerts many biological activities, including anti-oxidative, anti-inflammatory, anti-bacterial, and neuroprotective effects [12–15]. However, no previous study has examined the potential anti-asthmatic activity of MDGA. Here, we investigated the protective effects of MDGA on asthmatic responses using an ovalbumin (OVA)-induced mouse model of allergic asthma.

2. Materials and methods

2.1. Preparation of MDGA

MDGA was isolated from the ethyl acetate fraction of *S. chinensis* by silica gel chromatography. Briefly, dried roots of *S. chinensis* (9.7 kg) were extracted with 70% methanol by reflux for 24 h. The methanol extract was obtained 1.0 kg. The methanol extract was suspended with distilled water (1.4 L), and the resulting water layer was successively partitioned with *n*-hexane, ethyl acetate, and butanol (each 1.4 L $\times$ 3). The ethyl acetate fraction (130 g) was applied to a silica gel column (60 $\times$ 12 cm, Silica-gel 70–230 mesh; Merck, Germany), and the column was eluted with *n*-hexane and ethyl acetate solution (gradient from *n*-hexane 100% to ethyl acetate 100%) and then ethyl acetate and methanol (gradient from ethyl acetate 100% to methanol 100%). The eluent was combined on the basis of TLC, giving 39 fractions (SCE1–39) and obtained as a single compound. The purity of MDGA was determined to be $\geq 95\%$ by high-performance liquid chromatography (HPLC) analysis (Fig. 1). Green powder; $C_{20}H_{26}O_4$; mp 88–89.5 °C; $[\alpha]_D^{25}$ 0° (c 1.44, $CHCl_3$); ^1H -NMR ($CDCl_3$, 250 MHz) δ 6.77 (2H, d, J = 7.9 Hz, H-5, 5'), 6.58 (2H, d, J = 8.0 Hz, H-6, 6'), 6.50 (2H, d, J = 1.7 Hz, H-2, 2'), 3.78 (6H, s, $-OCH_3 \times 2$), 2.55–2.32 (4H, m, H-7, 7'), 1.72 (2H, m, H-8, 8'), 0.80 (6H, d, J = 6.7 Hz, H-9, 9'); ^{13}C -NMR ($CDCl_3$, 62.9 MHz) δ 146.2 (C-3, 3'), 143.4 (C-4, 4'), 133.5 (C-1, 1'), 121.5 (C-6, 6'), 113.8 (C-5, 5'), 111.2 (C-2, 2'), 55.7 ($-OCH_3 \times 2$), 41.0 (C-7, 7'), 37.4 (C-8, 8'), 13.8 (C-9, 9'); HRFABMS m/z 330.1842 ($[M]^+$, calcd. for $C_{20}H_{26}O_4$, 330.1831).

2.2. Animals

Specific pathogen-free (SPF) female BALB/c mice (5–6 weeks old) were purchased from Orient Bio (Seongnam, South Korea) and given a one-week period of quarantine and acclimatization prior to experiments. The animals were housed under environmentally controlled

conditions (22 ± 2 °C; relative humidity, $50 \pm 5\%$; and a 12 h light/dark cycle) and given standard rodent chow and sterilized tap water ad libitum. The use of animals in this study was approved by the Animal Experimental Ethics Committee of Chungnam National University (Daejeon, Republic of Korea).

2.3. OVA-induced allergic asthma

For OVA sensitization and challenge, the mice were divided into five groups (n = 7 mice per group). On days 0 and 14, mice were intraperitoneally sensitized with OVA (20 μ g) emulsified in aluminum hydroxide (2 mg) and suspended in 200 μ L phosphate buffered saline (PBS; pH 7.4). On days 21–23 after the initial sensitization, bronchial challenge with OVA (1%, in PBS) was delivered by ultrasonic nebulizer (NE-U12; Omron Corp., Tokyo, Japan) for 1 h. On days 18–23, MDGA was administered by once-daily gavage at doses of 10 mg/kg or 30 mg/kg dissolved in PBS. On days 21–23, this treatment was immediately followed by bronchial challenge. Positive control mice were orally treated with dexamethasone (DEXA, 200 μ g/mouse; Sigma, St. Louis, MO, USA), which is a standard glucocorticoid (GC)-class steroid drug that stands as one of the most effective therapies for asthma [16]. In addition, mice were sensitized/challenged with PBS and treated with MDGA (10 mg/kg or 30 mg/kg; n = 4 per group) on the above-described schedule.

In a previous study, mice were orally dosed with MDGA (1 or 10 mg/kg) for 2 weeks [17]. In preliminary studies, we tested the same doses (1 mg/kg and 10 mg/kg) using the procedures described herein. Inflammatory cell counts were obviously decreased in bronchoalveolar lavage fluid (BALF) from 10 mg/kg MDGA-treated mice compared with OVA-challenged mice. Those treated with 1 mg/kg also showed decreased inflammatory cell counts compared to OVA-treated mice, but the differences did not reach the level of significance. Based on these preliminary studies, we used 10 mg/kg of MDGA as the oral dose for the present study. To evaluate the dose-responses of MDGA, we used 30 mg/kg as the “high” dose.

2.4. Collection of blood and bronchoalveolar lavage fluid (BALF)

Mice were sacrificed 48 h after the last challenge (day 25). Blood samples were allowed to clot for 30 min at room temperature, and then centrifuged for 15 min at 200 g. Aliquots of serum were stored at -70 °C for biochemical analysis. To obtain BALF, ice-cold PBS (0.5 mL) was infused into the lung three times and withdrawn via tracheal cannulation (total volume, 1.5 mL). Each BALF aliquot was centrifuged, and the supernatants were harvested and stored individually at -70 °C for analysis. Each pellet was resuspended in 100 μ L PBS and centrifuged onto slides using a Cytospin centrifuge (Hanil Science Industrial, Seoul, Korea). The slides were dried and the cells were fixed and stained using the Diff-Quik staining reagent (IMEB Inc., Deerfield, IL, USA). Differential cell counting was performed based on the

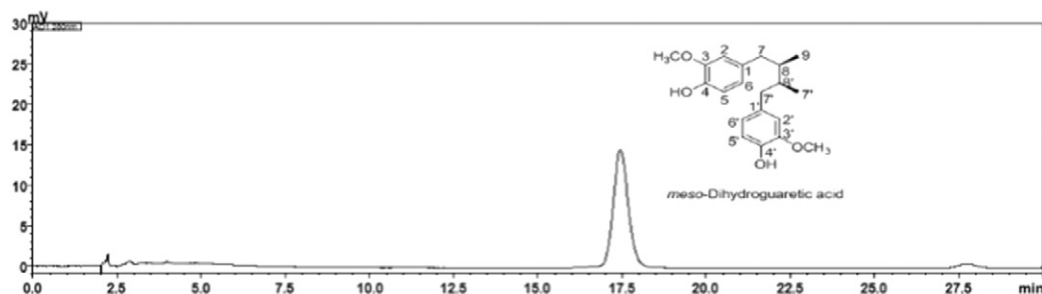


Fig. 1. HPLC chromatogram of MDGA at 280 nm. A SunFire C_{18} analytical column was used to separate the MDGA at 40 °C by isocratic elution with 0.1% (v/v) formic acid in distilled water (A) and 0.1% (v/v) formic acid in acetonitrile (B) (A:B = 4:6) as mobile phases. The flow rate was 1.0 mL/min and the injection volume was 10 μ L.

microscopic morphologies of the cells (Supplementary Fig. 1); five fields per slide (at least 100 leukocytes) were counted at $\times 400$ magnification.

2.5. Measurement of total and OVA-specific IgE levels in serum

The serum levels of total IgE and OVA-specific IgE were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). Briefly, 96-well microtiter plates were coated overnight with an isotype-specific coating (total IgE) or 10 $\mu\text{g}/\text{mL}$ OVA in PBS-Tween 20 (OVA-specific IgE). After the plate was washed and blocked, the samples were incubated for 2 h and HRP-conjugated goat anti-mouse IgE was added. After incubation of the plate for 1 h and four washes, 200 μL of o-phenylenediamine dihydrochloride (Sigma) was added to each well, the plate was incubated for 10 min, and absorbance was determined at 450 nm using a microplate ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.6. Measurement of cytokine, chemokine, and adhesion molecule levels

The concentrations of IL-4, IL-13, eotaxin and VCAM-1 in BALF were measured by specific ELISA kits (R&D Systems) according to the manufacturer's protocols. Whole lung tissue (40 mg) was homogenized in 500 μL of PBS with a protease inhibitor and centrifuged at 10,000 g for 20 min, and the supernatant was analyzed for pulmonary IL-5, TNF- α and MCP-1 using specific ELISA kits (R&D Systems). The results are presented as pg/mg protein.

2.7. Histopathology and periodic acid Schiff (PAS) staining

Lung tissues were embedded in paraffin and 4- μm -thick sections were cut using a microtome. The sections were deparaffinized using xylene, treated with a descending ethanol series, stained with hematoxylin and eosin (H&E), dehydrated in an ascending ethanol series, cleared with xylene, mounted in DPX mounting medium (Sigma) and analyzed under a microscope (Leica, Cambridge, UK). The degree of cell infiltration in the airway (Supplementary Fig. 2) was scored in a double-blind screen performed by two independent investigators. Peribronchiolar and perivascular inflammation were scored from 0 to 4, as described previously [18]. For each mouse, we analyzed five airway sections that were randomly distributed through the lung, and calculated their average scores. Sections were also assessed for mucus production by goblet cells, using a commercially available PAS staining kit (Merck Millipore, Guyancourt, France). The mucus production index

was calculated using an image analyzer (Leica), according to the previously published formula [19], as follows:

$$\text{Mucus production index (\%)} = \frac{\text{PAS-positive area}}{\text{Total area of airway epithelium}} \times 100\%.$$

2.8. Western blotting

Equal amounts of total lung proteins (35 μg) were resolved by 8% SDS-PAGE and transferred to nitrocellulose membranes at 30 V for 2 h. The membranes were blocked for 1 h with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% skim milk, and were incubated overnight at 4 $^{\circ}\text{C}$ with anti-NF- κB (Cell Signaling Technology, Beverly, MA, USA; 1:1000), anti-phospho-NF- κB (Cell Signaling Technology; 1:1000), anti-p38 MAPK (Cell Signaling Technology; 1:1000), anti-phospho-p38 (Cell Signaling Technology; 1:1000), anti-ERK1/2 (Cell Signaling Technology; 1:1000), anti-phospho-ERK1/2 (Cell Signaling Technology; 1:1000), or anti- β -actin (Sigma; 1:1500). The membranes were washed three times with TBST, and then incubated for 1 h at room temperature with a horseradish peroxidase (HRP)-conjugated secondary antibody (for NF- κB , p38 MAPK, and ERK1/2, we used an anti-rabbit secondary antibody at a 1:10,000 dilution; for β -actin, we used an anti-mouse secondary antibody at a 1:10,000 dilution; Sigma). The membranes were again washed three times with TBST, and then developed using an enhanced chemiluminescence kit (Thermo Scientific, Waltham, MA).

2.9. Statistical analysis

Data are expressed as the means $\pm$ standard error of the mean (SEM). Statistical comparisons were made with one-way analysis of variance (ANOVA) using SPSS 16.0 (IBM, Armonk, NY, USA), followed by a multiple comparison test with Tukey adjustment. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of MDGA on cell numbers in BALF

We examined changes in total cells, eosinophils, neutrophils, lymphocytes, and macrophages in BALF to determine the effects of MDGA on allergic asthma. Compared with BALF from negative control mice,

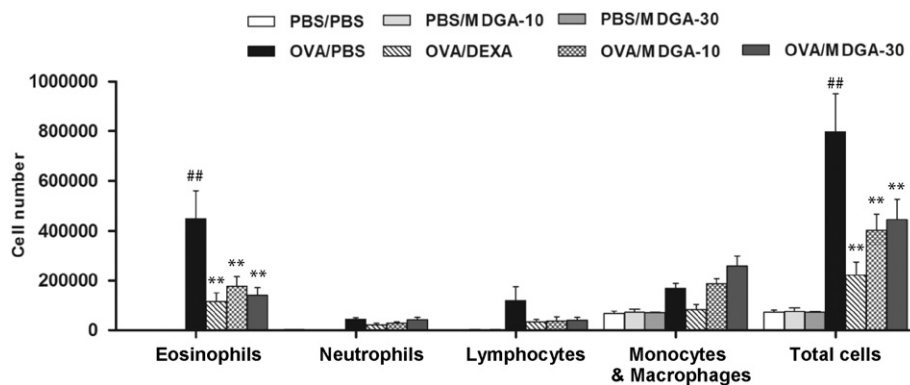


Fig. 2. Effects of MDGA on recruitment of inflammatory cells into BALF. BALF samples were collected 48 h after the last OVA challenge, and cell counting was performed on five fields per slide. Abbreviations: PBS/PBS, PBS-challenged mice; PBS/MDGA-10, PBS-challenged/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; PBS/MDGA-30, PBS-challenged/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice; OVA/PBS, OVA-sensitized/challenged mice; OVA/DEXA, OVA-sensitized/dexamethasone (200 $\mu\text{g}/\text{mouse}$)-treated mice; OVA/MDGA-10, OVA-sensitized/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; and OVA/MDGA-30, OVA-sensitized/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice. Data are expressed as means $\pm$ SEM; $n = 7$ (PBS/PBS, PBS/MDGA-10, PBS/MDGA-30, OVA/PBS, OVA/DEXA, OVA/MDGA-10, and OVA/MDGA-30) or 4 (PBS/MDGA-10 and PBS/MDGA-30) per group. Symbols: ##, $P < 0.01$ compared to the NC group; and **, $P < 0.01$ compared to the OVA group.

the eosinophils and total inflammatory cells were markedly higher in BALF from mice exposed to OVA sensitization and challenge (Fig. 2). BALF from the positive control DEXA-treated mice exhibited fewer eosinophils and total inflammatory cells than that from OVA-sensitized/challenged mice. Similarly, BALF from OVA-sensitized/challenged mice treated with MDGA displayed significantly fewer eosinophils and total cells than that from OVA-sensitized/challenged mice (Fig. 2).

3.2. Effect of MDGA on cytokines, chemokines, and adhesion molecules

Because MDGA reduced the number of inflammatory cells, we investigated its effects on the levels of the Th2-type cytokines, IL-4, IL-5, IL-13, and TNF- α . As shown in Fig. 3A and B, the levels of IL-4 and IL-13 in BALF were significantly higher in the OVA-sensitized/challenged mice compared with the negative controls. In contrast, MDGA-treated mice had a significantly lower level of IL-13 in BALF compared with

the OVA-sensitized/challenged mice. The levels of IL-5 and TNF- α in the lung were elevated in OVA/sensitized/challenged mice, but this increase was attenuated by MDGA treatment (Fig. 3C and D).

Similar to the above findings, the levels of eotaxin and MCP-1 were increased in the OVA-sensitized/challenged group, but this increase was significantly rescued in the MDGA-treated group (Fig. 3E and F). In addition, the significant increase of the level of VCAM-1 in the OVA-sensitized/challenged group was attenuated by MDGA treatment (Fig. 3G).

3.3. Effect of MDGA on total and OVA-specific IgE levels

Since antigen-specific Th2 responses trigger IgE antibody production, we examined whether MDGA influences total and OVA-specific IgE production in the OVA-induced allergic asthma mouse model. As shown in Fig. 4, the plasma levels of total and OVA-specific IgE were significantly increased in OVA-sensitized/challenged mice compared with

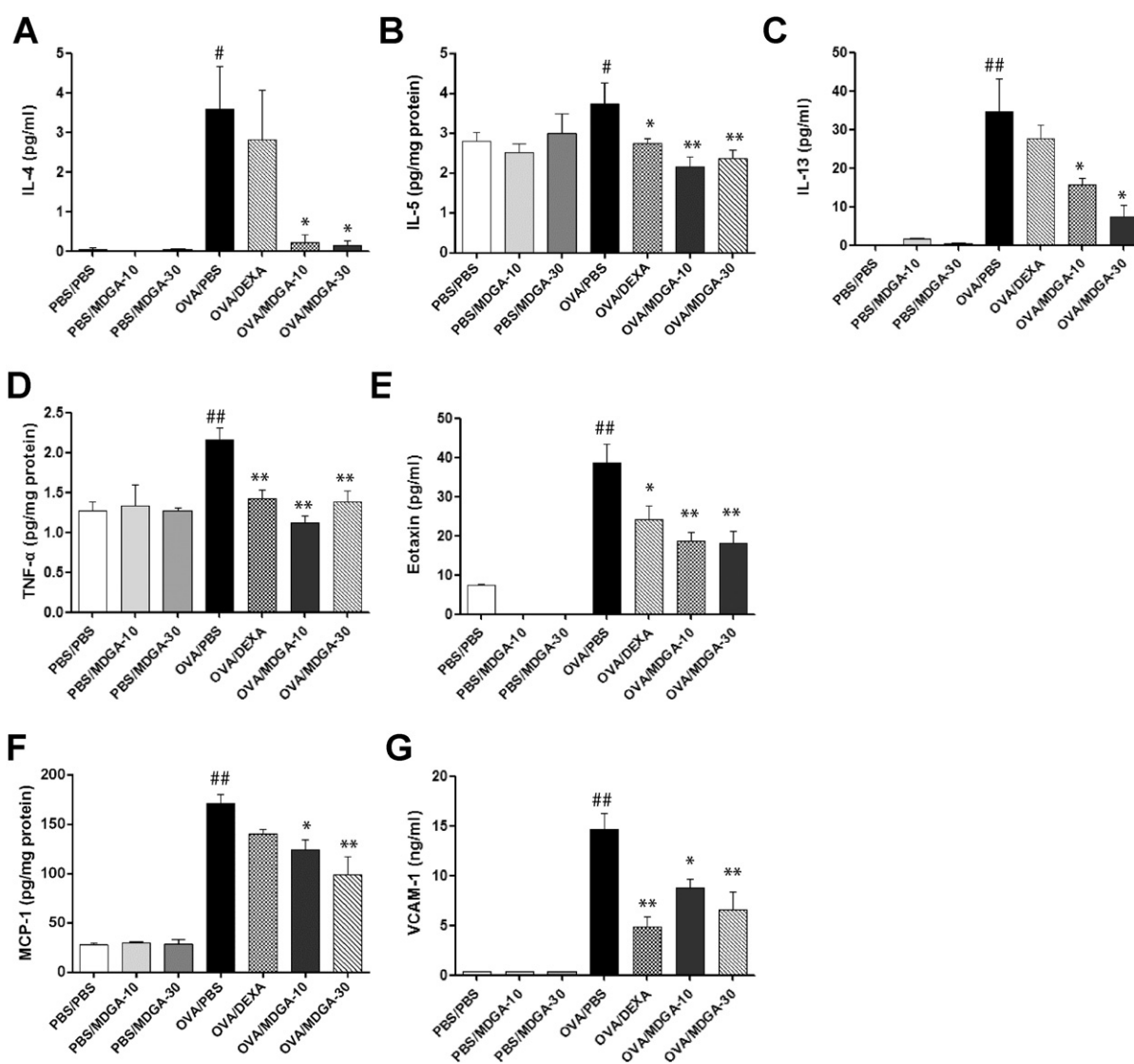


Fig. 3. Effects of MDGA on cytokines, chemokines, and adhesion molecules. BALF samples were collected 48 h after the last OVA challenge and assayed for the levels of IL-4, IL-13, eotaxin, and VCAM-1 using ELISA kits. Lung tissue homogenates were collected and used for analysis of IL-5, TNF- α , and MCP-1. (A) IL-4 levels in BALF. (B) IL-5 levels in lung tissue. (C) IL-13 levels in BALF. (D) TNF- α levels in lung tissue. (E) Eotaxin levels in BALF. (F) MCP-1 levels in lung tissue. (G) VCAM-1 levels in BALF. Abbreviations: PBS/PBS, PBS-challenged mice; PBS/MDGA-10, PBS-challenged/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; PBS/MDGA-30, PBS-challenged/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice; OVA/PBS, OVA-sensitized/challenged mice; OVA/DEXA, OVA-sensitized/dexamethasone (200 μ g/mouse)-treated mice; OVA/MDGA-10, OVA-sensitized/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; and OVA/MDGA-30, OVA-sensitized/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice. Data are expressed as means $\pm$ SEM; n = 7 (PBS/PBS, PBS/MDGA-10, PBS/MDGA-30, OVA/PBS, OVA/DEXA, OVA/MDGA-10, and OVA/MDGA-30) or 4 (PBS/MDGA-10 and PBS/MDGA-30) per group. Symbols: # and ##, $P < 0.05$ and $P < 0.01$, respectively, compared to the NC group; * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to the OVA group.

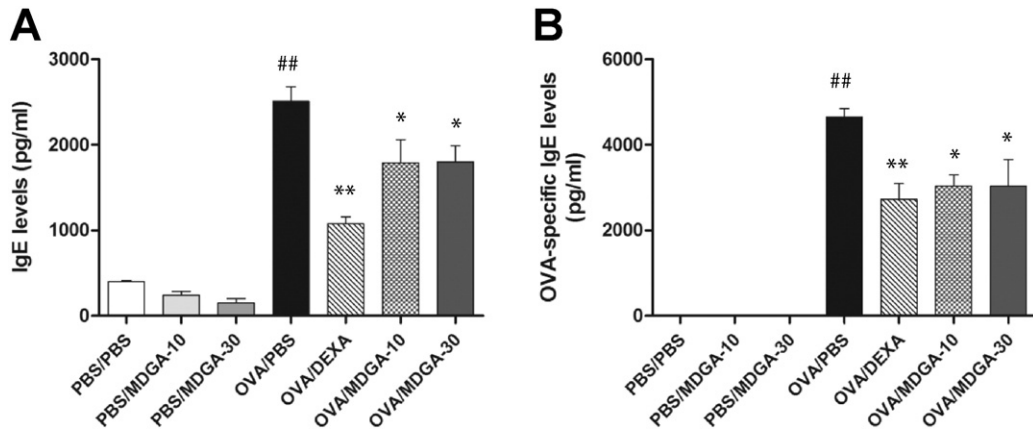


Fig. 4. Effects of MDGA on total and OVA-specific IgE levels in BALF. (A) Total IgE in BALF. (B) OVA-specific IgE in BALF. Abbreviations: PBS/PBS, PBS-challenged mice; PBS/MDGA-10, PBS-challenged/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; PBS/MDGA-30, PBS-challenged/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice; OVA/PBS, OVA-sensitized/challenged mice; OVA/DEXA, OVA-sensitized/dexamethasone (200 µg/mouse)-treated mice; OVA/MDGA-10, OVA-sensitized/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; and OVA/MDGA-30, OVA-sensitized/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice. Data are expressed as means ± SEM; n = 7 (PBS/PBS, PBS/MDGA-10, PBS/MDGA-30, OVA/PBS, OVA/DEXA, OVA/MDGA-10, and OVA/MDGA-30) or 4 (PBS/MDGA-10 and PBS/MDGA-30) per group. Symbols: # and ##, $P < 0.05$ and $P < 0.01$, respectively, compared to the NC group; * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to the OVA group.

negative control mice, whereas MDGA suppressed both total and OVA-specific IgE levels in BALF compared to those in the OVA-sensitized/challenged group. A similar suppressive effect was noted in DEXA-treated mice (Fig. 4).

3.4. Effect of MDGA on the recruitment of inflammatory cells to the airway

Lung inflammation is a characteristic hallmark of the allergic response to an allergen. Because MDGA was found to inhibit the

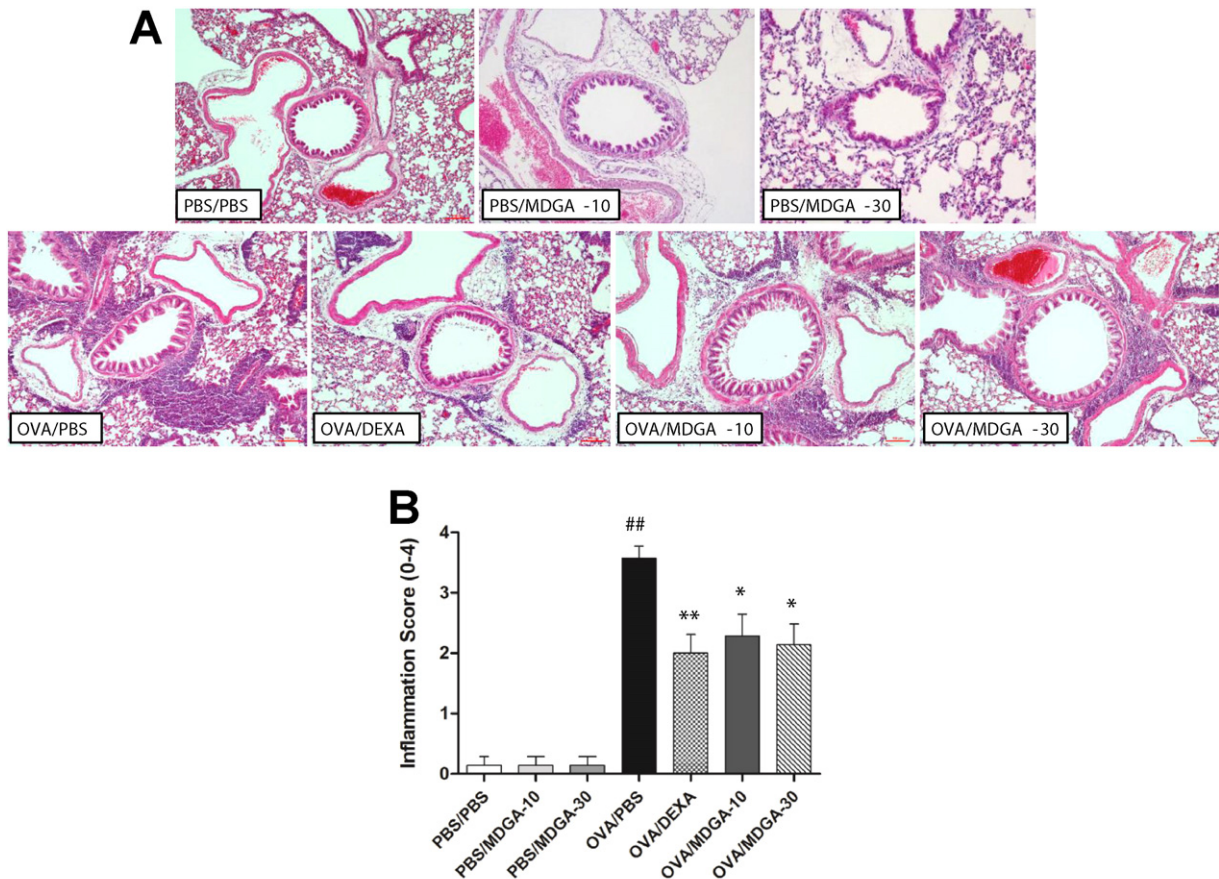


Fig. 5. Effects of MDGA on leukocyte recruitment in lung tissues. Histological examination was performed 48 h after the final OVA challenge. Lung tissues were fixed, sectioned at 4-µm thickness, stained with H&E, and examined under a microscope (X100). (A) H&E staining. (B) The extent of inflammation was scored via quantitative analysis of inflammatory cell infiltration. Abbreviations: PBS/PBS, PBS-challenged mice; PBS/MDGA-10, PBS-challenged/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; PBS/MDGA-30, PBS-challenged/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice; OVA/PBS, OVA-sensitized/challenged mice; OVA/DEXA, OVA-sensitized/dexamethasone (200 µg/mouse)-treated mice; OVA/MDGA-10, OVA-sensitized/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; and OVA/MDGA-30, OVA-sensitized/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice. Data are expressed as means ± SEM; n = 7 (PBS/PBS, PBS/MDGA-10, PBS/MDGA-30, OVA/PBS, OVA/DEXA, OVA/MDGA-10, and OVA/MDGA-30) or 4 (PBS/MDGA-10 and PBS/MDGA-30) per group. Symbols: ##, $P < 0.01$ compared to the NC group; * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to the OVA group.

recruitment of inflammatory cells into BALF, we used microscopic examination of lung tissues to examine the anti-asthmatic effects of MDGA. Lungs from the OVA-sensitized/challenged group showed widespread peribronchiolar and perivascular inflammation that comprised primarily eosinophils (Fig. 5). In contrast, mice treated with MDGA had substantially fewer eosinophils and macrophages in the peribronchial regions and airspaces compared with the OVA-sensitized/challenged mice (Fig. 5).

3.5. Effect of MDGA on airway mucus production and goblet cell hyperplasia

Although respiratory mucus protects the lower airways from dehydration and damage, excessive secretion of mucus by hyperplastic goblet cells contributes to the morbidity and mortality of many respiratory diseases, including allergic asthma. To determine whether MDGA could suppress the mucus overproduction induced by goblet cell hyperplasia, we stained lung sections with PAS. OVA-sensitized/challenged mice exhibited mucus overproduction in the bronchial airways compared with negative control mice, whereas the extent of mucus staining was markedly diminished in OVA-induced mice treated with MDGA (Fig. 6).

3.6. Effect of MDGA on the activities of NF- κ B p65 and MAPKs in lung tissues

To determine whether the therapeutic effect of MDGA on OVA-induced allergic asthma occurs via inactivation of the NF- κ B and/or MAPKs signaling pathways, we examined the protein expression levels of both phosphorylated and non-phosphorylated forms of various members of these pathways in mice subjected to OVA challenge. The phosphorylation of NF- κ B p65 in lung tissues was increased significantly in OVA-sensitized/challenged mice compared with the negative control group, but this induction was significantly smaller in the 30 mg/kg MDGA-treated group (Fig. 7A). Moreover, the phosphorylation levels of p38 MAPK and ERK1/2 were higher in OVA-sensitized/challenged mice than in PBS-treated mice, but these increases were reduced by MDGA treatment (Fig. 7B).

4. Discussion

We herein evaluated the protective effects of MDGA against airway inflammation in the OVA-induced mouse model of allergic asthma. The MDGA-treated mice displayed significantly fewer inflammatory cells (particularly eosinophils) in BALF and lung tissues, and lower levels of Th2-type cytokines (IL-4, IL-5, IL-13, and TNF- α), chemokines

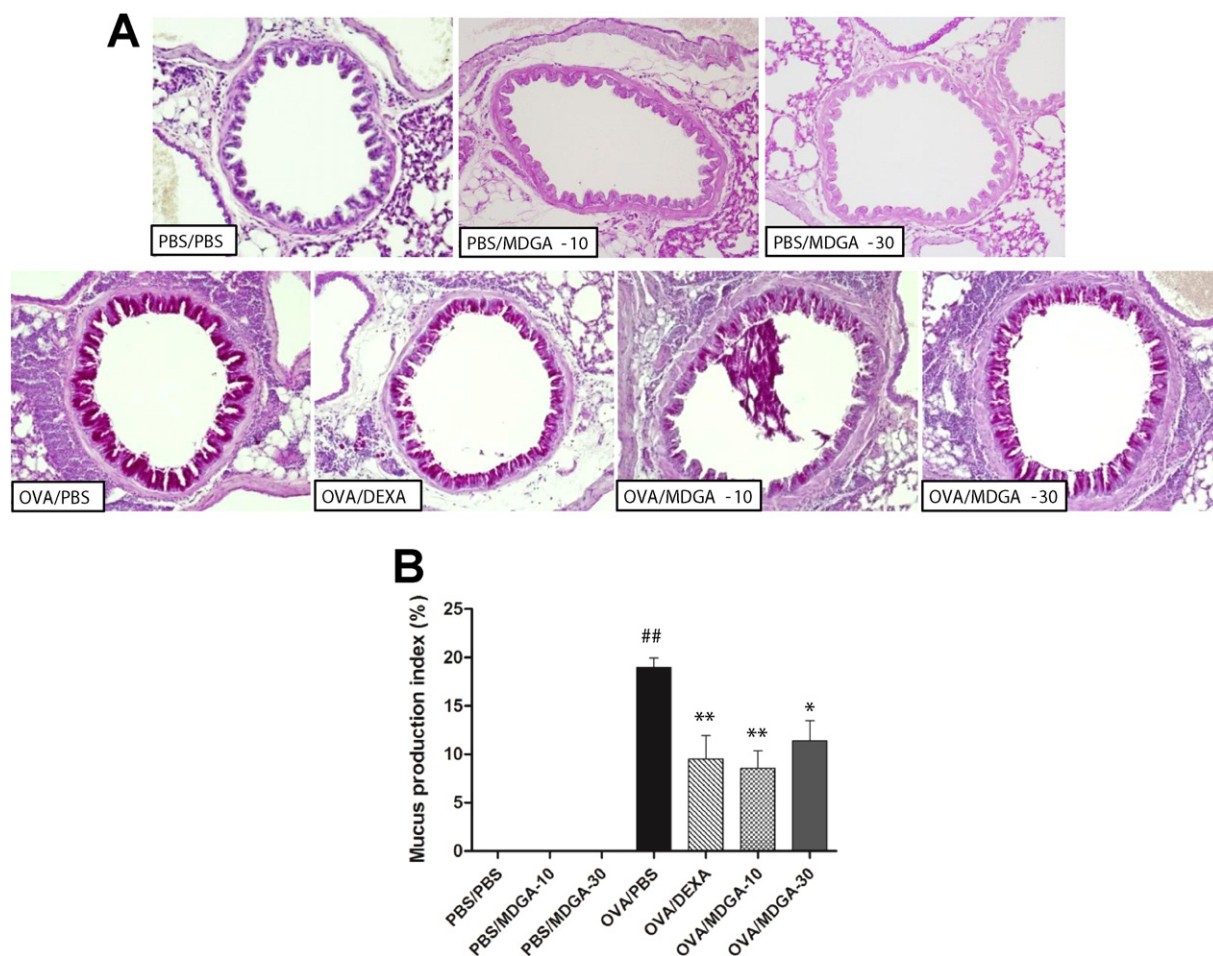


Fig. 6. Effects of MDGA on mucus production in lung tissues. Formalin-fixed lung sections were stained with PAS solution and examined under a microscope (A, X100). Mucus production was quantified in lung sections (B). Abbreviations: PBS/PBS, PBS-challenged mice; PBS/MDGA-10, PBS-challenged/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; PBS/MDGA-30, PBS-challenged/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice; OVA/PBS, OVA-sensitized/challenged mice; OVA/DEXA, OVA-sensitized/dexamethasone (200 μ g/mouse)-treated mice; OVA/MDGA-10, OVA-sensitized/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; and OVA/MDGA-30, OVA-sensitized/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice. Data are expressed as means $\pm$ SEM; $n = 7$ (PBS/PBS, PBS/MDGA-10, PBS/MDGA-30, OVA/PBS, OVA/DEXA, OVA/MDGA-10, and OVA/MDGA-30) or 4 (PBS/MDGA-10 and PBS/MDGA-30) per group. Symbols: ##, $P < 0.01$ compared to the NC group; * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to the OVA group.

(eotaxin and MCP-1), and the adhesion molecule (VCAM-1). Moreover, MDGA treatment decreased the serum levels of total IgE and OVA-specific IgE, abrogated mucus hypersecretion, and reduced the activations of NF- κ B and MAPKs in lung tissues, as compared with the OVA-sensitized/challenged mice.

Allergic airway inflammation, which is characterized by increased infiltration of leukocytes (e.g., eosinophils) and considerable secretion of mucus into the airways, is a major factor in the pathogenesis of asthma. Eosinophils have long been recognized as principal effector cells, and they are known to play pathogenic roles in asthma through the release of cytotoxic granule proteins [20,21]. Here, we report that MDGA prevents eosinophilic infiltration into the airways, as evidenced by significant decreases in the total cell and eosinophil counts of BALF. Likewise, histological examination showed that MDGA also inhibited tissue eosinophilia, as revealed by a marked reduction of inflammatory cell infiltration.

Previous studies have shown that the transmigration of eosinophils into the airways is a multistep process that is orchestrated by Th2 cytokines (e.g., IL-4, IL-5, and IL-13) and proinflammatory cytokines (e.g., TNF- α) and coordinated by the chemotactic cytokine (eotaxin) plus adhesion molecules (e.g., ICAM-1 and VCAM-1) [21,22]. IL-4

inhibits the apoptosis of eosinophils and promotes eosinophilic inflammation by inducing eosinophil chemotaxis/activation by up-regulating eotaxin [23]. Blockade of IL-4 by monoclonal antibodies has been shown to decrease IgE levels and airway eosinophilia in allergic mice [24], and IL-5 is known to be pivotal for the growth, differentiation, recruitment, and survival of eosinophils [25–27]. IL-13 potently induces mucus hypersecretion, eotaxin expression, airway inflammation, and airway hyperresponsiveness (AHR) [28,29]. TNF- α exerts similar responses, including the up-regulations of eosinophil chemoattractants and adhesion molecules, recruitment of eosinophils, elevation of cytokine release, and enhancement of AHR [30]. Eotaxin, which is a member of the chemotactic factor family, actively participates in the pathogenesis of allergic asthma by activating eosinophil recruitment [31,32]. Here, we report that MDGA decreases the levels of Th2 cytokines, proinflammatory cytokines, eotaxin, and adhesion molecules in the airway compared with those in OVA-sensitized/challenged mice, suggesting that the MDGA prevents eosinophilic airway inflammation by diminishing the secretion of the cytokines into lungs.

Mucus hypersecretion, which is another pathophysiological feature of many patients with allergic asthma, exacerbates asthma and chronic

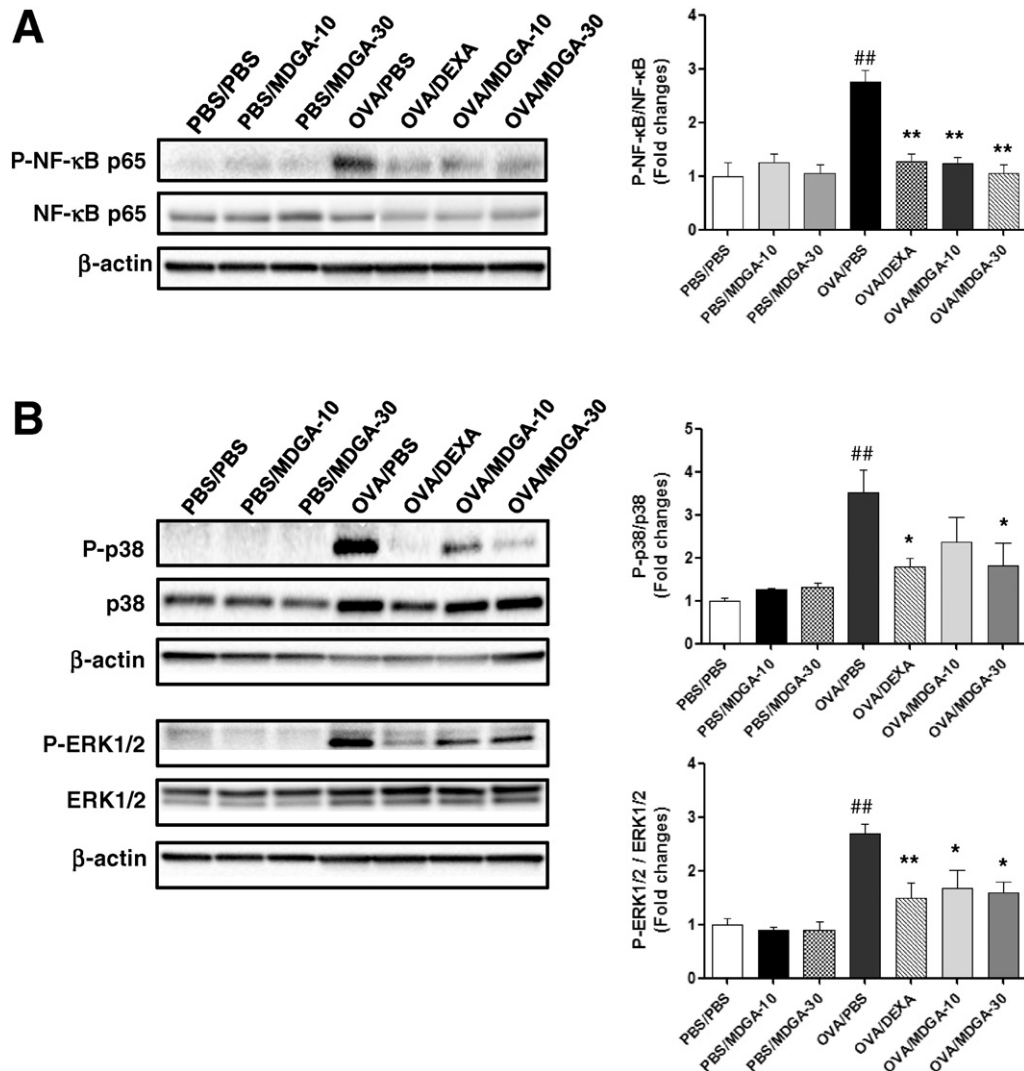


Fig. 7. Effects of MDGA on NF- κ B and MAPK activity in lung tissues. NF- κ B p65 (A), p38 MAPK, and ERK1/2 (B) protein levels were measured 48 h after the last challenge. Abbreviations: PBS/PBS, PBS-challenged mice; PBS/MDGA-10, PBS-challenged/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; PBS/MDGA-30, PBS-challenged/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice; OVA/PBS, OVA-sensitized/challenged mice; OVA/DEXA, OVA-sensitized/dexamethasone (200 μ g/mouse)-treated mice; OVA/MDGA-10, OVA-sensitized/*meso*-dihydroguaiaretic acid (10 mg/kg)-treated mice; and OVA/MDGA-30, OVA-sensitized/*meso*-dihydroguaiaretic acid (30 mg/kg)-treated mice. Data are expressed as means $\pm$ SEM; n = 7 (PBS/PBS, PBS/MDGA-10, PBS/MDGA-30, OVA/PBS, OVA/DEXA, OVA/MDGA-10, and OVA/MDGA-30) or 4 (PBS/MDGA-10 and PBS/MDGA-30) per group. Symbols: ^{##}, $P < 0.01$ compared to the NC group; * and **, $P < 0.05$ and $P < 0.01$, respectively, compared to the OVA group.

obstructive lung disease by causing severe narrowing of the airways [33]. As expected, OVA sensitized/challenged mice showed mucus over-production and goblet cell hyperplasia in their bronchial airways. The administration of MDGA reduced this mucus production, showing effects similar to that of DEXA. Excess mucus secretion can be induced by a variety of factors, such as Th2 cytokines, IgE, and eotaxin. In particular, IL-4 and IL-13 stimulate the production of IgE from B lymphocytes [34], and antigen-specific IgE binds to receptors on mast cells and sensitizes them to release mediators [35]. Activated mast cells produce diverse mediators and cytokines, which promote a hypersensitivity response characterized by bronchial smooth muscle contraction, mucosal edema, and mucus hypersecretion [36]. IL-4 can also trigger the differentiation of airway epithelial cells into mucus-producing goblet cells [37]. Our results demonstrate that MDGA may reduce Th2-mediated immune reactions and the subsequent IL-4- and IL-13-mediated inflammatory response and mucus hypersecretion. This suggests that MDGA could have therapeutic potential against human allergic asthma.

We also sought to explore the underlying mechanisms through which MDGA treatment ameliorates airway allergic diseases. Persistent activations of MAPKs and NF- κ B are known to play essential roles in immune and inflammatory processes, including those of asthma [38,39]. As a transcription factor, NF- κ B regulates a wide variety of target genes that encode multiple inflammatory cytokines (e.g., TNF- α , IL-1 β , IL-4, IL-5, IL-13, ICAM-1, and VCAM-1) that are closely implicated in the pathogenesis of asthma [40]. Here, we report that OVA challenge significantly increased the phosphorylation of NF- κ B, whereas pretreatment with MDGA blocked this OVA-induced NF- κ B activation. These results indicate that the anti-asthmatic effects of MDGA may be attributed to inhibition of the transcriptional activity of NF- κ B and subsequent reductions among the proinflammatory mediators. As MAPK family members reportedly contribute to mediating numerous biological functions, including cell proliferation, differentiation, apoptosis, and inflammation [41], we also investigated the effects of MDGA on MAPK signaling. Our results showed that MDGA treatment abolished the OVA-stimulated activations of p38 MAPK and ERK1/2. Since the MAPK signaling cascade is known to contribute to the activation of NF- κ B [42], our results suggest that the treatment of mice with MDGA suppresses MAPKs and subsequently disrupts NF- κ B activity, thereby reversing the pathophysiologic features of asthma.

Song et al. showed that MDGA inhibits platelet-derived growth factor (PDGF)-induced rat aortic vascular smooth muscle cell (VSMC) proliferation by suppressing the phosphorylation of MAPKs [43]. To further demonstrate the mechanism of action of MDGA, the same authors examined PDGF receptor beta (PDGFR β), which is a cell surface tyrosine kinase receptor for PDGF family members [44]. The binding of PDGF to PDGFR β is associated with activation of downstream signaling molecules, including p38, ERK1/2, and JNK [45]. Song et al. found that MDGA inhibits the phosphorylation of PDGFR β , suggesting that the anti-proliferative effects of MDGA in VSMCs are mediated by MAPK signaling through the suppression of PDGFR β phosphorylation. Although our present results show that the protective effects of MDGA were associated with its ability to suppress the activations of MAPKs and NF- κ B. Further studies are needed to identify the action mechanism of MDGA in ameliorating the OVA-induced murine model of asthma.

In conclusion, our data demonstrate that MDGA attenuates OVA-induced airway inflammation and mucus hypersecretion in a murine model of allergic asthma. The protective effects of MDGA were associated with the down-regulations of cytokines, IgE, and NF- κ B/MAPKs activities in lung tissues. These findings collectively suggest that MDGA has potential to be a useful medicine for the treatment of allergic asthma.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF-2010-0011450).

Appendix A. Supplementary data

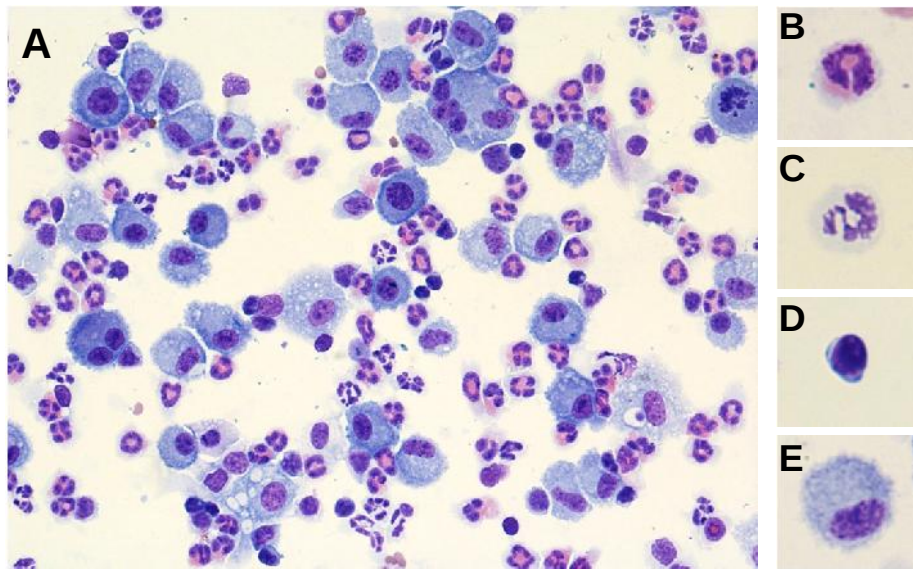
Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2015.12.033>.

References

- [1] D.K. Agrawal, Z. Shao, Pathogenesis of allergic airway inflammation, *Curr. Allergy Asthma Rep.* 10 (2010) 39–48.
- [2] R. Leigh, R. Ellis, J.N. Wattie, J.A. Hirota, K.I. Matthaie, P.S. Foster, et al., Type 2 cytokines in the pathogenesis of sustained airway dysfunction and airway remodeling in mice, *Am. J. Respir. Crit. Care Med.* 169 (2004) 860–867.
- [3] R. Afshar, B.D. Medoff, A.D. Luster, Allergic asthma: a tale of many T cells, *Clin. Exp. Allergy* 38 (2008) 1847–1857.
- [4] S.N. Georas, J. Guo, U. De Fanis, V. Casolaro, T-helper cell type-2 regulation in allergic disease, *Eur. Respir. J.* 26 (2005) 1119–1137.
- [5] F.Y. Goh, N. Upton, S. Guan, C. Cheng, M.K. Shanmugam, G. Sethi, et al., Fisetin, a bioactive flavonol, attenuates allergic airway inflammation through negative regulation of NF- κ B, *Eur. J. Pharmacol.* 679 (2012) 109–116.
- [6] D. Gras, P. Chanez, I. Vachier, A. Petit, A. Bourdin, Bronchial epithelium as a target for innovative treatments in asthma, *Pharmacol. Ther.* 140 (2013) 290–305.
- [7] X. Ci, X. Chu, X. Xu, H. Li, X. Deng, Short-term roxithromycin treatment attenuates airway inflammation via MAPK/NF- κ B activation in a mouse model of allergic asthma, *Inflamm. Res.* 61 (2012) 749–758.
- [8] J.S. Arthur, S.C. Ley, Mitogen-activated protein kinases in innate immunity, *Nat. Rev. Immunol.* 13 (2013) 679–692.
- [9] W. Duan, J.H. Chan, K. McKay, J.R. Crosby, H.H. Choo, B.P. Leung, et al., Inhaled p38alpha mitogen-activated protein kinase antisense oligonucleotide attenuates asthma in mice, *Am. J. Respir. Crit. Care Med.* 171 (2005) 571–578.
- [10] C.S. Ye, K.Y. Lee, Y.J. Kim, J.S. Jung, Y. Jahng, H.W. Chang, et al., Protective effect of lignans against sepsis from the roots of *Saururus chinensis*, *Biol. Pharm. Bull.* 31 (2008) 523–526.
- [11] H.S. Ryu, H.K. Lee, J.S. Kim, Y.G. Kim, M. Pyo, J. Yun, et al., Saucerneol D inhibits dendritic cell activation by inducing heme oxygenase-1, but not by directly inhibiting toll-like receptor 4 signaling, *J. Ethnopharmacol.* 166 (2015) 92–101.
- [12] W.S. Lee, Y.I. Baek, J.R. Kim, K.H. Cho, D.E. Sok, T.S. Jeong, Antioxidant activities of a new lignan and a neolignan from *Saururus chinensis*, *Bioorg. Med. Chem. Lett.* 14 (2004) 5623–5628.
- [13] T.C. Moon, C.S. Seo, K. Haa, J.C. Kim, N.K. Hwang, T.G. Hong, et al., Meso-dihydroguaiaretic acid isolated from *Saururus chinensis* inhibits cyclooxygenase-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells, *Arch. Pharm. Res.* 31 (2008) 606–610.
- [14] C.J. Ma, S.R. Kim, J. Kim, Y.C. Kim, Meso-dihydroguaiaretic acid and licarin A of *Machilus thunbergii* protect against glutamate-induced toxicity in primary cultures of a rat cortical cells, *Br. J. Pharmacol.* 146 (2005) 752–759.
- [15] C.J. Ma, S.H. Sung, Y.C. Kim, Neuroprotective lignans from the bark of *Machilus thunbergii*, *Planta Med.* 70 (2004) 79–80.
- [16] J. Liu, M. Zhang, C. Niu, Z. Luo, J. Dai, L. Wang, et al., Dexamethasone inhibits repair of human airway epithelial cells mediated by glucocorticoid-induced leucine zipper (GILZ), *PLoS One* 8 (2013), e60705.
- [17] W.C. Sim, S. Park, K.Y. Lee, Y.T. Je, H.Q. Yin, Y.J. Choi, et al., LXR- α antagonist meso-dihydroguaiaretic acid attenuates high-fat diet-induced nonalcoholic fatty liver, *Biochem. Pharmacol.* 90 (2014) 414–424.
- [18] K.G. Tournoy, J.C. Kips, C. Schou, R.A. Pauwels, Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness, *Clin. Exp. Allergy* 30 (2000) 79–85.
- [19] D. Adenuga, S. Caito, H. Yao, I.K. Sundar, J.W. Hwang, S. Chung, et al., Nrf2 deficiency influences susceptibility to steroid resistance via HDAC2 reduction, *Biochem. Biophys. Res. Commun.* 403 (2010) 452–456.
- [20] E.A. Jacobsen, S.I. Ochkur, N.A. Lee, J.J. Lee, Eosinophils and asthma, *Curr. Allergy Asthma Rep.* 7 (2007) 18–26.
- [21] N.W. Lukacs, Role of chemokines in the pathogenesis of asthma, *Nat. Rev. Immunol.* 1 (2001) 108–116.
- [22] J. Mattes, P.S. Foster, Regulation of eosinophil migration and Th2 cell function by IL-5 and eotaxin, *Curr. Drug Targets Inflamm. Allergy* 2 (2003) 169–174.
- [23] J.W. Steinke, L. Borish, Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists, *Respir. Res.* 2 (2001) 66–70.
- [24] Y.H. Kim, T.Y. Yang, C.S. Park, S.H. Ahn, B.K. Son, J.H. Kim, et al., Anti-IL-33 antibody has a therapeutic effect in a murine model of allergic rhinitis, *Allergy* 67 (2012) 183–190.
- [25] J. Corren, Inhibition of interleukin-5 for the treatment of eosinophilic diseases, *Discov. Med.* 13 (2012) 305–312.
- [26] P.S. Foster, S.P. Hogan, A.J. Ramsay, K.I. Matthaie, I.G. Young, Interleukin 5 deficiency abolishes eosinophilia, airways hyperreactivity, and lung damage in a mouse asthma model, *J. Exp. Med.* 183 (1996) 195–201.
- [27] M. Kopf, F. Brombacher, P.D. Hodgkin, A.J. Ramsay, E.A. Milbourne, W.J. Dai, et al., IL-5-deficient mice have a developmental defect in CD5⁺ B-1 cells and lack eosinophilia but have normal antibody and cytotoxic T cell responses, *Immunity* 4 (1996) 15–24.
- [28] L. Li, Y. Xia, A. Nguyen, Y.H. Lai, L. Feng, T.R. Mosmann, et al., Effects of Th2 cytokines on chemokine expression in the lung: IL-13 potently induces eotaxin expression by airway epithelial cells, *J. Immunol.* 162 (1999) 2477–2487.
- [29] M. Kudo, Y. Ishigatsubo, I. Aoki, Pathology of asthma, *Front. Microbiol.* 4 (2013) 263.

- [30] J.A. Boyce, B. Bochner, F.D. Finkelman, M.E. Rothenberg, Advances in mechanisms of asthma, allergy, and immunology in 2011, *J. Allergy Clin. Immunol.* 129 (2012) 335–341.
- [31] D.M. Conroy, T.J. Williams, Eotaxin and the attraction of eosinophils to the asthmatic lung, *Respir. Res.* 2 (2001) 150–156.
- [32] L. Hemelaers, R. Louis, [Eotaxin: an important chemokine in asthma], *Rev. Med. Liege* 61 (2006) 223–226.
- [33] R.M. Pascual, S.P. Peters, Airway remodeling contributes to the progressive loss of lung function in asthma: an overview, *J. Allergy Clin. Immunol.* 116 (2005) 477–486.
- [34] S.S. Deo, K.J. Mistry, A.M. Kakade, P.V. Niphadkar, Role played by Th2 type cytokines in IgE mediated allergy and asthma, *Lung India.* 27 (2010) 66–71.
- [35] S.J. Galli, M. Tsai, IgE and mast cells in allergic disease, *Nat. Med.* 18 (2012) 693–704.
- [36] H.C. Oettgen, R.S. Geha, IgE regulation and roles in asthma pathogenesis, *J. Allergy Clin. Immunol.* 107 (2001) 429–440.
- [37] K. Dabbagh, K. Takeyama, H.M. Lee, I.F. Ueki, J.A. Lausier, J.A. Nadel, IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo, *J. Immunol.* 162 (1999) 6233–6237.
- [38] C. Xinxin, C. Chi, C. Xiao, X. Xue, Y. Yongjun, C. Junqing, et al., Flufenicol inhibits allergic airway inflammation in mice by p38 MAPK-mediated phosphorylation of GATA 3, *Clin. Immunol.* 138 (2011) 231–238.
- [39] T. Asano, H. Kume, F. Taki, S. Ito, Y. Hasegawa, Thalidomide attenuates airway hyperresponsiveness and eosinophilic inflammation in a murine model of allergic asthma, *Biol. Pharm. Bull.* 33 (2010) 1028–1032.
- [40] A.A. Imanifooladi, S. Yazdani, M.R. Nourani, The role of nuclear factor-kappaB in inflammatory lung disease, *Inflamm. Allergy Drug Targets* 9 (2010) 197–205.
- [41] H. Tang, Y. Sun, Z. Shi, H. Huang, Z. Fang, J. Chen, et al., YKL-40 induces IL-8 expression from bronchial epithelium via MAPK (JNK and ERK) and NF-kappaB pathways, causing bronchial smooth muscle proliferation and migration, *J. Immunol.* 190 (2013) 438–446.
- [42] N. Sakai, T. Wada, K. Furuichi, Y. Iwata, K. Yoshimoto, K. Kitagawa, et al., p38 MAPK phosphorylation and NF-kappa B activation in human crescentic glomerulonephritis, *Nephrol. Dial. Transplant.* 17 (2002) 998–1004.
- [43] M.C. Song, E.C. Kim, W.J. Kim, T.J. Kim, Meso-dihydroguaiaretic acid inhibits rat aortic vascular smooth muscle cell proliferation by suppressing phosphorylation of platelet-derived growth factor receptor beta, *Eur. J. Pharmacol.* 744 (2014) 36–41.
- [44] C.H. Heldin, B. Westermark, Mechanism of action and in vivo role of platelet-derived growth factor, *Physiol. Rev.* 79 (1999) 1283–1316.
- [45] A.D. Hughes, G.F. Clunn, J. Refson, C. Demoliou-Mason, Platelet-derived growth factor (PDGF): actions and mechanisms in vascular smooth muscle, *Gen. Pharmacol.* 27 (1996) 1079–1089.

Song *et al.*, Supplementary Fig. 1



Song *et al.*, Supplementary Fig. 2

