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Macelignan attenuated allergic lung inflammation and airway hyper-responsiveness in murine experimental asthma

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ABSTRACT

Aims: Macelignan isolated from *Myristica fragrans* Houtt. is widely used for spice and flavoring for foods, and has been reported to have anti-inflammatory activity. The aim of this study was to investigate the effects of macelignan on allergic lung inflammation with a murine model of experimental asthma.

Main methods: Fungal protease mixed with chicken egg ovalbumin allergen was used as a challenge to induce murine experimental asthma. To determine its effects on allergy and inflammation, macelignan was administered orally during allergen challenge, and the symptoms of allergic asthma and its underlined mechanisms were examined.

Key findings: Treatment with macelignan attenuated eosinophilic airway inflammation and airway hyper-responsiveness. With the administration of macelignan, interleukin-4 (IL-4) producing cells, but not interferon- γ (IFN- γ) or IL-17 producing cells, were diminished in the lungs. Additionally, activation of the T helper type 2 (Th2) cell-specific master transcription factor, GATA3 was decreased with macelignan treatment. Finally, production of IL-4 but not IFN- γ or IL-17, by CD4⁺ T cells was reduced with stimulation when combined with the administration of macelignan.

Significance: Our data show that macelignan has anti-inflammatory effects on Th2 cell-mediated allergic lung inflammation and could potentially provide a novel preventative and/or therapy for the treatment of allergic diseases.

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Introduction

Asthma is a complex immune-related disorder characterized by chronic inflammation of the airways, airway hyper-responsiveness (AHR), and airflow obstruction (Busse and Lemanske, 2001; Umetsu et al., 2002). Many features of asthma are thought to reflect consequences of Th2 cell-dominated immune responses to allergens. Further, allergen-specific Th2 cells play a pivotal role in the pathogenesis of the disease. Lung homing Th2 cells, through secretion of IL-5 can recruit eosinophils resulting in the manifestation of tissue eosinophilia. In addition, Th2 effector cytokines, IL-4 and IL-13, can directly act on epithelial and smooth muscle cells of airway epithelium to induce mucus hyper-production, goblet cell hyperplasia, and AHR (Galli et al., 2008;

Wills-Karp, 1999). GATA3-deficient Th2 cells fail to produce IL-4, IL-5 or IL-13, or to induce Th2 memory responses (Pai et al., 2004; Zhu et al., 2004). Thus, GATA3 is regarded as a master regulator required for the induction of Th2 differentiation and the production of these cytokines (Zhang et al., 1997; Zheng and Flavell, 1997).

Macelignan (Fig. 1), isolated from *Myristica fragrans* (the seeds from which are commonly known as nutmeg), is structurally identified as a (8R, 8'S)-7-(3,4-methylene-dioxyphenyl)-7'-(4-hydroxy-3-methoxyphenyl)-8,8'-dimethylbutane (Woo et al., 1987). Macelignan has been reported to possess various biological properties including anti-oxidant, anti-inflammatory, and anti-cariogenic activities (Chung et al., 2006; Jin et al., 2005; Park et al., 2004). Nutmeg, derived from the dried seed kernel of *M. fragrans*, has been used for the treatment of several inflammatory diseases in traditional medicines of different countries (Joshi, 2000). However, the effects of macelignan in allergic asthma have not yet been investigated. Therefore, in this study, we investigated the anti-asthmatic effects of macelignan in combination with fungal protease and OVA in an allergen induced allergic asthma experimental model.

To evaluate a preventative and/or therapeutic potential of macelignan, we utilized a murine model of experimental asthma, and verified the ability of macelignan to prevent disease in this model. Here we show that

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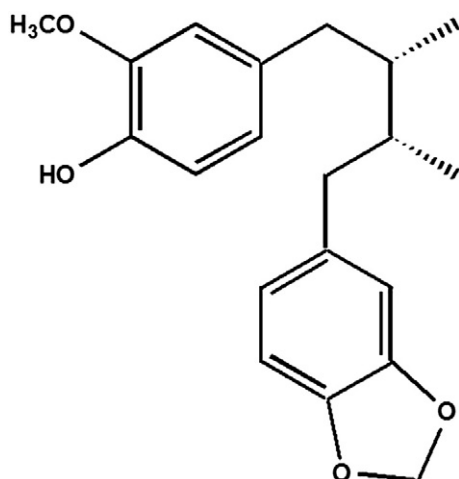


Fig. 1. The chemical structure of macelignan.

sensitizations and two intranasal challenges every 4 days (Days 0, 4, 8, 12, 16) with 50 μ l of allergen. For intranasal challenge, mice were lightly anesthetized using isoflurane inhalation (Abbott laboratory, Abbott Park, IL), and were administered 50 μ l of allergen mixture. Macelignan dissolved in Tween-80 (Sigma-Aldrich, 0 = vehicle only, 400 and 800 mg/kg) was given orally everyday during asthma induction. Low endotoxin levels in allergen and macelignan samples were confirmed before administration.

Analysis of asthma phenotypes

For all saline and allergen challenged mice, AHR, bronchoalveolar lavage (BAL) cytology, BAL glycoprotein assay, and lung histopathology were determined as previously described (Lee et al., 2003). Briefly, 16 h after final intranasal challenge, mice were anesthetized with pentobarbital sodium (Hanlim Pharma Co., Seoul, Korea, 60 mg/kg) and intubated by using 20-gage cannula. After intubation, mice were connected via the endotracheal cannula to a flexiVent system (SCIREQ Inc., Montreal, Canada). After initiating mechanical ventilation, mice were paralyzed with pancuronium bromide (Sigma-Aldrich, 1 mg/kg) and ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end expiratory pressure (PEEP) of 3 cmH_2O . To assess airway responsiveness, mice were given incremental doses of acetylcholine (Sigma-Aldrich) to the lateral tail vein (0 = vehicle (normal saline) only, 0.01, 0.03, 0.09, 0.27, and 0.81 mg/kg), and lung resistances were measured. Lung resistances were allowed to return to the baseline level between each dose of acetylcholine. The PC_{200} value ($\mu\text{g/g}$), in which the dose of acetylcholine required to induce a provocative 200% increase from base line, was calculated according to the lung resistance results. After AHR, BAL cytology, and the secreted glycoprotein assay were performed, lung cytokine profiles were produced by ELISpot analysis as described previously (Lee et al., 2004).

Briefly, ELISpot plates were prepared by coating with capture antibodies against IL-4 (clone: 11B11, BD Biosciences, San Diego, CA), IFN- γ (clone: AN18, eBioscience, San Diego, CA), and IL-17 (clone: eBio17CCK15A5, eBioscience) and by blocking with 0.2% I-block (Applied Biosystems, Foster City, CA). Single cell suspensions were prepared from the lungs of saline or allergen-challenged mice and were added to ELISpot plates and serially diluted (2-fold). After an overnight incubation, the plates were washed and the cells were incubated with biotin-conjugated detection antibodies against IL-4 (clone: BVD6-24G2, BD Biosciences), IFN- γ (clone: R4-6A2, eBioscience), and IL-17 (clone: eBio17B7, eBioscience). After washing, alkaline Phosphatase-conjugated streptavidin (BD Biosciences) diluted in PBS/Tween/BSA (1:1000 dilution) was added and the plates were incubated at room temperature for 30 min. After another wash, the plates were developed using 5-bromo-4-chloro-indolyl-phosphate in 0.1 M alkaline buffer (Sigma-Aldrich) suspended in 0.6% low melting agarose. Individual blue spots were counted after solidification of the agar using an ImmunoSpot® Analyzer (Cellular Technology Ltd., Shaker Heights, OH).

Measurement of secreted glycoprotein in BAL fluid

Secreted glycoprotein levels were measured by a modified ELISA that uses jacalin, a glycoprotein binding lectin as previously reported (Lee et al., 2004). Briefly, glycoprotein mucin standard (derived from porcine stomach, Sigma-Aldrich) dissolved in PBS was diluted serially 2-fold. BAL fluid samples were diluted in PBS serially 2-fold beginning at a 1:100 dilution, and 40 μ l from each sample was transferred to flat bottom ELISA plate (Greiner, Kremsmunster, Austria) and incubated at 37 $^{\circ}\text{C}$ for 2 h or at 4 $^{\circ}\text{C}$ overnight. After washing, plates were blocked by the addition of 200 μ l of 0.2% I-block and incubating at 37 $^{\circ}\text{C}$ for 2 h. After washing, 40 μ l of biotinylated jacalin (Vector Laboratories, Burlingame, CA) diluted in PBS/Tween with 0.1% BSA 178

macelignan attenuates the severity of murine experimental asthma by selectively suppressing IL-4 producing CD4^{+} Th2 cells through inhibition of GATA-3 activation.

Materials and methods

Animals

Wild type C57BL/6J mice and T cell receptor transgenic (OT-II) mice, in which CD4^{+} T cells are specific for chicken egg ovalbumin (OVA) peptides, were purchased from Jackson Laboratory (Bar Harbor, ME), and bred in our pathogen-free animal facility. All animals were fed a standard diet (PMI Lab Diet) ad libitum with free access to water. Female mice, 6 to 8 weeks old were used for this study. Animal care and experimental procedures were performed with the approval of the Animal Care Committee of KAIST.

Preparation of macelignan

Dried rhizomes of *M. fragrans* were collected in Jakarta, Indonesia. A voucher specimen has been deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). The ground nutmeg (100 g) was extracted with 100% ethanol (400 ml), and the extract (7 g) was further fractionated with ethyl acetate. The ethyl acetate fraction (4.2 g) was applied to silica gel open column chromatography (60, 70–230 mesh, Merck & Co., Whitehouse Station, NJ), and eluted with n-hexane and ethyl acetate solution (10:1, v/v) to obtain six fractions (Fractions I to VI). Fraction III was further fractionated with n-hexane and ethyl acetate solution (20:1, v/v), yielding Fraction III-B (0.52 g). Fraction III-B was eluted with 80% methanol using reversed-phase column chromatography (LiChroprep®, RP-18, 25–40 μm , Merck & Co.), yielding the compound, Fraction III-B-2 (0.5 g). Data obtained from ^1H nuclear magnetic resonance (NMR), ^{13}C NMR, and fast atom bombardment mass spectrometry (FABMS) analysis of compound Fraction III-B-2 were compared with published data (Woo et al., 1987), which suggested that the chemical structure was macelignan (Fig. 1).

Induction of experimental asthma

Asthma induction was performed as previously described (Corry et al., 1996). Briefly, *Aspergillus oryzae* protease allergen (Sigma-Aldrich, St. Louis, MO) was reconstituted to 1 mg/ml using sterile PBS. OVA allergen (Sigma-Aldrich) was reconstituted to 0.5 mg/ml using sterile PBS. *A. oryzae* protease and OVA allergens were mixed 1:9 (v/v) immediately before administration. Mice received three intraperitoneal

to 5 µg/ml was added and plates were incubated at 4 °C overnight. After another wash, 40 µl of alkaline phosphatase-conjugated streptavidin diluted in PBS/Tween/BSA (1:1000 dilution) was added and the plates were incubated at room temperature for 30 min. After a final wash, 70 µl of alkaline phosphatase substrate (5 mmol/l of p-nitrophenyl phosphate substrate in 0.1 mol/l alkaline buffer) was added and developed until the standard curve was readily determined. The reaction was terminated by the addition of 40 µl of 0.5 N sodium hydroxide, and the optical density was measured at 405 nm by an ELISA plate reader (BioRad, Hercules, CA).

Mucin production score

To validate the quantification of secreted glycoprotein in BAL fluid, we scored mucin production according to a previously reported method (Lee et al., 2004). Briefly, Periodic acid-Schiff (PAS)-stained sections of mouse lung were prepared and examined with light microscopy at 250× magnification. At least 50 consecutive airways from saline-challenged mice and 20 consecutive airways from allergen-challenged mice were examined. Histologic goblet cell scores were determined from the average of the total airway scores according to the abundance of PAS + goblet cells and assigned numeric scores (0: less than 5% goblet cells, 1: 5 to 25%, 2: 25 to 50%, 3: 50 to 75%, 4: greater than 75%, arbitrary Unit: U).

Real time RT-PCR

Total RNA was extracted using Ribospin™ (GeneAll, Seoul, Korea) according to the manufacturer's instructions and cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Invitrogen, Grand Island, NY). Semi-quantitative real-time PCR was performed with indicated primers using KAPA SYBR® FAST qPCR kit in an iCycler equipped with iQ™5 (Bio-Rad). The sequences for primers were as follows: GATA3 forward: CCGGTTCGGATGTAAGTCGAGG; GATA-3, GATA3 reverse: GGATGTCCTGCTCTCCTTGCTG; T-bet forward: CCAGCACCAGA CAGAGATGATCATCAC, T-bet reverse: GGGGAGTCTGGGTGGACATATA AGC; RORC forward: CTCTACACGGCCCTGGTTCATC, RORC reverse: GCACAGGCTCCGGAGTTTTC; and GAPDH forward: TGGTGAAGGTCG GTGTGAACGG, GAPDH reverse: TCTCGTCCTGGAAGATGGTGATG. Real-time PCR data were analyzed with Bio-Rad iQ™5 Optical System Software (Bio-Rad). The average threshold cycle (Ct) was determined from triplicate reactions and levels of gene expression relative to GAPDH were determined.

Protein extraction and western blot analysis

After allergen challenge with macelignan or vehicle administration, lungs were removed and lysed by PRO-PREP™ (Intron Biotechnology, Seoul, Korea). The lysates were separated by 10% SDS-PAGE, and transferred onto the nitrocellulose membranes. After blocking with 5% BSA for 1 h, the membranes were incubated with anti-GATA3 Ab (Cell Signaling, Beverly, MA, USA), anti-T-bet Ab (eBioscience) or anti-RORγt Ab (eBioscience) overnight at 4 °C, followed by incubation with HRP-conjugated secondary Ab (Cell signaling) for 1 h at room temperature. Signals were developed with West Save Up™ (AbFrontier, Seoul, Korea), and detected using LAS-1000 (Fujifilm, Tokyo, Japan), and the intensities of bands were quantified using Multi Gauge (Fujifilm).

Cell culture and in vitro T cell stimulation

Cell cultures were performed in RPMI 1640 medium supplemented with penicillin G (50 U/ml), 50 µg/ml of streptomycin, 2 mM L-glutamine (Invitrogen), and 10% fetal bovine serum (WelGene, Seoul, Korea) at 37 °C in a humidified atmosphere containing 5% CO₂. For antigen specific T cell stimulation, 2 × 10⁵ CD4⁺ T cells purified from OT-II mice splenocytes using positive selection and magnetic cell sorting (Miltenyi Biotec, Bergisch-Gladbach, Germany) were

simulated with irradiated (3000 rads) antigen presenting cells (APCs, CD4 T cell depleted splenocytes) and 10 µM of OVA_{323–339} peptide (ISQAVHAAHAEINEAGR) in round-bottom 96 well plates (BD Biosciences) (Lee et al., 2008).

T cell proliferation and cytokine assay

After 4 days of stimulation with OVA peptide or antibodies, cell pellets were collected for T cell proliferation analysis (MTT assay; Roche Applied Bioscience, Branchburg, NJ) according to the manufacturer's instruction. Before adding MTT reagent, supernatants were removed and the levels of IL-4, IFN-γ, and IL-17 were measured using a standard sandwich ELISA according to the manufacturer's instructions (BD Biosciences) (Corry et al., 2002).

Statistical analysis

Data are presented as the mean ± SEM. Non-parametric Mann-Whitney test was used for statistical analysis and *p* value (<0.05) was considered significant. The analyses were performed using GraphPad Prism Software V. 5.0 for Windows (GraphPad Software, La Jolla, CA).

Results

Macelignan attenuates phenotypes of murine experimental asthma

Structure of macelignan is shown in Fig. 1. The molecular weight is 328.4, density is 1.159, and melting point is 70–71 °C. To test the anti-inflammatory activities of macelignan, we examined the severity of murine experimental asthma induced by *A. oryzae* protease mixed with OVA allergen, which was characterized by increased inflammatory cells in BAL fluid and increased AHR. Although the *A. oryzae* protease acts as an outstanding Th2 and allergy-promoting adjuvant as described previously, the combined allergen is far superior compared to OVA/Alum in producing the symptoms of allergic asthma (Lee et al., 2004, 2008, 2003). Because lethal dose 50% (LD50) of macelignan in an oral toxicity test was shown to be 2000 mg/kg or more, we administered either low (400 mg/kg) or high (800 mg/kg) dose of macelignan (K. L. Han et al., 2012). Compared with the saline-challenged group (C), allergen-challenged groups (V, ML400 and ML800) had increased numbers of inflammatory cells in BAL fluid (Fig. 2A). In addition, compared to the vehicle treated group (V), the numbers of inflammatory cells in BAL fluid were decreased, and in particular, eosinophil recruitment was significantly reduced in the macelignan-fed group (ML400 and ML800). However, the numbers of neutrophils, macrophages and lymphocytes in BAL fluid were not affected. We also measured AHR to acetylcholine. AHR was the highest in vehicle-fed allergen-challenged mice and was decreased with macelignan treatment in a dose dependent manner (Fig. 2B). The PC₂₀₀ value (µg/g), in which the amount of acetylcholine required to induce a 200% provocative airway resistance increase from baseline was also calculated (vehicle group: 0.064 ± 0.0097; ML400: 0.103 ± 0.0229; ML800: 0.305 ± 0.145, data are presented as mean ± SEM and PC₂₀₀ values of both ML400 and ML800 are significant versus those of vehicle group as *p* value is less than 0.05). Furthermore, the secreted airway glycoprotein level was decreased with macelignan treatment although both low and high macelignan-administered groups did not reach statistical significance compared to vehicle treatment (Fig. 2C). Similar results were also confirmed with mucin production scores (Fig. 2D). Because high dose macelignan administration elicited a greater effect, we used this high dose in all subsequent experiments. Lastly, macelignan feeding (ML800) decreased the number of peribronchial inflammatory cells and mucus secreting goblet cells (Fig. 2E). Collectively, these data show that macelignan feeding diminishes the severity of murine experimental asthma. Furthermore, the effect of macelignan seems to be dose dependent, since high-dose

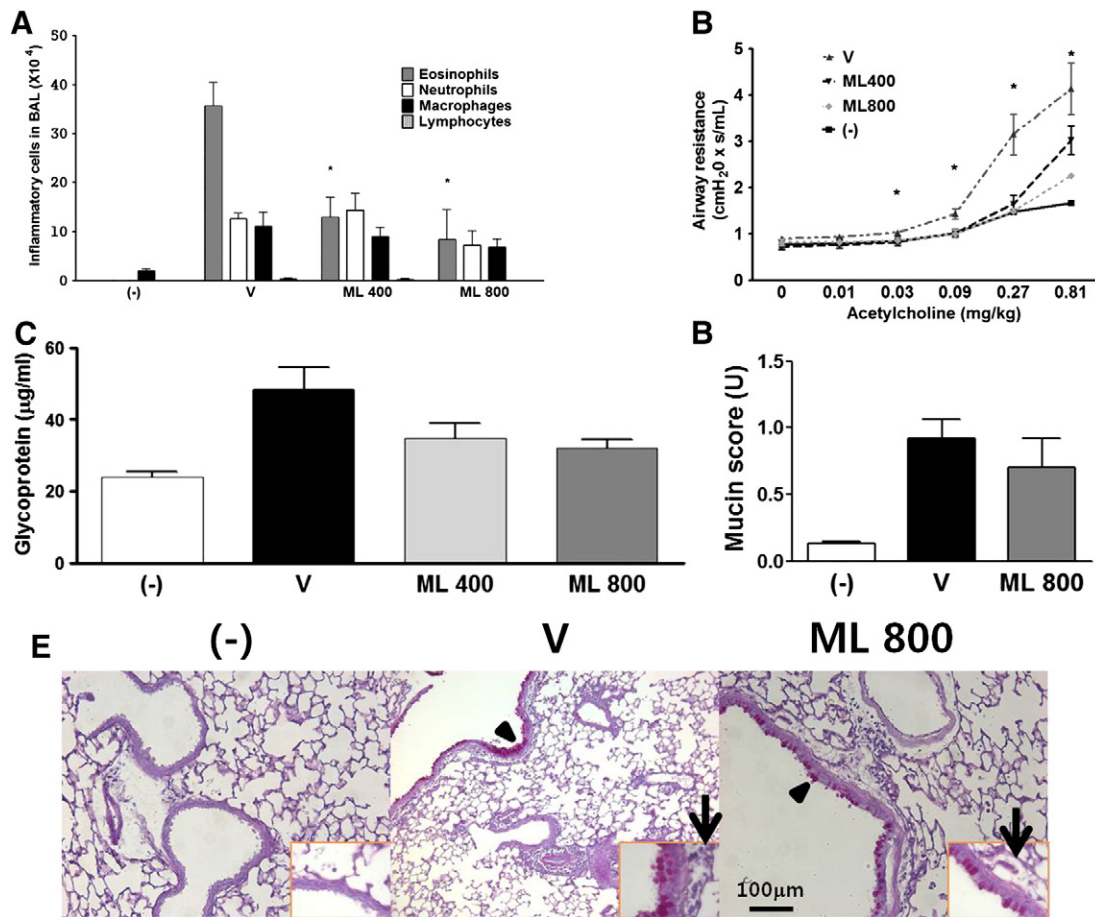


Fig. 2. Macelignan attenuated the severity of murine experimental asthma. Experimental asthma was induced with *A. oryzae* protease mixed with OVA allergen along with vehicle only (V), low-dose macelignan (ML400) or high-dose macelignan (ML800) treatment. (-) represents saline challenged control. Bronchoalveolar lavage (BAL) was performed and inflammatory cells in BAL fluid were quantified (A), and airway hyper-responsiveness (AHR) was measured with the flexiVent system (B). Both ML400 and ML800 treated groups showed statistical significance compared with vehicle treatment (* $p < 0.05$ versus vehicle treatment). Secreted airway glycoprotein in BAL fluid was quantified by modified ELISA (C). Mucin production scores were determined from PAS-stained lung sections (D). * $p < 0.05$ versus saline challenged group. Sections of the lungs were stained with PAS (E). Arrowheads indicate metaplastic goblet cells; arrows indicate inflammatory cells. Original magnification with $\times 200$; and insets with $\times 1000$. Data are presented as mean $\pm$ SEM and are representative of three independent experiments ($n = 4$ per group).

macelignan (ML800) treatment resulted in greatly reduced experimental asthma symptoms.

Decreased IL-4 producing cells and GATA3 expression in lungs of macelignan-treated mice

To investigate the mechanism of macelignan-mediated reduction of experimental asthma, we first examined pro-inflammatory cytokine profiles. Because IL-4 plays a key role in the pathogenesis of allergic asthma, we quantified IL-4-secreting cell numbers in lung by ELISpot analysis as performed previously (Lee et al., 2003). Asthmatic mice showed elevated numbers of lung IL-4-secreting cells compared with those of normal control. With macelignan administration, IL-4-producing cell numbers were significantly diminished (Fig. 3A). Because we have previously confirmed that direct lung IL-4 ELISpot is proportional to the number of lung Th2 cells in an allergic asthma model (Lee et al., 2003), this result suggests that macelignan directly inhibits the function of Th2 cells, and accordingly reduces the severity of experimental asthma. To understand whether macelignan treatment affects Th1, Th2, and Th17 immune responses, we quantified two other inflammatory cytokines, IFN- γ and IL-17. However, IFN- γ (Fig. 3B) and IL-17 (Fig. 3C) secreting cells in the lungs of macelignan-fed mice were not affected. In addition, we examined the levels of master transcription factors from subsets of Th cells from the lungs of macelignan-treated, allergen-challenged mice. Th1, Th2 and Th17 cells express distinct transcription

factors such as T-bet, GATA3, and ROR γ t for differentiation and effector function, respectively (Chatila et al., 2008; Schmidt-Weber et al., 2007; Wells et al., 2007). Thus, we first investigated the mRNA expression of these master transcription factors with real-time RT-PCR. GATA3, T-bet, and RORc mRNA expressions were not changed by treatment with macelignan (Fig. 3D). However, macelignan administration (ML800) reduced the level of GATA3 protein expression (densitometric analysis relative to β -actin, control (C): 1, vehicle (V): 1.33, macelignan (ML): 0.7, ML/V ratio is 0.53), but expressions of T-bet (densitometric analysis relative to β -actin, control: 1, vehicle: 1.29, macelignan: 0.95) and ROR γ t (densitometric analysis relative to β -actin, control: 1, vehicle: 0.91, macelignan: 1) were not affected (Fig. 3E). According to mRNA and protein results, GATA3 expression seemed to be controlled post-transcriptionally with a treatment of macelignan. Therefore, we could conclude that macelignan selectively regulates IL-4 production and/or Th2 cells via inhibition of GATA3 activation in the lungs of experimental allergic asthma.

Effect of macelignan on IL-4 production by in vitro-stimulated T cells

Of the many inflammatory cells, CD4⁺ Th2 cells are one of main producers of IL-4. Thus, we examined the effects of macelignan on in vitro-stimulated CD4⁺ T cells. Consistent with in vivo results, in which there was a reduced IL-4 and GATA3 expression with macelignan administration (Fig. 3), only IL-4 production was selectively decreased

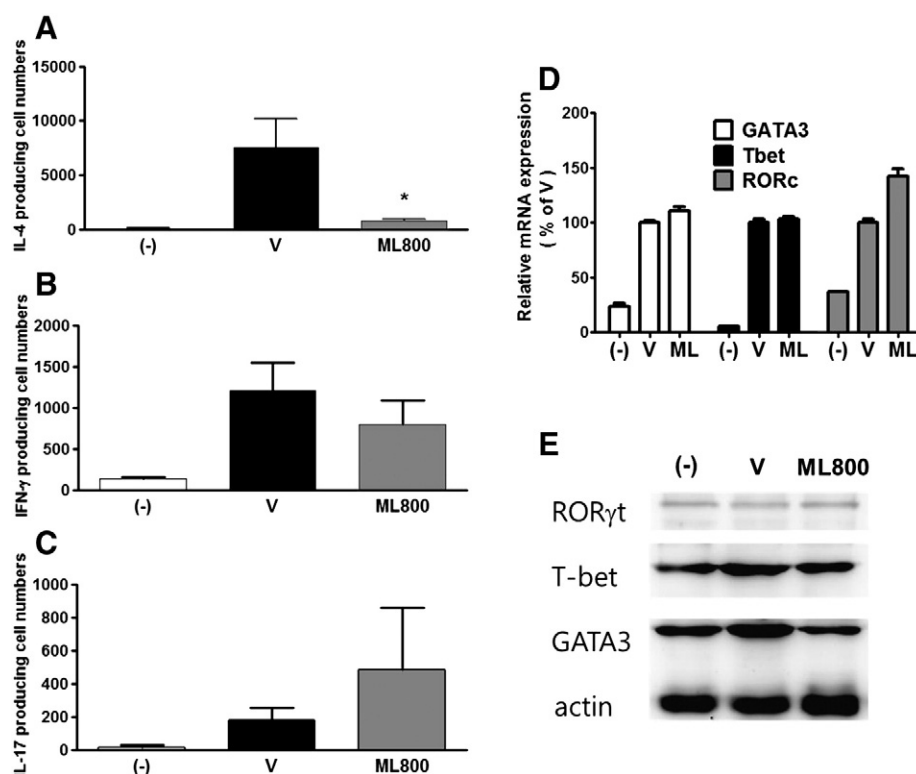


Fig. 3. IL-4-producing cells and GATA3 expression in the lungs of asthmatic mice were diminished with macelignan feeding. Experimental asthma was induced, and vehicle (V) and high-dose macelignan (ML800) were administered orally. Lung cells were collected and cytokine secreting profiles were examined with ELISpot analyses. Numbers of IL-4 (A), IFN- γ (B), and IL-17 (C) producing cells are presented as mean $\pm$ SEM. * $p < 0.05$ compared with vehicle treated group. Data are representative of three independent experiments ($n = 4$ per group). The levels of mRNA expression of GATA3, Tbet, and RORc were quantified with real-time RT-PCR (D). Protein levels of the master transcription factors (GATA3 for Th2 cells, Tbet for Th1 cells, and ROR γ t for Th17 cells) were assessed by western blot analysis of lungs from allergen-challenged mice (E). (-) saline challenge, (V) challenged with allergen along with vehicle treatment, and (ML800) challenged with allergen and high dose of macelignan administration. Data are representative of three independent experiments.

with macelignan treatment. However, production of IL-17 and IFN- γ was not affected in OVA peptide-stimulated CD4⁺ T cells from OT-II mice (Fig. 4A–C). Similar to OVA peptide stimulation, anti-CD3 and -CD28 antibody stimulation increased the secretion of IL-4, IL-17, and IFN- γ , and macelignan treatment reduced IL-4 production (data not shown). Consistent with the IL-17 and IFN- γ production results, T cell proliferation was not affected by macelignan administration (Fig. 4D). Again, anti-CD3 and -CD28 antibody stimulation also induced CD4⁺ T cell proliferation from wild type mice after macelignan treatment (data not shown). Thus, these results demonstrate that macelignan selectively inhibits the IL-4 producing capability of CD4⁺ T cells and Th2 cell differentiation probably through the down-regulation of GATA3 activation.

Discussion

In this study we investigated the effects of macelignan, which has shown anti-inflammatory effects in other model systems, on allergic asthma in a murine experimental asthma model (Jin et al., 2005). We specifically focused on Th2 cell-mediated allergic asthma because there was no study on the effects of macelignan on Th2 cell functions. In our murine experimental asthma model, macelignan administration reduced airway eosinophilia and AHR. Furthermore, macelignan selectively reduced IL-4 producing cell numbers through inhibition of Th2 master transcription factor, GATA3. Therefore, we conclude that macelignan functions as a Th2-specific immunosuppressor.

To understand the mechanisms of macelignan-mediated anti-inflammatory effects, we evaluated cytokine secretion profiles and levels of master transcription factors in different Th subsets following macelignan treatment. Macelignan administration reduced IL-4 production from CD4⁺ T cells activated with OVA peptide and/or

anti-CD3 and CD28 antibodies, but had no effect on the production of IL-17 and IFN- γ from similarly activated CD4⁺ T cells. Although IL-17 production from high dose macelignan-treated in vitro activated CD4⁺ T cells was a little decreased, in vivo macelignan administration did not decrease IL-17 producing cell numbers or ROR γ t expression in the lungs of allergen challenged mice. We also examined the effects of macelignan on activated CD4⁺ T cell proliferation and detected no difference compared with vehicle treatment. Thus, we conclude that macelignan does not affect Th17 immunity. Furthermore, only GATA3, a master transcription factor of Th2 cells, but not Tbet or ROR- γ t, was decreased in the lungs of allergen-challenged mice with macelignan administration. Without master transcription factor activation, it is unlikely that Th cells differentiate to the proper subsets, nor function fully by secreting specific effector cytokines. Interestingly, protein but not mRNA level of GATA3 was diminished by macelignan treatment, thus it is likely that macelignan inhibit the function of GATA3 post-transcriptionally. However, the exact mechanism of macelignan mediated inhibition of protein expression is not known yet. Future studies will be required to determine the mechanism by which macelignan inhibits GATA3 expression. Nonetheless, our data show that macelignan can function as a selective negative regulator of Th2 cell differentiation.

Recently, we reported that macelignan inhibits histamine release and inflammatory mediator production in activated rat basophilic leukemia mast cells (Y. S. Han et al., 2012). These inhibitory effects on mast cells might be involved in the attenuated asthma phenotypes of macelignan-administered mice because mast cells are well known effector cells in asthma. Although we did not investigate the therapeutic roles of macelignan in asthma in vivo, we expect that macelignan should be beneficial due to its suppressive effects on both T cells and mast cells in vitro.

There is an increasing evidence of the efficacy of lifestyle modification in the management of bronchial asthma. Furthermore, environmental

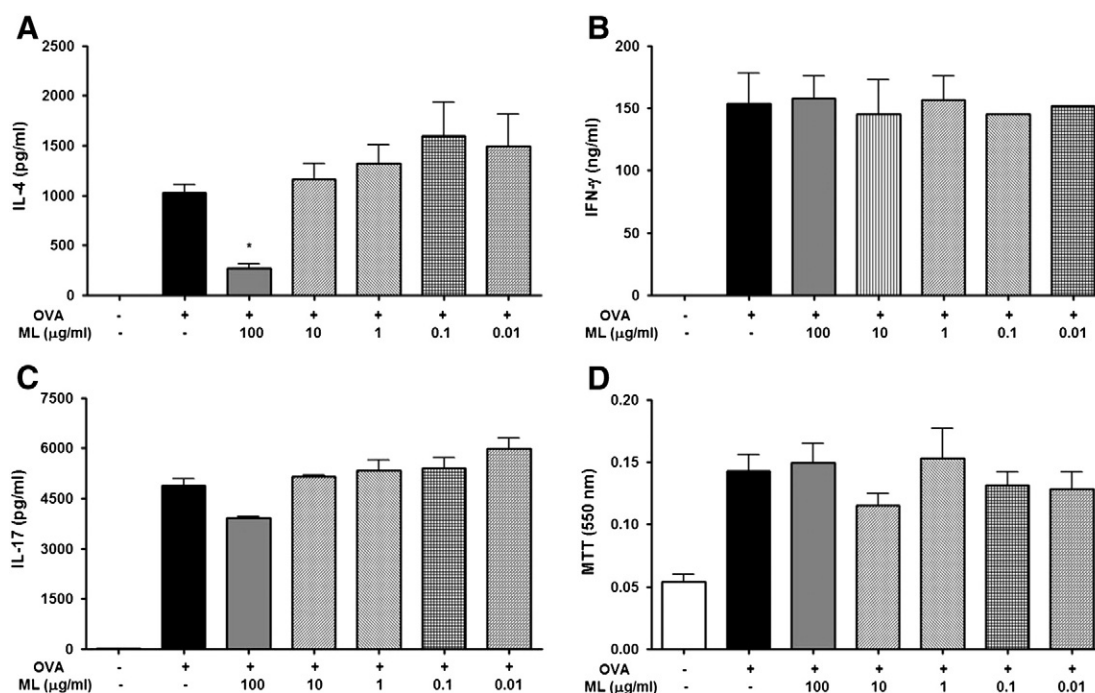


Fig. 4. Macelignan selectively inhibited the IL-4 producing capability of CD4⁺ T cells. CD4⁺ T cells from OT-II mice were stimulated with OVA peptide combined with different concentrations of macelignan. After 4 days of culture, levels of IL-4 (A), IFN-γ (B), and IL-17 (C) in supernatant were measured with ELISA. For T cell proliferation, an MTT assay was performed (D). Data are representative of two independent experiments (n = 3 per group) and presented as mean ± SEM (A–D). * p < 0.05 versus vehicle treatment.

factors such as dietary intake may play an important role in the onset and development of the disease (Arvaniti et al., 2011; Maes and Schlosser, 1988; Vempati et al., 2009). Nutmeg, which contains macelignan, is widely used as a juice, jam, and soup flavoring. In a view of a trend towards preventative management of asthma rather than merely treating symptoms and our data suggesting a preventive role of macelignan in experimental asthma, nutmeg intake might be recommended as a lifestyle modification.

In unpublished results, we have shown that macelignan administration reduced the symptoms of murine experimental atopic dermatitis, another Th2 immune-mediated disease. In addition, macelignan plays a role in experimental atopic dermatitis via a similar mechanism, with decreased Th2 cell differentiation and inhibition of GATA3 activation (manuscript in preparation). Thus, macelignan may show efficacy for other Th2 cell-mediated inflammatory diseases.

The study reported here employed an in vivo model of experimental asthma to investigate the anti-inflammatory effects of macelignan on allergic asthma, and the underlying mechanisms of its action. Here we demonstrate that macelignan attenuated the severity of allergic asthma with reduced lung inflammation and AHR by suppressing the IL-4 producing ability of CD4⁺ T cells and inhibiting activation of GATA3, which accounted for the effects on Th2 cells. Therefore, our study shows for the first time, the anti-asthmatic effects of macelignan.

Conclusion

Oral administration of macelignan attenuated the severity of murine experimental asthma. Macelignan treatment reduced IL-4 production from CD4⁺ T cells, but did not reduce the production of IL-17 and IFN-γ. The effects of macelignan were due to an inhibition of GATA3, which plays a critical role in the pathogenesis of asthma, but were not related to T-bet or RORγt activation. Thus, macelignan may be beneficial for the prevention and/or alleviation of asthma.

Conflict of interest

The authors declare that there are no conflicts of interest.

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