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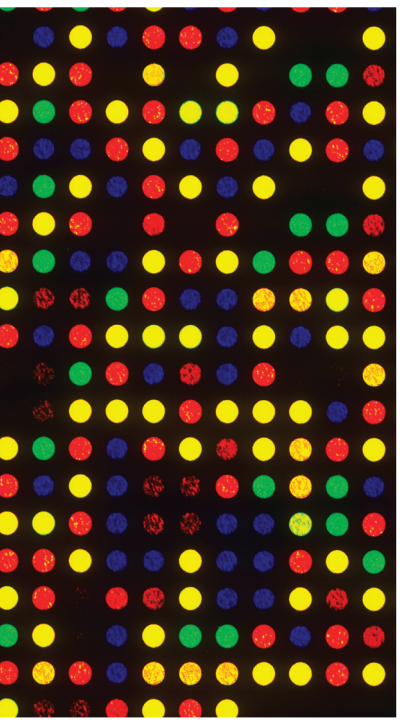
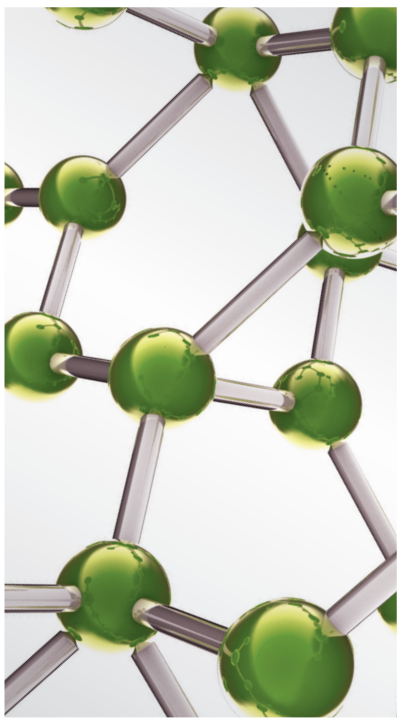
Traditional Herbal Medicine and Allergic Asthma

Guest Editors: Bi-Fong Lin, Bor-Luen Chiang, Yan Ma, Jin-Yuarn Lin, and Miaw-Ling Chen



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Editorial Board

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Editorial

Traditional Herbal Medicine and Allergic Asthma

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The frequency of allergic diseases such as asthma and allergic rhinitis has increased rapidly during the past decade; however, the exact mechanisms have still not been established. Both air pollution and change of diet habit have been thought to play an important role in increasing prevalence of atopic diseases. Atopic diseases were mediated predominantly by type 2 T helper- (Th2-) mediated activity including allergen-specific IgE antibody and eosinophils. Allergic asthma is a chronic disease with the characteristics of immune response mediated by type 2 T helper- (Th2-) related cytokines and IgE antibody. Allergic airway inflammation has been characterized by the infiltration of Th2 cells and eosinophils, subsequently followed by the bronchial constriction and mucus secretion.

Conventional treatments for allergic asthma include steroids, leukotriene antagonists, bronchodilators, and most recent anti-IgE antibody. All these drugs are still with certain shortcomings such as side effects, effectiveness, and cost. It has become more and more important to develop novel therapeutic approaches for the treatment of allergic asthma. Complementary medical approaches such Chinese herb medication and acupuncture have been suggested to play a role in the immune regulation of diseases [1]. More studies have focused on exploring the possibility of these complementary therapeutic approaches for the treatment of immunological diseases [2]. All these complimentary therapeutic approaches have been regarded as having less side effects and being used as the adjuvant therapy for the diseases [3]. Furthermore, many researchers also aim to identify the active components of herb medicine for the purification and development of drugs.

In this special issue, we have five articles including the survey of traditional Chinese medicine application for the treatment of allergic asthma and also the study on the traditional Tianjiu therapy for the treatment of asthmatic patients. Furthermore, three articles on studying the active components of herb medicine have been included in the issue. S.-I. Lin et al. analyzed the use of Chinese traditional medicine for the treatment of allergic asthma in Taiwan. They collected 20,800 newly diagnosed asthmatic children and found out 20% of them actually used traditional Chinese medicine as the treatment of their asthma. In addition, they also analyzed the most frequent used herbal medicine for allergic asthma in the article.

A variety of components purified from medicinal herbs have been found to exert the immune modulatory effect on many diseases [4]. Among the components, polyphenols, triterpenoids, and polysaccharides are found to be the most effective in the anti-inflammatory or immune modulation of the diseases. M.-L. Chen et al. have identified both triterpenoids and polysaccharide portion for the treatment of allergic diseases. The results showed that triterpenoids portion of *Ganoderma tsugae* exerted anti-inflammatory activity and polysaccharide portion had the immune stimulatory effect instead. C.-M. Ku and J.-Y. Lin also identified an active component of farnesol, a sesquiterpene alcohol,

exerting anti-inflammatory activity and alleviating airway inflammation in murine model of asthma. M.-L. Chen et al. also identified ethanol extract of *Perilla frutescens* which also exerted anti-inflammatory activity and also alleviated allergic airway inflammation. Finally, L. Zhu et al. studied the effect of Tianjiu therapy in Sanfu Days for the treatment of asthmatic children. The results suggested that Tianjiu therapy could decrease the dose of bronchodilator during the asthma attack. However, the symptoms of allergic asthma did not show significant improvement after treatment. More studies are needed for the application of Tianjiu therapy for the treatment of allergic asthma in the future.

The prevalence of clinical immunological diseases such as autoimmune diseases and allergic diseases is increasing in recent years. More and more efforts have been dedicated to develop novel therapeutic approaches for the treatment of these immune-mediated diseases. Complimentary medicine such as herb drugs or acupuncture has been suggested to be useful for the treatment of a variety of clinical immunological diseases [5]. Many of these herb medicinal components have been found to exert anti-inflammatory activity and modulation of immune response. Although more studies are needed to identify the novel compounds and the treatment, it might still be the future target for the novel therapeutic development. Particularly, these herb medicinal drugs are noted with fewer side effects, which might also be useful for possible adjuvant therapy.

Bi-Fong Lin
Bor-Luen Chiang
Yan Ma
Jin-Yuarn Lin
Miaw-Ling Chen

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Research Article

Ethanol Extract of *Perilla frutescens* Suppresses Allergen-Specific Th2 Responses and Alleviates Airway Inflammation and Hyperreactivity in Ovalbumin-Sensitized Murine Model of Asthma

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This study was to investigate the effects of different fractions of *Perilla frutescens* (Pf) leaves extracted by water or ethanol on asthma. BALB/c mice sensitized intraperitoneally and challenged with ovalbumin (OVA) were divided into six groups. Each group of mice was tube-feeding with 0 (control), 80 μg (PfWL), or 320 μg (PfWH) water extracts or 80 μg (PfEL) or 320 μg (PfEH) ethanol extracts of perilla leaves daily for 3 weeks. A negative control group (PBS) was neither sensitized nor treated with Pf. The effects of perilla leave extracts on allergic immune response were evaluated. The results showed that OVA-specific IL-5 and IL-13 secretions from OVA-stimulated splenocytes were significantly suppressed in the ethanol extract groups PfEL and PfEH. Serum level of anti-OVA IgE tended to be lower in the PfEH group. The inflammatory mediators, such as eotaxin and histamine, and total cells, particularly eosinophils in bronchoalveolar lavage fluid (BALF), were also decreased in the PfEL and the PfEH groups. Therefore, the PfEL and the PfEH groups had significantly lower methacholine-induced hyperresponsiveness (AHR). In conclusion, ethanol extracts, rather than water extract, of perilla leaves could significantly suppress Th2 responses and airway inflammation in allergic murine model of asthma.

1. Introduction

Allergic asthma is a chronic disease that clinically augments bronchial hyperresponsiveness and inflammation. The asthmatic inflammation is clearly associated with the high level of type 2 T cell (Th2) cytokines that induced immunoglobulin (Ig) E production and eosinophilic infiltration [1]. The Th2 cytokines IL-4, IL-5, and IL-13 are the major cytokines for development of atopic diseases, such as asthma, rhinitis, and dermatitis [2]. IL-4 promotes the immunoglobulin class switch from IgM to IgE [3]. IL-5 induces eosinophilia activity and infiltration, which is the critical response in the allergic asthma [4]. IL-13 acts to induce airway hyperresponsiveness (AHR) that contributes to atopic disease [5]. The suppression of Th2 responses is a feasible attempt to attenuate the symptoms of allergic asthma. Therefore, biologic targeted therapies have been developed to target the specific molecular pathways to treat asthma, especially those targets at IL-4, IL-5, IL-13, and IgE [6]. However, due to complex clinical symptoms and multiple mechanisms involved, the outcome of these trials has not been satisfied. Epidemiological studies indicate that patients often turn to complementary and alternative therapies, including dietary supplements [7]. Studies also showed that dietary oil, adlay, and medicinal herbs, such as Ganoderma and Andrographis, decreased Th2 cytokines productions and thus alleviated allergic responses in a murine model of asthma [8–12], suggesting the potential application of traditional herbal medicine for immunomodulation.

Perilla frutescens (Pf) leaf is a kind of aromatic vegetables, which is also used as the traditional medicine in Asia. Perilla has been demonstrated to exert antiobesity, anti-dyslipidemia, antioxidant, and anti-infammation [13–19]. Recently, some researches focused on its antiallergic effects [20–23]. The studies suggested water extract of Pf inhibited histamine release from mast cells [24]. Subcutaneous injection with perilla seed water/ethanol extract, as a traditional oriental therapeutic herbal acupuncture, seemed to reduce IgE, IL-4, IL-5, and IL-13 in BALF in OVA-induced asthma in mice [18].

However, they are either intraperitoneal injection [24], type II allergy evaluated by ear-passive cutaneous anaphylaxis or edema [20, 21], or lack of the important outcome of treatment in asthma, airway hyperresponsiveness (AHR) result [22]. There are few studies investigating the effects of oral supplementation of perilla extracts on overall allergic responses of asthmatic murine model. In addition, the crude polysaccharide isolated from Pf by hot water extraction was shown to increase nitric oxide and TNFα and IL-6 production both *in vitro* and *in vivo* [25]. Therefore, whether water or ethanol extracts of *Perilla frutescens* exert antiasthma effects *via* oral supplementation was investigated in this study using OVA-sensitized and challenged BALB/c mice.

2. Materials and Methods

2.1. Preparation of Extracts of Perilla frutescens. The crude extracts of *Perilla frutescens* (L.) Britt leaves were extracted as follows. Dried *Perilla frutescens* leaves were extracted with 5-fold (w/v) hot water for 60 min. The water extracts (PfW) were filtered through filter paper and concentrated using a rotary evaporator and then freeze-dried. The yield of PfW was 21.70% (w/w, dry basis). The ethanol extracts of dried *Perilla frutescens* leaves (PfE) were extracted with 15-fold (w/v) 95% ethanol for 24 hr. The PfE were filtered by filter paper and then evaporated in a rotary evaporator to remove the solvent. The yield of PfE was 9.22% (w/w, dry basis).

2.2. Animal Study, Sensitization, and Airway Challenge. Female BALB/c mice were purchased from the Animal Center at the National Taiwan University College of Medicine, Taipei, Taiwan, and maintained at the Department of Biochemical Science and Technology at the National Taiwan University. Animal care and handling conformed to accepted guideline [26].

The allergic asthma model, as well as the diet, was manipulated as described [11]. As shown in Figure 1, 8-week-old female BALB/c mice were sensitized intraperitoneally (i.p.) with 10 μg ovalbumin (OVA; Sigma, St. Louis, Mo) in alum adjuvant (Imject Alum; Pierce, Rockford, IL). After three times OVA-sensitization, sensitized mice were grouped randomly into six groups for tube-feeding with 0 μg (Control), 80 μg (PfWL), or 320 μg (PfWH) water extract or 80 μg (PfEL) or 320 μg (PfEH) ethanol extract of perilla leaves daily for 3 weeks. During these 3 weeks, the OVA-sensitized mice were challenged twice with 5% aerosolized OVA in PBS buffer by inhalation. In addition, mice without Pf supplement, that received alum without OVA, and that inhaled aerosolized PBS are used as the negative control group (PBS). After 3 weeks of Pf supplement, mice that received a third challenge were euthanized by CO_2 inhalation and the BALF, serum, and splenocytes of mice were collected for further analysis.

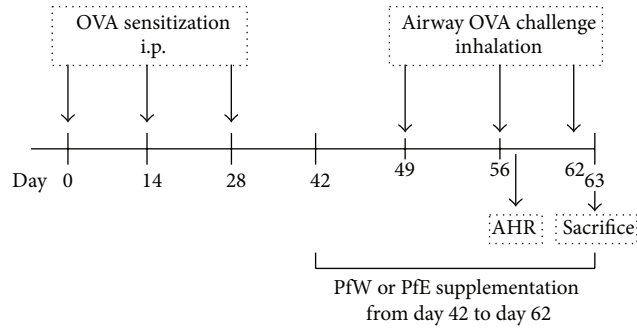

Figure 1: Schematic diagram of the experimental protocol. Eight-week-old female BALB/c mice were sensitized intraperitoneally (i.p.) by OVA for three times (10, 30, and 30 μg/mouse) on days 0, 14, and 28. OVA-sensitized mice were challenged with 5% aerosolized OVA three times on days 49, 56, and 62. After 3 weeks of supplementation, mice were sacrificed and the BALF, serum, and splenocytes of mice were collected for further analysis.

2.3. Determination of Airway Hyperresponsiveness (AHR). Airway function was measured by the whole-body plethysmography as described previously [11]. Twenty-four hours after the secondary challenge, AHR was measured when mice were stimulated with methacholine (Sigma) using whole-body plethysmography (Buxco, Wilmington, NC). Mice were placed in the main chamber of a whole-body plethysmography and challenged with aerosolized methacholine, increasing concentration from 12.5 to 50 mg/mL in PBS buffer. The AHR is expressed as the enhanced pause (Penh) values measured during each 3 min period.

2.4. Determination of Anti-OVA Antibodies. Serum anti-OVA IgE antibody titers were measured by ELISA method as previously described [27]. Briefly, 96-well plates were coated with 10 μg OVA/mL $NaHCO_3$ buffer. After overnight incubation at 4°C and being blocked with 1% bovine serum albumin (BSA)/PBS buffer for 2 hours at room temperature, the serum samples and positive control sera were appropriately diluted with blocking buffer and added to the 96-well plate. After 2 hours, the biotin-conjugated anti-mouse IgE (PharMingen, San Diego, CA) was added for 2 hours of incubation. Then, streptavidin-conjugated peroxidases and the enzyme substrate, 2,2′-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (ABTS), were added and incubated for 20 min at room temperature. The antibody levels of the samples were compared with the positive control sera. The positive control was a pool of serum collected from OVA-sensitized mice with strong response (optical density > 1). The results of the antibody titer were expressed in ELISA units (EU), EU = $(A_{\text{sample}} - A_{\text{blank}})/(A_{\text{positive}} - A_{\text{blank}})$.

2.5. Collection of BALF and Splenocytes. The BALF was collected with 5 instillations of 0.5 mL saline. Approximately 2.5 mL of fluid was recovered with each sample and the

volume did not differ significantly among groups. The fluid was collected and kept at −80°C for eotaxin and histamine analysis. The cell pellet was resuspended in 250 μL saline containing 10% BSA. The total cells were counted with a hemocytometer using the trypan blue dye exclusion method. BALF cells with a concentration of 2×10^5 were cytocentrifuged and then stained with Liu's stain for eosinophil counts.

The splenocytes were prepared by aseptically removing spleens from sensitized and challenged BALB/c mice [27]. Splenocytes were counted with a hemocytometer using the trypan blue dye exclusion method. A concentration of 5×10^6 cells/mL was cultured in 48-well plates in RPMI-1640 medium supplemented with TCM medium in the absence or presence of antigen, 50 and 100 μg/mL OVA, for 48 hours. The supernatants in cell cultures were collected and stored at −80°C for cytokines analysis.

2.6. Cytokines Assay. The cytokine levels in splenocytes culture supernatants were measured by sandwich ELISA methods. Briefly, the anti-cytokine antibody was coated in the 96-well plates (Nunc, Roskilde, Denmark). After overnight incubation at 4°C and being blocked with 1% BSA/PBS buffer for 30 min, the samples and standards were added to the 96-well plates for 2 hours of incubation. The biotin-conjugated antibodies were added and incubated. After washing, the streptavidin-conjugated peroxidase was added for 1 hour. The substrate ABTS was added to each well for 20 min. The plates were read in a microplate autoreader (microplate autoreader; Bio-Tek Instrument, Inc. Winooski, VT) at 405 nm.

2.7. Eotaxin and Histamine Assay. The eotaxin concentration in BALF was determined by mouse eotaxin sandwich ELISA kit (R&D Systems, Minneapolis, MN). The eotaxin concentration was assayed according to the manufacturer's instructions. After the color reagent was added, the plate was incubated at room temperature for 20 min for color development. The absorbance was measured at 630 nm in the microplate autoreader. The eotaxin level in BALF was determined using the standard curve.

The histamine level was determined by Histamine-ELISA kit (IBL Hamburg, Germany). The manipulation was according to manufacturer's instructions for use. In brief, the culture supernatants and plasma standards were acylated with acylation reagent first. Then, aliquots of 50 μL acylated sample and acylated standards were loaded into the 96-microplate wells, respectively. Aliquots of 50 μL enzyme conjugate and 50 μL antiserum were pipetted into each well to react for 3 hours. The plate was washed four times and 200 μL tetramethylbenzidine (TMB) substrate solution was added for 30 min. Then the reaction was stopped by adding 100 μL of stop solution into each well. The optical density was detected by the microplate autoreader at 450 nm. The histamine concentration is determined using the standard curve.

2.8. Statistical Analysis. Data were expressed as mean ± SD. The significance of difference between the Pf and the control groups was analyzed statistically by Student's t-test of the SAS program system (Strategic Application Software; SAS windows version 8.2, SAS Institute Inc., Cary, NC) throughout the study.

3. Results

3.1. Ethanol Extracts of Perilla frutescens Suppressed Th2 Responses of OVA-Sensitized Mice. After feeding of two doses of water extract of Pf (PfWL and PfWH) or ethanol extract of Pf (PfEL and PfEH) for 3 weeks, splenocytes were isolated from OVA-sensitized mice and stimulated with OVA to examine the effects of PfW and PfE on allergen-specific Th2 responses. The Th1 cytokines IL-2 and IFNγ were not significantly affected (data not shown). However, Th2 cytokines IL-5 and IL-13 productions of OVA-stimulated splenocytes in the PfEL and the PfEH groups were significantly lower than those of the control group, as shown in Figure 2. The OVA-stimulated IL-4 productions were low and not affected by either water or ethanol extracts. In addition, PHA-stimulated IL-13 productions by splenocytes were also suppressed by PfE (data not shown). These data suggested that ethanol extracts of Pf could inhibit allergen-specific stimulated Th2 cells activity.

The IgE antibody produced by Th2 cell-activated B cells tended to be lower in serum of OVA-sensitized mice supplemented with PfEH (Figure 3). The serum levels OVA-specific IgG_1 and IgG_{2a} were not significantly affected (data not shown). This data suggested that ethanol extracts of *Perilla frutescens* might have potential to suppress serum IgE levels in OVA-sensitized mice.

3.2. Ethanol Extracts of Perilla frutescens Decrease Cells Infiltration in Airway Allergic Inflammation of OVA-Sensitized Mice. The BALF was collected after OVA-inhalation challenge prior to sacrifice and the cell number in BALF was determined and shown in Figure 4. Few total cells and eosinophils were in the PBS negative control group. The infiltrated cells number increased after OVA-challenge as shown in the control group. The total cell number in BALF significantly decreased in asthmatic mice supplemented with PfEH (Figure 4(a)). Particularly, the number of eosinophils, the major cells contributing to airway inflammation of allergic asthma, was decreased by Pf supplement, significantly in both the PfEL and the PfEH groups (Figure 4(b)). These data suggested that *Perilla frutescens* inhibited the infiltration of inflammatory cells to BALF in asthmatic mice, especially the ethanol extracts.

The proinflammatory mediators such as histamine and eotaxin in BALF of OVA-sensitized mice were also suppressed by Pf, as shown in Figure 5. The histamine levels were significantly lower in both the PfEL and the PfEH groups. The eosinophil chemotactic protein eotaxin in BALF was also reduced in the PfEH group. Th2 cytokines in BALF of OVA-sensitized mice were also measured but were not found significantly lower by Pf in this experiment (data not shown). These results indicated that ethanol extracts of *Perilla frutescens* alleviated inflammatory cell infiltration and thus reduced the allergic inflammation in airway of OVA-sensitized and challenged mice.

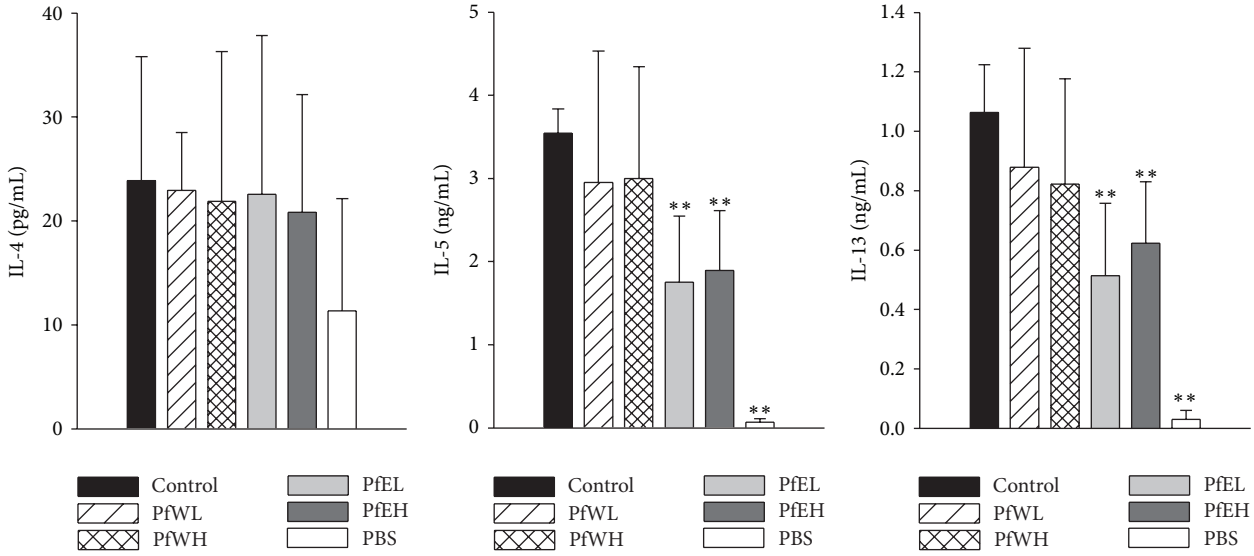

Figure 2: The Th2 cytokines produced by OVA-stimulated splenocytes from OVA-sensitized/challenged BALB/c mice supplemented with different extracts of *Perilla frutescens*. OVA-sensitized mice were fed with water (PfWL or PfWH) or ethanol (PfEL or PfEH) extracts of *Perilla frutescens* for 3 weeks, respectively. Splenocytes were isolated from OVA-sensitized/challenged mice and stimulated with OVA (100 μg/mL for IL-4, 50 μg/mL for IL-5 and IL-13) for 48 hours. The productions of Th2 cytokines in supernatant were determined by ELISA. Values represent mean ± SD, $n = 7$~8 for each OVA-sensitized group, and $n = 6$ for the PBS group as negative control. Statistical analysis was performed with Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

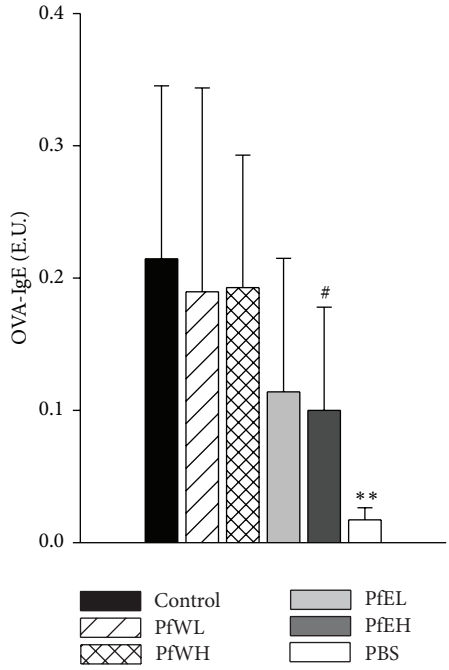

Figure 3: Serum levels of OVA-specific IgE in OVA-sensitized BALB/c mice supplemented with different extracts of *Perilla frutescens*. OVA-sensitized mice were fed with water or ethanol extracts of *Perilla frutescens* for 3 weeks, respectively. Sera of mice were collected and the OVA-IgE levels were determined by ELISA. Values represent mean ± SD, $n = 7$~8 for each OVA-sensitized group, and $n = 5$ for the PBS group as negative control. Statistical analysis was performed with Student's t-test, $^{**}P < 0.01$, $^{\#}0.05 < P < 0.1$ compared with the control group.

3.3. Ethanol Extracts of Perilla frutescens Decrease Airway Hyperresponsiveness (AHR) of OVA-Sensitized Mice. OVA-sensitized mice supplemented with two doses of water or ethanol extracts, respectively, were challenged with 50 mg/mL aerosolized OVA to further induce AHR. The results shown in Figure 6 demonstrated that the control group had significantly higher AHR, measured as Penh value, than the PBS negative control group after methacholine challenge. Ethanol extract of Pf significantly reduced AHR as significantly lower Penh values were detected in both the PfEL and the PfEH groups. Water extract of Pf tended to have low AHR ($P < 0.1$) at high dose (PfWH group) though the low dose PfWL group did not reach statistical significance. This data indicates that *Perilla frutescens* extracts possess inhibitory effects of airway hyperresponsiveness, possibly due to lower cell infiltration and inflammation in bronchiole and lung of allergic asthma.

4. Discussion

Allergic asthma is a chronic inflammatory disease in airway, which was induced by Th2-prone responses. The present allergic asthma murine model demonstrated that *Perilla frutescens* extracts, especially the ethanol extracts, decrease Th2 cytokines production, serum IgE level, cells infiltration, allergic mediator secretions, and AHR.

Early study examined the effects of different extracts of Pf on TNFα levels in inflammatory mice and found that water extracts had stronger inhibition than n-hexane or ethyl acetate extracts [28]. Water extract of Pf inhibited the histamine released from rat peritoneal mast cells *in vitro* [29]. Glycoprotein derived from the hot water extract of Pf demonstrated the effective component of *Perilla frutescens*

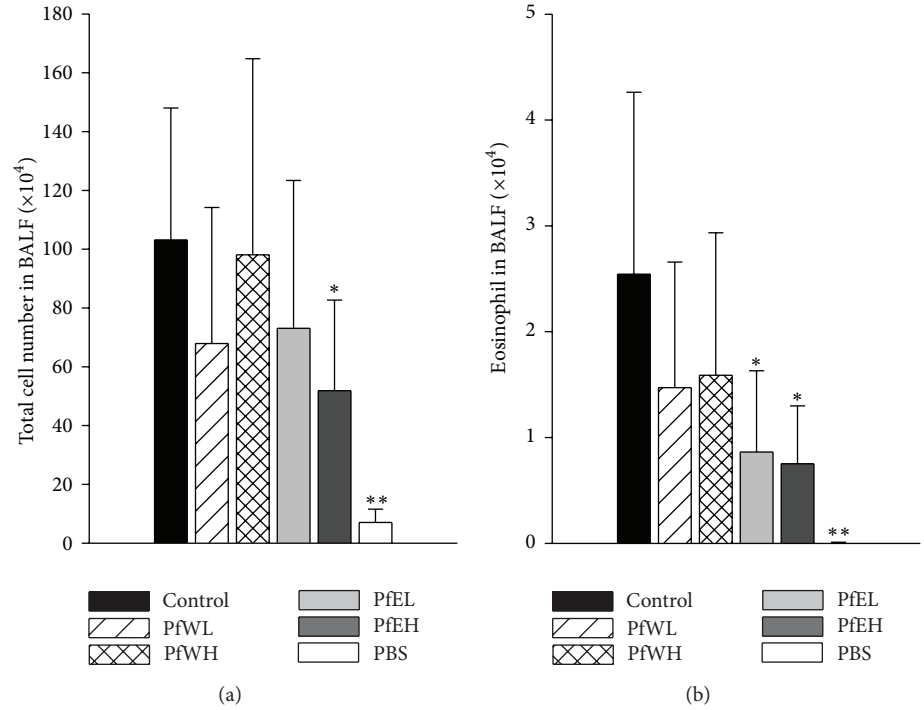

Figure 4: Total cells and eosinophils number in BALF of OVA-sensitized/challenged BALB/c mice supplemented with different extracts of *Perilla frutescens*. OVA-sensitized mice were fed with water or ethanol extracts of *Perilla frutescens* for 3 weeks, respectively, and BALF was collected after aerosolized OVA (50 mg/mL) inhalation challenge. Total cells (a) and eosinophils number (b) were determined. Values represent mean ± SD, n = 7~8 for each OVA-sensitized group, and n = 5 for the PBS group as negative control. Statistical analysis was performed with Student's t-test, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

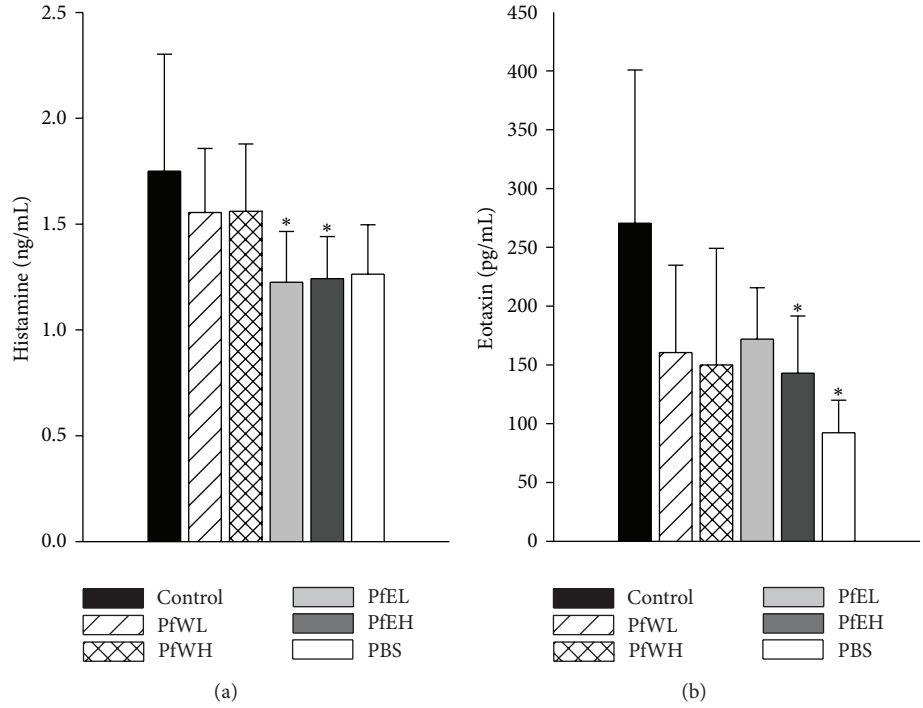

Figure 5: Histamine and eotaxin contents in BALF of OVA-sensitized/challenged BALB/c mice supplemented with the different extracts of *Perilla frutescens*. OVA-sensitized mice were fed with water or ethanol extracts of *Perilla frutescens* for 3 weeks, respectively, and BALF was collected after aerosolized OVA (50 mg/mL) inhalation challenge. The concentrations of histamine (a) and eotaxin (b) in BALF were determined by ELISA. Values represent mean ± SD, n = 7~8 for each OVA-sensitized group, and n = 5 for the PBS group as negative control. Statistical analysis was performed with Student's t-test, $^{*}P < 0.05$ compared with the control group.

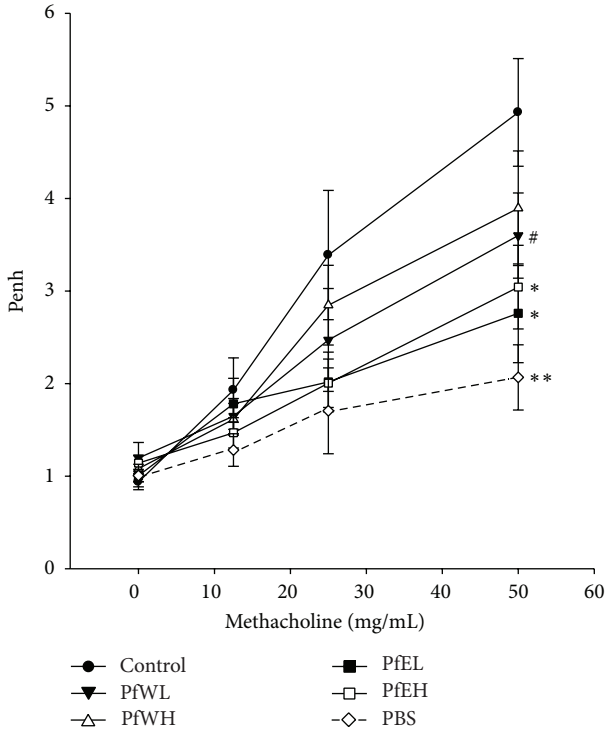

Figure 6: The AHR of OVA-sensitized/challenged BALB/c mice supplemented with different extracts of *Perilla frutescens*. OVA-sensitized mice were fed with water or ethanol extracts of *Perilla frutescens* for 3 weeks, respectively, and AHR was determined after aerosolized OVA (50 mg/mL) inhalation challenge. Values represent mean ± SEM, $n = 7\sim8$ for each OVA-sensitized group, and $n = 5$ for the PBS group as negative control. Statistical analysis was performed with Student's t-test, $^{\#}0.05 < P < 0.1$, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control group.

Britton to inhibit mast cells degranulation [30]. These studies suggested the potential antiallergic effect of Pf. Further, our study showed that the ethanol extract of Pf had more effective inhibition than water extract on the histamine release in BALF of OVA-challenged mice. We suggested that this inhibition might be related to the suppression of Th2 activities.

It has been known that IL-4 plays a critical role in IgE production. IL-4 receptor was significantly associated with asthma risk [31, 32]. Allergen-stimulated IL-4 secretions by splenocytes from sensitized mice were low in this study and not suppressed by Pf, which might explain the reason why sera IgE levels were not significantly lowered by Pf. The tendency of lower serum IgE in the PfEH group might be partially due to lower Th2 activities such as allergen-stimulated IL-5 and IL-13 productions. IL-13 polymorphisms were consistently associated with asthma and serum IgE in asthma populations [33]. Our study showed that the ethanol extracts of Pf significantly decreased IL-5 and IL-13 productions from splenocytes of OVA-sensitized mice. Blocking the binding of IgE to its receptors is the most effective therapy strategy for allergic disease [34]. The association between IL-13 and IgE productions in asthma [35, 36] suggests that ethanol extracts of Pf suppressed IL-13 secretion and thus tended to reduce IgE production [37].

IL-13 induced the eotaxin release by airway epithelial cells *in vitro* [38]. Our data also indicated that ethanol extracts of Pf decreased eotaxin production in BALF, consistent with its lower allergen-induced IL-13 secretion. The inflammatory cells, such as eosinophils, recruited into airway are the major clinical manifestations in allergic asthma. IL-5 is best characterized for eosinophilia that dominates airway inflammation on allergic asthma [39]. When asthmatic patients were given anti-IL-5 (mepolizumab), bronchial mucosal eosinophils decreased [40]. Furthermore, eosinophils not only act as terminal effector cells but also act to actively amplify allergic responses by promoting Th2 cell immunity [41], indicating the close relationship between eosinophils and airway hyperresponsiveness [42]. Th2-cell-derived cytokine IL-5, together with eotaxin, plays the critical roles in the induction of airway hyperreactivity and the development of chronic airway wall remodeling [43]. Present report indicated that high dose of ethanol extracts of Pf inhibited eosinophils and eotaxin levels in BALF and also decreased AHR. These data suggested that ethanol extracts of Pf exert attenuate airway hyperresponsiveness and inflammation in allergic asthma through inhibition of eosinophils and proallergic mediators.

However, the percentage of eosinophil in total cell in BALF was low in this study. It may be due to the OVA-challenge protocol. Continuous daily inhalation or intranasally challenge with allergen may drive more eosinophils in BALF. Our previous studies showed ~10% eosinophils in BALF of mice when challenged with OVA inhalation every three days [11], but only ~3% eosinophils were counted with challenge every seven days. The majority of cell populations in this study were neutrophils/basophils, which were 54% in the control group and 35–45% in the Pf groups.

Study showed that water extracts of perilla leaves improve atopic dermatitis [20] and identified the active constituent to be luteolin [44]. Rosmarinic acid extracts in Pf strongly inhibited hexosaminidase release from RBL-2H3 mast cells [45] and were shown to decrease the neutrophils and eosinophils recruitment in nasal lavage fluid of seasonal allergic rhinoconjunctivitis patients [46]. Recent report revealed that 80% ethanol extracts of purple perilla leaves contains 8.47% (w/w) of rosmarinic acid which is the major phenolic acid in perilla and commonly found in aromatic plants [47]. Our data suggested that the water extracts of Pf attenuated AHR in OVA-sensitized mice, but they are less effective than ethanol extract. It may be due to the same doses of different extracts used in our study. Recent report indicated that ethanol extracts of Pf significantly decreased TNFα production in BALF from LPS-induced airway inflammation and suggested that phenylpropanoids may contribute to the inhibitory activity of ethanol extracts of Pf on the lung inflammatory response [48]. As *Perilla frutescent* leaves are aromatic vegetables and can be consumed raw, cooked, or pickled [49], their application is feasible and expectable. In our study, the most effective dose for decreasing Th2 responses and AHR is 80~320 mg PfE daily for mouse. According to the dose translation from animal to human [50], it corresponds to 1.5~6 g (~5~20 pieces) of fresh *Perilla*

frutescens leaves daily for a 60 kg adult and 0.7~3 g (~2.5~10 pieces) for 20 kg child, respectively.

In conclusion, ethanol extracts of *Perilla frutescent* leaves could downregulate Th2 activities to secrete less IL-5 and IL-13 and thus lower serum IgE level when counting allergen challenge. The cell infiltration, particularly eosinophils, and proinflammatory mediators such as histamine and eotaxin in BALF were significantly suppressed. As a result, AHR is alleviated by the extracts of *Perilla frutescent* leaves, suggesting that *Perilla frutescent* leaves are a potential herbal medicine for immunomodulation.

Conflict of Interests

The authors have declared that no conflict of interests exists.

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Research Article

Characteristics Associated with Utilization of Asthma-Related Traditional Chinese Medicine Services among Asthma Children in Taiwan: A Nationwide Cohort Study

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Introduction. Previous studies have demonstrated the advantages of TCM use among asthmatic children. However, there is a paucity of epidemiologic reports on features of TCM users among asthmatic children. This cohort study aimed to investigate child's, parent's, and provider's characteristics associated with the use of asthma-related TCM services among newly diagnosed asthmatic children. *Materials and Methods*. A nationally representative cohort of one million National Health Insurance beneficiaries was used. The newly diagnosed asthma children who received asthma medication from western medicine providers from 2005 to 2010 were selected as our sample for analysis. Generalized estimating equation was applied to identify the child's, parents', and provider's characteristics associated with the use of asthma-related TCM among the newly diagnosed asthmatic children. *Results*. Of 20,080 children who were enrolled and followed up for one year, 4,034 children used TCM for asthma-related treatment. Children with prior experience of TCM, pre-school and school aged children, boys, those with more severe asthma or poorer health, with higher income parents were more likely to use asthma-related TCM. Herbal medicine was the most common modality among asthmatic children. *Conclusions*. There were only 20% newly diagnosed asthmatic children using TCM. The findings may shed light on possible integration of TCM with western medicine services.

1. Introduction

Asthma is one most prevalent chronic condition among children. Global prevalence ranges from 1% to 18% across countries [1]. In addition to the large financial burdens, asthma also leads to serious health consequences and compromises life quality of children [1]. Poorly controlled childhood asthma may increase use of steroids, risk of emergency room visits or hospitalizations, persistently decrease lung function, or even lead to COPD later in adulthood [1]. According to the guideline published by the Global Initiative for Asthma (GINA), current western medications of asthma such as steroid, beta-2 adrenergic agonist, leukotriene modifier, theophylline, and anti-IgE therapies are the mainstream treatments of asthma. Recent empirical evidences also show good comparative effectiveness of integrating traditional Chinese herbal medicines to asthma management as complementary therapies, particularly among children [2–6]. Traditional Chinese herbal medicines such as ASHMI [2], mMMDT [3], Ding-Chuan [4], and STA-1 [5, 6] are proven safe and having a positive effect on symptoms and/or lung function in children.

Recently, the rising popularity of complementary and alternative medicine (CAM) has facilitated the research and development of scientifically sound CAM therapies. Numerous studies have investigated the utilization patterns of CAM among children [7–11] and found that CAM

utilization is associated with children and parental characteristics, including age, gender, ethnicity, health conditions, household income, and parental use of CAM. Various CAM modalities children received included traditional Chinese medicine (TCM), herbal treatments, chiropractors, messages, and mind-body therapies [9]. However, not all CAM have been scientifically proven effective. Therefore, it is essential to investigate whether and to what extent some scientifically proven CAM therapies are used by children in addition to their mainstream medical therapies.

Of the existing literature, several studies focus specifically on CAM utilization of asthmatic children. The prevalence of CAM visits among asthmatic children ranged from 13% in Canada [12] to 89% in USA [13]. CAM treatments of asthma encompass many therapies, such as mind-body techniques, nutritional and herbal supplements, TCM, exercise, messages, and homeopathy [14]. CAM use in asthmatic children has been associated with children's age, asthma severity, parents' education level, parental income level, and insurance coverage [12, 13, 15–18].

Nevertheless, not only did characteristics of children and parents matter, but also the characteristics of their western medicine providers may influence their use of CAM [19–21]. Western medicine (WM) physicians may play a significant role in influencing parent's decision in seeking CAM therapies. WM providers' knowledge of, attitude towards, and practice of CAM may vary widely by their demographics, professional characteristics, and characteristics of the medical care system. Environmental factors such as availability of WM and CAM providers in the areas may create either a friendlier or a more hostile atmosphere for integration [22]. Hence, the role of providers in use of CAM among asthmatic children shall not be overlooked.

Furthermore, the existing findings on asthmatic children are mostly conducted in health care systems where CAM services are not covered by insurance, or in hospital settings. None of these studies are conducted in a health care system where a major domain of CAM, TCM, is covered by a social health insurance. It may be interesting to investigate the utilization pattern of asthma-related TCM among asthmatic children in the National Health Insurance (NHI) program in Taiwan, where TCM is comprehensively covered by the NHI program. This study aimed to investigate the use of TCM among the newly diagnosed asthmatic children and its associated characteristics of children, parents, and WM providers.

2. Materials and Methods

2.1. Data Source. The data source is the Taiwan National Health Insurance Research Database (NHIRD). The National Health Insurance program is a single-payer mandatory universal insurance system. In 2011, the enrollment rate was 99% of the total population [23]. It is well known for its comprehensive benefit coverage. It not only covers ambulatory and inpatient services of western medicine, but also reimburses TCM ambulatory care services. The NHI TCM coverage includes Chinese herbal medicine, acupuncture, traumatology, and massage. The dataset consists of individual's enrolment and claims information, including patients' identification number, gender, birthdate, date of service, diagnosis, medication, treatment procedure, and expenditures. The dataset also records provider's information, including gender, age, specialty of physicians, and ownership and accreditation level of physician's practice location. Individual and provider identifiers were encrypted before releasing to the researchers.

2.2. Study Design and Sample. This study was a retrospective cohort study. Of all the NHI enrollees in 2005 throughout Taiwan from the NHIRD, a representative cohort of 1 million randomly sampled enrollees was used. The newly diagnosed asthmatic children who were aged 0–18 years and received his/her primary diagnosis of asthma and asthma medication from western medicine providers from 2005 to 2010 were included for analyses. Asthma medications in western medicine were defined according to the GINA guideline. The children who had received any diagnosis of asthma within the five years before the index year were excluded. International Classification of Diseases, Ninth Revision, Clinical Modification codes 493.xx were used to identify asthma children. A total of 20,080 children were newly diagnosed with asthma in 2005–2010. Each child had been followed for one year since his/her first diagnosis of asthma for any asthma-related TCM visit.

2.3. Variables. Asthma-related TCM visits were defined as visits of primary TCM diagnostic code of respiratory diseases (ICD-code: 460~466, 470~478, 480~488, 490~496, 500~508, 510~519). Asthma is a chronic respiratory disease and may coexist with or have other related respiratory conditions, such as respiratory tract infection [24], allergic rhinitis [25], chronic rhinitis [26], chronic bronchitis, and emphysema [27]. According to the fundamentals of TCM theories, literatures, and clinical practices, TCM normally treat diseases from a more holistic perspective. TCM users were defined as those who had any asthma-related TCM visits during the 1-year follow-up period. TCM nonusers refer to those without any asthma-related TCM visits during the 1-year follow-up period. Sensitivity analyses were also conducted for various definitions of asthma-related TCM visits. The results remained robust. Furthermore, specific types of asthma-related TCM modalities children received and the top 10 commonly prescribed Chinese herbal medicinal formulas during the follow-up period among the asthmatic children were also analyzed.

Characteristics of children, parents, and WM providers were compared between TCM users and nonusers. Child's age, gender, health status (any hospitalization prior to the index year), previous TCM experience, level of medical resources of residential location, asthmatic WM medication used, frequency of WM visits, and incident year of asthma were constructed. Asthma medications used included controller medications, oral and systemic corticosteroids. Controller medications include LABAs, xanthines, ICS, leukotriene modifiers, immunomodulators, and cromolyn

sodium. Controller medications were used for controlling persistent asthma. Oral or systemic corticosteroids were used for quickly reliving asthma exacerbations. Level of medical resources was defined as densities of western medical doctors and TCM doctors. Density of TCM and WM doctors were defined as number of TCM/WM doctors per 10,000 population and classified into four categories: (1) high density of TCM doctors and high density of WM doctors, (2) high density of TCM and low density of WM doctors, (3) low density of TCM and high density of WM doctors, and (4) low density of TCM and low density of WM.

In addition, socioeconomic status of parents was defined using the insurance wage and category of the insured. For people with well-defined monthly wages, we categorized them into 3 groups: ≥40000 NTD, 20000–39999 NTD, <20000 NTD. For those without a clearly defined monthly wage, including fisherman and farmers, we categorize them in another group.

WM provider was defined as the most frequently visited western medicine doctor for asthma during the follow-up year. If the most frequently visited WM doctors were more than one, the provider was defined as the earliest one a child had visited. Characteristics of WM providers included WM physician's age, gender, specialty, ownership, and accreditation level of his/her practice location.

2.4. Statistical Analysis. Descriptive statistics were presented. A chi-square test was used to examine differences in characteristics between TCM and non-TCM users. To adjust for correlation among the care seeking behavior of children grouped into provider clusters, generalized estimating equation (GEE) was also applied to analyze the influence of children's, parental, and provider's characteristics on the concurrent use of TCM among the newly diagnosed children receiving WM medication. A two-tailed P value of less than 0.05 was considered statistically significant. SAS 9.3 was used for data management and statistical analysis.

3. Results

Of the 20,080 children who were enrolled and followed up for one year, 4,034 children (20.1%) had at least one asthma-related TCM visit; 16,046 (79.9%) did not. Table 1 shows the distribution of TCM users and TCM nonusers. TCM use among the group of infants and toddlers was the lowest (12.0%), whereas TCM use among the group of previous TCM users was the highest (46.8%). The variations of TCM use among children treated by WM providers were small, ranging from 16.9% to 21.6%.

Table 2 shows that children's age, gender, health status, and visits of WM doctor were significantly associated with TCM use. Preschool and elementary school children were 1.72 times and 1.5 times more likely to use asthma-related TCM than infant and toddler. Boys had 12.0% higher probability of using asthma-related TCM than girls. Prior TCM experience was the strongest predictor of using TCM when children were diagnosed with asthma (OR: 6.33). Children who had been hospitalized in the previous year (OR: 1.16) or

Table 1: Distribution of TCM users and nonusers in asthmatic children by different characteristics of children, parents, and providers.

Child and parental characteristics	TCM user (%)	TCM nonuser (%)	Chi-square P value
Age			
Infant and toddler	286 (11.9)	2,114 (88.1)	
Preschool	1,779 (22.2)	6,223 (77.8)	<0.001*
School	1,600 (21.5)	5,831 (78.5)	
Adolescent	369 (16.4)	1,878 (83.6)	
Gender			
Girls	1,675 (19.3)	6,990 (80.7)	0.019*
Boys	2,359 (20.7)	9,056 (79.3)	
Previous use of TCM			
Yes	1,952 (12.5)	13,683 (87.5)	<0.001*
No	2,082 (46.8)	2,363 (53.2)	
Previous hospitalization			
Yes	3,331 (19.7)	13,598 (80.3)	0.001*
No	703 (22.3)	2,448 (77.7)	
Controller medication used			
Yes	2,662 (21.3)	9,836 (78.7)	<0.001*
No	1,372 (18.1)	6,210 (81.9)	
Oral/systematic corticosteroid used			
Yes	1,851 (20.9)	7,000 (79.1)	0.010*
No	2,183 (19.4)	9,046 (80.6)	
Number of outpatient visits in one year			
<3	2,161 (18.6)	9,431 (81.4)	<0.001*
≥3	1,873 (22.1)	6,615 (77.9)	
Density of TCM and WM			
Low TCM and low WM	563 (19.6)	2,315 (80.4)	
Low TCM and high WM	326 (19.0)	1,393 (81.0)	0.006*
High TCM and low WM	857 (18.7)	3,717 (81.3)	
High TCM and high WM	2,288 (21.0)	8,621 (79.0)	
Incident year			
2005	841 (18.7)	3,648 (81.3)	
2006	710 (18.7)	3,080 (81.3)	
2007	806 (19.8)	3,269 (80.2)	<0.001*
2008	677 (21.7)	2,438 (78.3)	
2009	616 (23.2)	2,041 (76.8)	
2010	384 (19.7)	1,570 (80.4)	

Table 1: Continued.

Child and parental characteristics	TCM user (%)	TCM nonuser (%)	Chi-square *P* value
Parent's insurable wage/category			
<20000 NTD	1,099 (18.6)	4,802 (81.4)	0.002*
20000~39999 NTD	1,418 (20.5)	5,505 (79.5)	
≥40000 NTD	987 (21.6)	3,586 (78.4)	
Special group	530 (19.8)	2,153 (80.3)	
Provider characteristics			
Age			
≤40	1,772 (19.5)	7,336 (80.5)	0.108
41~50	1,672 (20.7)	6,391 (79.3)	
≥51	590 (20.3)	2,319 (79.7)	
Sex			
Female	691 (21.6)	2,515 (78.5)	0.024*
Male	3,343 (19.8)	13,531 (80.2)	
Specialty			
Family medicine	271 (16.9)	1,329 (83.1)	0.008*
Internal medicine	247 (19.8)	1,001 (80.2)	
Pediatrics	2798 (20.6)	10,815 (79.5)	
Other	718 (19.8)	2,901 (80.2)	
Ownership			
Private	3,694 (20.1)	14,729 (80.0)	0.649
Public	340 (20.5)	1,317 (79.5)	
Accreditation level			
Hospital	1,921 (20.8)	7,301 (79.2)	0.016*
Clinic	2,113 (19.5)	8,745 (80.5)	

*Statistically significance (*P*-value < 0.05).

who had more frequent WM outpatient visits (OR: 1.15) were significantly more likely to have asthma-related TCM visits. Children who received oral or systemic corticosteroids were associated with a higher likelihood of TCM visits (OR: 1.08) at the borderline significance (95% CI: 1.00, 1.18). Children of parents with monthly insurable wage below 20,000 NTD or without regular monthly wage were 13.0–16.0% less likely to seek TCM care for asthma.

Factors such as physician's gender, age, specialty, ownership and accreditation level of facility, and density of WM and TCM doctors were not significantly associated with asthma-related TCM use. However, asthmatic children whose main WM physicians aged 41~50 years were associated with a significantly higher likelihood of using TCM (OR: 1.09) at the borderline significance level (95% CI: 1.00, 1.18). Children who were treated by pediatricians were also significantly more likely to use TCM (OR: 1.17) at the borderline significance level (95% CI: 1.00, 1.38). No statistically significant difference was observed among children living in locations with varying density levels of WM and TCM doctors.

Herbal medicine was the most commonly used TCM modulation (99.6%) among the newly diagnosed asthma patients. Table 3 shows the top 10 commonly prescribed Chinese herbal formulas children used. Shin-Yi-Qing-Fei-Tang was the most commonly used herbal formula (21.4%). Shin-Yi-Qing-Fei-Tang composes *Magnolia liliiflora*, *Lilium brownie*, *Anemarrhena asphodeloides*, *Scutellaria baicalensis*, calcium sulphate, *Eriobotrya japonica*, *Cimicifuga foetida*, *Ophiopogon japonicus*, *Gardenia jasminoides*, and *Glycyrrhiza uralensis*. Xiao-Qing-Long-Tang was the second commonly used herbal formula (16.3%). Xiao-Qing-Long-Tang is composed of *Magnolia liliiflora*, *Lilium brownie*, *Anemarrhena asphodeloides*, *Scutellaria baicalensis*, calcium sulphate, *Eriobotrya japonica*, *Cimicifuga foetida*, *Ophiopogon japonicus*, *Gardenia jasminoides*, and *Glycyrrhiza Uralensis*. Cang-Er-Zi-San was the third commonly used herbal formula (14.5%). Cang-Er-Zi-San is composed of *Xanthium sibiricum*, *Magnolia liliiflora*, *Angelica dahurica*, and *Mentha haplocalyx*. Among three formulations, Xiao-Qing-Long-Tang had been scientifically proven for its effect on decreasing asthmatic Th2-related immune response and having the bronchodilating effect [28–30]. A formula of modified Cang-Er-Zi-San: Shi-Bi-Lin had been studied for its effect on relieving symptoms of nose blockage [31]. The major component of Shin-Yi-Qing-Fei-Tang, Magnoliae flos (Shen et al.), had been studied of its treatment effect on sinusitis and asthma [32].

4. Discussions

This study has revealed several statistically significant patterns regarding TCM utilization among asthmatic children under the NHI program in Taiwan. First, despite the minimal financial barriers to TCM services under the NHI program in Taiwan, only 20.1% of the newly diagnosed asthmatic children used asthma-related TCM, which was among the lower bound of estimates observed in other societies. This low prevalence of TCM use reflects a large room for improvement in integrating TCM modalities with the mainstream asthmatic care. Second, substantial variations were observed across different children groups. Consistent with previous literatures, in addition to preschool age [12] and boys, children with prior experience with TCM, more severe asthma, or poorer health [12, 17, 18, 33] were more likely to seek TCM care for better control of their asthma conditions. Unexpectedly, under the NHI program, socioeconomic differences were also observed in the use of TCM care among asthma children. Children of higher income parents were more likely to seek TCM care for asthma [7]. Third, the marginal statistically significant odds ratios observed across all provider characteristics ring a warning bell. The findings suggest that the utilization pattern of TCM among newly diagnosed asthmatic children did not vary dramatically across provider characteristics. More importantly, the findings imply that the integration of TCM care remained universally low among all WM providers. More specifically, previous use of TCM was the strongest influencing factor of TCM use. Similarly in Norway, adolescents' previous visits of

Table 2: Children, parent, and provider characteristics associated with TCM uses.

Child's characteristics	Crude OR (95% CI)	Adjusted OR (95% CI)
Age (ref.: infant and toddler)		
Preschool	2.11 (1.85, 2.42)*	1.72 (1.49, 1.99)*
School	2.03 (1.77, 2.32)*	1.50 (1.28, 1.75)*
Adolescent	1.45 (1.23, 1.72)*	0.91 (0.75, 1.11)
Sex (ref.: girls)		
Boys	1.09 (1.01, 1.17)*	1.12 (1.04, 1.20)*
Previous use of TCM (ref.: no)		
Yes	6.18 (5.73, 6.66)*	6.33 (5.83, 6.87)*
Previous hospitalization (ref.: no)		
Yes	1.17 (1.07, 1.29)*	1.16 (1.05, 1.29)*
Controller medication used (ref.: no)		
Yes	1.23 (1.14, 1.32)*	1.07 (0.98, 1.16)
Systematic or oral corticosteroid used (ref.: no)		
Yes	1.10 (1.02, 1.17)*	1.08 (1.00, 1.18)
Number of outpatient visits in one year (ref.: <3)		
≥3	1.24 (1.15, 1.33)*	1.15 (1.06, 1.25)*
Density of TCM and WM doctors (ref.: low TCM and low WM)		
Low TCM and high WM	0.96 (0.83, 1.12)	0.92 (0.77, 1.09)
High TCM and low WM	0.95 (0.84, 1.07)	0.90 (0.78, 1.03)
High TCM and high WM	1.09 (0.98, 1.21)	1.00 (0.89, 1.12)
Incident year (ref.: 2005)		
2006	1.00 (0.90, 1.12)	1.00 (0.89, 1.12)
2007	1.07 (0.96, 1.19)	1.03 (0.92, 1.15)
2008	1.21 (1.08, 1.35)*	1.03 (0.91, 1.17)
2009	1.31 (1.16, 1.47)*	1.17 (1.03, 1.33)*
2010	1.06 (0.93, 1.21)	0.91 (0.79, 1.06)
Parental characteristics		
Parent's insurance wage (ref.: ≥40000 NTD)		
<20000 NTD	0.83 (0.76, 0.92)*	0.84 (0.76, 0.94)*
20000~39999 NTD	0.94 (0.85, 1.03)	0.93 (0.84, 1.02)
Special group	0.89 (0.80, 1.01)	0.87 (0.76, 0.99)*
Provider's characteristics		
Age (≤40)		
41~50	1.08 (1.01, 1.17)*	1.09 (1.00, 1.18)
≥51	1.05 (0.95, 1.17)	1.10 (0.97, 1.24)
Sex (ref.: female)		
Male	0.90 (0.82, 0.99)*	0.90 (0.80, 1.02)
Specialty (ref.: family medicine)		
Internal medicine	1.21 (1.00, 1.47)	1.23 (0.99, 1.52)
Pediatrics	1.27 (1.11, 1.46)*	1.17 (1.00, 1.38)
Other	1.21 (1.04, 1.42)*	1.19 (1.00, 1.42)
Ownership (ref.: public)		
Private	0.97 (0.86, 1.10)	1.06 (0.92, 1.23)
Accreditation level (ref.: hospital)		
Clinic	0.92 (0.86, 0.98)*	0.93 (0.85, 1.01)

*Statistically significance (P-value < 0.05).

Table 3: Top 10 Chinese herbal medicines used.

Top 10	Herbal formulas	Frequency (%)
1	Shin-Yi-Qing-Fei-Tang	21.4
2	Xiao-Qing-Long-Tang	16.3
3	Cang-Er-Zi-San	14.5
4	Shin-Yi-San	13.9
5	Ma-Xing-Gan-Shi-Tang	12.4
6	Xing-Su-San	8.7
7	Ge-Gen-Tang	7.9
8	Yin-Qiao-San	7.4
9	Ding-Chuan-Tang	5.9
10	Zhi-Sou-San	5.5

CAM were also the strongest predictor of future CAM visits in the Young-HUNT studies [34]. Asthmatic patients accustomed to TCM therapies or believing in TCM treatments may lead to further integrative use of WM and TCM. Hence, it is important to increase awareness of or positive attitudes towards scientifically proved TCM care and build trust of TCM among asthmatic children and parents. More effective promotion or educational strategies or program about TCM may help to reduce nonfinancial barriers to TCM services.

Furthermore, in spite of low financial barriers to TCM care under the NHI program, children with higher income parents were significantly more likely to receive TCM care in addition to their WM asthmatic treatments. This may also be due to other barriers. A possible barrier is that parents' workload may be so hard that they did not have spare time to bring their children to visit other doctors. Further studies of other nonfinancial barriers (time or travel costs) are needed to facilitate better or more efficient integration of TCM and WM asthmatic treatments.

All of the provider factors studied are not significantly associated with TCM use among asthmatic children. Most importantly, the persistently low utilization of TCM across WM providers of different characteristics may reflect that the issue of integration of TCM and WM is generally overlooked in routine management of pediatric asthma. Furthermore, limited availability of clinical trials may be one plausible barrier to better integration of TCM and WM. Whether or not to use TCM to treat or control asthma may heavily depend on therapeutic effects and safety of TCM. Therefore, more clinical trials should be encouraged in this regard.

This study has several strengths. First, using the National Health Insurance data of a large national representative sample in Taiwan can help us avoid some of the known shortcomings of survey data. The National Health Insurance covered both TCM and WM universally. Using the medical records from National Health Insurance data may ameliorate the selection bias from the survey data. Second, in contrast to other studies on this topic, the large sample size and relatively comprehensive data allow us to go beyond prior research by investigating not only children and parental characteristics, but also WM provider characteristics in their influences on the utilization of TCM care among the newly diagnosed asthma children. Third, taking advantages of the detailed information on TCM modalities and herbal medications in the NHI databases, we were able to identify the common TCM medications and therapies prescribed to the newly diagnosed asthmatic children in Taiwan. The findings may serve as important references in clinical management of pediatric asthma and for future effectiveness researches in TCM.

Several limitations should also be noted. First, the NHI database only allows us to estimate the use of TCM services covered by the NHI program. The NHI program covers TCM services provided by the NHI contracted TCM providers, including extracted TCM powder preparations, acupuncture, moxibustion, and traumatology manipulative therapy. However, other TCM services or products including crude herbal medicine, herb tea bags, pills, capsules, and other CAM modalities are not covered by the NHI program and these insurance noncovered services are self-paid by patients. The prevalence of people who had used any self-paid TCM or CAM service in one year was 6.05% [35]. Those self-paid TCM services or CAM services are not included for analyses in this study. Not being able to include self-paid TCM or CAM services in this study may lead to an underestimation of the CAM use among asthmatic children in Taiwan. However, as the CAM ranges widely, not many modalities have been proven effective scientifically. As the TCM services covered by the NHI program are well accepted CAM modalities and patient's costs in using these services are very low, studying the TCM utilization patterns under the NHI program may help to go beyond the previous investigations of general CAM uses. Second, a possible misclassification bias may be of concern. Because a claim may have up to 3 diagnoses, defining asthma-related TCM visits based on one set of respiratory diagnoses may be questionable. Therefore, sensitivity analyses were conducted on various definitions of TCM visits containing only diagnoses of respiratory diseases, any diagnoses of respiratory diseases, diagnoses of asthma, and diagnoses of asthma and bronchitis. All major statistical results from sensitivity analyses remained significant. Third, due to data limitations, using a single measure (i.e., the NHI program's payroll and occupation-based categories) to analyze the association between a parent's SES and the decision on TCM care did not allow us to fully explore this association. In addition, possible confounding biases such as knowledge level may also exist.

5. Conclusion

Adopting TCM care for asthma management among the newly diagnosed children in Taiwan was low. The low prevalence of TCM use is commonly seen across children treated by different characteristics of WM providers. This may suggest there is still room to promote collaboration between WM and TCM providers. Instead of targeting specific physician groups, putting more efforts generally in integration between TCM and WM in the management of pediatric asthma is needed. More efforts in improving understanding of TCM such as the educational programs of TCM and more research efforts in showing evidence-based effectiveness of TCM

modalities may help to build more confidence or positive attitudes towards TCM among physicians and parents and to facilitate the integration TCM into the mainstream medicine in pediatric asthma management.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Triterpenoids and Polysaccharide Fractions of *Ganoderma tsugae* Exert Different Effects on Antiallergic Activities

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This study was to investigate antiallergic effects of triterpenoids (Gt-TRE) and polysaccharide (Gt-PS) extracts from *Ganoderma tsugae*, using mast cell line RBL-2H3, T cell line EL4, primary T cells, and transfected RAW264.7 macrophage cells. The results showed that histamine secreted from activated RBL-2H3 mast cells was significantly suppressed by Gt-TRE but not Gt-PS. Interleukin- (IL-) 4 secreted from activated EL4 cells was significantly suppressed by Gt-TRE but not Gt-PS. Further primary $CD4^+$ T cells cultures also confirmed that Gt-TRE (5 ~ 50 μg/mL) significantly suppressed Th2 cytokines IL-4 and IL-5 secretions but had no effect on Th1 cytokines IL-2 and interferon (IFN)-γ. Gt-PS did not affect IL-4 and IL-5 secretions until higher doses (400, 500 μg/mL) and significantly suppressed IFNγ secretions but enhanced IL-2 at these high doses. The reporter gene assay indicated that Gt-TRE inhibited but Gt-PS enhanced the transcriptional activity of NF-κB in activated transfected RAW264.7 cells and transfected EL4 cells. IL-4 secreted by this transfected EL-4 cells was also significantly decreased by Gt-TRE but not by Gt-PS, suggesting that these two fractions may exert different effects on NF-κB related cytokines expression. These data suggested that triterpenoids fraction of *Ganoderma tsugae* might be the main constituents to alleviate allergic asthma.

1. Introduction

The worldwide increase in prevalence of allergic diseases such as asthma [1, 2] poses a significant health problem and a demand for drug/diet therapy. Allergic asthma is characterized by histamine secretion by mast cell, bronchial hyperresponsiveness, and airway inflammation by accumulation of eosinophils, lymphocytes and mast cells, and higher serum IgE levels [3]. The allergic immune responses in asthma arise from an imbalance of helper T (Th) cells. Th1 cells and their cytokines IL-2 and IFNγ enhance Th1 generation and inhibit Th2 function, whereas Th2 cells and their cytokines inhibit Th1 generation and rise allergic responses. IL-4 secreted by Th2 cells stimulates B cells class switch to produce allergic immunoglobulin (Ig) E and promote neutrophil- and eosinophil-infiltrated inflammation. These cells were also activated by another Th2 cytokine IL-5. Therefore, downregulating Th2 cell differentiation by cytokine administration is frequently used as a therapy for allergic diseases [4]. Several studies indicated dietary factors, such as frying oil, adlay, and andrographolide from *Andrographis paniculata* suppressed Th2 immune responses in the Th2-skewed ovalbumin- (OVA-) sensitized BALB/c mice [5–7], and polysaccharide from fruiting bodies of *Ganoderma*

lucidum has been shown to mediate cytokines production [8]. Therefore, the immunomodulatory effects of *G. tsugae* rose our interest.

The polypore genus *Ganoderma* had been widely used for Chinese medicine in Asian countries for a long time. It has been known for many biological activities of *Ganoderma*, such as antitumor, immunoregulation, hepatoprotection, anticholesterol synthesis and anti-inflammation [9–14]. Although the major active ingredients are polysaccharides, triterpenoids and proteins, polysaccharides are most studied [15]. The polysaccharides with immunity enhancement effects have been isolated from the water extract of *G. lucidum* mycelia and fruiting bodies [16]. It has been demonstrated that polysaccharide extracts of *G. lucidum* exert immunomodulating activities by inducing cytokine expression *via* TLR4 signaling pathways, activation of dendritic cells, and innate immunity by NF-κB pathways [17–19]. The different ingredients may exert diverse bioactive functions [20]. Although recent review articles also summarized the health benefits of *Ganoderma*, especially triterpenoids [21], the immunomodulatory effects of triterpenoids still need to be clarified.

G. lucidum is the most studied species of the *Ganoderma*. However, another species of medicinal mushrooms, *G. tsugae*, is most widely cultivated in Taiwan and used as functional foods. Our previous study showed that *G. tsugae* supplementation significantly enhanced Th1/Th2 balance in the Th2-skewed OVA-sensitized and challenged BALB/c mice as an allergic inflammation model [22]. Further study showed that *G. tsugae* supplementation significantly alleviated histamine, prostaglandin (PGE) 2 and eotaxin, a protein that can activate eosinophils and airway hyperresponsiveness, levels in bronchoalveolar lavage fluid (BALF) in OVA-sensitized allergic BALB/c mice [23]. However, the eosinophils in BALF and Th2 cytokines IL-4 and IL-5 and were not significantly suppressed by *G. tsugae* [22, 23]. Since early *in vitro* study suggested that triterpenoids suppressed histamine release from mast cells [24], our study further demonstrated that triterpenoids fraction from *G. tsugae* significantly inhibited eosinophils, IL-4, IL-5, PGE2, eotaxin levels, and thus airway hyperactivity [25].

There are studies that indicated that polysaccharide component promotes Th1 immune responses [18, 26]. Therefore, whether histamine or IL-4 production was affected by polysaccharide or triterpenoids of *G. tsugae* were investigated in this study using a mast cell line and a murine T cell line. To further investigate whether cytokine productions were affected by polysaccharide or triterpenoids *via* T cell polarization, the primary $CD4^+$ T cells isolated from the DO11.10 transgenic mice were used in this study. The DO11.10 transgenic mice with high percentage of OVA peptide-specific $\alpha\beta$ T cell receptor can specifically respond to OVA stimulation [27] and it is a useful model for investigating the association between allergic disease and T cells. In addition, since inhibition of *in vitro* NF-κB transactivation activity was shown in accordance with *in vivo* anti-inflammation [28] and its association with T cells and dendritic cells activations [29], both RAW264.7 macrophage cell line and EL-4 murine T cell line were transfected with NF-κB driven reporter plasmid to determine the actions of polysaccharide or triterpenoids of *G. tsugae*. In this study, we provide the first comparison between the polysaccharide and triterpenoid extracts of *Ganoderma tsugae* on Th2 responses and its possible mechanism of NF-κB transactivation.

2. Materials and Methods

2.1. Materials and Chemicals. The polysaccharide and triterpenoid extracts were kindly provided by Double Crane Group (Yung-Kien Industry Corp., Taiwan). The fruiting bodies of *Ganoderma tsugae* YK-01 were fractionated into a polysaccharide fraction (Gt-PS) (alcohol-insoluble) as described previously [30], and an alcohol-soluble fraction. The alcohol-soluble fraction was extracted with acidic ethyl acetate to yield 4.2% triterpenoid rich extracts (Gt-TRE) as previously studied [31]. The purity of the Gt-TRE was identified by reverse-phase HPLC methods to be 38% and contained nine major peaks identified as ganoderic acids (ganoderic acids A, B, C, C5, C6, D, E, and G, and ganodermic acid D), reported previously by D.-H. Chen and W. K.-D. Chen [32].

2.2. Cell Lines Culture and Treatment. Cell lines used in this study were purchased from Food Industry Research and Development Institute (Taiwan). EL4 murine T cells (ATCC no. TIB-39) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY) plus 10% FBS (Gibco) at 37°C, 5% CO_2. EL4 T cells were cultured with Gt-TRE or Gt-PS and stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, St Louis, MO) plus 1 μg/mL ionomycin (Sigma) for 24 hours, and the supernatants were collected for IL-2 and IL-4 analysis.

RBL-2H3 mast cells (ATCC no. CRL-2256) were grown in minimum essential medium eagle (MEM, Gibco) plus 15% fetal bovine serum (FBS, Gibco), 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 0.1 mM nonessential amino acid. RBL-2H3 mast cells were pretreated with various doses of Gt-TRE or Gt-PS for 24 hrs and stimulated with ionomycin (500 ng/mL) for 5 hrs. After stimulation, supernatants were collected for histamine concentration analysis.

RAW264.7 macrophage cells (ATCC no. TIB-71) were grown in DMEM plus 10% FBS at 37°C, 5% CO_2. Cells were transfected with NF-κB responsive luciferase reporter plasmids, which are described in NF-κB responsive reporter assay section.

2.3. Determination of Histamine Release. The histamine level was determined by Histamine-ELISA kit (IBL Hamburg, Germany). The procedure was according to manufacturer's instructions. In brief, the culture supernatants and plasma standards were acylated with acylation reagent. Then, aliquots of 50 μL acylated sample and standards were loaded into the 96-microplate wells, and then 50 μL enzyme conjugate and 50 μL antiserum were added to each well. The plate was shaken carefully for 3 hours on an orbital shaker at room temperature. The plate was washed four times and 200 μL tetramethylbenzidine (TMB) substrate solution was added to each well for 30 min and stopped by 100 μL of TMB stop

solution. The optical density was measured with a microplate autoreader at 450 nm. The histamine concentrations were determined according to the standard curve.

2.4. Isolated and Cultured OVA-Specific Naïve CD4$^+$ T Cells. The CD4$^+$T cells were isolated from spleen of BALB/c DO11.10 OVA-specific T cell receptor transgenic mice with SpinSet kit (>95% CD4$^+$ purity; StemCell Technologies, Vancouver, BC, Canada). The CD4$^+$ T cells (5×10^5 cells/mL) were cultured in the presence of irradiated (2700 rad, 540 sec) antigen presenting cells (APC) at 2×10^6 cells/mL in a final volume of 1 mL with 1 μg/mL $OVA_{323-339}$ peptide and various concentrations of Gt-TRE or Gt-PS. After 72-hour incubation for naïve CD4$^+$ T cells differentiation, supernatants were collected and stored at −80°C until cytokines analysis. The IL-2 and IFNγ levels in the supernatants were the indicators for Th1 response, whereas IL-4 and IL-5 levels were the indicators for Th2 responses. The data is present as the relative cytokine production to the control cells without Gt-TRE or Gt-PS treatment in each experiment.

2.5. Determination of Cytokines Production. The cytokine levels in culture supernatants were measured by sandwich ELISA methods. Briefly, the anticytokine antibody, including purified rat anti-mouse cytokine monoclonal antibodies IL-2, IFNγ, IL-4, and IL-5 (PharMingen, San Diego, CA), were coated in the 96-well plates (Nunc, Roskilde, Denmark). After overnight incubation at 4°C and blocking with PBS containing 1% BSA for 30 min, the samples and standards (recombinant mouse cytokines, PharMingen) were added to the 96-well plates for 2 hours of incubation. The biotin-conjugated antibodies (biotinylated rat anti-mouse cytokine monoclonal antibodies, PharMingen) were added and incubated. After washing, the streptavidin-conjugated peroxidase (Thermo Fisher Scientific Inc., Rockford, IL) was added for 1 hour. The substrates, 2,2′-azino-bis-3-ethyl-benzthiazoline-6-sulfonic acid (ABTS, Sigma), were added to each well for 20 min to develop color. The plates were read in a microplate autoreader (Microplate autoreader; Bio-Tek Instrument, Inc. Winooski, VT) at 405 nm. The detection limits for IL-2, IFNγ, and IL-5 are 75 pg/mL and 3.9 pg/mL for IL-4.

2.6. NF-κB-Dependent Luciferase Activity Assay. The NF-κB-promoted luciferase reporter plasmid, 3x-κB-tk-luc [33], had three copies of an NF-κB binding site in the upstream thymidine kinase (tk) promoter and luciferase reporter gene in the pGL2 vector (Mock) was used to assay the activity of NF-κB transactivation as described previously [34]. Plasmids were grown in the JM109 strain of *E. coli* in Luria-Bertani medium containing 100 μg/mL ampicillin (Sigma), and plasmid DNA preparations were performed by the QIAGEN plasmid midi kit (QIAGEN, Hilden, Germany).

The 3x-κB-tk-luciferase and Renilla luciferase reporter plasmid pRL-tk-luciferase plasmids were introduced into RAW264.7 cells or EL4 T cells by liposome-mediated method. Transfection was performed in 24-well plates using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. Briefly, cells were grown in 24-well plates and transfected with the appropriate vector, 0.8 μg 3x-κB-tk-luciferase and 0.4 μg pRL-tk-luciferase (2 : 1), in serum-free OPTI-MEM medium (Gibco BRL, Gaithersburg, MD) containing 2 μL Lipofectamine 2000. After appropriate transfection times, 5 hrs for RAW264.7 cells and 24 hrs for EL4 T cells, the transfection mix was removed and replaced with complete medium. Cells were then either treated with Gt-TRE or Gt-PS or stimulated with mitogens, LPS plus IFNγ for RAW264.7 cells for 8 hrs, and PMA plus ionomycin for EL4 T cells for 24 hrs, respectively. The supernatants were collected for cytokine determination and the cells were for luciferase activity analysis.

Luciferase activity was determined using the Dual-Glo Luciferase assay system, according to the manufacturer's instructions from Promega (Madison, WI). Light emission was measured in a luminescence microplate counter (Wallac Victor-2 Perkin Elmer, Norwalk, MT). The efficiency of transfection, as determined by *Renilla* luciferase activity in the lysate by cotransfection with pRL-tk constructs, was used to normalize the activity of *firefly* luciferase [35]. Results were expressed as relative luciferase activity corrected for the differences in active efficiency by nonstimulated cells.

2.7. Statistical Analysis. The significance of difference between treatment and control groups was analyzed statistically by Student's t-test of the SAS program system (SAS/STATA version 8.2; SAS Institute, Cary, NC), in order to show the effect of each fraction's antiallergic properties. Differences were considered to be significant if P was <0.05.

3. Results

3.1. Triterpenoids, Not Polysaccharides, Suppressed Histamine Release from Mast Cells. Histamine is an important mediator of allergic disease. To evaluate whether triterpenoid-rich extracts or polysaccharide fractions of *Ganoderma tsugae* could inhibit the histamine secretion on allergic responses [23], histamine release from ionomycin-stimulated RBL-2H3 mast cells cultured with Gt-TRE or Gt-PS was investigated. The results as shown in Figure 1 indicated different effects of Gt-TRE and Gt-PS on histamine productions. Histamine levels were slightly decreased with increased doses of Gt-TRE and significantly suppressed by Gt-TRE at 50 μg/mL. On the contrary, histamine productions were not significantly affected by Gt-PS, though they were tended to increase.

3.2. Triterpenoids Decreased IL-4 Production from EL4 T Cells. In order to investigate the triterpenoids and polysaccharides on cytokines, IL-2 and IL-4, productions by activated murine EL4 T cells were cultured with Gt-TRE or Gt-PS (Figure 2). Under PMA plus ionomycin stimulation, Th1 cytokine IL-2 productions from EL4 T cell were not affected by Gt-PS and Gt-TRE treatment. On the other hand, IL-4 productions were decreased in a dose-dependent manner by Gt-TRE treatment but not by Gt-PS treatment. These data suggested that triterpenoid extracts of *G. tsugae* are the main constituent to suppress Th2 cytokine production.

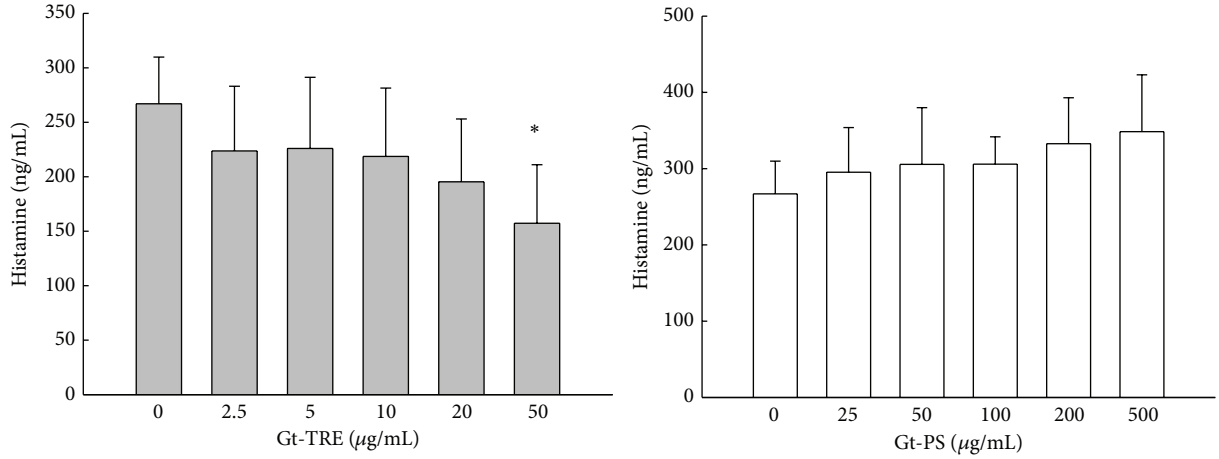


Figure 1: The histamine secretion from ionomycin-stimulated RBL-2H3 mast cells cultured with different doses of triterpenoids and polysaccharide extracts from *G. tsugae*. The RBL-2H3 mast cells (5×10^4 cells/mL) were pretreated with Gt-TRE or Gt-PS extracts from *G. tsugae* for 24 hr, and then stimulated with 500 ng/mL ionomycin for 5 hr. The supernatants were collected and assayed by histamine ELISA kit. The data are representative for three independent experiments. $^*P < 0.05$ as compared to the 0 μg/mL control cells.

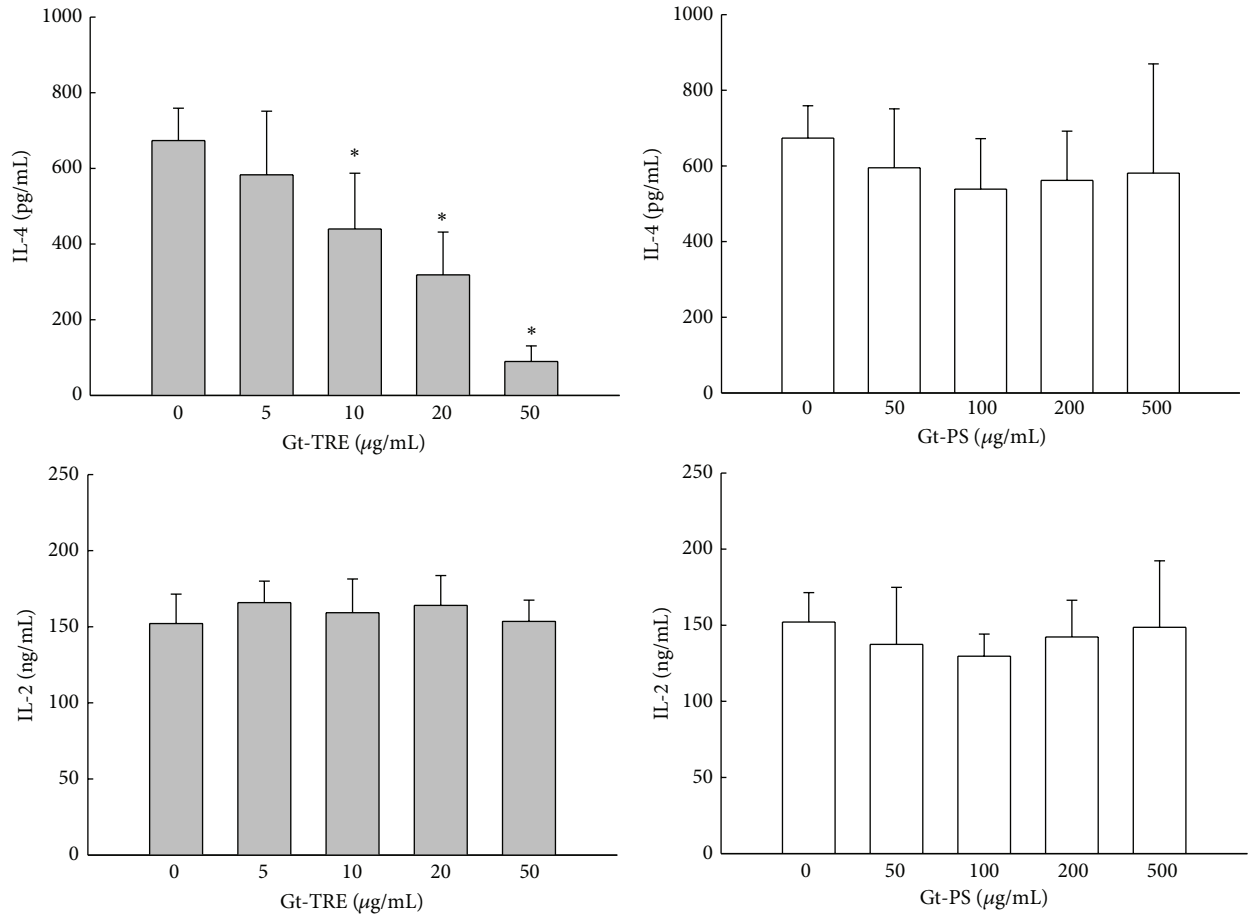
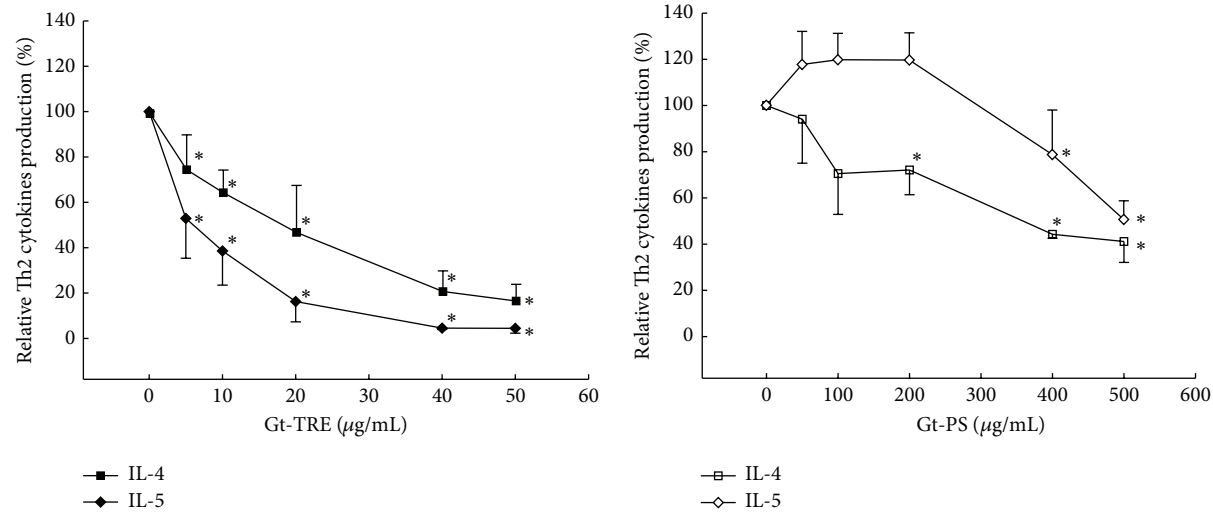
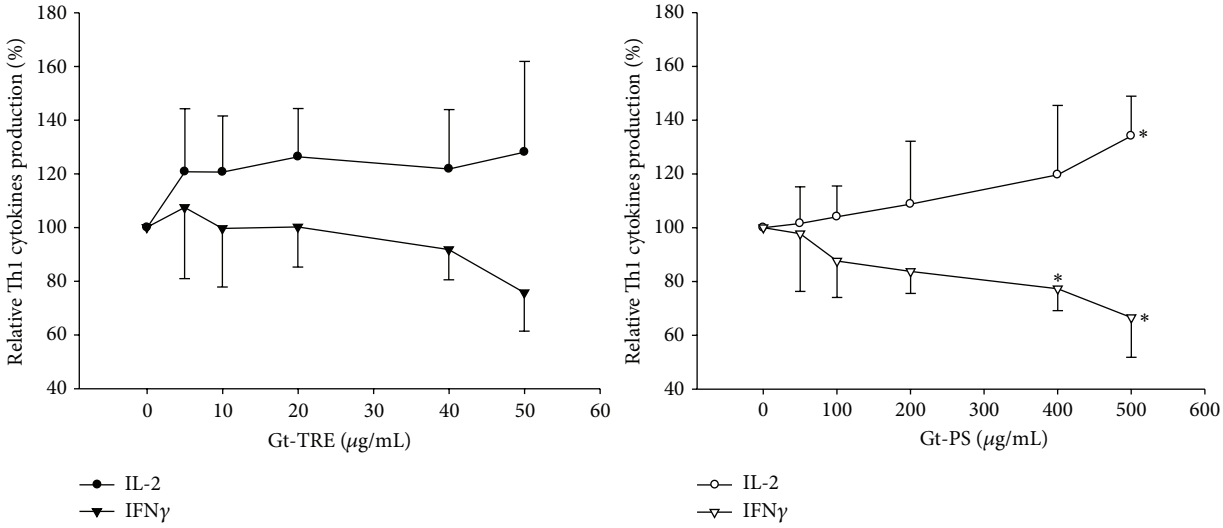


Figure 2: The IL-4 and IL-2 productions of activated EL4 T cells cultured with different doses of triterpenoids and polysaccharide extracts from *G. tsugae*. EL4 murine T cells (2×10^5 cells/mL) were cultured with Gt-TRE or Gt-PS extracts from *G. tsugae* and activated by 50 ng/mL PMA plus 1 μg/mL ionomycin for 24 hrs. The supernatants were collected and cytokine levels were determined by ELISA method. The data are representative for five independent experiments. $^*P < 0.05$ as compared to the 0 μg/mL control cells.



(a) Th2 cytokines production



(b) Th1 cytokine production

Figure 3: The relative Th2 cytokines (a) and Th1 cytokines (b) productions from primary T cells cultured with different doses of triterpenoids and polysaccharide extracts from *G. tsugae*. Isolated $CD4^+$ helper T cells (5×10^5 cells/mL) from the DO11.10 transgenic mice with high percentage of OVA peptide-specific T cell receptor were cultured with different doses of Gt-TRE or Gt-PS in the presence of OVA peptide and APC for 72 hrs. The supernatants were collected and cytokine levels were determined by ELISA method. The results are expressed as the mean ± SD of four independent experiments. $^*P < 0.05$ as compared to the 0 μg/mL control cells.

3.3. Triterpenoids Decreased Th2 Cell Development and Th2 Cytokine Production. To investigate whether triterpenoids and polysaccharides could affect the helper T cells polarization, we isolated $CD4^+$ T cells from the splenocytes of DO11.10 mice to obtain large population of T cells that can be activated by OVA peptide to induce higher antigen-specific cytokines production. The average levels of cytokines secreted from primary $CD4^+$ T cells under stimulation of 1 μg/mL $OVA_{323–339}$ peptide for 72 hrs from five independent experiments were 41.4 ± 13.0 ng/mL for IL-2, 19.9 ± 11.2 ng/mL for IFNγ, 1.19 ± 0.10 ng/mL for IL-4, and 1.47 ± 1.29 ng/mL for IL-5. The data in Figure 3 was present as the relative cytokine production compared to the 0 μg/mL control cells.

When these T cells were cultured with Gt-TRE or Gt-PS, as shown in Figure 3, the IL-4 and IL-5 productions were significantly decreased in a dose-dependent manner during T cells activation by APC and specific antigen OVA (Figure 3(a) left), but IL-2 and IFNγ productions were not significantly affected by Gt-TRE (Figure 3(b) left). However, the IL-4 tended to be increased by low dose of Gt-PS but significantly decreased by high doses (Figure 3(a) right), suggesting that Gt-PS exerts different effects depending on different doses. The IL-5 productions were dose dependently decreased by

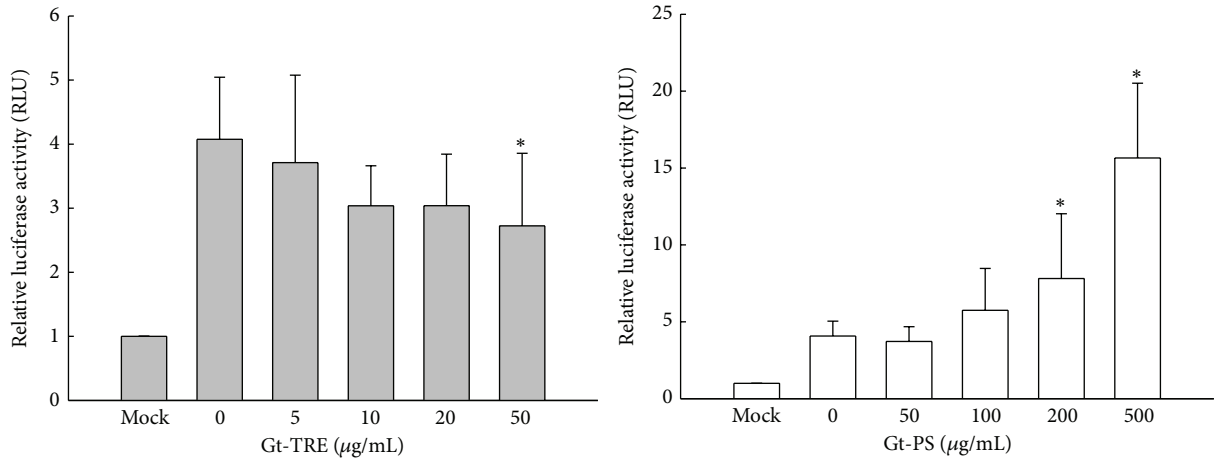


Figure 4: The NF-κB transcriptional activity of transfected RAW264.7 cells cultured with different doses of triterpenoid and polysaccharide extracts from *G. tsugae*. RAW264.7 macrophages (1×10^5 cells/mL) transiently transfected with NF-κB-promoted luciferase reporter plasmid were treated with Gt-TRE or Gt-PS and stimulated with LPS (100 ng/mL) plus IFNγ (1000 units/mL) for 8 hr. Mocks are the RAW264.7 cells transfected with empty vector as the negative control without Gt-TRE or Gt-PS treatment. The NF-κB responsive reporter luciferase activity was assayed as described in Materials and Methods. The data were representative for five independent experiments. $^*P < 0.05$ as compared to the 0 μg/mL control cells.

Gt-PS significantly decreased, reaching significantly lower at high doses. IL-2 productions were increased by Gt-PS dose dependently, reaching significantly higher at high dose of Gt-PS, whereas IFNγ productions were decreased by Gt-PS, especially significantly lower at higher doses of Gt-PS (Figure 3(b) right). These data suggested Gt-TRE suppressed Th2 cell polarization more than Gt-PS, whereas Gt-PS affected Th1 cell activity more than Gt-TRE.

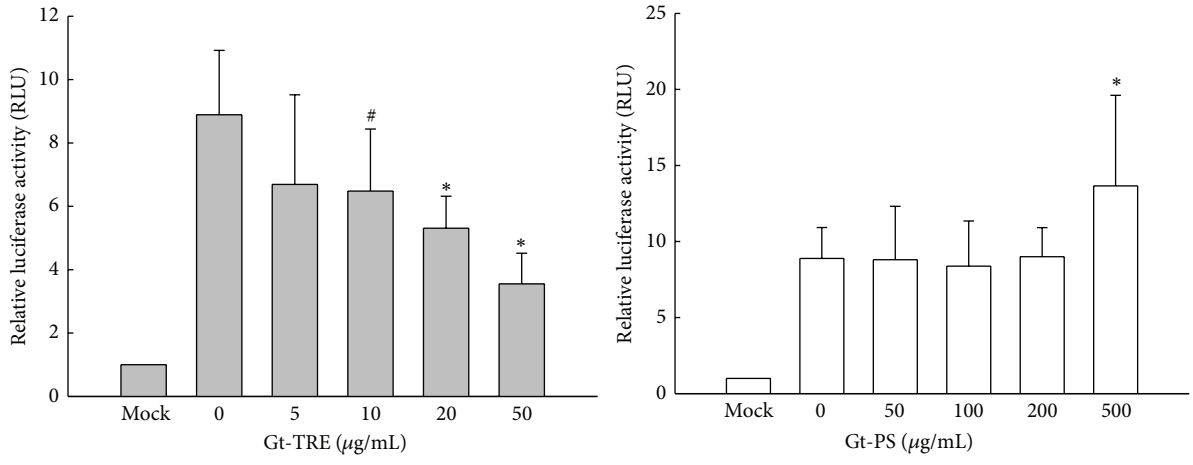
3.4. Triterpenoids Suppressed but Polysaccharides Enhanced NF-κB-Mediated Transcriptions. NF-κB is a converging transcription factor of various immune responses, such as cytokines expression. In order to investigate whether triterpenoids and polysaccharide may involve in NF-κB-activating cytokine gene expression in macrophages and T cells, both RAW264.7 and EL-4 cells were transiently transfected with NF-κB-promoted luciferase reporter plasmid. As shown in Figure 4, cells with Mock transfection showed barely reporter activity but NF-κB-promoted reporter transfection had 4-fold luciferase activity under stimulation. However, NF-κB-transactivation activities were gradually decreased by Gt-TRE, reaching statistical significance at the dose of 50 μg/mL. On the contrary, NF-κB-transactivation activities were elevated by Gt-PS in a dose-dependent manner with 16-fold luciferase activity (15.7 ± 4.9) at high dose of Gt-PS. These data suggested that polysaccharide fraction may activate macrophage through the NF-κB pathway.

When EL4 T cells were transfected with NF-κB-promoted reporter plasmid, as shown in Figure 5, 9-fold luciferase activity (8.89 ± 2.03) was observed under PMA plus ionomycin-stimulation but significantly decreased in a dose-dependent manner by Gt-TRE treatment. In contrast, luciferase activity was not suppressed by Gt-PS treatment but significantly elevated to 14-fold (13.67 ± 5.96) at high dose (500 μg/mL) of Gt-PS (Figure 5(a)). The cytokines secreted from transfected EL-4 cells were also measured to confirm the effects of Gt-TRE and Gt-PS (Figure 5(b)). Th2 cytokine IL-4 concentrations in Gt-TRE treated transfected EL4 T cells decreased with increased doses of Gt-TRE, which is consistent with the luciferase activity. The IL-4 levels in the Gt-PS treated were not significantly affected by Gt-PS. IL-2 productions from transfected cell were not significantly affected by Gt-TRE and Gt-PS treatments (data not shown). These data indicated that Gt-TRE decreased IL-4 production from activated-T cells, which were associated with suppression of transcription factor, NF-κB, and binding activity.

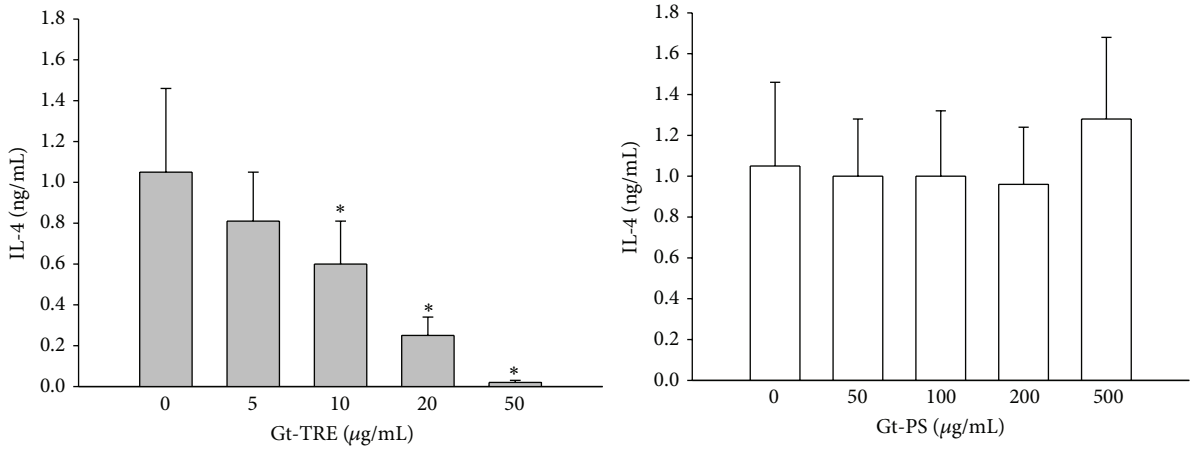
4. Discussion

Ganoderma has been used as traditional herbal medicine in Asia, particularly in China, for thousands of years. Though much attention has been focused on the beneficial effects, recently, *Ganoderma lucidum* is the species mostly studied and published, for example, 997 publications in PubMed, compared to 45 publications for *Ganoderma tsugae* so far. There are more publications of *Ganoderma* polysaccharides than triterpenoids (394 versus 76 publications in PubMed so far). Most studies on *G. tsugae* or triterpenoids are focused on anticancer activities [36]. In this study, we evaluated the immunomodulatory effects of triterpenoid and polysaccharides fractions of *G. tsugae* on allergic responses, using macrophage and T cell lines, primary T helper cells, and NF-κB-driven reporter plasmid transfected macrophage and T cells.

Our previous study demonstrated that *G. tsugae* supplementation decreased histamine release in the BALF and suppressed airway inflammatory responses in a murine model [23]. The present study showed that triterpenoid-rich extract of *G. tsugae* exerts anti-histamine release, but polysaccharide did not affect histamine release from mast cells. Triterpenoid extract possessing antihistamine effect might be the main constituent contributing to the suppression of airway



(a) Luciferase activity



(b) IL-4 production

Figure 5: The NF-κB transcriptional activity and IL-4 production by activated transfected EL4 T cells cultured with different doses of triterpenoids and polysaccharide extracts from *G. tsugae*. EL4 T cells (2×10^5 cells/mL) transiently transfected with NF-κB-promoted luciferase reporter plasmid were treated with Gt-TRE or Gt-PS and stimulated with 50 ng/mL PMA plus 1 μg/mL ionomycin for 24 hrs. Mocks are the EL4 cells transfected with empty vector as the negative control without Gt-TRE or Gt-PS treatment. The data are representative for five independent experiments. $^{\#}0.05 < P < 0.1$, $^{*}P < 0.05$ as compared to the 0 μg/mL control cells.

inflammation observed in asthmatic mice supplemented with *G. tsugae* [23]. Histamine, an inflammatory mediator, not only plays the important role in the pathogenesis of allergic asthma, but also can modulate the Th1/Th2 responses balance [37]. Histamine upregulates Th2 cells proliferation and production of Th2 cytokines, such as IL-4, IL-5, IL-10, and IL-13, and thus drives to development of allergies [38–40]. Recent study suggested that downregulation of the Th1 responses by histamine is one of the mechanisms for Th2 environment [41].

The Th2 immune response is characterized by the development of IL-4 and IL-5-producting effector T cells, which contribute to the allergic responses in several aspects, such as eosinophilia [42]. IL-4, a pleiotropic cytokine mainly produced by activated Th2 cells, basophils, and mast cells, is an important stimulus for the switching of antibody isotype to IgE, which is often found in patients with allergic diseases [43]. Furthermore, IL-4 is crucial for eosinophils and lymphocytes recruitment to the lung and for induction of inflammation [44]. The transcription factor NF-κB binds to its binding site on IL-4 promoter and upregulates IL-4 transcription [45]. Blocking NF-κB activation might suppress IL-4 transcription and reduce IL-4 production and thus attenuate allergic inflammation.

Our previous study also demonstrated that *G. tsugae* supplementation modulated the Th1/Th2 balance [22]. To enhance the frequency of antigen-specific T cells in OVA-sensitized animals for better detection of antigen-specific immune responses, a detectable number of $CD4^+$ T helper cells purified from DO11.10 mice were adopted in this study. Our present study demonstrated that Gt-TRE strongly decreases Th2 cytokines production not only by activated EL4 T cells but also these primary T helper cells during T cell polarization/activation. The dose-dependent decrease in IL-4 production and NF-κB transactivation activity of transfected EL4 T cells confirmed the suppressive effect of triterpenoids from *G. tsugae* on IL-4 production, as shown in *in vivo* asthmatic mice supplemented with Gt-TRE [25]. This

inhibition associated with NF-κB pathway not only in EL4 cells but also in RAW264.7 cells suggested that triterpenoids from *G. tsugae* exert its antiallergic effects *via* downregulation of NF-κB pathway not only in macrophage but also in T cells. Triterpenoid extracts, but not polysaccharide fraction, of *G. tsugae* did suppress NF-κB reporter gene expression and IL-4 production after T cell activation. These data suggested that triterpenoids of *G. tsugae* exert antiallergic effects by inhibiting Th2 cell development and Th2 cytokines production and might go through downregulating NF-κB transcriptional activity.

On the other hand, Gt-PS showed no effect on IL-4 secretion by activated EL-4 cells without or with NF-κB transactivated reporter plasmid but tended to slightly increase IL-4 secretion by primary T helper cells at low doses and significantly decreased IL-4 and IL-5 production at high doses. This might explain the reason why eosinophils in BALF and IL-4 and IL-5 levels were significantly suppressed in OVA-sensitized and challenged allergic mice fed with Gt-TRE alone [25], but not in those fed with *G. tsugae* with both Gt-TRE and Gt-PS [23].

This study indicated that Th1 cells were not affected by triterpenoids from *G. tsugae*. The cytokines IL-2 and IFNγ secreted by activated primary T helper cells and EL-4 T cells were not affected by Gt-TRE. This *in vitro* study is in accordance with observations made in OVA-sensitized mice supplemented with Gt-TRE [25]. In contrast, polysaccharides of *G. lucidum* have been reported to increase T cells proliferation and IL-2 secretion [46, 47]. Our results also showed that Gt-PS enhanced IL-2 secretion in activated primary T helper cells. NF-κB plays a key role on IL-2 expression during T cell development and activation [48]. The Rel A subunit of NF-κB activated IL-2 transcription, whereas it suppressed IL-4 transcription [49]. It demonstrated that NF-κB plays different regulatory role on Th1/Th2 cytokine expression. Other studies also demonstrated that polysaccharide of *G. lucidum* may activate dendritic cells and monocytes and promote Th1 responses *via* NF-κB signal pathway [17, 18, 26]. One study on *G. lucidum* observed that polysaccharide was merely the activator for macrophage but not T lymphocytes [20]. Our study also indicated that polysaccharide of *G. tsugae* enhanced NF-κB transactivation activity in both macrophage and T cells. These results suggested the different actions between Gt-PS and Gt-TRE. The more advanced study will be designed and done in the future to clarify the possible molecular mechanisms of NF-κB in the Th1/Th2 regulation.

In conclusion, triterpenoid extracts of *G. tsugae* inhibited histamine release from mast cells, Th2 cytokines IL-4, and IL-5 productions in T cells and NF-κB activation but did not affect Th1 cytokines IL-2 and IFNγ secretions. Polysaccharides of *G. tsugae* activated NF-κB pathway in macrophages and enhanced IL-2 secretion in T cells but did not affect IL-4 production in T cells until high doses. These data demonstrated that triterpenoid extract is the most effective fraction of *Ganoderma tsugae* that attenuated Th2 response, which is associated with NF-κB transcriptional activity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

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Research Article

Farnesol, a Sesquiterpene Alcohol in Herbal Plants, Exerts Anti-Inflammatory and Antiallergic Effects on Ovalbumin-Sensitized and -Challenged Asthmatic Mice

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To investigate the effect of farnesol on allergic asthma, three farnesol doses were extra-added into AIN-76 feed consumed by ovalbumin- (OVA-) sensitized and -challenged mice continuously for 5 weeks, at approximately 5, 25, and 100 mg farnesol/kg, BW/day. The results showed that there were no significant differences in body weight, feed intake, and visceral organ weights between the farnesol supplementation and dietary control groups. Farnesol supplementation decreased interleukin (IL)-6/IL-10 level ratios in bronchoalveolar lavage fluid (BALF). Farnesol supplementation significantly ($P < 0.05$) restored the cytokine secretion ability of peritoneal macrophages that was suppressed as a result of OVA sensitization and challenge and slightly decreased tumor necrosis factor (TNF-α)/IL-10 cytokine secretion ratios. Farnesol supplementation slightly ($P > 0.05$) decreased IL-4 but significantly ($P < 0.05$) increased IL-2 levels secreted by the splenocytes in the presence of OVA, implying that farnesol might have a systemic antiallergic effect on allergic asthmatic mice. Farnesol supplementation significantly ($P < 0.05$) increased IL-10 levels secreted by the splenocytes in the presence of OVA, suggesting that farnesol might have an anti-inflammatory potential to allergic asthmatic mice. Overall, our results suggest that farnesol supplementation may be beneficial to improve the Th2-skewed allergic asthmatic inflammation.

1. Introduction

Asthma that is an allergic disease estimated to affect at least 300 million people worldwide has attracted much attention recently [1, 2]. Allergic asthma is a chronic airway inflammatory disease accompanied with increased inflammatory cell infiltration, lung inflammation, and airway hyperresponsiveness. Asthma results from airway inflammation involving a diversity of activated cells including mast cells, eosinophils, T-lymphocytes, neutrophils, macrophages, and epithelial cells. These cells are recruited to the site and release proinflammatory cytokine mediators that augment and regulate airway inflammation, resulting in airway hyperresponsiveness responsible for the chronic symptoms of dyspnea, wheezing, and chest tightness [3]. Airway inflammation results in denudation of bronchial epithelium and thickening of subepithelial basement membrane due to deposition of collagen. In addition, severe asthma has been characterized by occlusion of the bronchial lumen by mucus, hyperplasia and hypertrophy of the bronchial smooth muscle, and goblet cell hyperplasia [3]. Therefore, the levels of mucus (possibly mucin) and other proteins in the bronchial lumen may be selected as an inflammatory marker.

There are two distinct helper T lymphocytes, type 1 helper T (Th1) and Th2 cells that synthesize differential cytokines to influence immune responses. Interleukin (IL)-1, tumour necrosis factor (TNF), and IL-6 produced by Th1, Th2 lymphocytes, or other inflamed cells that highlight the way and trigger local inflammation within injured tissues can be roughly classified as proinflammatory cytokines [4]. In contrast, IL-10 produced by Th2 cells, T regulatory cells (Th3 cells), macrophages, and some B cells to inhibit Th1 synthesis and other cytokines and macrophage functions during the late inflammation phase are recognized as anti-inflammatory cytokines [5]. An imbalance in Th1/Th2 immune response patterns and pro/anti-inflammatory cytokines produced and

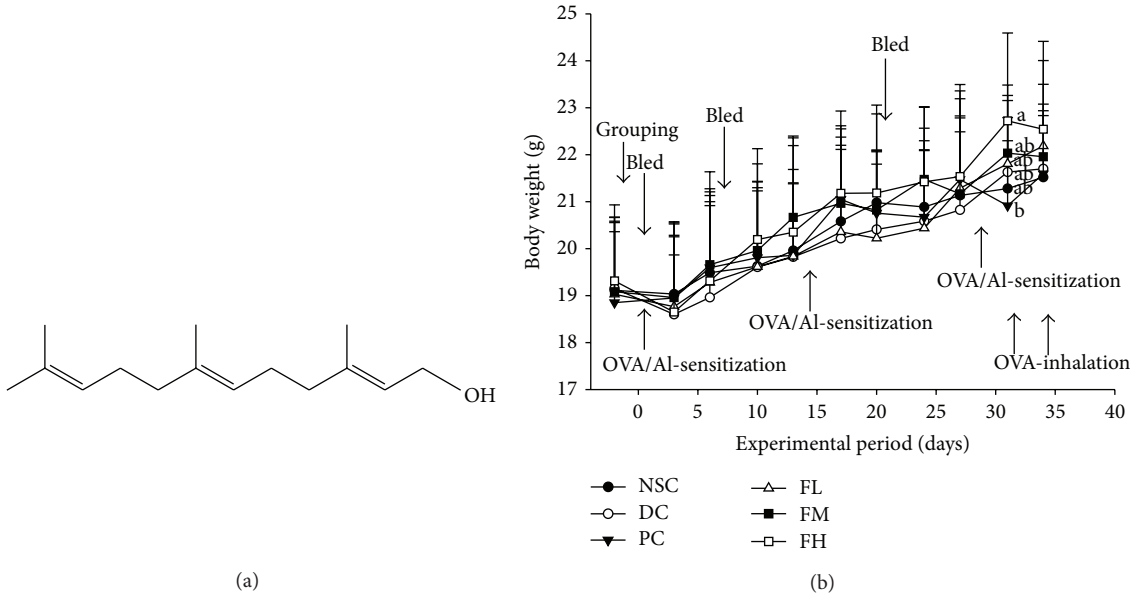

Figure 1: Farnesol structure (a) and its supplementation effects with different doses for 5 weeks on body weight changes of OVA/AL-sensitized and -challenged BALB/c asthmatic mice (b). Values are means ± SD (n = 12–15). Values among groups at the same experimental point not sharing a common small letter are significantly different ($P < 0.05$) from each other and assayed by one-way ANOVA, followed by Duncan's new multiple range test. NSC, nonsensitized control; DC, dietary control; PC, positive control (dexamethasone, 3 mg/kg BW, 0.3 mL/mouse by gavage); FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

generally accompanied with stress hormones may result in differential diseases, for example, persistent infections, severe immunosuppression, autoimmunity, allergy/atopy, tumour growth, and chronic graft-versus-host disease [6, 7]. Allergic asthma is characterized as a Th2-skewed disease. Regulation of the Th1/Th2 imbalance and anti/proinflammatory cytokine expression profiles in the host may avoid immune disorder diseases [8]. Potential phytochemicals from different food materials or herbs recently shed light on immunomodulation and may be beneficial for the corresponding human diseases [9].

Many drugs are used to treat asthma, such as inhaled corticosteroids, leukotriene inhibitors, mast cell stabilizers, and β2-adrenergic agonists that control the inflammation responses resulting from nitric oxide (NO), proinflammatory enzymes, and cytokines produced by macrophages [10]. The overaccumulation of proinflammatory mediators may cause severe damage to the heart, lung, and nerve system. At present, inhaled glucocorticoid is widely used to treat asthma; however, about 50% of asthma patients are not improved by the drug, which may induce adverse side effects, suggesting that alternative agents need to be developed [11]. Asthma exacerbations and early manifestations of the disease must be prevented to stop the diseases' evolution to severe asthma [12]. Therefore, natural traditional herbal medicines, health foods, and their possible active compounds show potential in treating asthma.

Among the possible active compounds against asthma, farnesol was found to have potential. Farnesol is a sesquiterpene alcohol that exists widely in fruits such as peaches, vegetables such as tomatoes and corn, herbs such as lemon grass and chamomile, and in the essential oils of ambrette seeds and citronella [13, 14]. Farnesol is found to alleviate massive inflammation, oxidative stress, and lung injury induced by the intratracheal instillation of cigarette smoke extract in rats [15]. Farnesol ameliorates 1,2-dimethylhydrazine induced oxidative stress, inflammation, and apoptotic in the colon of Wistar rats [14]. Farnesol has been shown to be potent in treating antimetabolic disorders, anti-inflammation, showing antioxidant, anticancer, and antibiotic effects [14, 16, 17]. Recently, we found that farnesol exhibited a relative Th1-inclination and anti-inflammatory property on immune cells that may be applied to improve Th2-skewed allergic asthmatic inflammation *in vivo* [18].

We hypothesized that farnesol has immunomodulation potential against allergic asthmatic inflammation. To validate this assumption, farnesol at different doses was administered to ovalbumin- (OVA-) sensitized and challenged mice for 5 weeks. The anti-inflammatory effects of farnesol supplementation on the experimental mice were determined.

2. Materials and Methods

2.1. Chemicals. Farnesol that is a sesquiterpene alcohol ($C_{15}H_{26}O$) in many plants was purchased at the highest available purity (>95%, a mixture of isomers) (Sigma, St. Louis, MO, USA). The chemical structure is shown in Figure 1(a).

2.2. Animal Grouping and Feeding. The experimental feed was prepared according to the American Institute of

Nutrition AIN-76 recommendation that satisfies the nutritional requirement for mouse growth and varied only in farnesol composition [19]. Three farnesol doses, low dose (0.003%), medium dose (0.017%), and high dose (0.067%), were added to the AIN-76 feed (Table 1) [20]. Each feed was prepared by thoroughly mixing in farnesol and storing at −20°C. Approximately, 3 grams of AIN-76 feed was consumed per day by each individual mouse with 20 grams of body weight (BW). Farnesol low (FL), medium (FM), and high (FH) doses, respectively, corresponding to 5, 25, and 100 mg farnesol/kg BW/day, were designed for the experimental mice. It could be estimated that farnesol supplementation at the indicated doses might not produce significant energy *in vivo*. The energy contribution of each experimental diet was 67.5% from carbohydrate, 20.8% from protein, and 11.7% from fat. The calorie density of each diet was 3.85 Kcal/g. The animal use protocol listed below was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan.

In our preliminary similar experiments, we found that the results from either male or female mice had same trend; however, female mice were more sensitive to treatments than male mice. Therefore, we selected female mice as our experimental mice in our following studies. The results from female mice in immunology have been accepted in many published papers. The female BALB/cByJNarl mice (7 weeks old) were obtained from the National Laboratory Animal Center, National Applied Research Laboratories, National Science Council in Taipei, and maintained in the Department of Food Science and Biotechnology at National Chung Hsing University College of Agriculture and Natural Resources in Taichung, Taiwan. The animal room was kept on a 12 h light and 12 h dark cycle. Constant temperature (25 ± 2°C) and humidity were maintained. The mice were housed and kept on a chow diet (laboratory standard diet, Diet MF 18, Oriental Yeast Co., Ltd., Osaka, Japan) to acclimatize for 1 week before feeding the experimental diet. After this equilibrium period, the mice were divided randomly into six groups ($n = 15$) varied by farnesol doses and sensitized treatments. The treatments were nonsensitized control (treated with phosphate-buffered saline (PBS) and alum ($Al(OH)_3$), namely, PBS/AL, coded as NSC), dietary control (treated with OVA and alum, namely, OVA/AL, coded as DC), farnesol low dose (treated with OVA/AL, supplemented with low dose farnesol about 5 mg/kg BW/day, coded as FL), farnesol medium dose (treated with OVA/AL, supplemented with medium dose farnesol about 25 mg/kg BW/day, coded as FM), farnesol high dose (treated with OVA/AL, supplemented with high dose farnesol about 100 mg/kg BW/day, coded as FH), and positive control (treated with OVA/AL, treated with dexamethasone, coded as PC). NSC group is nonsensitized control group that is intraperitoneally injected with PBS and alum. NSC group is not suitable for a negative group in this study because it still induced mild inflammatory effects. Unfortunately, the experiment neglected to select normal mice that were not treated with any agents for a negative group. The initial average body weight of each group showed no significant differences among groups. Mice in each group were fed with the specified experimental diet *ad libitum* for 35 consecutive days. Mouse food intake and body weight were measured twice a week during the study period [20].

Table 1: AIN-76 and farnesol feed formula ingredients.

Ingredients (g/kg diet)	Formulas			
	AIN-76	FL	FM	FH
Casein	200	200	200	200
DL-methionine	3	3	3	3
Corn starch	150	150	150	150
Sucrose	500	500	500	500
Fiber	50	50	50	50
Soybean oil	50	50	50	50
Mineral mixture	35	35	35	35
Vitamin mixture	10	10	10	10
Choline bitartrate (41% choline)	2	2	2	2
Farnesol	0	38 μL	188 μL	753 μL
Farnesol (%)	0	0.003	0.017	0.067
Suggested mg farnesol/kg BW/day	0	5	25	100
Calorie density (Kcal/g)	3.85			
Nutrients (% of total calories)				
Carbohydrate	67.5%			
Protein	20.8%			
Fat	11.7%			
Total	**100%**			

FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

2.3. OVA-Sensitized and -Challenged Allergic Asthmatic Inflammation Mouse Model. The mice (8 weeks old) were sensitized and challenged to induce allergic airway inflammation. Mice were sensitized using an intraperitoneal injection (i.p.) of 0.2 mL alum-precipitated antigen containing 8 μg of ovalbumin (OVA, albumin chicken egg grade III, Sigma-Aldrich Co., St. Louis, MO, USA) and 2 mg $Al(OH)_3$ (Sigma-Aldrich Co., St. Louis, MO, USA) to induce primary immunity and started the specified experimental diets (at day 0). Two booster injections of this alum-OVA mixture were given 14 and 28 days later, respectively. Nonsensitized control mice received alum-phosphate-buffered saline (PBS, 137 mM NaCl (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2.7 mM KCl (Sigma-Aldrich Co., St. Louis, MO, USA), 8.1 mM Na_2HPO_4 (Sigma-Aldrich Co., Steinheim, Germany), 1.5 mM KH_2PO_4 (Sigma-Aldrich Co., St. Louis, MO, USA), pH 7.4, 0.2 μm filtered) only. At days 31 and 34, the OVA-sensitized mice were challenged using aerosolized OVA at a concentration of 5 mg OVA per milliliter PBS for 60 min (namely, 9:00 am for 30 min and 4:00 pm for 30 min). The aerosolized OVA was produced using an ultrasonic nebulizer (sw918, Shinmed, Taipei, Taiwan). Nonsensitized control mice received only PBS [21]. Two hours before aerosolized OVA was administered, the PC group was treated with dexamethasone (DEX, 3 mg/kg BW, 0.3 mL/mouse, Sigma, St. Louis, MO, USA) by gavage to reduce allergic asthmatic inflammation [22]. Two days later (at day 36), all of

the animals were anesthetized, exsanguinated using retroorbital venous plexus puncture, and immediately euthanized by CO_2 inhalation (Figure 1(b)). The bronchoalveolar lavage fluid (BALF), peritoneal macrophages, spleen, and visceral organs were collected and assayed for cytokines and other inflammatory mediators [20].

2.4. BALF Collection. After the mice were euthanized, the airways and the lungs were immediately lavaged aseptically using a cannula through the trachea with 5 aliquots of 0.6 mL Hank's balanced salts solution (HBSS), free of ionized calcium and magnesium (HyClone Laboratories Inc., Logan, UT, USA). The BALF was centrifuged at 400 ×g for 10 min at 4°C. The supernatant (BALF) volume was determined and stored at −80°C for future assay [23].

2.5. Peritoneal Macrophage Isolation and Culture. After BALF collection, peritoneal macrophages from the experimental mice were collected according to the method described by Lin et al. [24]. Peritoneal cells were prepared by lavaging the peritoneal cavity with 2 aliquots of 5 mL sterile Hanks' balanced salts solution (HBSS) (50 mL of 10x HBSS (HyClone Laboratories Inc., Logan, UT), 2.5 mL of antibiotic-antimycotic solution (100x PSA) containing 10,000 units/mL of penicillin, 10 mg/mL of streptomycin, 25 μg/mL of amphotericin B in 0.85% saline (Atlanta Biologicals Inc., Norcross, GA), 20 mL of 3% bovine serum albumin (BSA, Sigma-Aldrich Co., St. Louis, MO) in phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.20 μm filtered), 2.5 mL of 7.5% $NaHCO_3$ (Wako, Osaka, Japan), and 425 mL of sterile water) for a total of 10 mL through peritoneum. The peritoneal lavage fluid was centrifuged at 400 ×g for 10 min at 4°C. The cell pellets were collected and resuspended in tissue culture medium (TCM, a serum replacement; Celox Laboratories Inc., Lake Zurich, IL), suspended in a medium consisting of 10 mL TCM, 500 mL RPMI 1640 medium (Atlanta Biologicals Inc.), and 2.5 mL antibiotic-antimycotic solution (100x PSA) (Atlanta Biologicals Inc.). The peritoneal adherent cells (>90% of macrophages) from each animal were adjusted to 2×10^6 cells/mL in TCM medium with a hemocytometer using the trypan blue dye exclusion method. The peritoneal macrophages (0.5 mL/well) in the absence or presence of lipopolysaccharide stimulus (LPS) (L-2654, Sigma-Aldrich Co., St. Louis, MO; at the final concentration of 2.5 μg/mL, 0.5 mL/well) were cultured in 48-well plates. LPS, an endotoxin from Gram-negative bacteria, was selected to augment macrophages' inflammation *in vitro* [25, 26]. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for 48 h. The plates were then centrifuged at 400 ×g for 10 min to obtain the cell culture supernatants. The cell culture supernatants were collected for cytokine assay using enzyme-linked immunosorbent assay (ELISA).

2.6. Tissue Collection and Analysis. The thoracic and abdominal cavities of the experimental mice were opened aseptically. The organs such as heart, lung, liver, spleen, and kidney were removed and weighted. To evaluate the possible effects of farnesol supplementation on the body and visceral organs, the visceral organs weights in each experimental group were expressed as absolute and relative visceral organ weights, respectively [27]. The relative organ weight (%) was computed using the following equation: relative tissue (or organ) weight (%) = (individual tissue (or organ) weight (g)/body weight (g) of the experimental mouse) × 100.

2.7. Splenocytes Isolation and Cultures. The splenocytes were prepared by aseptically removing spleens from the experimental BALB/c mice. The spleen was ground with the flat bottom of a syringe piston to homogenize the splenocytes. Splenocytes were centrifuged at 400 ×g for 7 min at 25°C. The cell pellets were resuspended in 10 mL of red blood cell (RBC) lysis buffer (0.017 M Trizma Base (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.144 M NH_4Cl (Sigma-Aldrich Co., St. Louis, MO, USA) in deionized water, pH 7.2, 0.2 μm filtered) for 3 minutes and centrifuged at 400 ×g for 7 min at 25°C. The cell pellets were washed with HBSS three times. Splenocytes were resuspended in TCM medium that contained 20% of TCM Serum Replacement (Protide Pharmaceuticals Inc., Illinois, USA) and 0.5% of Penicillin-Streptomycin Amphotericin B Solution in RPMI 1640 medium (HyClone, Utah, USA). The cells were counted with a hemocytometer using the trypan blue dye exclusion method. The cell density was adjusted to 1×10^7 cells/mL in TCM medium. The isolated splenocyte suspensions (5×10^6 cells/mL) were plated into 48-well plates and, respectively, incubated in the absence or presence of OVA (30 μg/mL). The plates were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air for 48 h. The cell culture supernatants were collected and stored at −80°C for cytokine assays.

2.8. Inflammatory Mediators and Markers Assay in BALF

2.8.1. Nitric Oxide (NO) Assay. Eighty μL aliquots of BALF samples and standards (0–100 μM sodium nitrite (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in double distilled water) were pipetted into the 96-microplate wells (Nunc, Thermo Fisher Scientific, Rockford, IL, USA). One hundred sixty μL aliquots of Griess reagent were then added into each well to develop the color. The Griess reagent was freshly prepared from Reagents A and B at a ratio of 1 : 1 (Reagent A: 1% (w/v) sulfanilamide (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in 5.0% (v/v) phosphoric acid (Riedel-de Haen, Seelze, Germany); Reagent B: 0.1% (w/v) N-(1-naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich Co., St. Louis, MO, USA) dissolved in deionized water). After incubation for 5 min, the plate was read on a plate reader (ELISA reader, ASYS Hitech, GmbH, Austria) at 550 nm. Using the standard curve, the NO concentration for each unknown sample was determined [20].

2.8.2. Protein Level Assay. The BALF protein content was analyzed using bicinchoninic acid (BCA) protein assay (Thermo Scientific Inc., Rockford, USA), according to the accompanying instructions using a 96-well microtiter plate [23].

Table 2: Effects of farnesol supplementation with different doses for 5 weeks on initial and final body weights and food and energy intake, as well as feed and energy efficiencies of OVA/AL-sensitized and -challenged BALB/c asthmatic mice.

Groups	n	Initial body weight (g)	Final body weight (g)	Body weight gain (g/d)	Feed intake (g/d)	Energy intake (kcal/day)	Feed efficiency (g gain/100 g feed)	Energy efficiency (g gain/100 kcal feed)
DC	13	19.1 ± 1.6	21.7 ± 0.8	0.07 ± 0.02	4.28 ± 0.62	16.5 ± 2.4	1.74 ± 0.57	0.45 ± 0.06
FL	14	19.1 ± 1.4	22.3 ± 1.5	0.09 ± 0.02	4.37 ± 0.46	16.8 ± 1.8	2.06 ± 0.49	0.54 ± 0.13
FM	12	19.2 ± 1.5	22.0 ± 1.4	0.08 ± 0.02	4.81 ± 0.27	18.5 ± 1.0	1.67 ± 0.27	0.43 ± 0.07
FH	14	19.2 ± 1.5	22.4 ± 1.8	0.09 ± 0.02	4.70 ± 0.34	18.1 ± 2.0	1.97 ± 0.38	0.51 ± 0.10
PC	14	19.2 ± 1.6	21.8 ± 0.9	0.07 ± 0.02	4.55 ± 0.31	17.5 ± 1.2	1.65 ± 0.60	0.43 ± 0.16
NSC	15	19.1 ± 1.4	21.5 ± 1.0	0.07 ± 0.02	3.82 ± 0.40	14.7 ± 1.5	1.81 ± 0.78	0.47 ± 0.20

Values are means ± SD. There are no significant differences among groups within same column assayed by one-way ANOVA, followed by Duncan's new multiple range test. NSC, nonsensitized control; DC, dietary control; PC, positive control; FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

2.8.3. Eotaxin Concentration. The BALF eotaxin concentration was determined using the mouse eotaxin sandwich ELISA kit (Quantikine M murine, R&D Systems, Minneapolis, MN, USA). The eotaxin concentration was assayed according to the manufacturer's instructions. The sensitivity of this assay was <3.9 pg/mL [20].

2.9. Measurement of Cytokine Levels in BALF and Peritoneal Macrophage Cultures Using ELISA. Cytokine (IL-4, IL-5, IL-2, IFN-γ, IL-1β, IL-6, TNF-α, and IL-10) levels in BALF, peritoneal macrophages, or splenocytes cultures were determined using sandwich ELISA kits, respectively. These cytokine concentrations were assayed according to the cytokine ELISA protocol of manufacturer's instructions (mouse DuoSet ELISA Development system, R&D Systems, Minneapolis, MN, USA). The sensitivity of these cytokine assays was <3.9 pg/mL [28].

2.10. Statistical Analysis. Data are expressed as mean ± SD. Data among groups were analyzed using analysis of variance (ANOVA), followed by Duncan's new multiple range test. Differences among groups were considered statistically significant if $P < 0.05$. Statistical tests were performed using SPSS version 12.0.

3. Results

3.1. Effects of Farnesol Supplementation on Intake and Growth of OVA-Sensitized and -Challenged Mice. The body weight and experimental procedure during the experimental period are given in Figure 1(b). The mean body weight of all experimental mice increased slightly as the experimental period was extended. There were no significant differences in mean body weight between the DC and other groups at each same experimental point. The feed efficiency and body weight changes in the experimental mice are shown in Table 2. There were no significant differences in the initial and final body weight, gain in body weight, feed intake, feed efficiency, and energy efficiency among groups. In the farnesol-treated mice, their mean feed intakes were 4.37 (FL), 4.81 (FM), and 4.70 g feed/day/mouse (FH), respectively. Three farnesol doses, low dose (0.003%), medium dose (0.017%), and high dose (0.067%), were added to the AIN-76 feed (Table 1). Therefore, the actual delivered doses to the farnesol-treated mice were 0.1311 (FL), 0.8177 (FM), and 3.149 mg farnesol/mouse/day (FH). Our results indicated that the farnesol treatment doses adopted in this study had no apparent toxic effects.

3.2. Effects of Farnesol Supplementation on Body and Visceral Organ Weights of OVA-Challenged Mice. The OVA-challenged mice were fed with different doses of farnesol for 5 weeks to evaluate farnesol effects on asthmatic inflammation. The results showed that farnesol supplementation did not significantly affect the liver, kidney, thymus, and heart weight among groups, indicating no toxic effect from farnesol treatments on the visceral organs (Table 3). However, OVA sensitization and challenge resulted in a slight increase ($P > 0.05$) in the spleen weight of BALB/c mice, indicating that a mild systemic inflammation was induced in the OVA-challenged mice. Importantly, DEX treatment (PC group) significantly decreased ($P < 0.05$) absolute and relative spleen and thymus weights compared to those in the DC group, indicating that DEX treatment effectively decreased systemic inflammation in the OVA-challenged mice. The spleen and thymus, particularly the spleen, are immune organs in the body that reflect systemic inflammation status. Farnesol treatments (FL, FM, and FH groups) just slightly, but not significantly ($P > 0.05$), decreased absolute and relative spleen and thymus weights, implying that farnesol treatments might alleviate systemic inflammation a little in the OVA-challenged mice. Interestingly, OVA sensitization and challenge caused a slight decrease ($P > 0.05$) in the epididymal fat weight of BALB/c mice, indicating a decrease in fat deposition resulting from allergic asthma in the OVA-challenged mice. However, farnesol and DEX treatment obviously increased the epididymal fat weight in the OVA-challenged mice, indicating that both farnesol and DEX treatments might alleviate the epididymal fat loss and improve nutrition status in the experimental mice.

Table 3: Effects of farnesol supplementation with different doses for 5 weeks on absolute and relative weights of visceral organs of OVA/AL-sensitized and -challenged BALB/c asthmatic mice.

Organ	Group	DC	FL	FM	FH	PC	NSC
	n	13	14	12	14	14	15
Spleen	ATW (g)	0.15 ± 0.03^{a}	0.14 ± 0.01^{a}	0.14 ± 0.02^{a}	0.14 ± 0.03^{a}	0.12 ± 0.01^{b}	0.15 ± 0.03^{a}
	RTW (%)	0.76 ± 0.16^{a}	0.64 ± 0.21^{ab}	0.68 ± 0.11^{ab}	0.70 ± 0.15^{ab}	0.58 ± 0.38^{b}	0.72 ± 0.11^{a}
Liver	ATW (g)	1.29 ± 0.11	1.33 ± 0.11	1.29 ± 0.13	1.29 ± 0.17	1.31 ± 0.11	1.36 ± 0.21
	RTW (%)	6.56 ± 0.32	6.51 ± 0.28	6.31 ± 0.51	6.25 ± 0.61	6.67 ± 0.08	6.61 ± 0.68
Kidney	ATW (g)	0.30 ± 0.03	0.30 ± 0.06	0.32 ± 0.02	0.31 ± 0.03	0.30 ± 0.02	0.30 ± 0.04
	RTW (%)	1.53 ± 0.12	1.44 ± 0.20	1.42 ± 0.46	1.52 ± 0.10	1.54 ± 0.05	1.49 ± 0.18
Heart	ATW (g)	0.12 ± 0.02	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01
	RTW (%)	0.61 ± 0.07	0.58 ± 0.06	0.61 ± 0.04	0.57 ± 0.18	0.61 ± 0.01	0.58 ± 0.03
Thymus	ATW (g)	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.01^{a}	0.02 ± 0.00^{a}	0.01 ± 0.00^{b}	0.03 ± 0.01^{a}
	RTW (%)	0.11 ± 0.03^{a}	0.12 ± 0.04^{a}	0.11 ± 0.03^{a}	0.12 ± 0.02^{a}	0.04 ± 0.16^{b}	0.13 ± 0.04^{a}
Epididymal fat	ATW (g)	0.34 ± 0.13^{b}	0.40 ± 0.08^{ab}	0.40 ± 0.09^{ab}	0.40 ± 0.12^{ab}	0.47 ± 0.12^{a}	0.42 ± 0.16^{ab}
	RTW (%)	1.71 ± 0.60^{b}	1.70 ± 0.79^{b}	1.95 ± 0.41^{ab}	1.94 ± 0.57^{ab}	2.38 ± 0.06^{a}	2.02 ± 0.78^{ab}

Values are means ± SD (n = 12–15). Values within same row not sharing a common small letter are significantly different ($P < 0.05$) from each other and assayed by one-way ANOVA, followed by Duncan's new multiple range test. NSC, nonsensitized control; DC, dietary control; PC, positive control; FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed); ATW, absolute tissue weight; RTW, relative tissue weight.

Table 4: Effects of farnesol supplementation with different doses for 5 weeks on proinflammatory and anti-inflammatory cytokine levels in BALF of OVA/AL-sensitized and -challenged BALB/c asthmatic mice.

Groups	Proinflammatory and anti-inflammatory cytokines secreted in BALF				Pro/anti-inflammatory cytokine ratios	
	IL-1β (pg/mL)	IL-6 (pg/mL)	TNF-α (pg/mL)	IL-10 (pg/mL)	TNF-α/IL-10 (pg/pg)	IL-6/IL-10 (pg/pg)
DC	35.2 ± 22.8	34.6 ± 8.6	57.2 ± 13.0	60.0 ± 18.4	0.98 ± 0.27	0.60 ± 0.22^{ab}
FL	32.8 ± 3.9	20.9 ± 18.3	50.6 ± 15.1	60.5 ± 14.8	0.84 ± 0.15	0.36 ± 0.24^{b}
FM	34.3 ± 13.4	28.9 ± 22.0	48.9 ± 22.9	60.2 ± 24.2	0.81 ± 0.16	0.48 ± 0.32^{ab}
FH	35.8 ± 5.6	23.4 ± 12.7	49.5 ± 9.6	55.5 ± 12.6	0.95 ± 0.10	0.37 ± 0.12^{b}
PC	32.0 ± 3.3	21.4 ± 12.8	39.7 ± 7.9	51.8 ± 14.1	0.83 ± 0.19	0.40 ± 0.18^{b}
NSC	28.3 ± 8.7	39.7 ± 21.0	54.3 ± 19.2	58.9 ± 15.7	0.91 ± 0.24	0.64 ± 0.26^{a}

Values are means ± SD (n = 9–13). Values within same column not sharing a common small letter are significantly different ($P < 0.05$) from each other and assayed by one-way ANOVA, followed by Duncan's new multiple range test. The limit of detection (LOD) of these kits used in this study was <3.9 pg/mL. NSC, nonsensitized control; DC, dietary control; PC, positive control; FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

3.3. Effects of Farnesol Supplementation on Cytokine and Inflammatory Mediator and Marker Levels in BALF of OVA-Challenged Mice. Table 4 shows proinflammatory and anti-inflammatory cytokine levels in BALF of OVA-sensitized and -challenged asthmatic mice. The results showed that there were no significant differences in IL-1β, IL-6, TNF-α, and IL-10 levels, as well as TNF-α/IL-10 (pro/anti-inflammatory) cytokine level ratios in BALF among the differential treatments, suggesting that there was just a mild inflammation induced in the airways and lungs in this animal model. Further comparison with proinflammatory and anti-inflammatory cytokine level ratios showed that both farnesol and DEX treatments slightly, but not significantly ($P > 0.05$), decreased IL-6/IL-10 (pro/anti-inflammatory) cytokine level ratios in BALF. These results suggest that both farnesol and DEX treatments just slightly alleviate inflammation status in the lungs and airways of allergic asthmatic mice through decreasing pro/anti-inflammatory cytokine level ratios.

Table 5 shows the nitric oxide (NO), protein, and eotaxin levels in BALF. Unfortunately, there were no significant differences between the farnesol treatments and DC group. We presumed that there was just a mild inflammation induced in the airways and lungs in this animal model. Therefore, there is no significant difference in the eotaxin level in BALF between NSC and DC groups.

3.4. Effects of Farnesol Supplementation on Proinflammatory and Anti-Inflammatory Cytokine Secretion Levels in Peritoneal Macrophage Cultures from OVA-Challenged Mice. Table 6 shows the secretion levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α as well as an anti-inflammatory cytokine IL-10 in peritoneal macrophage cultures from

Table 5: Effects of farnesol supplementation with different doses for 5 weeks on inflammatory mediator and marker levels in BALF of OVA/AL-sensitized and -challenged BALB/c asthmatic mice.

		Inflammatory mediator levels in BALF		
Groups	n	NO (μM)	Protein (μg/mL)	Eotaxin (pg/mL)
DC	9	5.89 ± 1.66	99.9 ± 12.4^{abc}	278 ± 130^{ab}
FL	11	5.22 ± 1.42	103 ± 22^{ab}	382 ± 109^{a}
FM	9	5.40 ± 1.33	106 ± 10^{a}	323 ± 159^{ab}
FH	11	6.42 ± 1.51	94.7 ± 12.7^{abc}	298 ± 73^{ab}
PC	11	5.68 ± 0.85	86.4 ± 7.6^{c}	241 ± 79^{b}
NSC	13	5.40 ± 1.42	90.0 ± 15.6^{bc}	270 ± 96^{ab}

Values are means ± SD. Values within same column not sharing a common small letter are significantly different ($P < 0.05$) from each other and assayed by one-way ANOVA, followed by Duncan's new multiple range test. NSC, nonsensitized control; DC, dietary control; PC, positive control; FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

OVA-sensitized and -challenged mice through 5 weeks of feeding. In general, macrophages that are typical inflammatory cells should secret diverse cytokines when stimulated. However, our results showed that sensitization and challenge with OVA significantly inhibited ($P < 0.05$) proinflammatory and anti-inflammatory cytokine secretion levels by peritoneal macrophages in the absence or presence of LPS, implying that the decreased immunity might appear in allergic asthmatic mice. Importantly, both farnesol and DEX treatments significantly increased ($P < 0.05$) proinflammatory and anti-inflammatory cytokine secretion levels by peritoneal macrophages in the absence or presence of LPS as compared to those in the DC group, implying that both farnesol and DEX treatments increased immunity that might be suppressed as a result of allergic inflammation in allergic asthmatic mice. Further comparison with proinflammatory and anti-inflammatory cytokine secretion level ratios showed that farnesol treatments obviously decreased TNF-α/IL-10 (pro/anti-inflammatory) cytokine secretion level ratios. These results suggest that farnesol treatments alleviate systemic inflammation status through decreasing pro/anti-inflammatory cytokine secretion level ratios in peritoneal macrophages from allergic asthmatic mice. The farnesol treatment effects were better than DEX treatment (Table 6), implying that farnesol may be used to improve allergic inflammation in asthma patients in the future.

3.5. Effects of Farnesol Supplementation on Th1/Th2 Cytokine Secretion Levels in Splenocyte Cultures from OVA-Challenged Mice. To evaluate the effects of farnesol supplementation on systemic immune response in asthmatic subjects, Th1/Th2 cytokine levels in the splenocyte cultures from OVA-challenged mice were determined. The levels of Th1 (IL-2 and IFN-γ) and Th2 (IL-4, IL-5, and IL-10) cytokines in the splenocyte cultures in the absence or presence of OVA from OVA-challenged mice fed different experimental diets through 5 weeks are shown in Figure 2. The results showed that spontaneous cytokine secretion levels of Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-5), except IL-10, were too low to be detected. However, both Th2 (IL-4, IL-5, and IL-10) and Th1 (IL-2 and IFN-γ) cytokine secretion levels in splenocyte cultures in the presence of OVA were significantly ($P < 0.05$) increased as compared to those in the absence of OVA. All Th1 and Th2 except IL-10 cytokine secretion levels in DC group were significantly ($P < 0.05$) higher than those in NSC group. Moreover, spontaneous cytokine secretion profiles exhibited that Th2 (IL-4 + IL-5 + IL-10)/Th1 (IL-2 + IFN-γ) secretion ratios in DC group were significantly higher than those in NSC group (Figure 2(f)). These data indicated that OVA sensitization and challenge indeed induced a Th2-skewed systemic OVA-specific immune response, reflecting in the spleen. Farnesol supplementation slightly ($P > 0.05$) decreased IL-4 (a Th2 cytokine) (Figure 2(a)) but significantly ($P < 0.05$) increased IL-2 (a Th1 cytokine) (Figure 2(d)) levels secreted by the splenocytes in the presence of OVA, implying that farnesol supplementation might have an antiallergic effect on allergic asthmatic mice. The IL-10 level in NSC group was slightly higher than that in DC group, implying that the decreased immunity might appear in allergic asthmatic mice (Figure 2(c)). Furthermore, farnesol supplementation significantly ($P < 0.05$) increased IL-10 (a Th2 and anti-inflammatory cytokine) levels secreted by the splenocytes in the presence of OVA, suggesting that farnesol supplementation might also have an anti-inflammatory potential to allergic asthmatic mice (Figure 2(c)). Unfortunately, high dose farnesol supplementation (FH group) just slightly ($P > 0.05$) decreased Th2 (IL-4 + IL-5 + IL-10)/Th1 (IL-2 + IFN-γ) secretion ratios by the splenocytes in the absence or presence of OVA as compared to those in DC group (Figure 2(f)). Consequently, DEX treatments significantly ($P < 0.05$) decreased IL-4, IL-5 (Th2), and IFN-γ (Th1) but increased IL-10 (anti-inflammatory) cytokine secretion levels by the splenocytes in the presence of OVA as compared to those in DC group, implying that DEX treatments might inhibit immunity but have strong anti-inflammatory potential in allergic asthmatic mice (Figure 2). However, DEX treatments increased Th2 (IL-4 + IL-5 + IL-10)/Th1 (IL-2 + IFN-γ) secretion ratios by the splenocytes in the absence or presence of OVA as compared to those in DC group (Figure 2(f)), suggesting that DEX treatments might aggravate the Th2-skewed inclination in allergic asthmatic mice.

4. Discussion

In the present study, there was no significant influence of farnesol supplementations on intake and growth, indicating

Table 6: Effects of farnesol supplementation with different doses for 5 weeks on proinflammatory and anti-inflammatory cytokine secretions by peritoneal macrophages of OVA/AL-sensitized and -challenged BALB/c asthmatic mice.

Cytokines secreted by splenocytes	Groups	Treatment	
		Spon.	LPS
IL-1β (pg/mL)	DC	8.39 ± 3.88^{b}	ND
	FL	14.0 ± 3.4^{ab}	11.8 ± 8.4^{ab}
	FM	11.9 ± 3.4^{b}	18.5 ± 13.2^{a}
	FH	12.4 ± 8.0^{b}	18.5 ± 13.2^{a}
	PC	11.5 ± 6.2^{b}	8.3 ± 8.0^{bc}
	NSC	$\mathbf{18.1 \pm 10.6}^{a}$	$\mathbf{18.4 \pm 16.1}^{a}$
IL-6 (pg/mL)	DC	159 ± 95^{d}	537 ± 156^{c}
	FL	270 ± 124^{cd}	1047 ± 640^{bc}
	FM	943 ± 528^{a}	1915 ± 1066^{a}
	FH	477 ± 278^{bc}	1215 ± 786^{b}
	PC	515 ± 292^{bc}	1076 ± 619^{bc}
	NSC	$\mathbf{595 \pm 338}^{b}$	$\mathbf{1231 \pm 824}^{b}$
TNF-α (pg/mL)	DC	192 ± 73^{b}	332 ± 126^{b}
	FL	250 ± 44^{ab}	399 ± 74^{ab}
	FM	271 ± 85^{ab}	506 ± 96^{a}
	FH	213 ± 141^{ab}	356 ± 178^{b}
	PC	301 ± 98^{a}	505 ± 177^{a}
	NSC	$\mathbf{301 \pm 148}^{a}$	$\mathbf{501 \pm 215}^{a}$
IL-10 (pg/mL)	DC	48.6 ± 35.1^{c}	91.0 ± 41.0^{b}
	FL	91.7 ± 36.7^{ab}	166 ± 59^{a}
	FM	129.3 ± 83.9^{a}	193 ± 79^{a}
	FH	65.9 ± 47.2^{bc}	151 ± 80^{a}
	PC	80.1 ± 46.0^{bc}	159 ± 85^{a}
	NSC	$\mathbf{80.9 \pm 32.0}^{bc}$	$\mathbf{150 \pm 63}^{a}$
TNF-α/IL-10 (pg/pg)	DC	3.89 ± 1.38^{ab}	3.18 ± 1.36^{ab}
	FL	2.46 ± 0.52^{c}	2.54 ± 0.98^{ab}
	FM	2.89 ± 1.60^{bc}	2.13 ± 0.27^{b}
	FH	3.18 ± 0.88^{abc}	2.15 ± 1.05^{b}
	PC	4.29 ± 1.98^{a}	3.07 ± 1.04^{ab}
	NSC	$\mathbf{3.63 \pm 1.22}^{abc}$	$\mathbf{3.52 \pm 1.92}^{a}$

Values are means ± SD (n = 12–15). Values within same column not sharing a common small letter are significantly different ($P < 0.05$) from each other and assayed by one-way ANOVA, followed by Duncan's new multiple range test. The LOD of these kits used in this study was <3.9 pg/mL. "ND" means not detectable. NSC, nonsensitized control; DC, dietary control; PC, positive control; FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

that the farnesol treatment doses adopted in this study had no apparent toxic effects (Table 2). Actual delivered doses to the farnesol-treated mice were 0.1311 (FL), 0.8177 (FM), and 3.149 mg farnesol/mouse/day (FH). Mean body weights of farnesol-treated mice calculated by their initial and final body weights during the experimental period were 20.7 (FL), 20.6 (FM), and 20.8 g/mouse (FH), respectively (Table 2). Thus, the actual delivered doses to the farnesol-treated mice were 0.1311 mg/20.7 g BW/day (FL), 0.8177 mg/20.6 g BW/day (FM), and 3.149 mg/20.8 g BW/day (FH), namely, 6, 40, and 151 mg/kg BW/day, respectively. The actual supplemented high dose of farnesol (151 mg/kg BW of mouse/day = 3.02 mg/20 g BW of mouse/day) to mice is equal to 1171 mg/day in humans according to an appropriate conversion ratio at 1 : 387.9 for mice (20 g) to human (70 kg) [20]. Horn et al. indicated that supplementing rats with 500 mg farnesol/kg BW by gavage for 28 days did not significantly influence their body weight, feed intake, and liver weights [29]. We found that supplementing experimental mice with farnesol at the indicated high doses for 5 weeks showed no apparent toxic side effects (Tables 2 and 3). Farnesol is a sesquiterpene alcohol that widely exists in fruits, vegetables, herbs, and essential oils [13, 14]. This study further provides farnesol safety data for food and possible pharmacological uses for anti-inflammatory and antiallergic effects. Our study suggests that farnesol supplementation at the indicated high dose is acceptable; however, the bioavailability of farnesol supplementation and its relative distribution in the body remain to be further clarified.

Asthma is a Th2-skewed allergic disease accompanied with systemic and airway inflammation [30]. In this study, we established a mild asthmatic animal model using OVA sensitization and challenge and evaluated farnesol anti-inflammatory and antiallergic effects *in vivo* [31]. Both Th2 (IL-4, IL-5, and IL-10) and Th1 (IL-2 and IFN-γ) cytokine secretion levels in splenocyte cultures in the presence of OVA were significantly ($P < 0.05$) increased as compared to those in the absence of OVA (Figure 2). In addition, all Th1 and Th2 except IL-10 cytokine secretion levels in DC group were significantly ($P < 0.05$) higher than those in NSC group. OVA sensitization and challenge markedly ($P < 0.05$) increased spontaneous secretion ratios of Th2 (IL-4 + IL-5 + IL-10)/Th1 (IL-2 + IFN-γ) by the splenocytes of the experimental mice (Figure 2(f)). The results evidenced that OVA sensitization and challenge successfully induced a Th2-skewed systemic OVA-specific immune response, reflecting in the spleen. We found that levels of NO, protein, and eotaxin in BALF showed no significant differences between the farnesol treatments and DC group (Table 5), indicating that the asthmatic animal model used in this study is still a mild model that did not cause severe injury or inflammation to the lungs and airways. Based on our results, OVA sensitization 3 times with subsequent 3 challenges is recommended to induce more severe asthmatic response in the lungs and airways. Eosinophils infiltration into the airways was detected but farnesol supplementation did not affect eosinophil numbers in BALF (data not shown). However, farnesol supplementation slightly ($P > 0.05$) decreased IL-4 (a Th2 cytokine) (Figure 2(a)) but significantly ($P < 0.05$) increased IL-2 (a Th1 cytokine) (Figure 2(d)) levels secreted by the splenocytes in the presence of OVA, implying that farnesol supplementation might have an antiallergic effect on Th2-skewed allergic asthmatic mice. High dose farnesol supplementation (FH group) slightly ($P > 0.05$), but not significantly, decreased Th2 (IL-4 + IL-5 + IL-10)/Th1 (IL-2 + IFN-γ) secretion ratios by the splenocytes in the absence or presence of OVA as compared to those in DC group,

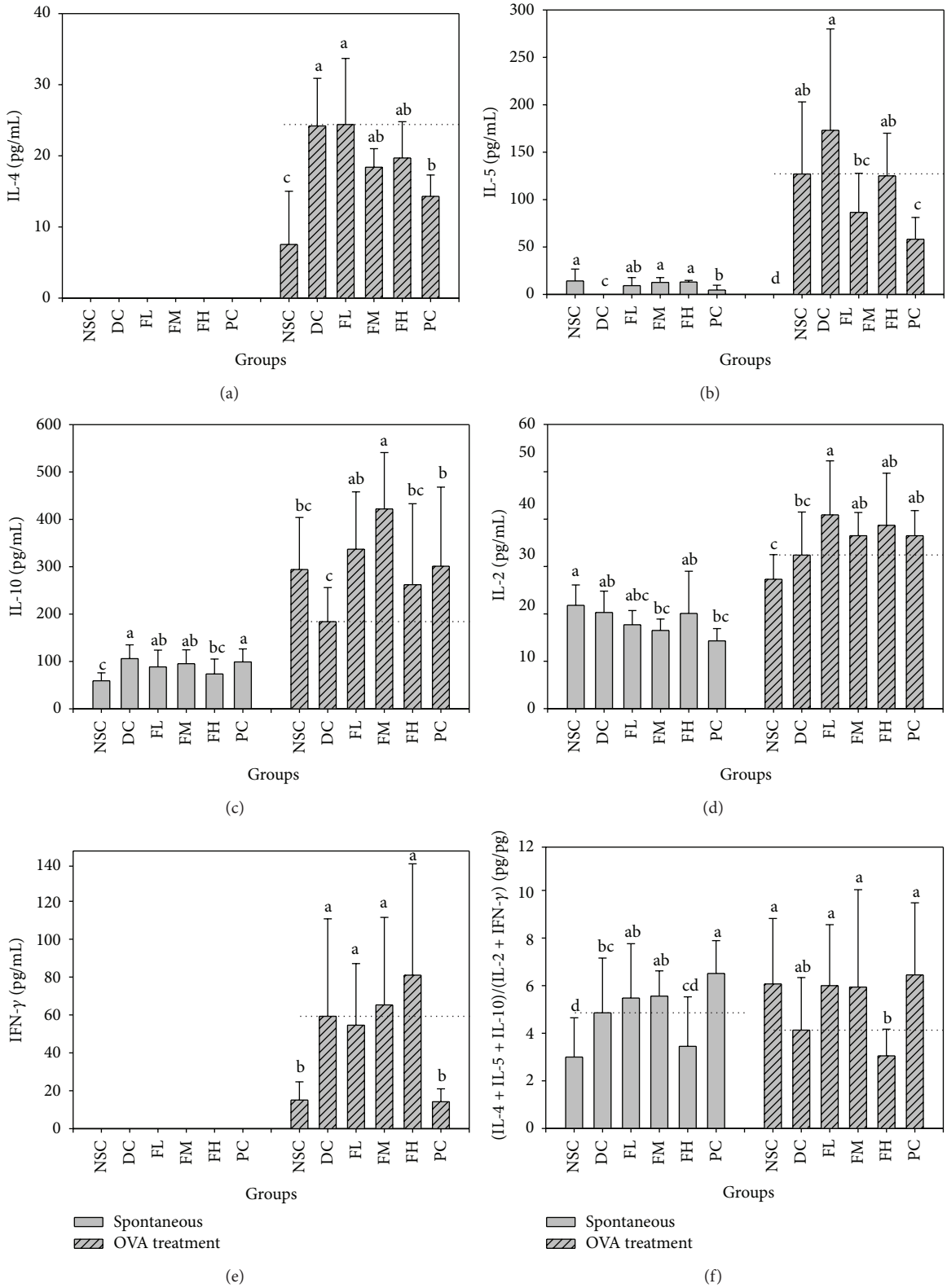

Figure 2: Farnesol supplementation effects with different doses for 5 weeks on IL-4 (a), IL-5 (b), IL-10 (c), IL-2 (d), IFN-γ (e), and (IL-4 +IL-5 + IL-10)/(IL-2 + IFN-γ) ratios (f) secreted by splenocytes of OVA/AL-sensitized and -challenged BALB/c asthmatic mice. Values are means ± SD (n = 12–15). Values among groups within the same treatment not sharing a common small letter are significantly different ($P < 0.05$) from each other and assayed by one-way ANOVA, followed by Duncan's new multiple range test. The limit of detection (LOD) of these kits used in this study was <3.9 pg/mL. NSC, nonsensitized control; DC, dietary control; PC, positive control (dexamethasone, 3 mg/kg BW, 0.3 mL/mouse by gavage); FL, low dose farnesol (0.003% in AIN-76 feed); FM, medium dose farnesol (0.017% in AIN-76 feed); FH, high dose farnesol (0.067% in AIN-76 feed).

further suggesting a mild effect of farnesol supplementation against Th2 responses (Figure 2(f)). In addition, sera from the experimental mice were collected to measure antibody titers. The results showed that farnesol supplementation significantly increased ($P < 0.05$) OVA-specific IgG2a/IgE antibody titer ratios but decreased total IgE levels (data not shown). Our results evidenced that farnesol supplementation ameliorated allergic status and reversed Th2-skewed immune responses in the allergic asthmatic mice via decreasing serum OVA-specific IgE titers but increasing IgG2a/IgE titer ratios. Farnesol supplementation significantly ($P < 0.05$) reduced IL-4 levels in BALF that increased due to OVA sensitization and challenge, suggesting that farnesol supplementation may have potential to modulate Th1/Th2 immune balance toward Th1 pole in the airways and lungs (data not shown). Unfortunately, farnesol supplementation did not have significant effects on infiltrations of total cells and eosinophils into the airways and lungs (data not shown). Importantly, farnesol supplementation significantly ($P < 0.05$) increased IL-10 levels secreted by the splenocytes in the presence of OVA, implying that farnesol supplementation might also have an anti-inflammatory potential to allergic asthmatic mice (Figure 2(c)). Th2 cells are the major source of IL-10 production. However, Th2 cytokines, particularly IL-10, may inhibit the production of Th1 cytokines such as proinflammatory IL-1β and TNF-α cytokines. In addition, IL-10 is produced in late stage inflammation by immune effector cells to inhibit the synthesis of other cytokines. Therefore, IL-10 has been recognized as a Th2 and anti-inflammatory cytokine. Unfortunately, many cytokines production (IL-2, IL-4, IL-5, and IL-10) in farnesol-administered groups did not show dose-response phenomena (Figure 2). It is a universal mechanism to induce low- and high-zone tolerances of immunomodulation. Thus, the optimal dose for farnesol administration is difficult to ascertain. Farnesol administration should be carefully considered to achieve the best effect for various purposes. Our results suggested that the most effective dose of farnesol *in vivo* might be low dose administration for long term.

To compare the farnesol effects, dexamethasone (DEX), a potent synthetic member of the glucocorticoid family, was selected in this study as the positive control for its anti-inflammatory and immunosuppressant activities. We found that both farnesol supplementation and DEX treatment decreased enlarged spleen weights (Table 3), IL-6/IL-10 level ratios in BALF (Table 4), and TNF-α/IL-10 (pro/anti-inflammatory) cytokine secretion ratios by peritoneal macrophages (Table 6), indicating significant anti-inflammatory effects of farnesol and DEX on the airways and systemic inflammation. However, the anti-inflammatory effects of farnesol supplementation were much better than DEX treatment through decreasing TNF-α/IL-10 (pro/anti-inflammatory) cytokine secretion ratios by peritoneal macrophages (Table 6). To cure asthma, preventing the early manifestations of the disease and thus preventing its evolution into severe asthma are most important [12]. Our results suggest that farnesol may be used as a food supplement to prevent and improve allergic inflammation in asthma patients in the future. We found that OVA sensitization and challenge significantly inhibited IL-1β, IL-6, TNF-α, and IL-10 productions by peritoneal macrophages (Table 6); however, farnesol supplementation significantly restored the cytokine secretion levels, indicating that farnesol supplementation may enhance the inhibited immunity but inhibit inflammation in asthmatic mice. We assume that farnesol might exert its anti-inflammatory effect through modulating nuclear factor (NF)-κB pathway [32]; however, its possible anti-inflammatory mechanisms remain to be further clarified. In addition, farnesol is considered to be a significant contact allergen and it was recommend that it should be included in a fragrance patch-test preparation and that its use should be regulated for consumer safety reasons [33]. The safety of dietary farnesol should be further studied.

5. Conclusions

Our results showed that actual farnesol supplementation at the indicated high dose of 151 mg/kg BW/day for 5 weeks had no toxic effect on the experimental mice. Farnesol supplementation decreased IL-6/IL-10 level ratios in BALF, suggesting an anti-inflammatory effect of farnesol on the lungs and airways. Farnesol supplementation significantly restored the secretion ability of peritoneal macrophages and slightly decreased TNF-α/IL-10 cytokine secretion ratios, indicating farnesol might enhance systemic immunity but inhibit inflammation in the lungs and airways in asthmatic mice. Farnesol supplementation slightly decreased IL-4 but significantly increased IL-2 levels secreted by the splenocytes in the presence of OVA, implying that farnesol supplementation might have a systemic antiallergic effect on allergic asthmatic mice. Furthermore, farnesol supplementation significantly increased IL-10 levels secreted by the splenocytes in the presence of OVA, suggesting that farnesol supplementation might also have an anti-inflammatory potential to allergic asthmatic mice.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Two Years versus One Year of Tianjiu Therapy in Sanfu Days for Chronic Asthma: A Clinical Efficacy Observation Trial

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Background. Tianjiu therapy has established efficacy against chronic asthma with related symptoms or the medication need during asthma attack. This study aimed to explore the optimal duration of Tianjiu therapy for asthma. *Methods.* This study was a self-comparison-to-the-baseline study, which comparing treatment with Tianjiu therapy for 1 year and 2 years in the same 102 chronic asthma patients. Totally 6 sessions of Tianjiu treatment were provided, 3 sessions in a year as a course of treatment and totally two years treatment. The primary endpoint was the number of asthma related symptoms which frequently appeared in asthma patients and the frequency of bronchodilator used during asthma attack. *Results.* The frequency of bronchodilator used during asthma attack significantly improved ($\chi^2 = 46.276$, $P = 0.000$). But the number of asthma related symptoms which frequently appeared in asthma patients added by 1.38 points (95% CI, 0.25 to 2.51), 2.93 ± 0.41 in 1-year group and 4.31 ± 0.41 in the 2-years group ($P < 0.05$). *Conclusions.* The effect of 2 years Tianjiu therapy was not as effective as 1 year such treatment for asthma, but the second year Tianjiu therapy was still needed because it has a role to consolidate the curative effect of Tianjiu therapy for asthma.

1. Introduction

Asthma is one of the most common chronic diseases in the world. Currently, β-agonist as the first-line drug in treating acute asthma attack and glucocorticoids is still used widely to treat chronic asthma [1]. From the perspective of immunology, asthma is a systemic allergic disease with immune disturbance. Allergic airway inflammation is only a local show, and inhaled glucocorticoids also only pay attention to the local anti-inflammatory therapy while overlooking the systemic immune dysfunction in asthma patients. Hence, current therapy for asthma is still imperfect; it needs us to keep improving the therapy for asthma, and immunotherapy is likely to be one of the important ways to improve asthma therapy [2]. Traditional Chinese medicine (TCM) has a long history of treating asthma and it claimed that the mechanism of asthma mainly lies in two aspects: one is "deficiency in origin" and another is "enrichment in symptom." The saying of "deficiency in origin" is similar to "systemic immune dysfunction," and "enrichment in symptom" is similar to "allergic airway inflammation." TCM pays more attention to the "preventive treatment of disease," which means conduct prevention treatment for asthma patients when there is no asthma attack. Tianjiu Therapy in Sanfu Days is a classic prevention treatment for asthma. Sanfu Days means the three hottest days in a year which are calculated by ancient calendar. Both Positive-qi in human body and nature are in a most exuberant status in Sanfu Days, so Sanfu Days is a good time for cold-insufficiency patients to tonic Positive-qi. As a result, patients can have a strong body-resistance to against exogenous pathogen because they have already accumulative enough Positive-qi inside. Just as Chinese medicine said: "when there is sufficient Positive-qi inside, the Pathogenic-qi has no way to invade the health body." Tianjiu Therapy means applying herbs patches on special acupoints in order to stimulate skin to form blisters, hyperemia, and even suppuration. As a result, Sanfu Tianjiu Therapy can attain the goal of strengthening Yang-qi, removing cold pathogen,

and enhancing body resistance through the combination effect of drug infiltration absorption, acupoints stimulation, and time effect. Sanfu Tianjiu Therapy for asthma aims to improve the body immunity which in turn can get a purpose of preventing and reducing respiratory viral or bacterial infection, reducing airway inflammation injury, and reducing airway hyperresponsiveness which in turn reduces the times of asthma attacks [3–12]. Tianjiu Therapy substantially improves asthma control level and the quality of life in patients with asthma and is used widely in Mainland China as an adjuvant setting [3–12]. We have already conducted an initial trial which compared 1 year of Tianjiu treatment with a placebo control group. In that study, it was found that Tianjiu Therapy can really reduce the need for medications to control asthma, improved the quality of participants' life, and significantly reduced the level of asthma. Previous study has preliminary confirmed the effect of Tianjiu Therapy in Sanfu Days for asthma, while the optimal treatment duration is still unknown. Hence this study was conducted to compare the different effect of 2-year Tianjiu Therapy with 1-year such treatment in asthma patients.

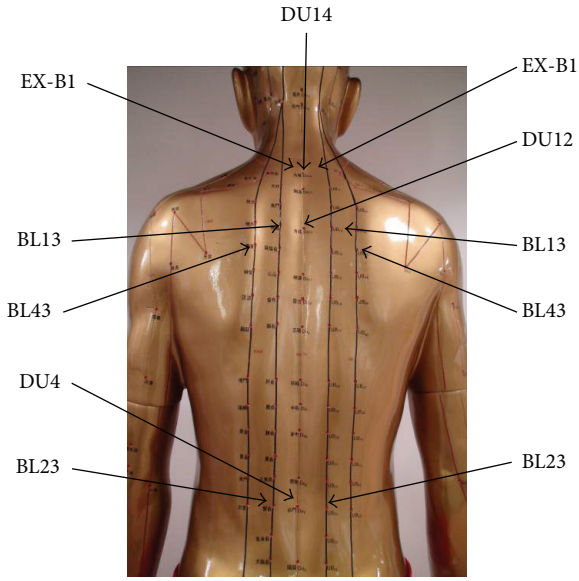

Figure 1: Acupoints.

2. Methods

2.1. Enrollment Criteria. This study was a self-control and clinical efficacy observation trial in 102 Hong Kong citizens (above 13 years of age) with chronic asthma. Participants need to provide physician's diagnosis of asthma or documentation of asthma-related symptoms preceding study visit. Besides, all participants should provide evidence of asthma attack as indicated by hospitalization or unscheduled urgent care twelve months before study entry. In addition, all patients were required to meet the following inclusion criteria: cough (worse at night), difficulty in breathing, chest tightness, awakening the patient, and symptoms occurring or worsening with exercise or at night; episodic symptoms of airflow obstruction, viral infections, strong emotions, and changes in weather. Patients would be assessed ineligibility according to one or more of the following exclusion criteria: severe cardiac and pulmonary diseases; fever, pharyngitis, acute asthma attack, and pregnancy. Diabetes mellitus, tuberculosis, and hypersensitive skin condition were also forming the excluded reasons. Additionally, allergy to topical medication, severe heart diseases, keloid, bleeding disorders or participants with pacemaker will also be excluded.

This study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (HKU/HA HKW IRB) with coding UW 13-316. All participants were informed about this study and written informed consent was obtained from every patient or patient's legal guardian or the patient's parent.

2.2. Study Design. In this study, participants from 2010 to 2013 who totally receive two courses of Tianjiu Therapy (one course means completing three-time Tianjiu Therapy in a year, so two-course treatment needs at least two years and six-time Tianjiu Therapy) will constitute study subjects for final analysis. For example, patients who have already received 1-course Tianjiu Therapy in 2010 need to receive another course treatment in 2011 or 2012 or 2013. Patients from placebo group in 2010 still need to receive other 2-course treatment in the following three years. As a result, 102 patients who totally received two-course treatment were included for the final analysis. These 102 patients must complete two-course pre-assessment and postassessment which means that they have completed data in four time points. Among these 102 patients, there were 68 continuous participants (attended in 2010 and 2011, 2011 and 2012, or 2012 and 2013) plus 34 discontinuous participants (attended in 2010 and 2012, 2010 and 2013, or 2011 and 2013). This study conducted a subgroup analysis for continuous group and discontinuous group, but nothing different was found in most of the outcome measurements. Hence, this study combined continuous participants with discontinuous participants as study subjects.

Each year Asthma Control Test (ACT), self-made questionnaire, and pulmonary function will be assessed for participants before treatment and after treatment. Also three-time Tianjiu Therapy with 2-hour duration in a year was conducted for all participants from 2010 to 2013 and every time Tianjiu Therapy was conducted in Sanfu Days. In other words, eligibility patients participated in a certain year study then that year's three treatments and pre and post assessment were conducted for them. The three treatment times from 2010 to 2013 were July 19, July 29, and August 8, 2010; July 14, July 24, and August 13, 2011; July 18, July 28, and August 7, 2012; July 13, July 23, and August 12, 2013.

Eleven acupoints will be applied by Tianjiu patches: BL13 (both sides), BL23 (both sides), BL43 (both sides), EX-B1 (both sides), Du14, Du12, and Du4 (Figure 1).

One patch was applied on one acupoint and every patch weighted 2 grams (Figure 2).

The formula of Tianjiu patch was a combination of the formula frequency used in clinical trials and the record in

Figure 2: Tianjiu patch.

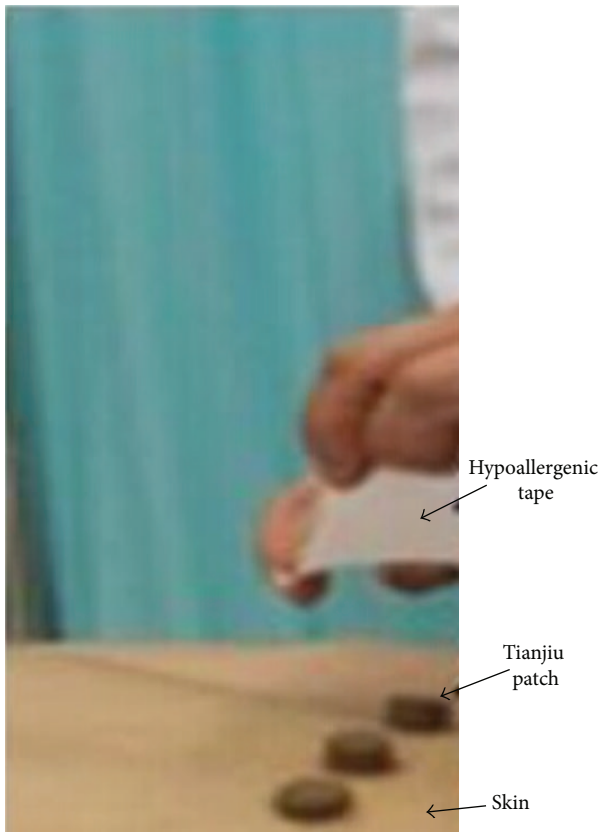

Figure 3: Tianjiu Therapy schematic diagram.

"Zhang Shi Yi Tong." The final formula used consisted of *Sinapis alba, Radix Corydalis Yanhusuo, processed Euphorbia kansui, Asari Herba cum Radice, Ephedrae Herba, processed Radix Aconiti Praeparata, Cinnamomum cassia,* and *Eugenia caryophyllata*. The ratio of selected herbal was 2 : 1 : 1 : 1 : 1 : 1 : 1 : 1 and all this herbal powder will be mixed together with ginger juice. Finally, approximately 4 cm × 4 cm hypoallergenic tape (3M Micropore Tape 1535-3) will be used to stick Tianjiu patches on skin after the patch was applying on acupoints (Figure 3).

2.3. Outcome Measures. The primary outcome was the number of asthma-related symptoms which frequently appeared in asthma patients and the frequency of bronchodilator used during asthma attack. Other outcomes included the number of days with asthma-related symptoms in 1 month before visit; spirometric measurements; asthma-related health care use and the score on the ACT. Scores on the ACT range from 5 to 25, with scores of 20 or more indicating disease control and 3 points are the minimally important difference for the ACT score [13].

2.4. Statistical Analysis. Continuous variables in this study from 1st year baseline to 2nd year posttreatment were examined using a linear mixed-effects model with time as fixed effects. There were totally four time points in this linear mixed-effects model: 1st year baseline as the 1st time point, 1st year posttreatment as the 2nd time point, 2nd year baseline as the 3rd time point, and 2nd year posttreatment as the 4th time point. The continuous variables included the score of ACT, the number of asthma-related subhealthy symptoms, the number of days with asthma-related symptoms, and lung function: FEV and FEV_1/FVC (%). All analysis was conducted for the same 102 people. Generalized estimated equation (GEE) analysis was performed for the frequency of bronchodilator used during asthma attack and asthma-related health care used during asthma attack in order to repeat measures in different time points. Descriptive analysis was conducted for the percentage of patients with twenty-three asthma-related symptoms which frequently appeared in asthma patients. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS) software program (version 19) for windows XP.

3. Results

From June 2010 through July 2010, 323 patients were recruited in 2010 and finally 242 patients completed treatment. 274 patients were recruited as study subjects in 2011, of which 84 participants were from 2010 Tianjiu Therapy group, 82 were from 2010 placebo group, and 108 were new recruited subjects. During the treatment period and postassessment in 2011, 104 patients did not complete all parts. As a result, 170 participants completed all treatments and postassessment in 2011. Totally 132 patients completed all treatments and pre- and postassessments in 2012, and 150 patients lastly completed preassessments, postassessments, and three-time Tianjiu Therapy in 2013. Patients would be ineligible for final statistical analysis due to the incomplete data. The cause of incomplete data included incomplete treatments, during treatment period participants losing contact, illnesses, pregnancy, asthma exacerbation, inability to participate timely, refusing to participate, or incomplete questionnaires, ACT, or lung function test. The patients can be included for final analysis only when they have four time points' data (1st year pre- and postassessments, 2nd year pre- and postassessments). Overall, from 2010 to 2013, patients who received two-course treatment were 102.

For the baseline characteristics of these 102 participants, the average age was 50.4 years (interquartile range, 13 to 78); 53% were female (Table 1). The average number of days on which patients had asthma-related symptoms was 9.35. Patients had a mean ACT score of 19 or less which indicated a lack of disease control [14]. In the previous year, 22.5% patients received emergency treatments at least once

Table 1: Baseline characteristics of study participants.

Characteristic	Tianjiu Therapy (N = 102)
Age—year	50.35 ± 17.17
Gender—number (%)	
Male	48 (47.1)
Female	54 (52.9)
Duration of asthma—year	20.22 ± 14.14
Asthma-related symptoms—number of[a]	9.35 ± 11.90
Lung function	
FEV_1	1.86 ± 0.98
FEV_1:FVC × 100	85.24 ± 27.76
Asthma-related health care—number (%)	
≥1 Admitted to A and E[b]	23 (22.5)
≥1 Hospitalization	19 (18.6)
≥1 Outpatient visit	60 (58.8)
≥1 Prescription	72 (70.6)
≥1 Buy medication	11 (10.8)
≥1 Not to be processed	10 (9.8)
The frequency of bronchodilator used during asthma attack—number (%)	
More than twice per day	25 (24.5)
Once to twice per day	38 (37.3)
2-3 times per week	5 (4.9)
Less than once per week	16 (15.7)
Never	18 (17.6)
Symptoms which frequently appeared in asthma patients—number of symptoms[c]	8.20 ± 4.40

[a]Plus-minus values are means ± SD, unless noted otherwise. PIF denotes peak inspiratory flow; PEF: peak expiratory flow; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity.
[b]The total number of symptoms which frequently appeared in asthma patients is 23 and its scale ranges from 0 to 23.
[c]Accident and Emergency Departments (A and E).

which were associated with an asthma-related event, 18.6% had been hospitalized, 58.8% went to clinic, 70.6% treated themselves following the prescription, 10.8% buy medication for themselves, and 9.8% did not need medical treatment. The mean (±SD) FEV_1 was 1.86 ± 0.98, and the mean ration of FEV_1 to the forced vital capacity (FVC) was 85.24 ± 27.76. For the frequency of bronchodilator used during asthma attack, 24.5% used bronchodilator more than twice per day, 37.3% once to twice per day, 4.9% 2-3 times per week, and 15.7% less than once per week, and 17.6% never used bronchodilator during asthma attack. The number of symptoms which frequently appeared in asthma patients was 8.20 ± 4.40.

3.1. Response to Intervention. The number of symptoms which frequently appeared in asthma patients was 2.93 ± 0.41 in end of 1-course treatment and 4.31 ± 0.41 in end of 2-course treatment; one course has 5.27-point reduction and 2 courses have 3.88-point reduction, respectively, comparing with 8.20 ± 0.41 in baseline ($P < 0.05$) (Figure 4 and Table 2).

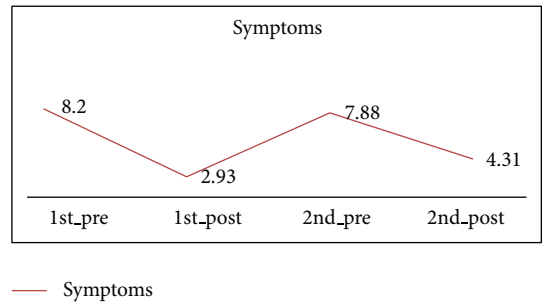

Figure 4: The number of symptoms which frequently appeared in asthma patients in 1 course and 2 courses.

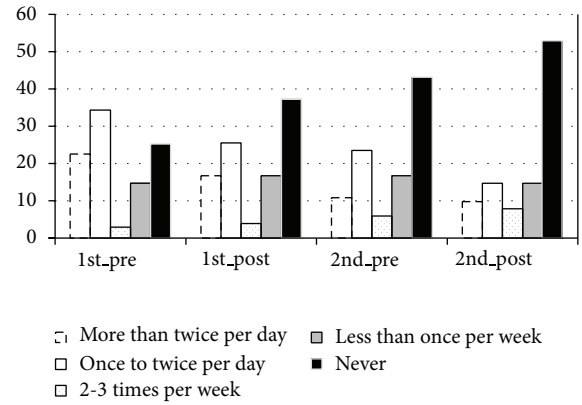

Figure 5: The frequency of bronchodilator used during asthma attack.

But 1-course treatment reduced more points than 2-course treatment ($P < 0.05$).

The frequency of bronchodilator used during asthma attack in 2 courses was better than 1 course: 16.7% more than twice per day in 1 course and 9.8% in 2 courses, 25.5% once to twice per day in 1 course and 14.7% in 2 courses, 3.9% 2 to 3 times per week in 1 course and 7.8% in 2 courses, and 16.7% less than once per week in 1 course and 14.7% in 2 courses, and 37.3% never used it in 1 course and 52.9% in 2 courses, indicating that the frequency of bronchodilator used during asthma attack was decreasing (Table 3 and Figure 5, $P < 0.05$).

The score of ACT improved by 1.53 points (95% CI, −0.31 to 3.37) after one-course treatment (19.80) and 3.53 points (95% CI, 1.69 to 5.37) after two courses of treatment (21.80) (Figure 6 and Table 4). In other words, the score of ACT after two-course treatment was better than one-course treatment and the score of ACT showed an increasing trend ($P < 0.05$).

Different from the improvement like the score of ACT and the frequency of bronchodilator used during asthma attack, as compared with 1-year treatment, 2-year treatment with Tianjiu increased the mean number of days on which patients had asthma-related symptoms from 1.98 to 5.03 ($P < 0.05$) (Table 2 and Figure 7); even both 2-year treatment and 1-year treatment compared with baseline have a significant improvement ($P < 0.05$).

No changes occurred in pulmonary function for both 1-year treatment and 2-year treatment comparing with no

Table 2: Variable on asthma patients in 1 course and 2 courses.

Variable	1st baseline	1st posttreatment	2nd baseline	2nd posttreatment	1st posttreatment versus 1st baseline (95% CI)/P value	2nd posttreatment versus 1st baseline (95% CI)/P value	2nd posttreatment versus 1st posttreatment (95% CI)/P value
Asthma-related symptoms—number of days[a]	8.44 ± 0.99	1.98 ± 0.99	6.94 ± 0.99	5.03 ± 0.99	−6.46 (−9.21 to −3.72)$^{\#\#\#}$	−3.41 (−6.16 to −0.67)$^{\#}$	3.05 (0.30 to 5.80)$^{\#}$
Lung function							
FEV	1.86 ± 0.09	2.01 ± 0.09	1.80 ± 0.09	1.76 ± 0.09	0.15 (−0.11 to 0.40)	−0.10 (−0.36 to 0.15)	−0.25 (−0.51 to 0.003)
FEV_1:FVC × 100	85.24 ± 1.87	84.72 ± 1.87	81.89 ± 1.87	84.56 ± 1.50	−0.52 (−5.70 to 4.67)	−0.68 (−5.86 to 4.51)	−0.16 (−5.35 to 5.03)
Symptoms which frequently appeared in asthma patients—number of symptoms[b]	8.20 ± 0.41	2.93 ± 0.41	7.88 ± 0.41	4.31 ± 0.41	−5.27 (−6.40 to −4.13)$^{\#\#\#}$	−3.88 (−0.50 to −2.75)$^{\#\#\#}$	1.38 (0.25 to 2.51)$^{\#}$
Asthma-related health care use—number (%)							
≥1 Admitted to A and E[c]	23 (23)	1 (1)	16 (16)	6 (6)	0.000$^{\#\#\#}$	0.000$^{\#\#\#}$	0.054
≥1 Hospitalization	19 (19)	0 (0)	11 (11)	2 (2)	0.000$^{\#\#\#}$	0.000$^{\#\#\#}$	0.153
≥1 outpatient visit	60 (59)	9 (9)	52 (51)	50 (49)	0.000$^{\#\#\#}$	0.000$^{\#\#\#}$	0.000$^{\#\#\#}$
≥1 Prescription	72 (71)	61 (60)	79 (77)	70 (69)	0.066	0.758	0.102
≥1 Buy medication	11 (11)	1 (1)	13 (13)	8 (8)	0.001$^{\#\#}$	0.437	0.006$^{\#\#}$
≥1 Not to be processed	10 (10)	2 (2)	15 (15)	10 (10)	0.018$^{\#}$	1.000	0.018$^{\#}$

$^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$.

[a]Plus-minus values are means ± SE, unless noted otherwise. PIF denotes peak inspiratory flow; PEF: peak expiratory flow; FEV_1: forced expiratory volume in one second; FVC: forced vital capacity.

[b]The total number of symptoms which frequently appeared in asthma patients is 23 and its scale ranges from 0 to 23.

[c]Accident and Emergency Departments (A and E).

Table 3: The frequency of bronchodilator used during asthma attack—n (%).

Variable	Time			
	1st_pre	1st_post	2nd_pre	2nd_post
More than twice per day	23 (22.5)	17 (16.7)	11 (10.8)	10 (9.8)
Once to twice per day	35 (34.3)	26 (25.5)	24 (23.5)	15 (14.7)
2-3 times per week	3 (2.9)	4 (3.9)	6 (5.9)	8 (7.8)
Less than once per week	15 (14.7)	17 (16.7)	17 (16.7)	15 (14.7)
Never	26 (25.2)	38 (37.3)	44 (43.1)	54 (52.9)

Time main effect: Wald $\chi^2 = 46.276$, $P = 0.000$.

Table 4: ACT score before and after treatment in 1 course and 2 courses.

1st baseline	18.27 ± 0.66
1st posttreatment	19.80 ± 0.66
2nd baseline	20.43 ± 0.66
2nd posttreatment	21.80 ± 0.66
1st posttreatment versus 1st baseline (95% CI)/P value	1.53 (−0.31 to 3.37)
2nd posttreatment versus 1st baseline (95% CI)/P value	3.53 (1.69 to 5.37)$^{\#\#\#}$
2nd posttreatment versus 1st posttreatment (95% CI)/P value	2.00 (0.16 to 3.84)$^{\#}$

Notes: $^{\#}P < 0.05$; $^{\#\#\#}P < 0.001$.

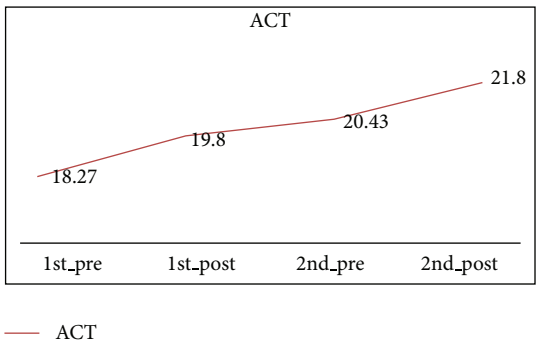

Figure 6: The score of ACT in 1 course and 2 courses.

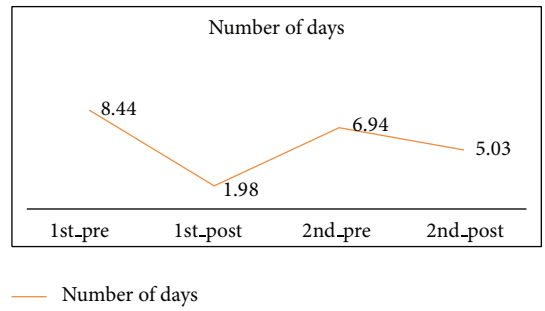

Figure 7: The number of days with asthma-related symptoms in 1 year and 2 years.

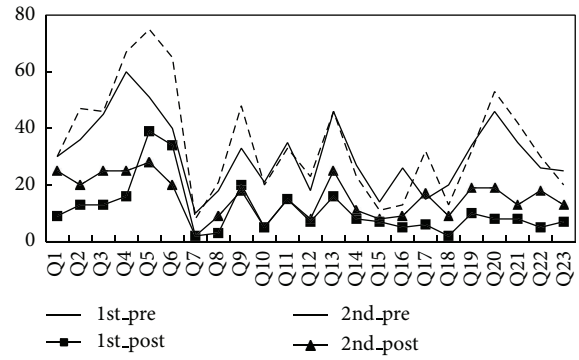

Figure 8: The proportion of participants with twenty-three symptoms which frequently appeared in asthma patients in 1 course and 2 courses.

treatment status. With respect to asthma-related health care use during asthma attack, the percentage of participants admitted to Accident and Emergency Departments (A and E) was 1% in year 1 and 6% in 2 years; comparing with baseline both have a significant improvement ($P < 0.05$). Similarly, the proportion of patients who were hospitalized due to asthma was 0% in 1 year and 2% in 2 years, with both being better than no treatment ($P < 0.05$). The percentage of participants who visited outpatient clinic was 9% in 1 year and 49% in 2 years; both of them have a significant improvement in comparison with no treatment; however, the effect of 1 year was much better than 2 years ($P < 0.05$). The ratio of subjects who took the previous prescription by doctors during asthma attack was 60% in 1 year and 69% in 2 years, and no difference occurred in both groups comparing with no treatment ($P > 0.05$); the scale of patients who only need to take medication by themselves was 1% in 1 year and 8% in 2 years; 1-year treatment was better than no treatment while 2-year treatment was not different from baseline, indicating that 1-year treatment was better than 2-year treatment ($P < 0.05$); last but not least, the percentage of patients who do not need to process during asthma attack was 2% in 1 year and 10% in 2 years; 2-year treatment has nothing different from baseline, while 1-year treatment was worse than no treatment and 2 years performed better than 1 year ($P < 0.05$) (Table 2).

Figure 8 showed the percentage of participants with twenty-three symptoms which frequently appeared in asthma patients. Among these twenty-three symptoms, the top three frequently occurring symptoms were as follows: easy onset during quarter turn (67% in baseline, 16% after 1-course treatment, and 25% after 2-course treatment), rapid or difficult

breathing (75% in baseline, 39% after 1-course treatment, and 28% after 2-course treatment), and waking up with asthma symptoms (65% in baseline, 34% after 1-course treatment, and 20% after 2-course treatment), indicating that the majority of participants owned the lung-qi-deficiency syndromes. Nearly all patients have a significant reduction in the twenty-three symptoms after both 1-course Tianjiu Therapy and 2-course such treatment. What is more, it showed that the status of patients in the 2nd course baseline was better than 1st course baseline which indicated that Tianjiu Therapy has a cumulative effect after one course of treatment. Additionally, from Figure 8, it can be found that the reduction of 1 course was much more than 2 courses. It seems that the effect of 2 courses was not as sensitive as 1 course, regardless of the fact that 2-course treatment still has a significant improvement compared with 1st course baseline.

3.2. Adverse Event. The observation procedures followed after Tianjiu Therapy were conducted in both 1 course and 2 courses. During the treatment, most patients would show cutaneous reaction such as skin warm feeling, itching, pain, and blisters due to the stimulation of herbal patch. All these appearances were a normal skin reaction after Tianjiu Therapy and also a key condition of Tianjiu Therapy taking effect in the treatment of asthma. Most of them would disappear after several hours or several days. In 1st course Tianjiu Therapy, there were five serious adverse events reported at the end of three-time treatment: three patients showed asthma exacerbation, one patient showed the rash due to being allergic to Tianjiu patch, and one subject tended to vomit blood and automatically stopped after the 2nd Tianjiu Therapy, which had confirmed that it has nothing to do with Tianjiu Therapy. Besides, in 2nd course Tianjiu Therapy, the majority of patients also showed skin reaction after Tianjiu Therapy as that in 1st course. There were also five serious adverse events in the 2nd course treatment: three were skin sensibility which cannot disappear after stopping treatment even several days later. And finally, his skin returned to normal through dermatologist's appropriate process. one person was caused by the smell of paint because his house was mopping during the treatment period; another was induced by flu. Overall, there was nothing different between 1st course Tianjiu Therapy and 2nd course Tianjiu Therapy in adverse events.

4. Discussion

In the previous study (HKCTR-1128), Tianjiu Therapy has shown substantial improvements in the number of days with asthma-related symptoms, the number of symptoms which frequently appeared in asthma patients, and the medication need after treatment. Although there was nothing different between placebo group and Tianjiu Therapy group after the 3rd treatment immediately, the effect of Tianjiu Therapy tended to be superior to placebo group in four-time followup. This study was conducted to explore how long the curative effect of Tianjiu Therapy lasts and the optimum duration of treatment for chronic asthma based on previous finding. This study tested the hypothesis that continuation of Tianjiu Therapy beyond 1 course would be more effective than 1 course of such treatment. As a result, this study found that two courses of Tianjiu Therapy showed a significant improvement in the score of ACT as compared with 1-course treatment and the score of ACT showed an increasing trend. In addition, the frequency of bronchodilator used during asthma attack in 2 courses was also superior to the effect of 1 course. Patients' asthma control situation in 2nd pretreatment was better than that in the 1st pretreatment. Which means patients' immunity improved and kept well after one-course Tianjiu Therapy? While, it was arbitrary to make conclusion that the effect of 2-course Tianjiu Therapy was superior to 1 course by only judging from the change of the score of ACT and the bronchodilator used during asthma attack between 1 course and 2 courses. Nevertheless, different from the findings above, the result of the mean number of days on which patients had asthma-related symptoms shows no such additional benefit; the effect of 2-course treatment was close to 1 course such treatment. Likewise, results about the number of symptoms which frequently appeared in asthma patients, asthma-related health care use during asthma attack, and the percentage of participants with symptoms which frequently appeared in asthma patients were not mostly different between the 1-course treatment and 2-course such treatment. Last but not least, no changes occurred in pulmonary function for both 1-course treatment and 2-course treatment comparing with 1st course baseline.

Although the result was inconsistent in all outcome measurements, it was found that the effect of 2-course Tianjiu Therapy was less sensitive than 1 course of such treatment for chronic asthma, but both 1-course and 2-course Tianjiu Therapy can get a significant improvement comparing with the situation in 1st pretreatment. And the baseline status of the same people in the 2nd course remained well even one year later after the first course of treatment, indicating that the effect of Tianjiu Therapy can last for a quite long time. For safety issue, the number of adverse events in 2nd course treatment was nearly the same as the number in 1st course treatment. There is no doubt that all these findings will be beneficial for health policy makers and asthma patients.

There is a limitation of this study because this study did not set up followups and only takes the second course's baseline as the followup of first course treatment. However, Sanfu Days in every year were around July and August which was a stable period for asthma patients, so it was easy to produce bias without followup in asthma high incidence and research staff cannot track the asthma control situation of participants. Hence, it is urgent and necessary to add followups. Table 5 showed the temperature record of 2010 to 2013. The average temperature in Sanfu Days was 28.7°C from 2010 to 2013; the average humidity was 81% from 2010 to 2013; the average duration of sun exposure was 6.6 hours from 2010 to 2013.

5. Conclusion

Both 2-course Tianjiu Therapy and one-course Tianjiu Therapy significantly reduced the number of symptoms which

Table 5: Temperature record in Sanfu Days from 2010 to 2013.

Item	Year											
	2010			2011			2012			2013		
	1st hottest day (July 19)	2nd hottest day (July 29)	3rd hottest day (Aug. 8)	1st hottest day (July 14)	2nd hottest day (July 24)	3rd hottest day (Aug. 13)	1st hottest day (July 18)	2nd hottest day (July 28)	3rd hottest day (Aug. 7)	1st hottest day (July 13)	2nd hottest day (July 23)	3rd hottest day (Aug. 12)
Maximum temperature (°C)	31.3	31.2	31.4	31.6	31.3	30.9	31.4	31.2	31.3	31.6	31.3	30.9
Temperature (°C)	28.8	28.6	28.8	28.9	28.8	28.4	28.8	28.6	28.8	28.9	28.8	28.5
Minimum temperature (°C)	26.8	26.6	26.8	26.8	26.9	26.4	26.8	26.7	26.8	26.8	26.8	26.5
Relative humidity (%)	81	82	82	80	80	83	81	81	81	80	81	83
Sun exposure (hour)	6.7	6.1	6.5	7.5	7.1	5.8	6.7	6.1	6.6	7.5	7.3	5.8

frequently appeared in asthma patients and medication need in participants with chronic asthma as compared with baseline. The asthma control situation in 2nd course pretreatment was better than 1st course pretreatment. Although the second course Tianjiu Therapy was less sensitive than first course Tianjiu Therapy for chronic asthma, the second course treatment still plays a role in consolidating the curative effect of Tianjiu Therapy in the treatment of asthma.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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