Macmoondongtang modulates Th1-/Th2-related cytokines and alleviates asthma in a murine model

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Abstract

Objective

Macmoondongtang has been used as a traditional medicine to treat pulmonary disease in Korea. However, the mechanism underlying its therapeutic effect has yet to be reported. In the present study, the role of macmoondongtang as a respiratory medicine, especially as an anti-asthmatic agent, has been attributed to the down-regulation of interleukin (IL)-4 and tumor necrosis factor (TNF)-α.

Materials & methods

BALB/c mice were divided into five groups: control, asthma-induced control, dexamethasone treatment, treatment with 150 mg/kg macmoondongtang, and treatment with 1500 mg/kg macmoondongtang. To evaluate the anti-asthmatic effect of macmoondongtang, we investigated its suppressive or inhibitory effects against typical asthmatic changes such as differential cell count in bronchioalveolar fluid (BALF), serum IgE levels, lung morphology, expression of Th1/Th2 cell transcription factors such as T-bet and GATA-3, and Th1-/Th2-/Th17-related cytokines such as interferon (IFN)-γ, IL-12p40, IL-4, -5, -13, TNF-α, and IL-6. The active ingredients in macmoondongtang were further analyzed.

Results

Macmoondongtang treatment down-regulated serum IgE level, a very important marker of hyper-responsiveness. It reversed typical morphological changes such as mucus hypersecretion, lung epithelial cell hyperplasia, and inflammatory cell infiltration near bronchioalveolar space and veins. Macmoondongtang significantly decreased neutrophil count in BALF, as well as reduced T-bet, IFN-γ, and TNF-α expression in the lung. It also showed a dose-dependent control of inflammatory cells in BALF, controlled the expression of IL-12, IL-4, and IL-5 genes in the lung, and the protein expression of IL12p40, GATA-3, IL-4, IL-5, and IL-13. The component analysis revealed glycyrrhizin and liquiritin as the active ingredients.
Conclusions

Macmoondongtang treatment alleviates asthma symptoms and modulate the Th1-/Th2-related cytokines. Glycyrrhizin and liquiritin could be the major the active therapeutic components.

Introduction

In 2013, the World Health Organization reported that at least 200 million individuals worldwide were diagnosed with asthma. The allergens were classified into two categories: indoor agents (such as pet dander, dust mites, tobacco smoke, and so forth) and outdoor agents (e.g., pollen, environmental pollutants, cold temperature, and so forth) [1]. Asthma is a type I allergy, and anaphylaxis. It is hard to completely eradicate and its symptoms are very diverse, ranging from cough to apnea. Patients with severe allergy may die from pulmonary obstruction and lack of gas exchange due to hypersecretion of mucus, overgrowth of pulmonary subepithelial cells, enlarged basement membrane, and accumulation of inflammatory cells near bronchioles and vessels [2,3].

Among several theories of asthma occurrence, the imbalance between Th1 and Th2 cells plays a significant role in increasing the risk [2]. Cytokines such as interferon gamma (IFN-γ) that induce the release of Th1 cells are associated with the severity of asthma [4]. Interleukin 12 (IL-12) is an important cytokine that mediates Th1 cell differentiation and decreases Th2 cell proliferation [5–7]. Th2 cells release specific cytokines such as IL-4, IL-5, IL-13, and tumor necrosis factor-alpha (TNF-α) while IL-6 and IL-1β are Th17-related cytokines [8]. IL-4 is induced by GATA-3, a transcription factor with several bioactive functions, including stimulation of Th2-related cytokine expression [9], regulation of IgE levels [10], and so on. The level of IL-6 in asthma patients is substantially higher than in normal persons [11]. As one of the important factors that regulate asthma severity, IL-6 induces IL-4 up-regulation and stimulates Th17 cell differentiation [12]. IL-13 is linked to morphological changes in respiratory organs and B cell activation [13–16]. TNF-α is synthesized by macrophages [17]. It recruits neutrophils and eosinophils [18], stimulates T cell activation [19], and finally establishes airway hyperresponsiveness [20].

Inhaled corticosteroids are usually used to inhibit clinical asthma symptoms such as cough, respiratory depression, etc [21]. Asthma occurrence is higher in early childhood and elderly population than in the young and grown-up groups [1]. Inhaled corticosteroids are associated with serious adverse effects such as growth inhibition [22], cataracts, glaucoma, hypertension, hyperlipidemia, peptic ulcers, myopathy, and immunological suppression [23]. Therefore, efforts to discover new anti-asthmatic agents are ongoing and trials to investigate the mode of action on suppression or inhibition against asthma occurrence among the traditional medicines-related pulmonary diseases have increased.

Several hundred years ago in Korea, a medical encyclopedia known as Donguibogam was composed [24]. Donguibogam introduced macmoondongtang, a traditional prescription for the treatment of respiratory illness. Macmoondongtang is composed of plants such as Glycyrrhizae radix, Oryza sativa, Zizyphus jujube Inermis, Ophiopogon japonicas, Pinellia ternate, and Panax ginseng. Although macmoondongtang has been used to treat pulmonary disease recently, its mode of action has yet to be reported. Therefore, the objective of this study was to analyze the possible anti-asthmatic effect of macmoondongtang and the underlying mechanism of action.
Materials and methods

Preparation of macmoondongtang

Macmoondongtang (Batch No. 14010, hot water extract-powder form) was manufactured by Hankuk Inspharm, Ltd. (Jeonnam, Korea) based on the prescription indicated in Donguibogam [24] and used in the present study. Macmoondongtang is generally available as an over-the-counter (OTC) drug. It contains G. radix, O. sativa, Z. jujube Inermis, O. japonicas, P. ternate, and P. ginseng. The dosage used in this study was calculated based on the drug composition. The human equivalent dose (HED) for mice is 12.3 and the safety factor is 10×[25]. In this study, we applied 1/100 and 1/10 dosages for mouse based on therapeutic human dosage.

Identification of anti-asthmatic compounds in macmoondongtang

To analyze the levels of anti-asthmatic compounds in macmoondongtang, appropriate amounts of glycyrrhizin and liquiritin were accurately weighed and dissolved in methanol in 100 mL volumetric flasks to obtain stock solutions of 100 μg/mL. Solutions were subsequently serially diluted two-fold to 3.125 μg/mL. A sample measuring 0.5 g was dissolved in 10 mL methanol and sonicated to expedite the dissolution of particles. Subsequently, 1 mL was transferred to a volumetric flask and diluted with 9 mL of mobile phase A. The final concentration of macmoondongtang was 50 mg/mL. HPLC analysis was performed using an Alliance 2695 HPLC system (Waters, Milford, MA, USA) equipped with a photodiode array detector. A reverse phase column (C18, 5 μm, 150 mm × 5 mm) was used with a mobile phase consisting of a mixture of solvent A (acetonitrile) and B (0.2% acetic acid). The mobile phase conditions for glycyrrhizin were as follows: 0–35 min, gradient elution from 15/85 to 35/65 v/v; 35–50 min, gradient elution from 35/65 to 100/0 v/v; and 50–55 min, from 100/0 to 15/85 v/v. The mobile phase conditions for liquiritin were as follows: 0–8 min, gradient elution from 10/90 to 20/80 v/v; 8–20 min, gradient elution from 20/80 to 25/75 v/v; 20–21 min, from 25/75 to 100/ v/v; and 21–30 min, from 100/0 to 10/90 v/v. The flow rate was 1.0 mL/min, and the injection volume was 10 μL.

Animal experiments

The animal study investigating the anti-asthmatic effect of macmoondongtang was followed by a confirmative study. For each study, 35 female BALB/c mice were purchased from Samtako Korea (Osan, Korea) and divided into five groups according to the treatment: vehicle control (tap water), ovalbumin (OVA)-treated group, OVA-treated group exposed to 1 mg/kg dexamethasone, OVA-treated group exposed to 150 and 1500 mg/kg macmoondongtang. The following doses were injected intraperitoneally into all animals except the control group: 20 μg OVA (Sigma-Aldrich, St. Louis, MO, USA) and 1 mg aluminum hydroxide hydrate (Sigma-Aldrich) in 500 μL saline. A week after injection of the second booster dose, all animals except the control group were exposed to OVA including a first inhalation dose for 5 days after OVA challenge. The animals were treated orally with tap water, dexamethasone, and 2 doses of macmoondongtang.

Ethics statement

This animal study was conducted following approval by the Institutional Animal Care and Use Committee of Dongshin University (Animal Study Approval No. 2014-08-02).
Bronchoalveolar fluid (BALF) and serum analysis

Bronchoalveolar fluid (BALF) was collected from all mice under anesthesia on a day after the final treatment. BALF was collected using 0.4 mL cold phosphate-buffered saline (PBS). The white blood cell (WBC) count and differential blood count were performed with the collected fluid using Hemavet Multipurpose Hematology System (Drew Scientific Inc, Waterbury, CT, USA). Inflammatory cells in BALF were stained with Diff-Quick method [26]. Serum IgE levels were evaluated using ELISA.

Histopathological analysis

In order to observe the morphological changes, lungs were fixed in formaldehyde, dehydrated in ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. In order to compare the levels of released glycoprotein, periodic acid Schiff (PAS) stain was used. Subsequently, the images were acquired with AxioScope A1 (Carl Zeiss, Gottingen, Germany).

Reverse transcription polymerase chain reaction (RT-PCR) and enzyme-linked immunoassay (ELISA)

In order to evaluate changes in the cDNA levels of IFN-γ, IL-12p40, IL-4, IL-5, IL-13, TNF-α, and IL-6, which are related to asthma induction, RT-PCR analysis was conducted as reported in our previous study protocol (Lee et al., 2017). Total RNA was extracted from the lung using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Total RNA (100 ng) was used as a template for the reaction. Primers were synthesized for RT-PCR as shown in Table 1. The RT-PCR cycles consisted of denaturation at 95 °C for 5 s and annealing/extension at 65 °C for 30 s for 40 cycles using with QTOVER2.2. (Analytik Jena AG, Thuringia, Germany). The serum levels of IgE were measured using a specific mouse IgE enzyme-linked immunosorbent assay kit (BD bioscience, 555248, San Jose, CA, USA) according to the manufacturer’s protocols.

Table 1. Primer sequences for RT-PCR.

| Genes    | Primer sequences                      |
|----------|---------------------------------------|
| IFN-γ    | Forward 5'-GGCCATCGCAACACATAAG-3'     |
|          | Reverse 5'-GTTGACCCATCAACCTG-3'       |
| IL-12p   | Forward 5'-GGACCAAAGGGACTATGAGAAG-3' |
|          | Reverse 5'-CTTCCAACGCCCAGTTGATAG-3'  |
| IL-4     | Forward 5'-ACAGGAGAAGGGCACCCAT-3'    |
|          | Reverse 5'-GAAGCCTCAGACGCAGGCT-3'    |
| IL-5     | Forward 5'-TGCTTACGTTCACAGTTTC-3'    |
|          | Reverse 5'-GGATGCTAAGGTTTGATGTA-3'   |
| IL-13    | Forward 5'-CAGCCCTCAGCAGAAT-3'       |
|          | Reverse 5'-CTTGGATGTGTAAACAGCATTCT-3'|
| IL-6     | Forward 5'-GATAAGCTGGAGCTACAGAAGG-3' |
|          | Reverse 5'-TTGCCAGGATGATTCACAAAGG-3' |
| TNF-α    | Forward 5'-CTGACATCAGCAGAAGGAG-3'    |
|          | Reverse 5'-CCTCAGGAGAAATCTGGAAAG-3'  |
| GAPDH    | Forward 5'-GTGGAGTACATCTGACATGAG-3'  |
|          | Reverse 5'-AATGGTGAGGTCGGTGTTG-3'    |

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Immunofluorescent analysis

In order to localize the expression of Th1 cell transcription factor, T-bet, and Th2 cell transcription factor, GATA-3 immunofluorescent analysis was conducted. Only four groups were evaluated: control, OVA, dexamethasone plus OVA treatment, and 1500 mg/kg macmoon-dongtang plus OVA treatment. Prior to the antibody binding step, the same materials were used for immunohistochemical analysis; however, rabbit anti-mouse T-bet (Biorbyt, orb7075, Cambridge, UK) or goat anti-mouse GATA-3 (OriGene, TA305795, Rockville, MD, USA) were used as primary antibodies for 1 h at room temperature. The slides were incubated for 2 h with FITC-conjugated anti-rabbit IgG (Jackson Immunoresearch, 315-095-003, West Grove, PA, USA) or Alexa Fluor®-555-conjugated anti-goat IgG (ThermoFisher Scientific, A-21127, Waltham, MA, USA). The cells were counterstained with DAPI (ThermoFisher Scientific, 62249, Waltham, MA, USA). The images were obtained using a K1-Fluo confocal microscope (Nanoscope System, Daejeon, Korea).

Immunohistochemical analysis

In order to analyze the changes in the levels of asthma-related protein, immunohistochemistry was performed using antibodies such as IFN-γ (Santa Cruz, sc-74104), IL-12p40 (Santa Cruz, sc-57258), IL-4 (Santa Cruz, sc-73318), IL-5 (Santa Cruz, sc-7887), IL-13 (Santa Cruz, sc-1776), IL-6 (Santa Cruz, sc-1265), and TNF-α (BioVision, 3053R-100, Milpitas, CA, USA) and the dilution rate ranged from 1:100 to 1:200. The slides were incubated with a biotinylated pan-specific secondary antibody for 10 min and reacted with the streptavidin-peroxidase complex for 5 min (Vector Laboratories Universal Quick Kit, Burlingame, Canada). Signals were detected using 3,3-diaminobenzidine tetrahydrochloride substrate chromogen solution. Cells were counterstained with Mayer’s hematoxylin. Subsequently, the cells were imaged using Axioscope A1 (Carl Zeiss).

Statistical analysis

Results are expressed as the mean ± standard deviation (SD). Group differences were evaluated via one-way analysis of variance followed by Dunnett’s multiple comparison tests. Significance was considered at \( p < 0.01 \) or \( p < 0.05 \).

Results

**Macmoon-dongtang contains two anti-asthmatic markers: Glycyrrhizin and liquiritin**

HPLC analyses were performed to identify the anti-asthmatic compounds and immune modulators in *macmoon-dongtang*. Typical HPLC chromatograms and their retention times are shown in Fig 1. Glycyrrhizin (0.38 ± 0.002%), and liquiritin (0.02 ± 0.0002%) were identified using HPLC analysis.

**Macmoon-dongtang suppresses ovalbumin-induced hyperproliferation of inflammatory cells and immune cells**

OVA treatment significantly boosts the levels of inflammatory and immune cells such as white blood cells, eosinophils, and neutrophils in the lung [27]. *Macmoon-dongtang* induced a dose-dependent suppression of inflammatory cells, which were increased by OVA treatment (Fig 2A and S1 File). We used dexamethasone as a positive control during the treatment with macmoon-dongtang as it is generally used to manage acute asthma exacerbation [28]. As shown in...
Fig 2B–2D, the WBC, eosinophil, and neutrophil counts in the dexamethasone treatment group were significantly decreased compared with the OVA-induced asthma group. However, macmoondongtang treatment suppressed their proliferation dose-dependently. Especially, the number of neutrophils in the group treated with 1500 mg/kg macmoondongtang was significantly decreased (Fig 2D) similar to the group treated with dexamethasone.

**Macmoondongtang induces dose-dependent suppression of IgE**

IgE levels usually surge in allergy and increase significantly in asthma [29]. Serum IgE levels in OVA-induced asthma group (60.17 ± 8.135 ng/mL) were significantly increased compared to the levels (13.98 ± 3.877 ng/mL) in the control (Fig 3 and S1 File). Although the serum IgE level in dexamethasone-treated group was higher than in the control, the increased level of IgE induced by OVA treatment was down-regulated to 34.00 ± 8.351 ng/mL (57% compared with OVA treatment) after dexamethasone treatment. Macmoondongtang controlled the IgE level in a dose-dependent manner: 54.91 ± 10.141 ng/mL in the group treated with 150 mg/kg and 44.35 ± 9.618 ng/mL in the group exposed to 1500 mg/kg. There was no significant difference in serum IgE levels between groups treated with dexamethasone and 1500 mg/kg macmoondongtang, which suggested that 1500 mg/kg macmoondongtang was adequate to treat asthma patients.

**Macmoondongtang dose-dependently inhibits typical morphological changes in OVA-induced asthma model**

To evaluate morphological changes in the lung, H&E staining was performed (Fig 4A). The OVA-treated group showed typical morphological changes such as inflammatory cell infiltration (purple color) near bronchioles and vessels, pulmonary hyperplasia (thickening inner bronchiole), and mucous hypersecretion (purple color) in the bronchiole (Fig 4Bb) compared with the control (Fig 4Aa). Dexamethasone controlled OVA-induced morphological changes in the lung by decreasing inflammatory cell infiltration near bronchioles and vessels and inhibiting basal membrane thickening of inner bronchioles and mucous secretion (Fig 4Ac). It Fewer differences in lung morphology were detected between groups treated with OVA (Fig 4Ab) and 150 mg/kg macmoondongtang (Fig 4Ad). However, OVA-induced asthmatic
Macmoondongtang treatment suppressed infiltration of both inflammatory cells and immune cells such as white blood cells (WBCs), eosinophils, and neutrophils. (A) Macmoondongtang effectively decreased OVA-induced proliferation of inflammatory cells. (B) Macmoondongtang dose-dependently down-regulated the population of WBCs. (C) Macmoondongtang effectively suppressed the number of eosinophils. (D) Macmoondongtang significantly and dose-dependently decreased neutrophil proliferation. A, vehicle control; B, asthma induction; C, dexamethasone; D, 150 mg/kg/day macmoondongtang; E, 1500 mg/kg/day macmoondongtang. Each bar represents the mean ± SD (n = 8). *p < 0.05 vs. control; **p < 0.001 vs. control; $p < 0.05$ vs. asthma induction; $^*$p < 0.01 vs. asthma induction; $^**p < 0.05$ vs. dexamethasone. Scale Bar = 100 μm. Magnification, ×400.

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Macmoondongtang treatment effectively suppressed serum IgE level in a dose-dependent manner. Serum IgE level in the group treated with 1500 mg/kg macmoondongtang was compared with that of dexamethasone-treated group. Each bar represents the mean ± SD (n = 16). *p < 0.05 vs. control; **p < 0.001 vs. control; $p < 0.05$ vs. asthma induction; $^*$p < 0.01 vs. asthma induction; $^**p < 0.05$ vs. dexamethasone.

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changes in the lung were reversed by treatment with 1500 mg/kg macmoondongtang (Fig 4Ae), although the recovery was less than that induced by dexamethasone.

In the case of asthma patients, breathing difficulty is usually observed due to factors such as pulmonary base membrane thickening due to epithelial cell hyperplasia and mucous hypersecretion [16]. Mucous hypersecretion in the bronchiole can directly inhibit breathing, and therefore, is a very important parameter for the evaluation of asthma severity. To determine the effect of macmoondongtang on mucous secretion, PAS staining was conducted (Fig 4B). In the control, no mucus was detected in the bronchiole (Fig 4Ba). However, in the OVA-treated group (Fig 4Bb) and the group treated with 150 mg/kg/day macmoondongtang (Fig 4Bd), the
bronchiole was almost obstructed by mucus (purple color). There was little difference in mucus levels between the groups treated with dexamethasone (Fig 4Bc) and 1500 mg/kg macmoondongtang (Fig 4Be). These results indicate that macmoondongtang dose-dependently suppresses mucous secretion.

**Macmoondongtang effectively induces inactivation of both Th1 and Th2 cell transcription factors**

Asthma is caused by the imbalance of Th1 and Th2 cells [2]. T-bet is a Th1 cell transcription factor [30, 31] while GATA-3 is a Th2 cell transcription factor [9]. GATA-3 immunofluorescent assay was conducted to evaluate the changes in the activation levels of Th1 cell transcription factor, T-bet, and Th2 cell transcription factor (Fig 5). The activated transcription factors are translocated from the cytoplasm to nuclei. The distribution of T-bet (green color, Fig 5B) was similar to that of GATA-3 (red color, Fig 5C). OVA treatment activated both Th1 and Th2 transcription factors in the nuclei (Fig 5Db). However, in the other groups, both T-bet and GATA-3 remained localized to the cytoplasm (Fig 5Da, 5Dc & 5Dd). These results indicate that macmoondongtang inhibited the activation of both Th1 and Th2 cell transcription factors.

**Macmoondongtang significantly suppresses both gene and protein expression of IFN-γ and IL-12**

To analyze the levels of Th1-related cytokines such as IFN-γ and IL-12, we conducted RT-PCR (Fig 6A and S1 File) and IHC staining (Fig 6B and 6C). As shown in Fig 6A, macmoondongtang effectively and dose-dependently inhibited IFN-γ gene expression, which was dramatically up-regulated by OVA treatment. IFN-γ gene expression in the group treated with 1500 mg/kg macmoondongtang was similar to that of the group treated with dexamethasone. Similar to changes in the expression of IFN-γ gene, the protein expression induced by OVA treatment was also suppressed by macmoondongtang (Fig 6B). Changes in gene and protein expression of IL12p40 were similar to those of IFN-γ (Fig 6A and 6C).

**Macmoondongtang controls the expression of IL-4 and IL-5, but not IL-13, in a dose-dependent manner**

OVA treatment significantly suppressed the expression of IL-5 and IL-13 genes, but not that of IL-4 gene, although it varied depending on treatment materials such as sterilized tap water (control), dexamethasone, and macmoondongtang (Fig 7A and S1 File). However, macmoondongtang significantly down-regulated the expression of IL-5 gene alone. The gene expression of Th2-related cytokines differed from their protein levels in the respiratory system (Fig 7B–7D). Although the IL-5 gene expression was statistically significant after macmoondongtang treatment, the protein expression of all Th2-related cytokines in the present study such as IL-4, IL-5, and IL-13 was dose-dependently suppressed by macmoondongtang treatment. The levels of IL-4 (Fig 7Be), IL-5 (Fig 7Ce) and IL-13 proteins (Fig 7De) in the group treated with 1500 mg/kg macmoondongtang were significantly decreased compared with those of the dexamethasone-treated group (Fig 7Cc & 7Dc).

**Macmoondongtang modulates the expression of TNF-α, but not IL-6**

OVA treatment increased the levels of only TNF-α gene (Fig 8A and S1 File). Dexamethasone significantly decreased the expression of TNF-α gene while macmoondongtang dose-dependently suppressed its level. Especially, treatment with 1500 mg/kg macmoondongtang suppressed the expression of TNF-α gene similar to that of dexamethasone-treated group.
Macmoondongtang down-regulated TNF-α expression in a dose-dependent manner (Fig 8B). However, it did not suppress the level of IL-6, which was increased by OVA treatment (Fig 8Cc, 8Cd & 8Ce). The TNF-α level was decreased by treatment with 1500 mg/kg macmoondongtang (Fig 8Be), similar to that of dexamethasone treatment (Fig 8Bc).

**Discussion**

Environmental allergens inducing asthma are classified into two types: indoor allergens (for e.g., pet dander, dust mite, tobacco smoke, etc.) and outdoor allergens (for e.g., pollen, environmental pollutants, cold temperature, etc.) [32]. At first, antigen-presenting cells (APCs) recognize repeated invasion by allergens, leading to asthma. Studies have been performed to
Fig 6. Treatment with macmoondongtang significantly down-regulated gene and protein expression of IFN-γ and IL-12p40. Macmoondongtang dose-dependently controlled both IFN-γ gene expression quantified by RT-PCR (A) and IFN-γ protein levels assessed by IHC (B). Macmoondongtang also regulated IL-12 gene expression and up-regulated gene and protein expression of IL-12p40 in the lung (C). a, vehicle control; b, asthma induction; c, dexamethasone; d, 150 mg/kg/day macmoondongtang; e, 1500 mg/kg/day macmoondongtang. Each bar represents the mean ± SD (n = 8). "p < 0.05 vs. control; ""p < 0.001 vs. control; "p < 0.05 vs. asthma induction; "#p < 0.05 vs. dexamethasone. Scale Bar = 50 μm. Magnification, ×200.

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Fig 7. Macmoondongtang dose-dependently suppressed Th2-related cytokines such as IL-4, IL-5, and IL-13. Macmoondongtang significantly and effectively inhibited the gene expression of IL-5 (A) and IL-5 protein level (C). The treatment dose-dependently controlled the IL-4 protein expression (B). Similar changes at the gene (A) and protein levels of IL-13 (D) were observed as those for IL-5. a, vehicle control; b, asthma induction; c, dexamethasone; d, 150 mg/kg/day macmoondongtang; e, 1500 mg/kg/day macmoondongtang. Each bar represents the mean ± SD (n = 8). "p < 0.05 vs. control; ""p < 0.001 vs. control; "p < 0.05 vs. asthma induction; "#p < 0.05 vs. dexamethasone. Scale Bar = 50 μm. Magnification, ×200.

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verify the relation between type 1 allergy and IgE, an important and representative biomarker [29, 33]. Accordingly, IgE increase is a very important factor that triggers asthma, suggesting the need to decrease IgE expression induced by OVA exposure. Immune response is critical to maintain the homeostasis of Th1 and Th2 cells in living organisms. The hypothesis of Th1/Th2 balance for immune system was introduced in the 1980s [34]. Asthma is one of the diseases triggered by Th1/Th2 imbalance and according to the theory [35], the balance between Th1 cells and Th2 cells is generally maintained. However, an up-regulation of Th2 cells including related cytokines may trigger asthma. IFN-γ and IL-12 are Th1 cytokines, whereas IL-4, IL-5, and IL-13 are Th2 cytokines, and IL-6 and TNF-α are Th17-related cytokines [10].

T-bet is a factor associated with Th1 cell transcription. T-bet and IFN-γ show a positive feed-back [30, 36]. IL-12p40 is one of the key factors regulating Th1-related cytokines in that it specifically stimulates IFN-γ production in asthma [5]. As shown in Fig 6, changes in IFN-γ gene and protein levels were very similar to those of IL-12p40 while Macmoondongtang effectively inhibited their increases.

In asthma patients, the Th2-related cytokines such as IL-4, IL-5, and IL-13 are usually increased [37–39]. GATA-3 is a Th2 cell transcription factor. IL-4 increases its activation. Consequently, the regulation of GATA-3 and IL-4 produces Th2-related cytokines [9]. Macmoondongtang not only significantly inhibited the gene (A) and protein expression (B) of TNF-α but not that of IL-6 expression (C). a, vehicle control; b, asthma induction; c, dexamethasone; d, 150 mg/kg/day macmoondongtang; e, 1500 mg/kg/day macmoondongtang. Each bar represents the mean ± SD (n = 8). *p < 0.05 vs. control; †p < 0.05 vs. asthma induction. Scale Bar = 50 μm. Magnification, ×200.

Fig 8. Macmoondongtang treatment significantly inhibited TNF-α gene and protein expression, but not that of IL-6. Macmoondongtang dose-dependently suppressed the gene (A) and protein expression (B) of TNF-α but not that of IL-6 expression (C). a, vehicle control; b, asthma induction; c, dexamethasone; d, 150 mg/kg/day macmoondongtang; e, 1500 mg/kg/day macmoondongtang. Each bar represents the mean ± SD (n = 8). *p < 0.05 vs. control; †p < 0.05 vs. asthma induction. Scale Bar = 50 μm. Magnification, ×200.

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the levels of all Th2-related cytokines investigated such as IL-4, IL-5, and IL-13. It specifically suppressed the expression of IL-4 and IL-5 (Fig 7A–7C). These results explain the anti-asthmatic effect of macmoondongtang mediated via suppression of serum IgE level (Fig 3) to reverse the typical morphological changes associated with asthma such as epithelial hyperplasia, mucous hypersecretion, and airway obstruction (Fig 4).

Th17-related cytokines such as TNF-α, IL-6, and IL-1β are important in the control of asthma onset [41]. TNF-α is one of the chemo-attractants for neutrophils and eosinophils [18]. It induces death of eosinophils via oxidative stress in pulmonary epithelial cells [42]. Although eosinophil numbers are increases in many asthma patients, other patients show a surge in neutrophil levels [43]. In the present study, macmoondongtang effectively suppressed the proliferation of eosinophils and neutrophils in BALF and inhibited eosinophil infiltration near bronchioles and alveoli, and vessels via TNF-α modulation (Fig 2). The expression of IL-6 in asthma patients is significantly increased [44]. The IL-6 induced by allergens stimulates the release of IL-4 and IL-13 from Th2 cells [12]. IL-6 may be one of the key factors maintaining the balance of helper T cells to control Treg cells or Th17 cells [44].

Several reports suggest the anti-asthmatic effects of herbal extracts [45–47] or compounds derived from natural products [48]. However, the bioactivity of may be associated with specific compounds derived from herbs or mushrooms. The anti-asthmatic effect was attributed to bioactive ingredient(s) in the natural product. Macmoondongtang used in this study was obtained as an extract from several herbs such as Glycyrrhizae radix, Oryza sativa, Zizyphus jujube, Ophiopogon japonicas, Pinellia ternate, and Panax ginseng. A review of literature suggests that macmoondongtang contains anti-asthmatic markers such as glycyrrhizin and anti-inflammatory markers such as liquiritin. To determine the bioactive compounds in macmoondongtang, HPLC analysis was conducted. Results showed that glycyrrhizin (0.38%) and liquiritin (0.02%) were present in the macmoondongtang extract. Glycyrrhizin is one of the major compounds in Glycyrrhiza uralensis and Glycyrrhiza glabra. Ram et al. (2006) reported that glycyrrhizin exhibits an immunomodulatory effect in a mouse model of asthma [49]. Oral administration of glycyrrhizin (2.5 to 20 mg/kg) to OVA-induced mice resulted in a suppression of IL-4, IL-5, IFN-γ, and IgE levels. In the present study, we identified liquiritin content in macmoondongtang extract. Liquiritin modulates inflammation via inhibition of pro-inflammatory mediators such as inducible nitric oxide synthase, cyclooxygenase (COX)-2, TNF-α, IL-1β, and IL-6 [50, 51]. Thus, liquiritin occurring in macmoondongtang may alleviate the inflammatory symptoms caused by asthma. Although G. uralensis contains glycyrrhizin and G. glabra has liquiritin, each herb exhibits a typical anti-asthmatic effect.

Ram et al. (2006) have reported that an oral dose starting with 5 mg/kg of liquiritin was effective in mice [49]. In the present study, we found that macmoondongtang resulted in an anti-asthmatic effect at a dose of 1500 mg/kg in our animal model. The dose of 1500 mg/kg corresponds to 5.7 mg/kg of glycyrrhizin. Thus, the curative dose in the present study was consistent with that of the previous report, suggesting that glycyrrhizin contained in macmoondongtang is a possible therapeutic candidate for the management of asthma. Furthermore, we evaluated a daily oral dose of 7.3 g macmoondongtang for treatment of asthma. The therapeutically effective oral dose of macmoondongtang in mouse was 1500 mg/kg/day. The conversion factor between human and mouse is 12.3 [21]. Therefore, if the effective dose of macmoondongtang in mice is 1500 mg/kg/day, the human equivalent dose is 7300 mg/60kg/day. The recommended daily intake of macmoondongtang for human is 9 g. Thus, the optimal dose for asthma treatment is less than its daily intake and then we could confirm that oral intake of macmoondongtang at a dose of 7.3 g might prevent the occurrence of asthma.
From the results we concluded that Macmoondongtang treatment alleviates asthma symptoms and modulate the Th1-/Th2-related cytokines. Glycyrrhizin and liquiritin could be the major the active therapeutic components.

**Supporting information**

S1 File. This file is raw data for making graphs in Fig 2, Fig 3, Fig 6, Fig 7 and Fig 8. (XLSX)

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