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ARTICLE

Astragalus membranaceus modulates Th1/2 immune balance and activates PPARγ in a murine asthma model

Shih-Ming Chen, Yau-Sheng Tsai, Su-Wen Lee, Ya-Hui Liu, Shuen-Kuei Liao, Wen-Wei Chang, and Pei-Jane Tsai

Abstract: *Astragalus membranaceus*, a traditional Chinese herb, has been used to improve airway inflammation and asthma. The present study investigated whether *A. membranaceus* has immunotherapeutic effects on asthma, a chronic inflammatory mucosal disease that is associated with excess production of IgE, eosinophilia, T helper 2 (Th2) cytokines, and bronchial hyperresponsiveness. An ovalbumin (OVA)-induced, chronic inflammatory airway murine asthma model was used to examine the status of pulmonary inflammation after the administration of *A. membranaceus*. The IgE levels in serum and bronchoalveolar lavage fluid showed a tendency to decrease after the administration of *A. membranaceus*. The number of eosinophils decreased and infiltration of inflammatory cells and collagen deposition declined in lung sections after *A. membranaceus* administration. The RNA and protein levels of Th2 cytokines and the ratio of the GATA3/T-bet mRNA levels decreased after *A. membranaceus* treatment. Furthermore, the mRNA level of peroxisome proliferator-activated receptor γ (PPARγ), a nuclear hormone receptor, increased in the lung tissues of *A. membranaceus*–treated mice. Finally, an *A. membranaceus* water extract activated PPARγ activity in either human embryonic kidney 293 (HEK293) or A549 cells in a PPARγ-responsive element-containing luciferase reporter assay. These results indicate that *A. membranaceus* has an inhibitory effect on airway inflammation in a murine model of asthma through modulating the imbalanced relationship between Th1 and Th2 cytokines.

Key words: Astragalus membranaceus, PPARγ, asthma, murine model.

Résumé : *Astragalus membranaceus* (AM), une plante médicinale traditionnelle chinoise, a été utilisée pour atténuer l'inflammation des voies respiratoires et l'asthme. La présente étude a examiné si AM exerçait des effets immunothérapeutiques sur l'asthme, une maladie inflammatoire chronique de la muqueuse associée à une production excessive d'IgE, une éosinophilie, une production de cytokines Th2 et une hyperréactivité bronchique. Un modèle d'asthme consistant en une inflammation chronique des voies aériennes induite par l'OVA a été utilisé chez la souris afin d'examiner l'état d'inflammation pulmonaire à la suite de l'administration d'AM. Les niveaux d'IgE dans le sérum et le liquide de lavage broncho-alvéolaire tendaient à diminuer après l'administration d'AM. Le nombre d'éosinophiles diminuait, et l'infiltration des cellules inflammatoires et le dépôt de collagène étaient abaissés dans les coupes de poumons à la suite de l'administration d'AM. Les niveaux des cytokines Th2 et de leur ARNm correspondant, ainsi que le rapport des niveaux d'ARNm de GATA3/T-bet diminuait après l'administration d'un traitement avec AM. De plus, le niveau d'ARNm de PPARγ, un récepteur hormonal nucléaire, était accru dans les tissus pulmonaires des souris traitées avec AM. Finalement, un extrait aqueux d'AM activait PPARγ chez les cellules HEK293 ou A549 selon un dosage de l'activité luciférase d'un gène rapporteur comportant un élément de réponse au PPARγ. Ces résultats indiquent que AM exerce un effet inhibiteur de l'inflammation des voies respiratoires dans un modèle d'asthme chez la souris en modulant la relation débalancée entre les cytokines Th1 et Th2. [Traduit par la Rédaction]

Mots-clés : Astragalus membranaceus, PPARγ, asthme, modèle chez la souris.

Introduction

Asthma is the most common chronic inflammatory airway disease and is characterized by bronchoconstriction, pulmonary inflammation, and airway remodeling (Hansel and Barnes 2001). The prevalence of asthma is increasing every year and is higher in industrialized countries. The causes of asthma are quite complex and include genetic factors (Dijk et al. 2013), allergen exposure (Custovic and Simpson 2012), and some specific infections of microorganisms (superantigens) (Gilstrap and Kraft 2013). The imbalance between T helper (Th)1 and Th2 immunity is believed to play a major role in asthma (Ngoc et al. 2005). The predominance of Th2 immunity makes Th2 proinflammatory cytokines, such as interleukin (IL)-4, IL-5, IL-9, and IL-13, highly expressed in patients with asthma (Barnes 2001). These Th2-mediated cytokines are responsible for functional and pathological changes, including airway inflammation, methacholine responsiveness, mucosal hyperplasia, and airway remodeling (Wohlfert et al. 2007).

Although corticosteroids and β2-agonists are effective in managing asthma symptoms, a curative therapy for asthma is still

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lacking (Li and Brown 2009). Therefore, we decided to investigate a complementary and alternative treatment for asthma. The application of GATA3, the key transcription factor for differentiation of Th2 cells and a specific DNAzyme, can decrease the inflammatory responses and accumulation of eosinophils in asthmatic airways (Sel et al. 2008). Other immunomodulators, including Chinese herbs, are also applied and possibly act through anti-inflammation (Wang et al. 2013*b*), inhibition of airway smooth muscle contraction (Yang et al. 2013*a*), or modulation of the Th1/2 balance (Yuan et al. 2013).

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that function as zinc-finger transcription factors and bind to the PPAR-response element (PPRE) in the initiating area (Schoonjans et al. 1996). Three related PPAR isoforms (PPARα, PPARβ, and PPARγ) have been found in vertebrates (Desvergne and Wahli 1999), and PPARα and PPARγ have been especially noted for their anti-inflammatory effects. The agonists of PPAR ligands have been shown to decrease the production of eosinophils (Ueki et al. 2004), the proliferation of T cells, and the secretion of their derived cytokines (IL-4, IL-5, and IL-13) (Lee et al. 2005; Sugiyama et al. 2000), and B cell apoptosis (Ray et al. 2005). These actions make PPAR ligands candidates for the treatment of asthma.

Astragalus membranaceus, a traditional Chinese medicinal herb, has long been used to treat allergic diseases, such as asthma and nasal allergy, in Chinese medicine (Cho and Leung 2007; Block and Mead 2003). The major active constituents of *A. membranaceus* include flavonoids, saponins, polysaccharides, and amino acids (Ma et al. 2002). *A. membranaceus* extracts can improve airway inflammation, hyperresponsiveness, and remodeling in murine models of asthma via upregulating the regulatory T cells (Treg) ratio (Jin et al. 2013; Qu et al. 2010). In addition, the anti-inflammation activity of *A. membranaceus* acts by inhibiting nuclear factor (NF)-κB (Yang et al. 2013*b*) and increasing the expression and activity of PPAR proteins (Shen et al. 2006) have been reported. In this study, we investigated the effects of *A. membranaceus* on allergic asthma by measuring Th2-type cytokines, eosinophil infiltration, and PPARγ expression in an aluminum hydroxide/ovalbumin (OVA)–induced murine asthma model.

Materials and methods

Materials and chemicals

A. membranaceus, a water-extracted single-herb powder (pharmaceutical name: Radix Astragli), was purchased from KO DA Pharmaceutical Co., Ltd. (Taoyuan County, Taiwan) and suspended in water at 30 mg/mL. OVA, aluminum hydroxide, and methacholine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI, USA) and dissolved in DMSO at a stock of 10 mmol/L.

Mice sensitization, challenge, and drug treatment

Female C57BL/6JNarl mice aged 6 to 8 weeks were purchased from the National Laboratory Animal Center (Tainan, Taiwan) and used in this study. All mice were maintained and handled according to the guidelines of the Animal Care Committee of Cheng Kung University for the Care and Use of Laboratory Animals. All the animal studies were performed following a protocol approved by Institutional Animal Care & Use Committee of National Cheng Kung University (Approval No. NCKU-IACUC-103-015). The murine model of asthma that was used in this study was modified from Pinelli et al. (Pinelli et al. 2009). Briefly, the mice were rendered allergic by intraperitoneal (IP) injection of OVA (0.4 μg/μL; grade V; Sigma, St. Louis, MO, USA), which was complexed with aluminum potassium sulfate on days 0 and 7. From day 7 to 21, the mice were challenged with inhalation (intranasal) of 4 μg/10 μL OVA. In each experiment, the mice were divided into 2 groups: (1) sensitized and challenged with OVA and treated with oral forced feeding of normal saline (OVA/saline group); (2) sensitized and challenged with OVA and treated with oral forced feeding of 3 μg/kg *A. membranaceus* (OVA/*A. membranaceus* group). Starting on day 21, the mice received saline (OVA/saline group) or *A. membranaceus* (OVA/*A. membranaceus* group) every other day for the following 8 consecutive days.

Collection of bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was immediately collected from euthanized mice on day 29 using 3 consecutive instillations of PBS (1 mL) at room temperature and was centrifuged at 110*g* at 4 °C for 5 min. The supernatants were collected and stored at −80 °C to measure the cytokine and antibody levels.

Cytokines and antibody analysis

The concentrations of cytokines and antibodies in BALF and serum were measured. Serum IL-4 and IL-13 were measured using enzyme-linked immunosorbent assays (ELISAs) from R&D Systems (Minneapolis, MN, USA). Total IgE in the BALF or serum was measured using ELISAs from BD Biosciences (San Jose, CA, USA). To measure the OVA-specific IgE in BALF or serum, plates were coated overnight at 4 °C with 20 μg/mL OVA in PBS. After being blocked with 20% fetal bovine serum (FBS), serum samples or cell culture supernatants were added and incubated for 2 h at room temperature, followed by detection with biotinylated anti-mouse IgE (BD Biosciences), avidin–horseradish peroxidase (HRP; BD Biosciences), and tetramethylbenzidine substrate. Purified mouse IgE (BD Biosciences) was used as a relative standard.

Lung histology and Masson's trichrome staining

Lungs were inflated with 10% formalin-phosphate buffer (Sigma-Aldrich, St. Louis, MO, USA) at a fixed pressure (10 cm H_2O) via the trachea. The lungs were then removed from the chest cavity and placed in 10% formalin overnight at 4 °C. Tissues were embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E). Inflammatory infiltrates and lung architecture were assessed using light microscopy. To determine the collagen content in the lung sections, a Masson's trichrome staining kit (Sigma-Aldrich) was used per the manufacturer's recommendation.

Measurement of airway hyperresponsiveness

The airway resistance of the mice was measured using a single-chamber, whole-body plethysmograph (Buxco Electronics Inc., Troy, NY, USA) as described in the manual. Briefly, mice were exposed to normal saline and methacholine for 3 min followed by incremental dosages (6.25, 12.5, 25, and 50 mg/mL) of aerosolized methacholine. A pulmonary airflow obstruction was demonstrated as an enhanced pause (P_{enh}) by the software provided by Buxco Electronics.

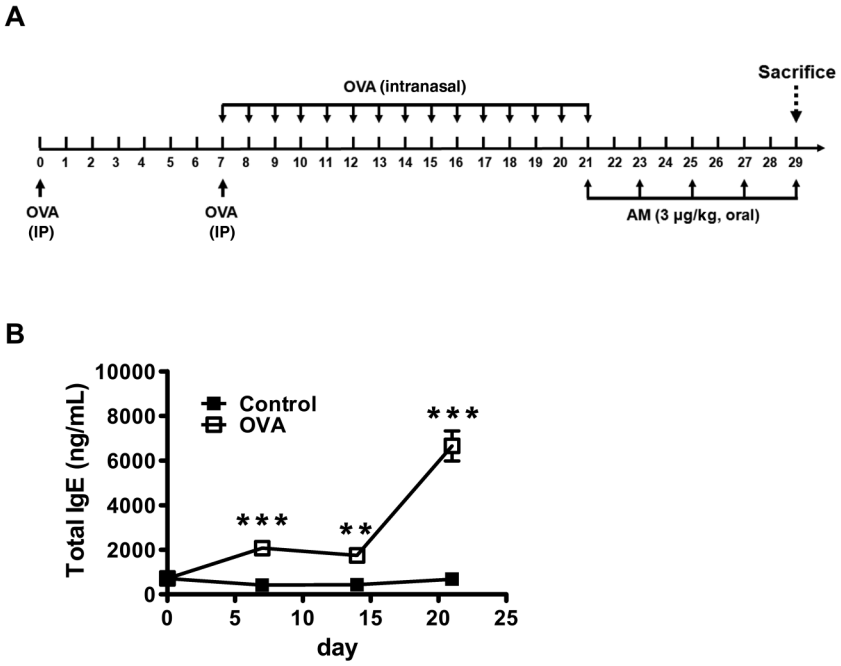
Quantitative real-time PCR analysis

Total RNA of lung homogenates was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was generated using the oligo dT18 primer and SuperScriptII according to the manufacturer's instructions (Life Technologies, Grand Island, NY, USA). A SYBR Green kit (Takara Bio Inc., Shiga, Japan) was used in real-time quantitative RT–PCR assays (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in triplicate and normalized to the respective β-actin measurement. The sequences of the primers and reaction conditions are available upon request.

PPER luciferase assay

Human embryonic kidney 293 (HEK293) cells and A549, which are adenocarcinomic human alveolar basal epithelial cells, were transiently transfected using Lipofectamine 2000 reagent (Life Technologies, Grand Island, NY, USA) and the described protocol with modification. For each transfection, cells were grown to 80% confluence in 6-well plates, transfected with the hPPREgamma-Luc (a kind gift from Dr. Chih-Hao Lee at Harvard Medical School) and Renilla-Luc plasmids at a ratio of 10:1 in a total amount of 1 μg

Fig. 1. The murine model of OVA-induced asthma. (A) Schematic diagram illustrating the murine asthma model. The OVA sensitization, challenge, and *A. membranaceus* (AM) administration protocols in this study. IP, intraperitoneal. (B) The concentrations of IgE in sera from mice sensitized with OVA (□) or saline (■) (n = 5/group) on days 7, 14, and 21 were detected by ELISA. The experiments were repeated 3 times. Asterisks indicate significant difference from the saline-challenged control group (**$p < 0.01$, ***$p < 0.001$).



DNA. For the luciferase activity assay, the cells were cotransfected with a Renilla reporter plasmid to normalize for transfection efficiencies. After transfection, the cells were treated with 10 μmol/L rosiglitazone (a PPARγ ligand) and *A. membranaceus* water extract at 3 mg/mL for 24 h. The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA).

Statistical analysis

Data were analyzed by two-way ANOVA; the Bonferroni correction was used for multiple comparisons. A p value less than 0.05 was considered statistically significant.

Results

Oral administration of *A. membranaceus* attenuates the IgE levels in experimental allergic mice

To examine whether *A. membranaceus* has an immunomodulatory effect on asthma, we established a murine model of asthma induced by OVA (protocol is illustrated in Fig. 1A). An elevated IgE level is critical for the pathogenesis of allergic disorders. As shown in Fig. 1B, the total IgE level in sera was significantly induced in OVA-challenged mice on days 7, 14, and 21. To evaluate the attenuation ability of *A. membranaceus* on the development of OVA-induced allergic responses, the levels of total IgE in sera or BALF were measured after the oral administration of *A. membranaceus* to allergic mice. The levels of total IgE in the sera of OVA-challenged/saline–administered mice were increased at day 29, but they were significantly decreased in the *A. membranaceus*–administered group (Fig. 2A). The reduction in total IgE that was induced by *A. membranaceus* administration was more obvious in BALF ($p < 0.01$, Fig. 2B). We then examined if administration of *A. membranaceus* could cause an increase in OVA-specific IgG in OVA-allergic mice. Although there was no significant difference in the OVA-specific IgG in the sera of saline- and *A. membranaceus*–administered mice (Fig. 2C), OVA-specific IgG was significantly induced in the BALF of *A. membranaceus*–administered mice (Fig. 2D). These results indicate that *A. membranaceus* has an immunomodulatory effect that reduces the IgE level in experimental allergic mice.

A. membranaceus decreases eosinophilia in experimental allergic mice

Cellular inflammation of the airways with eosinophilia is a characteristic feature of asthma and is considered relevant to the pathogenesis of the disease. The analysis of the complete cell counts of the blood drawn from the mice after OVA induction showed significantly increased numbers of white blood cells (WBC), lymphocytes, neutrophils, and eosinophils compared with the vehicle control mice. There were no significant differences between the mice treated with *A. membranaceus* or the saline group in total WBC and lymphocytes (Table 1). Significantly decreased eosinophilia, but not neutrophilia, was observed in *A. membranaceus*–treated mice (Table 1).

A. membranaceus improves airway functions in experimental allergic mice

We next examined if administration of *A. membranaceus* could improve airway inflammation in OVA-induced allergic mice. On the day after final *A. membranaceus* administration, the histology of the lung tissues of the mice was examined by H&E staining. As shown in Fig. 3A, OVA challenge induced the infiltration of inflammatory cells in saline-administered mice (upper central panel, Fig. 3A). In contrast, oral administration of *A. membranaceus* could massively reduce the infiltration of inflammatory cells in OVA-challenged mice (upper right panel, Fig. 3A). An increased thickness of the reticular basement membrane in asthma patients that results from the deposition of collagen has been reported (Saetta and Turato 2001). We then used Masson's trichrome staining to evaluate the collagen depositions in the lung tissues of the mice. The deposition of collagen was observed in OVA-challenged and saline-administered mice (lower central panel, Fig. 3A), but it could be reduced to a level similar to that of the sham control after *A. membranaceus* administration (lower right panel, Fig. 3A).

Fig. 2. Effects of *A. membranaceus* on the antibody levels of allergic mice. Serum IgE (A) and BALF IgE (B) in the saline-challenged control, OVA-sensitized and saline-treated (OVA/saline), and OVA-sensitized and *A. membranaceus*–treated (OVA/AM) groups (n = 5/group) were measured on day 29 by ELISA. The OVA-specific IgG (OVA-IgG) concentrations in mouse sera (C) and BALF (D) were detected in the 3 groups on day 29 using a self-prepared ELISA, read at an optical density of 450 nm. Experiments were repeated 3 times. The results are expressed as the mean ± SEM. Asterisks indicate a significant difference from the saline-challenged control group (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

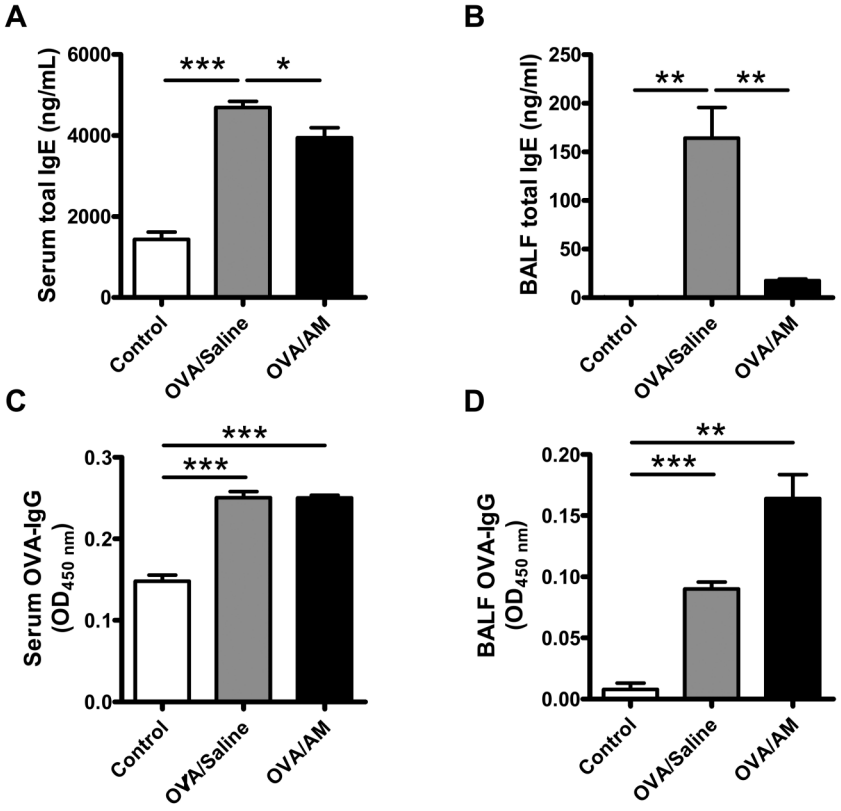


Table 1. The analysis of the complete cell counts of mice blood.

	WBC (K/mL)	Lymphocyte (K/mL)	Neutrophil (K/mL)	Eosinophil (K/mL)
Control	08.99±0.33**	6.25±0.30***	1.25±0.13***‡	0.26±0.02**†
OVA/Saline	11.45±0.60*	8.70±0.46†	1.51±0.08***‡	0.60±0.08*†
OVA/AM	11.39±0.63*	7.80±0.68***	2.38±0.18†§	0.33±0.04‡**

Data expressed as mean ± SEM. (n = 5).
*, $p < 0.01$ versus control.
†, $p < 0.001$ versus control.
‡, $p < 0.01$ versus OVA/Saline.
§, $p < 0.001$ versus OVA/Saline.

We next examined the patterns of breathing of allergic mice to an increasing dose of methacholine using a noninvasive whole-body plethysmograph (P_{enh}), which could be used to screen overall lung function (Bates et al. 2004). The P_{enh} values of OVA-challenged and saline-administered mice were significantly increased with 12.5, 25, and 50 mg/mL methacholine, whereas *A. membranaceus*–administered mice had P_{enh} values similar to the control mice (Fig. 3B). The change of P_{enh} in *A. membranaceus*–administered allergic mice may reflect the improvement of airway inflammation. Taken together, these results indicate that *A. membranaceus* may improve airway function in experimental airway allergic mice.

A. membranaceus decreases the production of Th2 cytokines

Th2 cytokines, especially IL-4 and IL-13, promote the differentiation of IgE-producing B cells (Punnonen et al. 1993). We next examined if *A. membranaceus* administration in OVA-sensitized mice could reduce the expression of IL-4 and IL-13. By quantitative RT–PCR, the IL-4 and IL-13 mRNA levels were significantly reduced in *A. membranaceus*–administered mice when compared with the saline-administered group ($p < 0.05$, Fig. 4A). In addition to mRNA, we determined the protein levels of IL-4 and IL-13 in the sera. The secretion of IL-4 and IL-13 in the sera was also reduced in *A. membranaceus*–administered mice when compared with the saline-administered group ($p < 0.05$, Fig. 4C). These results suggest that the reduction in the total IgE level in the sera of allergic mice by *A. membranaceus* could result from the inhibition of IL-4 and IL-13.

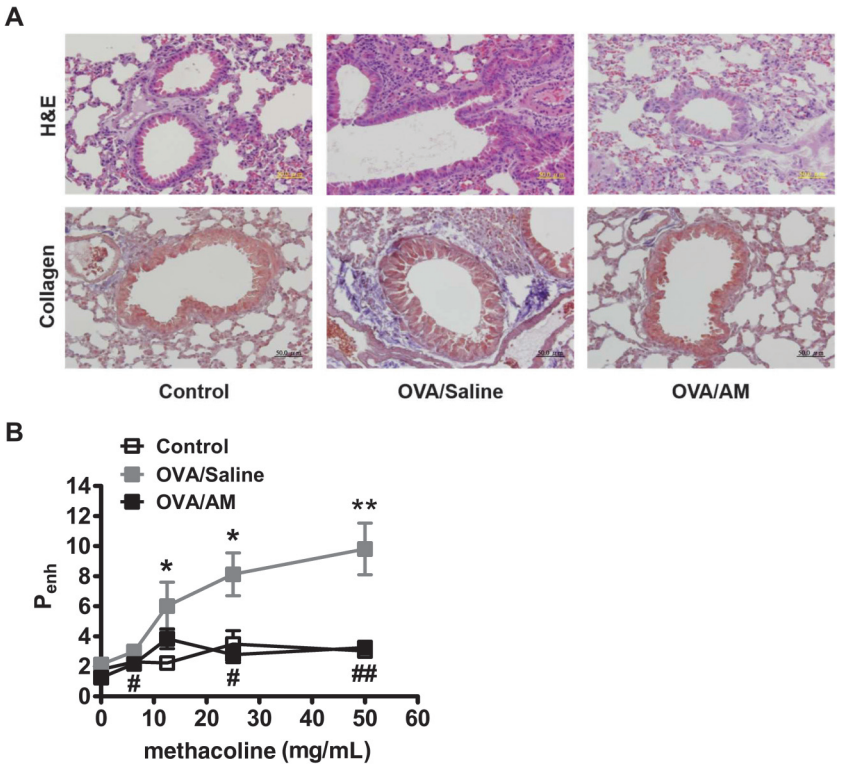
A. membranaceus reduces the level of GATA3 and increases the activity of PPARγ

Because Th2 cytokines in allergic mice could be reduced by *A. membranaceus* administration, we hypothesized that the antiallergic effect of *A. membranaceus* may act through the regulation of transcriptional factors (i.e., T-bet and GATA-3) in Th1 and Th2 cell differentiation (Ho et al. 2009). To investigate the balance of Th2/Th1, we determined that the ratio of GATA3/T-bet expression in lung tissues of allergic mice was reduced by administration of *A. membranaceus* ($p < 0.05$, Fig. 5A). PPARγ has been reported to decrease the expression of Th2 cytokines; therefore, we were also interested in whether *A. membranaceus* could induce the expression and activation of PPARγ. We found that the mRNA level of PPARγ was decreased in saline-administered allergic mice ($p < 0.05$, Fig. 5B), but increased when the mice were administered *A. membranaceus* ($p < 0.05$, Fig. 5B). Using a luciferase-based reporter assay, the activity of PPARγ was increased in *A. membranaceus*–treated HEK293 or A549 cells ($p < 0.05$, Fig. 5C) to a level similar to that by rosiglitazone, which is a PPARγ agonist.

Discussion

In this study, an OVA-induced murine asthma model was used to assess the anti-inflammatory effect and mechanism of *A. membranaceus*

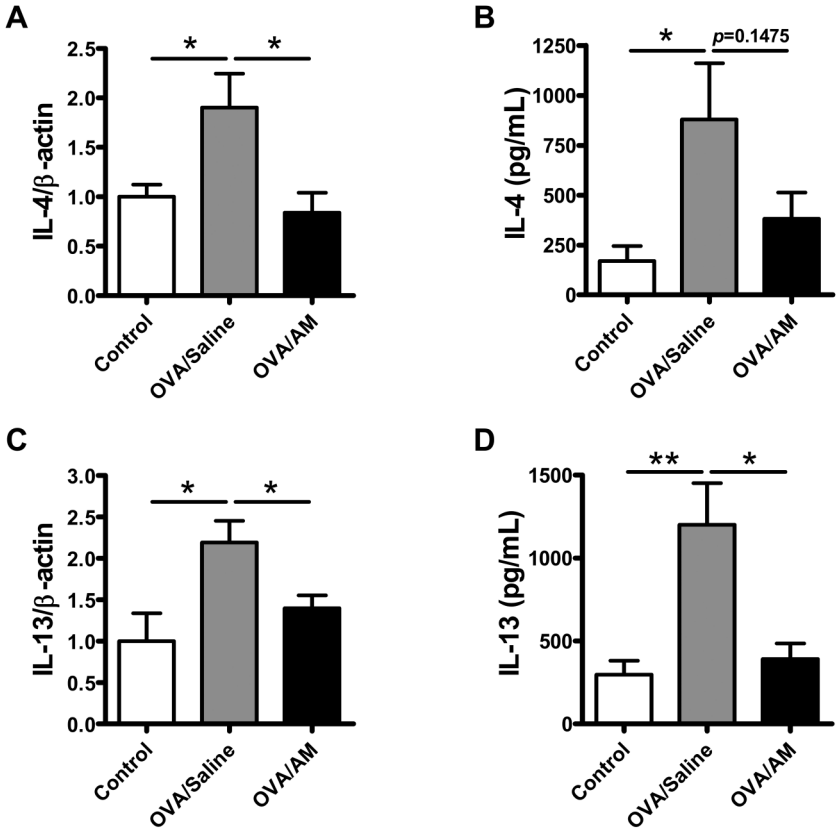
Fig. 3. Effects of *A. membranaceus* on lung pathology and airway responsiveness of allergic mice. (A) Paraffin-embedded lung tissues were processed with H&E staining (upper panel) and Masson's trichrome staining (lower panel). Data are representative of results from 3 separate experiments. (B) The airway responsiveness was determined using a noninvasive whole-body plethysmograph. Open squares indicate mice that were sensitized with saline, gray squares indicate mice that were sensitized with OVA and administered saline (OVA/saline), and black squares indicate mice sensitized with OVA and administered *A. membranaceus* (OVA/AM). All mice were treated with methacholine and the airway responsiveness was detected by measurement during the enhanced pause (P_{enh}). The results are expressed as the mean ± SEM. Asterisks indicate a significant difference of saline-treated group from the control group ($^{*}p < 0.05$, $^{**}p < 0.01$). Hashtags indicate a significant difference between *A. membranaceus* treated group with saline-treated group ($\#p < 0.05$, $\#\#p < 0.01$).



on chronically inflamed airways. We found that *A. membranaceus* could effectively decrease airway allergic responses, including specific IgE (Fig. 2C and 2D), eosinophilia, lymphocyte infiltration (Table 1), airway inflammation, abnormal increases in airway resistance (Fig. 3), Th2 cytokine polarization (Fig. 4), and the transcription of GATA3 (Fig. 5A). In *A. membranaceus*–treated mice, a significantly increased neutrophil count was observed (Table 1). The role of neutrophils in asthma remains controversial. Although no reports have indicated that neutrophils are the predominant inflammatory cells in asthma patients with low numbers of eosinophils and poor responses to inhaled corticosteroids (Green et al. 2002; Jatakanon et al. 1999; Fahy et al. 1995), others have found no differences in the neutrophil numbers in the BALF (Boulet et al. 1993) or sputum (Taha et al. 2001) of patients with slight to moderate asthma. The role of neutrophils in asthma has been suggested to attract and activate eosinophils to produce superoxide or degranulation (Monteseirin 2009). The recruitment of eosinophils in allergic asthma has been suggested to be initiated by Th2 cytokines (Possa et al. 2013). Here we observed that the eosinophilia was decreased in *A. membranaceus*–treated mice but increased with neutrophilia (Table 1). The effect of *A. membranaceus* in airway allergy is most likely to downregulate the infiltration of eosinophils through inhibition of Th2 cytokines. The involvement of neutrophils in *A. membranaceus*–modulated airway allergic responses warrants further investigation. Furthermore, we found that in the lung tissues of mice sensitized with OVA and treated with *A. membranaceus*, the expression of PPARγ was significantly increased (Fig. 5B). From these results, we hypothesize that *A. membranaceus* could be used to treat allergic asthma through its inhibition of GATA3 expression by the activation of PPARγ.

We observed that IgG (Fig. 2C and 2D), but not anti-OVA IgE (Fig. 2A and 2B), was significantly decreased in the blood and BALF of mice treated with *A. membranaceus*. This observation corresponds to the result showing that *A. membranaceus* increased the expression of IgG that was noted in another study (Yang et al. 2005). It was also confirmed in our study that *A. membranaceus* preferentially and specifically inhibited the allergic response rather than universal immune responses, which indicated that *A. membranaceus* could selectively reverse the allergic asthmatic response whereby shift Th2 to Th1 immunodominance. We also observed that the ratio of GATA3/T-bet in OVA-sensitized lungs was decreased by *A. membranaceus* administration (Fig. 5A). T-bet is known to be required for optimal interferon-γ (IFN-γ) production in CD4+ T cells and dendritic cells (Lugo-Villarino et al. 2003). It is also known that IFN-γ inhibits the production of IgE and IgG1, whereas IL-4 blocks the secretion of IgG2a (Boehm et al. 1997). Actually, the levels of IgG1 or IgG2a are also widely used as markers for the Th2 or Th1 responses, respectively. It is possible that *A. membranaceus*–induced downregulation of OVA-specific IgE in BALF is due to an increase in IFN-γ; however, we did not measure the level of IFN-γ in the *A. membranaceus*–treated mice. Recently, Oefner et al. detected sialylated, allergen-specific IgG in allergic patients with successful specific immunotherapy (Oefner et al. 2012). Anti-inflammatory sialylated IgGs are suggested to play a novel immunomodulatory role in the induction of T cell–dependent antigen tolerance (Oefner et al. 2012). Further investigation

Fig. 4. Effects of *A. membranaceus* on the IL-4 and IL-13 levels of OVA-sensitized mice. The expression of IL-4 (A) and IL-13 (C) in lung homogenates on day 29 in the saline-challenged control, OVA-sensitized and saline-treated (OVA/saline), and OVA-sensitized and *A. membranaceus*–treated (OVA/AM) groups (n = 5/group) were determined by quantitative RT–PCR. The expression level of β-actin was used as an internal control. The relative expression was based on the expression ratio of the target gene versus that of β-actin. (A and C) The serum levels of IL-4 (B) and IL-13 (D) in 3 groups of mice (n = 5/group) were detected by ELISA. The results are expressed as the mean ± SEM. ($^{*}p < 0.05$, $^{**}p < 0.01$).

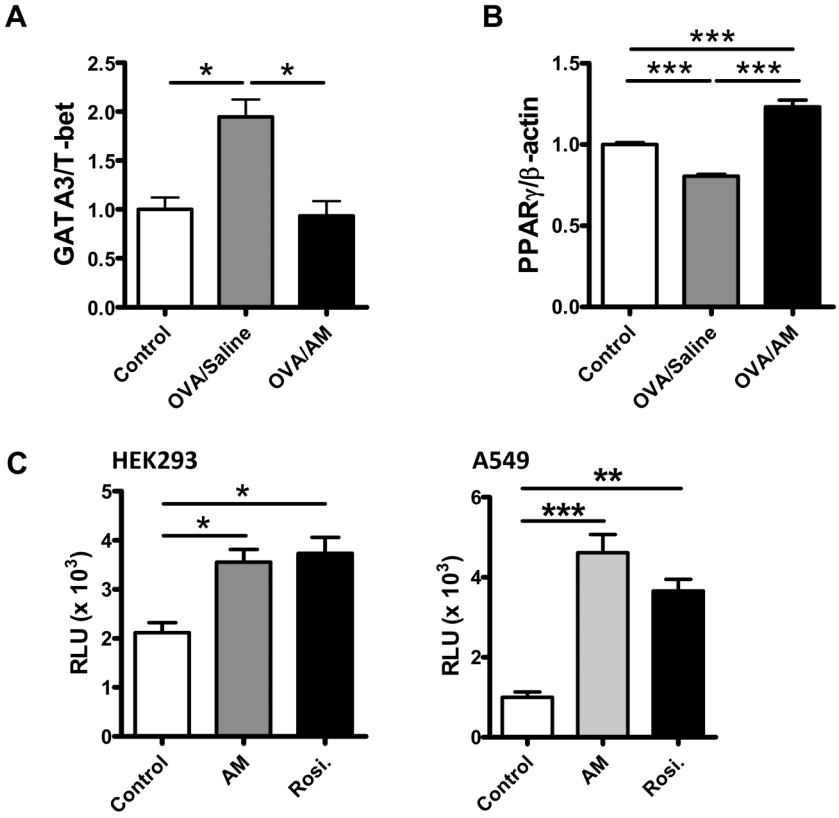


is required to determine whether *A. membranaceus* can increase the production of OVA-specific, sialylated IgG.

Fang et al. demonstrated that *A. membranaceus* administration to T cells recovered from the splenocytes of BALA/c mice ex vivo could increase the expression of IL-4 in Th2 lymphocytes and augment the allergic response (Kang et al. 2004). Higher levels of IL-1, IL-6, IL-8, and TNFα were found in children with nephrotic syndrome when treated with *A. membranaceus* (Peng et al. 2005). In asthmatic patients treated with *A. membranaceus*, the mRNA expression of the Th1 transcription factor T box in T cells was significantly increased and the level of IL-4 was decreased (Wang et al. 2006). Although controversial results exist regarding the treatment of allergic patients with *A. membranaceus*, most reports favor its Th1-polarizing effect on asthma, cancer, herpes simplex keratitis, or type I diabetes (Tomas 2011). These controversial results might be due to different mouse strains used for the induced murine models. The strain used as the mouse model to induce chronic airway inflammation by OVA and to evaluate the inhibitory mechanisms of *A. membranaceus* in this study was C57BL/6JNarl, which is considered a prototypical Th1 strain (Watanabe et al. 2004). It has been demonstrated that C57BL6/JNarl mice display a positive correlation between peribronchial inflammation and the eosinophil count in BALF of an OVA-induced asthma model; however, BALB/c mice, considered a prototypical Th2 strain (Watanabe et al. 2004), did not do the same (Gueders et al. 2009). These 2 strains of mice displayed a comparable level of OVA-specific IgE in BALF (Gueders et al. 2009). Based on previous literature, we decided to use C57BL/6Narl mice as a model to investigate the effect of *A. membranaceus* in airway inflammation.

Here, we demonstrated that the mRNA expression and activity of PPARγ was increased in OVA-induced mice treated with *A. membranaceus*. This result was similar to that of a report of similarly treated type 2 diabetic mice (Li et al. 2007). Many investigations showed that agonists of PPARγ could decrease the allergic response (Lee et al. 2005; Sugiyama et al. 2000) and inhibit inflammation (Clark 2002; Daynes and Jones 2002; Benayoun et al. 2001; Delerive et al. 2001) in respiratory airways. According to these results, we suggest that *A. membranaceus* could inhibit murine airway inflammation through the activation of PPARγ. However, how PPARγ decreases airway inflammation remains unclear. PPARγ activation through rosiglitazone could prime human monocytes into alternative M2 macrophages with anti-inflammatory properties both in vitro and in vivo (Bouhlel et al. 2007). PPAR-γ appears to regulate IL-4 through a concomitant decrease in GATA-3 expression (Woerly et al. 2003). PPAR-γ activation might also influence lung infiltration through inflammatory cells and, among them, eosinophils, which are responsive to IL-5. PPARγ could control the inflammatory response by negatively interfering with proinflammatory signaling pathways such as activator protein 1 (AP-1), NF-κB, or STAT3 in activated M1 macrophages (Van Ginderachter et al. 2008). Enhancing the effect in inducible and natural, thymic-derived Tregs using a PPARγ agonist was also demonstrated (Wohlfert et al. 2007). Administration of PPARγ agonists or adenovirus-mediated PPARγ expression could improve the asthmatic features via regulation of IL-10 expression/IL-10 receptor activation (Kim et al. 2005), which implies that PPARγ may have therapeutic potential for the treatment of airway inflammation and hyperresponsiveness. In

Fig. 5. Effects of *A. membranaceus* on the GATA3/T-bet ratio and PPARγ level in the lungs of allergic mice and on the transcriptional activity of PPARγ in epithelial cells. (A) The ratios of GATA3/T-bet were determined in lung homogenates on day 29 in the saline-challenged control, OVA-sensitized and saline-treated (OVA/saline), and OVA-sensitized and *A. membranaceus*–treated (OVA/AM) groups (n = 5/group) by quantitative RT–PCR. (B) The expression level of PPARγ was determined by quantitative RT–PCR. The expression level of β-actin was used as an internal control. (C) The activation of PPARγ by *A. membranaceus* and rosiglitazone (Rosi) was determined using a PPRE-luciferase reporter assay in HEK293 and A549 cells. The relative light units (RLUs) are expressed as the mean ± SEM. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).



summary, we conclude that *A. membranaceus*–induced PPARγ activation exerted an anti-inflammatory mechanism and modulated the immune response.

Although *A. membranaceus* has been demonstrated to prevent hyperresponsiveness related to Th2 response inhibition in the airways of mice, the exact mechanism remains to be elucidated. Many studies showed that the extract of *A. membranaceus*, including saponins (Lee et al. 2013), polysaccharides (Liu et al. 2013; Lu et al. 2013) and flavonoids (Li et al. 2013), could suppress lipopolysaccharide (LPS)-induced inflammation through inhibiting NF-κB activation and adhesion molecule expression (Yang et al. 2013*b*). We also demonstrated that the aqueous extract of *A. membranaceus* could increase the activity of PPARγ in both HEK293 and A549 cells (Fig. 5C). Here, we demonstrated that *A. membranaceus* treatment might activate PPARγ to modulate airway hyperresponsiveness in a murine model. It has been reported that the activation of PPARγ in immune cells may inhibit NF-κB activation and further lead to the downregulation of proinflammatory cytokine expression. Using pancreatitis-associated ascitic fluid to treat THP-1 cells, a human monocytic cell line, the levels of NF-κB-p65 and proinflammatory cytokines (i.e., TNF-α and IL-6) were increased, whereas the downregulation of PPARγ was observed (Wang et al. 2013*a*). Pretreatment with pioglitazone, a ligand of PPARγ, could protect rats from acute pancreatitis through the downregulation of NF-κB–induced proinflammatory cytokines (TNF-α and IL-6) (Xu et al. 2013). Taken together from our results and a previous report from Yang et al. (Yang et al. 2013*b*), it is suggested that the modulatory activity of *A. membranaceus* in airway hyperresponsiveness may act through PPARγ-induced downregulation of NF-κB and this remains to be investigated in the future.

Collectively, our results demonstrate that *A. membranaceus* treatment improves hyperresponsiveness in a murine model of asthma through the PPARγ pathway. Due to the recently reported failure of monotherapies targeting eosinophils (e.g., anti-IL-5) in the treatment of eosinophilia, asthma, and airway hyperresponsiveness (Leckie et al. 2000), activation of the nuclear receptors expressed on many immune (including T cells) and nonimmune cell types in inflammatory airways by *A. membranaceus* might represent an attractive alternative therapeutic strategy for asthma. In the future, it will be interesting to verify the involvement of PPARγ in the immunomodulatory effect of *A. membranaceus* in an OVA-induced asthma model of PPARγ-knockout mice.

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