Mycoleptodonoides aitchisonii suppresses asthma via Th2 and Th1 cell regulation in an ovalbumin-induced asthma mouse model

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Abstract. Asthma is a chronic respiratory disease related to hyper-responsiveness. The majority of patients suffer mild symptoms, however, some cases, especially in the young and the elderly, can lead to death by apnea. *Mycoleptodonoides atichisonii* (*M. atichisonii*) is an edible mushroom that has previously been reported to possess several bioactive properties, such as the synthesis of nerve growth factors, anti-obesity effects and the ability to prevent cell death. In the current study, the authors evaluated the anti-asthmatic effects of *M. atichisonii* using an ovalbumin-induced asthma mouse model. *M. atichisonii* dose-dependently suppressed the levels of white blood cells, eosinophils and immunoglobulin (Ig)E in BALB/c mice, resulting from ovalbumin-induced asthma. *M. atichisonii* recovered the typical asthmatic morphological changes in lungs, such as mucous hyper-secretion, epithelial layer hyperplasia, eosinophil infiltration and various cell surface molecules, such as CD3, CD4, CD8, CD19 and major histocompatibility complex class II. With the exception of CD19+ cells and IL-12p40, *M. atichisonii* affected almost all factors related to asthma induction including the T helper (Th)1/Th2 transcription factors, T-bet and GATA-3, Th1-related cytokines, Th2-related cytokines and proinflammatory cytokines. In addition, *M. atichisonii* significantly inhibited the expression of IL-5, IL-13 and IL-6. The authors concluded that *M. atichisonii* may be a promising drug candidate against asthma.

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Introduction

Asthma is a severe respiratory disease classified as one of the hyper-responsive immune diseases. According to the 2013 report from the World Health Organization, asthma patients numbered ~235 million worldwide in 2013 (1). The United States Environmental Protection Agency (EPA) estimated in 2013 that there were 25.9 million asthma patients in the USA alone. In addition, the EPA stated that asthma was among the most serious respiratory diseases that leads to hospitalization, especially in children under 15 years of age (2). Various allergens can trigger asthma, such as dust mites, cockroaches, pet dander, viral infections, pollen, mold, fungi, tobacco smoke and pollutants (1). Asthma is an incurable disease that is related to the pulmonary system, and presents itself with various symptoms ranging from coughing to constructive apnea, all resulting from mucous secretion, goblet cell hyperplasia, epithelial cell shedding, basement membrane thickening and eosinophil infiltration (3,4).

Asthma may be induced by an imbalance of T helper (Th)1 and Th2 cells, as well as various other factors related to disease occurrence. The transcription factor, T-bet, serves a role as a Th1 cell transcription factor (5,6), producing Th1-related cytokines including interferon (IFN)-γ, which is stimulated by T-bet to produce more T-bet in a positive feedback loop. Interleukin (IL)-12 is a key cytokine that regulates the balance between Th1 and Th2 cells (7). It consists of a p35 subunit expressed ubiquitously and a p40 subunit that is restricted to IL-12-producing cells (8). IL-12 stimulates Th1-related immunoglobulins, including IgG2a, and it influences Th cell trafficking (9).

GATA-3 is a Th2 cell transcription factor (10). In a manner similar to T-bet, GATA-3 regulates Th2-related cytokines and accelerates the differentiation of Th2-related cytokines, including IL-4. Following IL-4 differentiation by GATA-3, IL-4 stimulates the production of more GATA-3 in a positive feedback loop. IL-5 increases the eosinophil population (11). IL-13 and IL-4 enhance B cell activation and Ig E production (12). IL-6 is a proinflammatory cytokine (13) and, because it is a regulatory factor for CD4+ cell balance (14), IL-6 is an important factor in asthma regulation. TNF-α is

a proinflammatory cytokine induced by mast cells that has several regulatory functions that are related to asthma, such as smooth muscle contraction (15), neutrophil and eosinophil attractions (16) and T cell activation, which involves cytokine release (17).

Symptomatic relievers have been investigated in the past as asthma is a hyper-responsive disease and is difficult to completely eradicate/cure (18). For several decades, steroids were commonly prescribed for asthma, but the drugs have many severe adverse effects such as inhibiting the growth of children (19,20), cataracts and glaucoma, hypertension, hyperlipidemia, peptic ulcers, myopathy and immunological suppression (21). Consequently, there have been many efforts to find more effective candidates from natural products with fewer adverse effects (22,23). *M. atichisonii* is a mushroom that has been consumed for a long time in East Asia (24) and has been reported to have several medicinal effects, such as the synthesis of nerve growth factors (25), anti-obesity (26) and the prevention of cell death (27).

In the present study, the authors attempted to identify anti-asthmatic drug candidates from natural products to reduce the adverse effects of existing therapies, and to develop a drug that is more effective against asthma. Using immunohistochemical studies, M. atichisonii was determined to reduce the physiological and histopathological changes related to asthma in the bronchoalveolar lavage fluid (BALF), including the number of white blood cells, differential cell count and immunoglobulin (Ig)E in the lungs, as well as the associated morphological changes and asthma-inducing factors.

Materials and methods

Mycoleptodonoides (M.) aitchisonii preparation. *M. atichisonii* used in the present study was provided by the Jeollanam-do Wando Arboretum (Wando, Korea). The fresh fruiting bodies were hot air-dried and ground into a powder. *M. atichisonii* extract was prepared by boiling 1 kg of mycelium powder in 10 l filtered sterile water for 8 h at 50˚C. The supernatant was saved and the pellet was re-boiled with 10 l water for 8 h. Insoluble material was removed by filtration and a two-fold volume of cold ethanol was added to the filtrate to precipitate the polysaccharide. Following standing the mixture at 4˚C overnight, the precipitate was collected by centrifugation (4,000 x g, 30 min at room temperature). The precipitate was washed with ethanol and resuspended in water. The resuspended solution was then freeze-dried.

Ovalbumin-induced asthma mouse model. Six-week-old female BALB/c mice (n=80; body weight, 22±2 g) were purchased from Samtako Bio Co. (Osan, Korea), fed with an *ad libitum* diet and water, and housed in a controlled environment (22±3˚C, 12 h light/dark cycle). They were then divided into six treatment groups: (i) Control, (ii) sterilized tap water with ovalbumin (OVA) induction, (iii) 1 mg/kg/day dexamethasone (DEX; Sam Nam Pharm, Chungcheongnam, Korea) with OVA induction, (iv) 10 mg/kg/day *M. atichisonii* with OVA induction, (v) 100 mg/kg/day *M. atichisonii* with OVA induction and (vi) 1,000 mg/kg/day *M. atichisonii* with OVA induction. On days 1 and 8, all mice were sensitized via intraperitoneal injection of 20 μg OVA (cat no. A5503-50G; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1 mg aluminum hydroxide hydrate (Prod #77161; Thermo Fisher Scientific, Inc. Waltham, MA, USA) in 500 μl saline. From days 21 to 25, all mice, except for those used as control, were challenged once daily with 5% OVA for 30 min using a nebulizer (3 ml/min, NE-U17; Omron Corporation, Kyoto, Japan). During the same 5 day period, the treatment groups were also administered once daily with oral doses of sterilized tap water, DEX, 10, 100 or 1,000 mg/kg/day *M. atichisonii* 1 h prior to the OVA challenge.

Ethics statement. All experiments were approved by the Institutional Animal Care and Use Committee at Dongshin University (Naju, Korea; approval no. 2014-08-04).

BALF analysis. A total of one day following the final treatment, the mice were anesthetized with intraperitoneal injections of 50 mg/kg Zoletil (Virbac, Carros, France), and their tracheas were cannulated with disposable animal feeding needles. Lavages were performed with three 0.4 ml aliquots of cold phosphate-buffered saline (PBS; cat no. 17-516F; Lonza, Walkersville, MD, USA). BALF samples were collected and immediately centrifuged at 900 x g for 5 min at room temperature (Sorvall Legend Micro 17R; Thermo Fisher Scientific, Inc.). The cell pellets were resuspended in PBS for total and differential cell counts. The numbers of total and differential cells were counted using the Hemavet Multispecies Hematology system (n=8 per group; Drew Scientific Inc., Miami Lakes, FL, USA). Levels of IgE in the BALF were measured using a specific mouse IgE enzyme-linked immunosorbent assay kit (cat no. 555248; BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's protocol (n=8 per group).

Histopathological analysis. Lung tissue was fixed in 10% (v/v) formaldehyde solution for 10 days at room temperature, dehydrated in a graded ethanol series (99.9, 90, 80 and 70%), and embedded in paraffin. Paraffin-embedded lung tissue was then sectioned (4 μm) longitudinally and stained with hematoxylin and eosin. In addition, the sections were stained with Periodic Acid-Schiff (PAS; periodic acid; cat no. P7875; Sigma-Aldrich; Merck KGaA; Schiff's reagent; cat no HX54780633; EMD Millipore, Billerica, MA, USA) for the semi-quantitative analysis of glycoproteins.

Immunohistochemical analysis. Deparaffinized tissue sections were treated with 3% hydrogen peroxide in methanol for 10 min to remove endogenous peroxidase. Antigen retrieval was carried out with sodium citrate buffer (0.1 M) using a microwave. The slides were incubated with normal serum to block non-specific binding and then incubated overnight at 4˚C with the following primary antibodies (diluted 1:100 to 1:200): Rabbit anti-mouse CD3 polyclonal (cat no. ab5690; 1:100; Abcam, Cambridge, MA, USA), rat anti-mouse CD4 monoclonal (cat no. 14-9766; 1:200; eBioscience, Inc., San Diego, CA, USA), rat anti-mouse CD8 monoclonal (cat no. sc-18913; 1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-mouse CD19 polyclonal (cat no. 250585; 1:100; Abbiotec, San Diego, CA, USA), rat anti-mouse MHC class II monoclonal (cat no. sc-59318; 1:100; Santa Cruz Biotechnology, Inc.), rabbit anti-mouse Tbx21/T-bet

Table I. Operation parameters for the GC/MS.

Conditions	GC/MS[a]		
Column	J&W Scientific, DB-5 cross-linked 5% phenyl methyl silicone		
Carrier	Helium		
Split/splitless	Split 10:1		
Injection volume	1 μl		
Detector	MS		
MS source	230˚C		
MS quad	150˚C		
Conditions	Rate (˚C/min)	Value (˚C)	Hold time (min)
Analytical temperature			
Initial	-	65	-
Step 1	-	65	10
Step 2	10	300	22
Total		55.5 min	
Electron ionization		70 ev	
Mass range		50-550 amu	
Scan method		Full scan	

[a]HP Agilent GC:7890A MS:5975C. GC/MS, Gas chromatography/mass spectrometry.

polyclonal (cat no. bs-3599R; 1:100; BIOSS, Beijing, China), goat anti-mouse GATA-3 (cat no. TA305795; 1:100; OriGene Technologies, Inc., Rockville, MD, USA), rat anti-mouse IFN-γ monoclonal (cat no. sc-74104; 1:100), goat anti-mouse IL-12p35 polyclonal (cat no. sc-9350; 1:100), rat anti-mouse IL-12p40 monoclonal (cat no. sc-57258; 1:100) (all from Santa Cruz Biotechnology, Inc.), rabbit anti-mouse TNF-α polyclonal (cat no. 3053R-100; 1:200; BioVision Milpitas, CA, USA), rat anti-mouse IL-4 monoclonal (cat no. sc-73318; 1:100), rabbit anti-mouse IL-5 polyclonal (cat no. sc-7887; 1:100), goat anti-mouse IL-6 polyclonal (cat no. sc-1265; 1:100), and goat anti-mouse IL-13 polyclonal (cat no. sc-1776; 1:100) (all from Santa Cruz Biotechnology, Inc.). The slides were incubated for 10 min with a biotinylated secondary antibody (cat no. PK-7800; Vector Laboratories, Inc., Burlingame, CA, USA) and horseradish-peroxidase conjugated streptavidin. Signals were detected using a 3,3-diaminobenzidine tetrahydrochloride substrate chromogen solution (cat no. SK-4105; Vector Laboratories), and cells were counterstained with Mayer's hematoxylin. To determine the number of positively stained cells, five random, non-overlapping fields of view were selected, and cells were counted (magnification, x200) from three separately immunostained lung sections per animal (n=8 per group).

Gas chromatography/mass spectrometry (GC-MS) analysis. GC-MS (Agilent 5975C MSD and 7890A GC; Agilent Technologies, Inc., Santa Clara, CA, USA) was tuned by perfluorotribuylamine (cat no. 442747; Sigma-Aldrich; Merck KGaA) using three mass fragments (m/z) of 69.0, 219.0 and 502.0 in the condition of electron ionization. A 5MS GC column (DB-5 cross-linked 5% phenylmethyl silicone; Agilent Technologies, Inc.) was used for the analysis as this column has low bleed that improves sensitivity for constituent identification. The GC oven was heated using the following program: Isothermal at 65˚C for 10 min and increasing by 10˚C every min to 300˚C with He as the carrier gas. The summarized operation parameters for the GC-MS are presented in Table I. In order to analyze the quality of extract in *M. aitchisonii*, the certain amount of dried samples by dehydrofreezing procedure was prepared. The samples were extracted using 10 min sonication at room temperature, twice with dichloromethane, followed by sonication twice with acetone using a sonicator (Bransonic® CPXH; Branson Ultrasonics Corporation, Dallas, TX, USA). The composited extract was evaporated by high volume nitrogen blowdown (TurboVap II; Caliper Life Sciences, Hopkinton, MA, USA) to reach 5-10 ml and was further evaporated to a final volume of 100 μl using low volume nitrogen blowdown (MGS-2200; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). A final extract volume of 100 μl was sialylated with N,O-bis(trimethylsilyl) trifluoroacetamide (Supelco; Sigma-Aldrich; Merck KGaA) to derivatize the constituents to their trimethylsilyl-derivatives (TMS-derivatives) prior to GC-MS analysis.

Statistical analysis. Data are presented as the mean ± standard deviation. Group differences were evaluated by one-way analysis of variance followed by Dunnett's multiple comparison tests. Statistical significance was set at $P<0.05$ or $P<0.01$.

Results

M. atichisonii dose-dependently suppressed the populations of WBCs and eosinophils in BALF. In the OVA-induced asthma

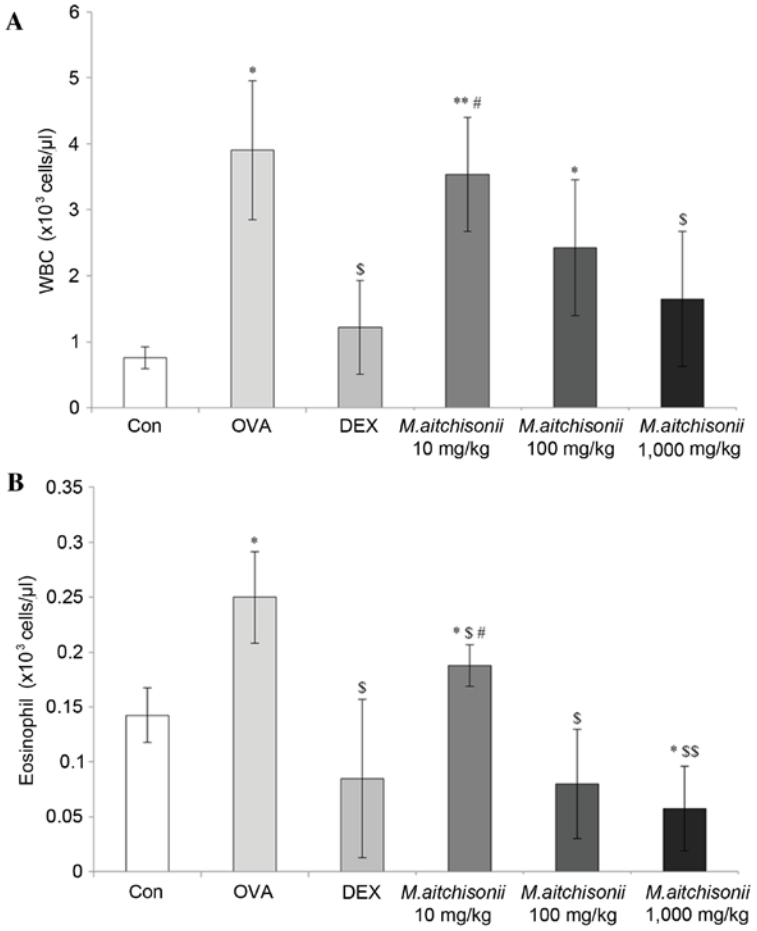

Figure 1. *M. atichisonii* suppressed the OVA-induced increase of WBC and eosinophil levels. (A) *M. atichisonii* dose-dependently suppressed the number of WBCs. (B) Treatment with 100 to 1,000 mg/kg *M. atichisonii* significantly decreased the level of eosinophils similarly to treatment with DEX. Each bar represents the mean ± standard deviation (n=6). *P<0.05 vs. Con; **P<0.001 vs. Con; $^{\$}$P<0.05 vs. OVA-induced asthma, $^{\$\$}$P<0.001 vs. OVA-induced asthma; $^{\#}$P<0.05 vs. DEX treatment. WBC, white blood cell; Con, control; OVA, ovalbumin; Dex, dexamethasone.

group, *M. atichisonii* dramatically increased WBC count, when compared with the level of WBCs in the control group. However, in the DEX treated group, WBC number decreased by a similar amount in the control group (Fig. 1A; P<0.05 or P<0.01). *M. atichisonii* suppressed WBC proliferation induced by OVA in a dose-dependent manner. The number of WBCs in the 1,000 mg/kg *M. atichisonii* treatment group was similar to that in the DEX treatment.

Eosinophil number was dramatically was more affected by *M. atichisonii* treatment than WBC number (Fig. 1B; P<0.05 or P<0.01). *M. atichisonii* dose-dependently suppressed eosinophil proliferation and the levels of eosinophils in the 1,000 mg/kg/day *M. atichisonii* treatment group, and this was similar to that in DEX treatment.

M. atichisonii effectively decreased the quantity of IgE in the BALF of OVA-induced asthma mice. IgE is related to the hyperresponsiveness of asthma and serves an important role in asthma occurrence (28). *M. atichisonii* effectively decreased the level of IgE in a dose-dependent manner, which was increased by OVA treatment, when compared with that the in OVA treatment group (8.2±0.69 ng/ml). In addition, the quantity of IgE in the 1,000 mg/kg treatment group (2.7±1.02 ng/ml) was similar to that in the DEX treatment group (2.4±0.54 ng/ml; Fig. 2; P<0.05 or P<0.01).

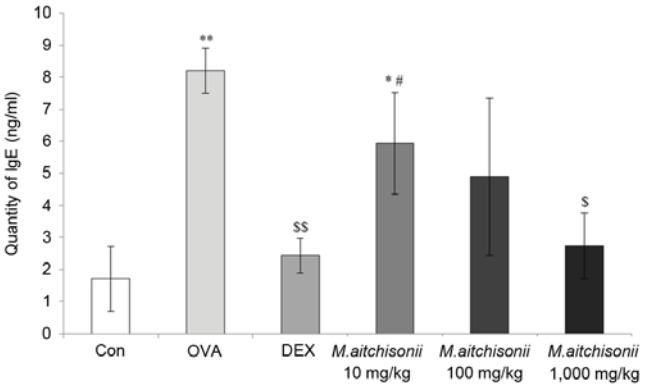

Figure 2. *M. atichisonii* decreased the quantity of IgE in bronchoalveolar lavage fluid in OVA-induced asthma animals in a dose-dependent manner. Each bar represents the mean ± standard deviation (n=6). *P<0.05 vs. Con; **P<0.001 vs. Con; $^{\$}$P<0.05 vs. OVA-induced asthma, $^{\$\$}$P<0.001 vs. OVA-induced asthma; $^{\#}$P<0.05 vs. DEX treatment. IgE, immunoglobulin E; Con, control; OVA, ovalbumin; Dex, dexamethasone.

Mycoleptodonoides atichisonii recovered the typical asthmatic morphological changes in the lung. To analyze the morphological changes in the lung that were induced by OVA treatment, hematoxylin and eosin staining was used, in

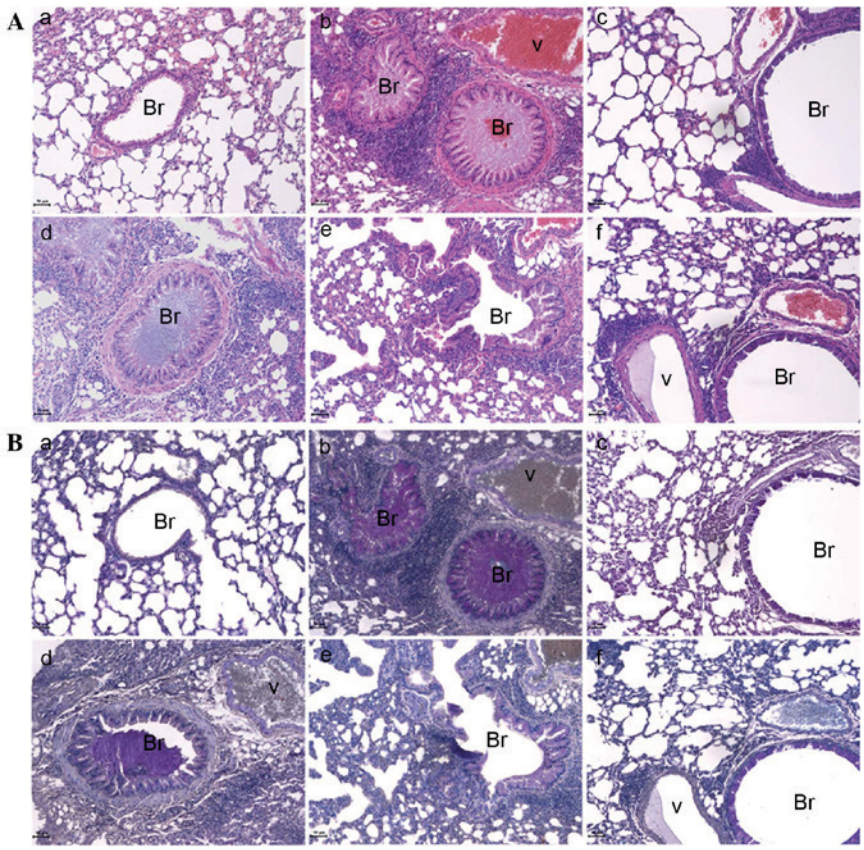

Figure 3. *M. atichisonii* recovered the asthmatic changes in the lung. (A) *M. atichisonii* effectively and dose-dependently recovered the ovalbumin-induced morphological changes such as mucous secretion in bronchioles, epithelial hyperplasia and eosinophil infiltration. This is a representative image of a lung stained by hematoxylin and eosin. (B) *M. atichisonii* decreased ovalbumin-induced mucous secretion similar to dexamethasone. This is a representative image of a lung stained by Periodic Acid-Schiff. Scale bar (bottom-left of each image)=50 μm. a, control; b, ovalbumin; c, dexamethasone; d, 10 mg/kg/day *M. atichisonii*; e, 100 mg/kg/day *M. atichisonii*; f, 1,000 mg/kg/day *M. atichisonii*. Br, bronchiole; V, vessel.

addition to PAS staining (Fig. 3). In the lungs of mice with OVA-induced asthma, typical histopathological changes were observed such as airway remodeling, mucous hyper-secretion, epithelial hyperplasia and eosinophil infiltration (Fig. 3A-b). In particular, PAS stained mucous hyper-secretion in the bronchioles was clearly observed using the specific staining method (Fig. 3B-b). DEX, which is typically used as a representative anti-asthmatic drug, significantly suppressed the morphological changes in the lung (Fig. 3A-c) and completely prevented mucous secretion (Fig. 3B-c). *M. atichisonii* not only recovered the asthmatic alterations (Fig. 3A-d-f) but also suppressed mucous secretion (Fig. 3B-d-f). These results suggested that *M. atichisonii* may ameliorate airway obstruction.

Mycoleptodonoides atichisonii downregulated the expressions of MHC class II molecules and T cells. In the 100 mg/kg/day *M. atichisonii* treatment and 1,000 mg/kg/day *M. atichisonii* treatment, expression of CD3+ total T cell (Fig. 4A; P<0.05 or P<0.01) was almost fully diminished. The expression of CD4+ helper T cells was suppressed in a dose-dependent manner (Fig. 4B; P<0.05 or P<0.01). A total of 1,000 mg/kg/day *M. atichisonii* treatment effectively inhibited the expression of CD4+ helper T cells (Fig. 4B-f; P<0.05 or P<0.01) when compared with the DEX treatment group (Fig. 4B-c; P<0.05 or P<0.01). In the 100 mg/kg/day or more *M. atichisonii* treatment group, the expression pattern of CD8+ cytotoxic T cells (Fig. 4C; P<0.05 or P<0.01) was the same as that observed for the CD4+ helper T cells, which was that they were almost fully diminished. Although *M. atichisonii* downregulated the expression of CD19+ B cells, the decreased level of CD19+ B cells was lower compared with other groups including CD3+ total T cell, CD4+ helper T cell, and CD8+ cytotoxic T cell (Fig. 4D; P<0.05 or P<0.01). The expression of MHC class II+ molecule (Fig. 4E; P<0.05 or P<0.01) almost disappeared in the 100 mg/kg/day *M. atichisonii* treatment and in the 1,000 mg/kg/day *M. atichisonii* treatment groups. The 1,000 mg/kg/day *M. atichisonii* treatment effectively inhibited the expression of MHC class II+ molecules (Fig. 4E-f; P<0.05 or P<0.01) more, when compared with the DEX treatment group (Fig. 4E-c; P<0.05 or P<0.01).

Although *M. atichisonii* appears to control levels of both T cells (CD3+, CD4+, CD8+) and B cells (CD19+), the potency to modulate B cell (CD19+) proliferation may be smaller than that of T cell (CD3+, CD4+, CD8+) proliferation, however it downregulated CD3+ positive cells (total T cell) and CD8+ positive cells (cytotoxic T cell) and dose-dependently regulated CD4+ positive cell (helper T cell).

M. atichisonii controlled T-bet and GATA-3. In order to compare the suppressive effects of the Th1 cell transcription

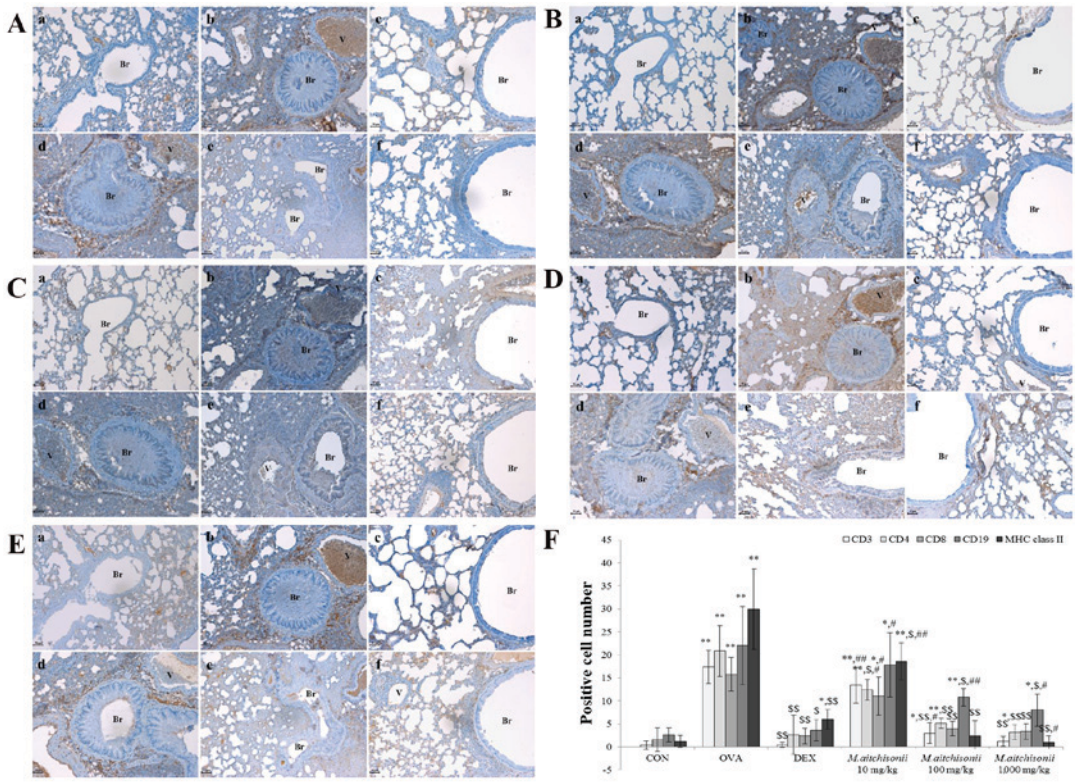

Figure 4. *M. atichisonii* almost completely inhibited the expressions of several cells related to asthma occurrence. (A) *M. atichisonii* completely inhibited the expression of CD3+ cell levels. (B) *M. atichisonii* dose-dependently suppressed CD4+ cells. (C) *M. atichisonii* almost fully diminished CD8+ cell levels. (D) *M. atichisonii* downregulated the expression of the CD19+ cell, although the effect of *M. atichisonii* was smaller than CD3+, CD+8 and MHC class II. (E) The effect of 100 or 1,000 mg/kg *M. atichisonii* treatment against MHC class II was greater than that of DEX treatment. (F) Quantification of expression. Each bar represents the mean ± standard deviation (n=6). a, Con; b, OVA; c, DEX; d, 10 mg/kg/day *M. atichisonii*; e, 100 mg/kg/day *M. atichisonii*; f, 1,000 mg/kg/day *M. atichisonii*. *P<0.05 vs. control; **P<0.001 vs. Con; $^{\$}$P<0.05 vs. OVA-induced asthma, $^{\$\$}$P<0.001 vs. OVA-induced asthma; $^{\#}$P<0.05 vs. DEX treatment, $^{\#\#}$P<0.01 vs. DEX treatment. Br, bronchiole; V, vessel; MHC, major histocompatibility complex; Con, control; OVA, ovalbumin; DEX, dexamethasone.

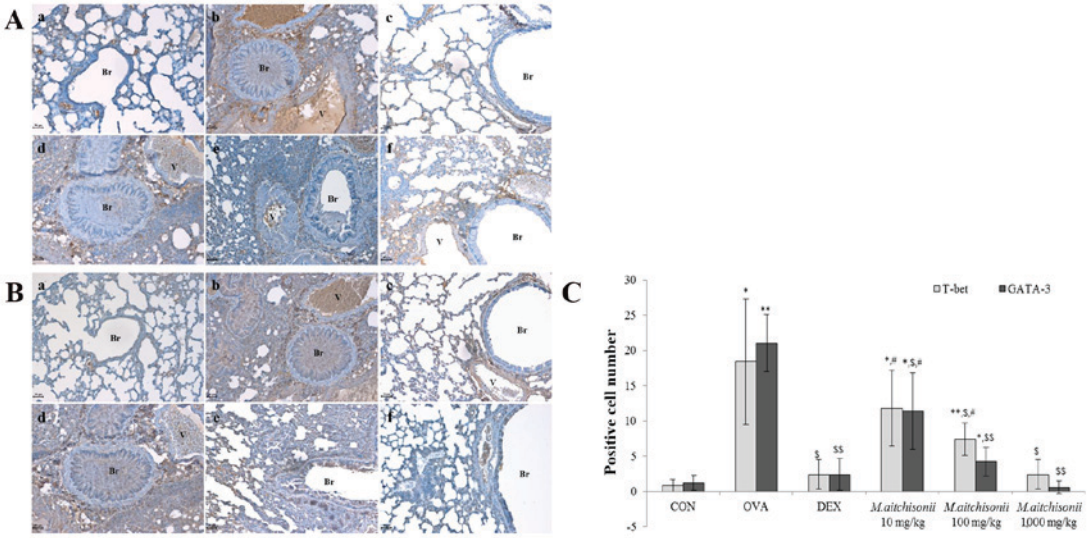

Figure 5. *M. atichisonii* controlled T-bet and GATA-3 expression levels. (A) *M. atichisonii* dose-dependently suppressed the expression of T-bet. (B) *M. atichisonii* downregulated GATA-3 more than T-bet, and the effect of *M. atichisonii* was similar the effect of dexamethasone. (C) Quantification of expression. Each bar represents the mean ± standard deviation (n=6). a, Con; b, OVA; c, DEX; d, 10 mg/kg/day *M. atichisonii*; e, 100 mg/kg/day *M. atichisonii*; f, 1,000 mg/kg/day *M. atichisonii*. *P<0.05 vs. Con; **P<0.001 vs. Con; $^{\$}$P<0.05 vs. OVA-induced asthma, $^{\$\$}$P<0.001 vs. OVA-induced asthma; $^{\#}$P<0.05 vs. DEX. Br, bronchiole; V, vessel; Con, control; OVA, ovalbumin; DEX, dexamethasone.

factor, T-bet, and the Th2 cell transcription factor, GATA-3, the changes of expressions on T-bet and GATA-3 were measured via immunohistochemical staining (Fig. 5). The expressions of T-bet and GATA-3 in the OVA treatment

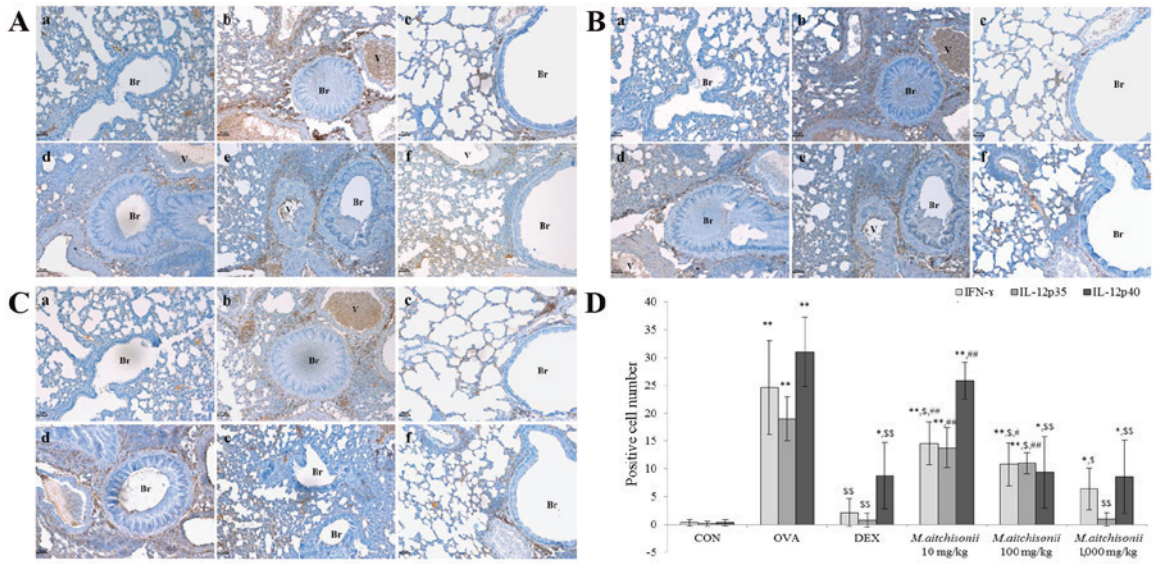

Figure 6. *M. atichisonii* suppressed the expressions of IFN-γ and IL-12p35. (A) In a dose-dependent manner, *M. atichisonii* suppressed the expression of IFN-γ, however, the effect of *M. atichisonii* is smaller than the effect of DEX. (B) Although *M. atichisonii* inhibited the expression of IL-12p35, the effect of *M. atichisonii* in 10 mg/kg treatment and 100 mg/kg treatment groups was small. The 1,000 mg/kg *M. atichisonii* treatment suppressed the expression of IL12-p35 similarly to DEX treatment. (C) IL-12p40 may be affected by DEX and *M. atichisonii* as the changes of expression level were not large following treatment. (D) Quantification of expression. Each bar represents the mean ± standard deviation (n=6). a, Con; b, OVA; c, DEX; d, 10 mg/kg/day *M. atichisonii*; e, 100 mg/kg/day *M. atichisonii*; f, 1,000 mg/kg/day *M. atichisonii*. *P<0.05 vs. Con; **P<0.001 vs. Con; $^{\$}$P<0.05 vs. OVA-induced asthma, $^{\$\$}$P<0.001 vs. OVA-induced asthma; $^{\#}$P<0.05 vs. DEX treatment, $^{\#\#}$P<0.01 vs. DEX treatment. Br, bronchiole; V, vessel; IFN-γ; interferon-γ; IL, interleukin; Con, control; OVA, ovalbumin; DEX, dexamethasone.

group was significantly increased, when compared with the control, however DEX decreased the expressions of T-bet and GATA-3, which was induced by OVA, to a similar level as the control groups (Fig. 5A and B). Although *M. atichisonii* dose-dependently downregulated the expression of T-bet, there was no significant difference between 100 mg/kg *M. atichisonii* treatment and 1,000 mg/kg *M. atichisonii* treatment (Fig. 5C; P<0.05 or P<0.01). Conversely, from treatment with 10 mg/kg *M. atichisonii* to 1,000 mg/kg, the changes in GATA-3 expression were dependent on the dose-response relationship (Fig. 5C; P<0.05 or P<0.01).

M. atichisonii suppressed the expression of Th1-related cytokines. M. atichisonii dose-dependently suppressed the expression of IFN-γ and the effect of *M. atichisonii* on INF-γ was less than DEX (Fig. 6A). The results of IFN-γ by *M. atichisonii* were similar to that of T-bet (Fig. 5A). *M. atichisonii* inhibited the expression of IL-12p35 (Fig. 6B). The effect of 1,000 mg/kg *M. atichisonii* treatment on IL-12p40 was similar to that of dexamethasone. DEX and *M. atichisonii* effectively suppressed the expression of IL-12p40 (Fig. 6C). Although there was no significant difference between 10 mg/kg *M. atichisonii* treatment and 100 mg/kg *M. atichisonii* treatment (P>0.05), in the 1,000 mg/kg *M. atichisonii* treatment group, IL-12p35 levels were almost fully diminished (Fig. 6D).

M. atichisonii almost fully inhibited the expression of Th2-related cytokines such as IL-5, IL-6, and IL-13. In order to measure the downregulation effect of *M. atichisonii* on Th2-related cytokines, such as TNF-α (Fig. 7A), IL-4 (Fig. 7B), IL-5 (Fig. 7C), IL-6 (Fig. 7D) and IL-13 (Fig 7E), immunohistochemical analysis was conducted. Although *M. atichisonii* did not completely prevent the expression of TNF-α, it was similar to that of DEX (Fig. 7A). *M. atichisonii* dose-dependently suppressed the expression of IL-4, but the suppression was less than DEX (Fig. 7B and F). The expression of IL-5 was controlled by *M. atichisonii* and the effect was similar to DEX. In particular, IL-6 and IL-13 were dose-dependently suppressed by *M. atichisonii* treatment more than by DEX (Fig. 7F).

Nicotinic acid (niacin), oleic acid and linoleic acid may act as anti-asthmatic compounds in M. aitchisonii. To identify the compounds in *M. aitchisonii* that may possess anti-asthmatic properties, GC-MS analysis was conducted. Not all compounds in *M. aitchisonii* were isolated, however nicotinic acid (niacin), oleic acid and linoleic acid were identified as candidate compounds. Fig. 8 presents the identification of silylated fatty acids. Of the identified fatty acids, niacin, linoleic acid and linoleic acid were analyzed at retention times of 18.21, 28.01 and 28.06 min respectively.

Discussion

Eosinophils are a key mediator of innate and adaptive immunity (29,30). The differentiation and activation of eosinophils is strongly related to the IL-5 gene; if the *IL-5* gene is depleted, airway eosinophilia cannot occur (31). Eosinophils are increased in number in asthma, and this is induced by IL-5 (11). In allergic situations, eosinophils remain for 8-12 h in circulatory blood, and for an additional 8-12 days in tissue following the removal of the stimuli (32). In the present study, although the expression of IL-5 was more suppressed by *M. atichisonii*

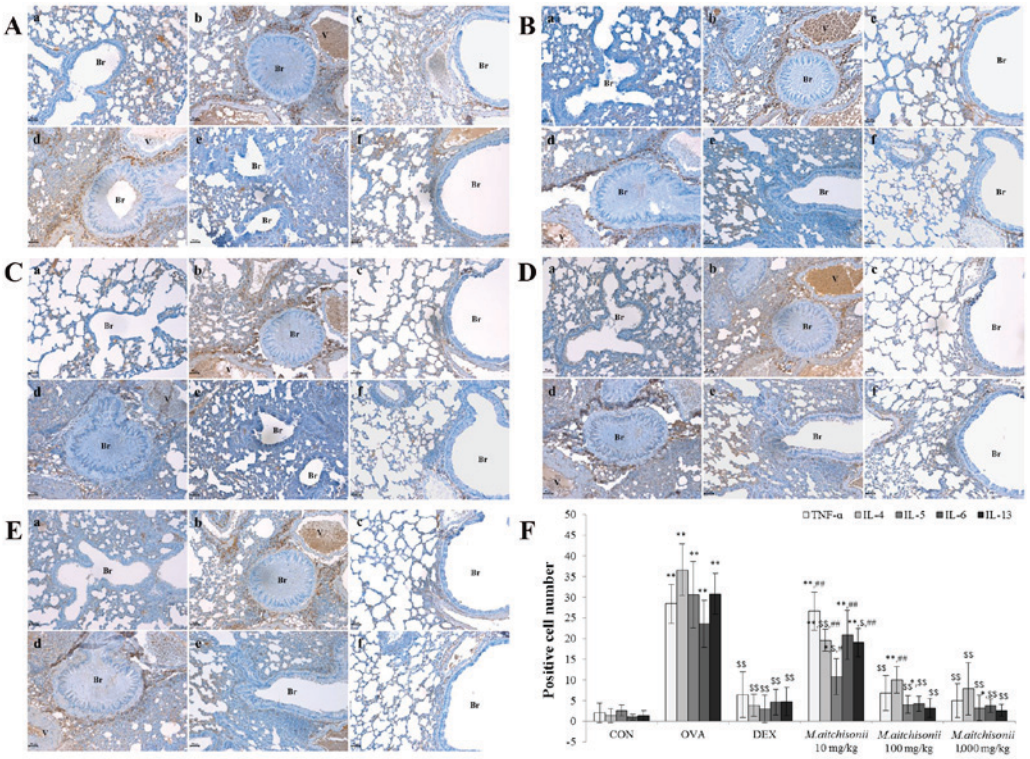

Figure 7. *M. atichisonii* significantly inhibited the expression of Th2-related cytokines in a dose-dependent manner. (A) *M. atichisonii* suppressed the expression of TNF-α similarly to dexamethasone treatment. (B) *M. atichisonii* downregulated the expression of IL-4. (C) *M. atichisonii* significantly inhibited the expression of IL-5, similar to treatment with dexamethasone. (D) *M. atichisonii* dramatically inhibited the expression of IL-6 more than treatment with dexamethasone. (E) The expression of IL-13 dramatically inhibited the expression of IL-6 more than treatment with dexamethasone. (F) Quantification of expression. Each bar represents the mean ± standard deviation (n=6). a, Con; b, OVA; c, DEX; d, 10 mg/kg/day *M. atichisonii*; e, 100 mg/kg/day *M. atichisonii*; f, 1,000 mg/kg/day *M. atichisonii*. *P<0.05 vs. control; **P<0.001 vs. control; $^{\$}$P<0.05 vs. OVA-induced asthma, $^{\$\$}$P<0.001 vs. OVA-induced asthma; $^{\#}$P<0.05 vs. DEX treatment, $^{\#\#}$P<0.01 vs. DEX treatment. Th, T helper; TNF-α, tumor necrosis factor-α; IL, interleukin; Br, bronchiole; V, vessel; Con, control; OVA, ovalbumin; DEX, dexamethasone.

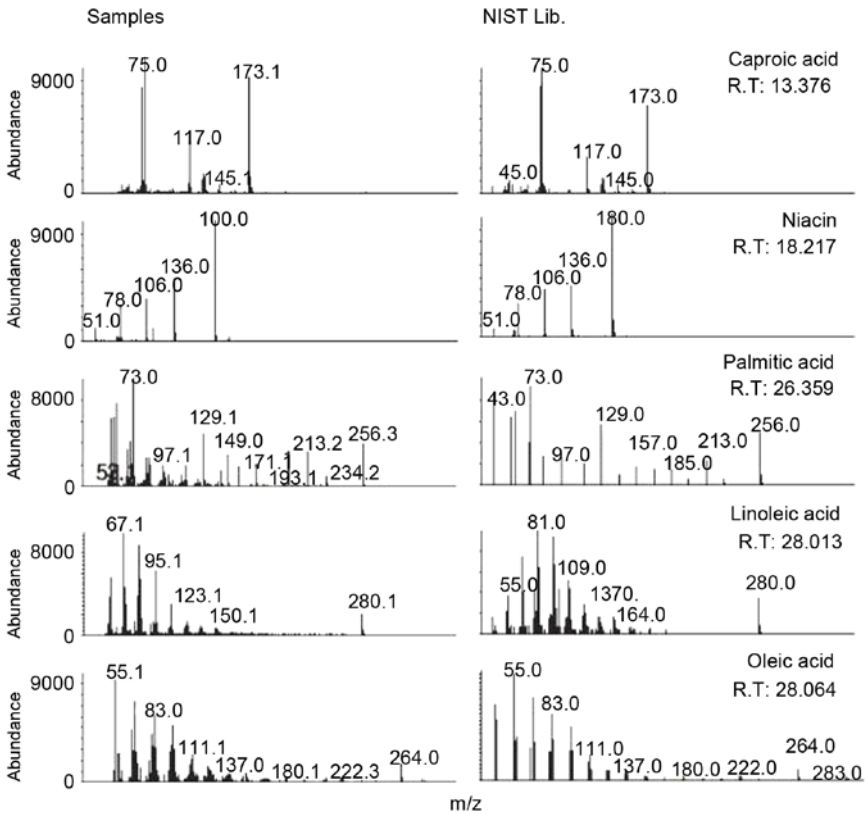

Figure 8. Gas chromatography/mass spectrometry analysis of *M. atichisonii*.

than that of eosinophils, the downregulatory patterns were similar for both, indicating that *M. atichisonii* may suppress eosinophils through IL-5. As the level of IL-5 suppression by *M. atichisonii* was larger than the other effects, IL-5 might be one of the key mediators in the preventive mechanism of *M. atichisonii* against asthma.

MHC class II molecules, which are made by antigen presenting cells, have the function of presenting processed antigens to helper T cells and triggering acquired immunity (33). MHC class II+ expression was almost completely eliminated by 100 mg/kg *M. atichisonii* treatment, confirming that *M. atichisonii* may completely control MHC class II molecule expression, which serves an important role in asthma occurrence.

Based on the changes in IFN-γ levels, a controversial hypothesis regarding the mechanism underlying asthma was previously proposed that implicated the Th1/Th2 cell imbalance and T cell priming induced by allergens (34). *M. atichisonii* slightly decreased not only the expressions of Th2-related factors, but also that of IFN-γ increased by OVA (Fig. 4D) in the present study and this result may support the T cell priming theory. IL-12 is produced by cells including dendritic cells, macrophages and monocytes (35) and is considered an IFN-γ-inducing factor because it stimulates the production of IgM and IFN-γ (8). Although *M. atichisonii* suppresses or inhibits most asthma-induced factors, the key factors for the anti-asthmatic effect may be IL-5, IL-6 and IL-13 (Fig 7C-E). The ratio of Th17 cells and Treg cells is related to the late phase asthma induction and, in the case of late phase asthma, the analysis of Th17 cells and Treg cells may be important (36).

In the current study, the authors determined that *M. atichisonii* lowered the OVA-induced level of WBCs and eosinophils in BALF and the level of IgE in serum, recovered respiratory changes, such as mucous secretion, epithelial cell hyperplasia and eosinophil infiltration and finally ameliorated airway obstruction. This was controlled by T cell-related molecules, such as CD3+, CD4+ and CD8+, as well as by MHC class II+ molecules that were increased following OVA treatment. Ultimately, this led to downregulated T-bet and GATA-3 levels, and a dose-dependent decreased to the level of Th1-related cytokines, IFN-γ and IL-12p40, and Th2-related cytokines, TNF-α, IL-4, IL-5, IL-6 and IL-13. In particular, the expression of IL-5, IL-6 and IL-13 increased by OVA treatment were almost completely eliminated by *M. atichisonii* application.

In this study, niacin, oleic acid and linoleic acid in *M. aitchisonii* were identified and isolated. Melton (37) previously reported that nicotinic acid reduced the frequency of asthmatic paroxysm. In addition, oleic acid has beneficial effects in inflammatory related diseases (38) and linoleic acid is one of the mediators that regulate inflammations and asthma (39).

The present study is one of the ongoing efforts to identify appropriate anti-asthmatic drug candidates. From the results, it may be concluded that *M. atichisonii* has an anti-asthmatic effect and that the pharmacological effect may be based on the suppression or inhibition of various factors related to Th1 and Th2. In particular, IL-5, IL-6 and IL-13 may be some of the most important factors related to asthma occurrence, and they are modulated by *M. atichisonii*. *M. atichisonii* is a promising drug that is believe to be key in the control of asthma.

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