Ganoderma tsugae supplementation alleviates bronchoalveolar inflammation in an airway sensitization and challenge mouse model

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Abstract

Ganoderma tsugae (a Chinese mushroom Songshan lingzhi) cultivated in Taiwan is extensively used in Chinese traditional medicine to treat different diseases. To determine whether *G. tsugae* has anti-inflammatory effects on bronchoalveolar inflammation in vivo, we investigated the anti-inflammatory effects of *G. tsugae* products, YK01 and YK07, on bronchoalveolar inflammation using an airway sensitization and challenge mouse model. Female BALB/c mice were weekly sensitized by intraperitoneal injection of ovalbumin (OVA) three times and challenged with aerosolized OVA twice. Differential cell counts of infiltrating leukocytes, inflammatory mediators, cytokines in bronchoalvelor lavage fluid (BALF) of OVA-challenged mice were examined after continuously consuming *G. tsugae* diets for 5 weeks. We found that supplementation of *G. tsugae* significantly decreased total infiltrating leukocytes and lymphocyte percentage in BALF in the experimental groups. Supplementation of *G. tsugae* also significantly reduced inflammatory mediators in BALF including histamine, prostaglandin E_2, eotaxin, and protein levels, however the levels of pro-inflammatory cytokines, interleukin (IL)-1β and IL-6, in BALF did not significantly change. These results suggest that both *G. tsugae* supplementation diets YK01 and YK07 might alleviate bronchoalveolar inflammation via decreasing the infiltration of inflammatory cells and the secretion of inflammatory mediators into the local tissues of lungs and airways. Further, these results indicate that the relief of bronchoalveolar inflammation in an airway sensitization murine model provides a possible therapeutic application for *G. tsugae* in allergic asthma.
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Keywords: Ganoderma tsugae; Bronchoalvelor lavage fluid (BALF); Anti-inflammatory effects; Airway sensitization and challenge mouse model

1. Introduction

Allergic diseases, especially asthma, have risen as a public health problem worldwide, particularly in developed countries [1]. Allergic asthma is a chronic disease accompanied with airway hyperresponsiveness (AHR) associated with bronchial inflammation [2], mucus protein overproduction in airway [3,4], chronic cough [5],

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airway obstruction [6,7] and elevated serum IgE [8]. The time course for asthmatic responses includes immediate asthmatic response (IAR), late asthmatic response (LAR), and airway inflammation [9]. IAR may occur within 1 min after antigen inhalations, however LAR results from repeated airway inflammation such as eosinophilia [10,11] and mediator releases including histamine, thromboxane A_2 (TXA$_2$) [9], leukotrienes [12], T helper 1 (Th1)/T helper 2 (Th2)-type cytokines [11], nitric oxide (NO), and eotaxin [13,14]. Eotaxin, produced by epithelial cells, endothelial cells, and lung fibroblast, is a small inducible chemokine involved in trafficking and activation of leukocytes such as eosinophils, Th2 cells, basophils and mast cells [14]. Single or multiple chemical mediator releases in bronchoalvelor mucosa may cause leukocytes infiltration into lungs and airways and subsequently lead to severe airway inflammation damage. Airway inflammation plays an important role in asthma, especially in the late asthmatic phase reaction. Decreases in leukocyte infiltration and inflammatory mediator levels of bronchoalveolar tissues might reduce airway inflammation and alleviate allergic asthma [15,16].

Mouse models of allergic asthma are extensively used to evaluate the status of airway inflammation [1,2,10–17]. Mice can be sensitized by ovalbumin (OVA) using alum as an adjuvant, and then mice are challenged by aerosolized 5% OVA to induce severe allergic airway inflammation [16]. Allergic airway inflammation to OVA has been established in BALB/c [18,19], C57BL/6 [19], and DBA/2 mice [11]. However, differential susceptibilities to OVA may exist in different genotypes of laboratory strains and different antigen doses [11,19]. However, OVA-sensitized and -challenged mouse model is one of the most popular allergic asthma models to test the possibility of reversing allergic airway inflammation. Moderate allergic inflammation in a murine model for asthma may offer different approaches for the treatment of allergic disorders.

More research has been focused on exploring a possible therapeutic application for traditional medicines in allergic asthma. Traditional Chinese medicine has been applied to treat diseases including asthma for centuries and is still extensively used in modern medical practice around the world [20–22]. Traditional Chinese herbal medicine consists of herbal formulas or herbal derivatives and provides a great potential in treating asthma and allergy. Although basic researches concerning herbal interventions for allergy and asthma in well-controlled scientific studies are still limited, searching and developing an alternative medicine in asthma therapy from traditional medicine or health food is incessant [20–22]. Recently, a Chinese herbal formula, MSSM-

002, exhibits a therapeutic effect on established airway hyperresponsiveness through its immunomodulatory actions on Th2 cells in a murine model of allergic asthma [20]. A Chinese and Japanese herbal medicine Xiao-Qing-Long-Tang (Sho-seiryu-to) modulates Th1/Th2 balance and increases nerve growth factor (NGF) in the lungs of OVA-sensitized animals [21]. However, the traditional Chinese medicine Bu-zhong-yi-qi-tang (Hochu-ekki-to) demonstrates dichotomous effects on allergic asthma in OVA-sensitized mice following OVA inhalation [22]. Oral administration of Bu-zhong-yi-qi-tang reduces eosinophilia and Th2-type cytokine production in the airways at the induction phase immediately after OVA sensitization, however Bu-zhong-yi-qi-tang given in the eliciting phase induces a predominant Th2 response with increased IgE production [22]. Therefore, circumspective uses of traditional medicines to treat allergic asthma are essential.

Mushrooms consist of a large variety of bioactive substances, including biologically active immunostimulatory polysaccharides, triterpenes, proteins, lipids, cerebrosides, and phenols [23]. Ganoderma are traditionally introduced into Chinese medicine and widely used as medicinal mushrooms in Asian countries. Anti-allergic constituents chloroform-extracted from Ganoderma lucidium, including oleic acid and cyclooctasulfur, inhibit histamine release from rat peritoneal mast cells through membrane-stabilization [24,25]. However, the sensitization to G. lucidium in the atopic population of India has been reported [26]. The use of G. lucidium for allergy and asthma therapy is controversial, however Ganoderma tsugae, most widely cultivated species of medicinal mushrooms in Taiwan, might offer an alternative chance to treat allergic diseases. G. tsugae, one of Chinese traditional medicines, is extensively used to treat different diseases such as tumors [27,28], wound healing [29], liver fibrosis [30], and autoimmune diseases [31]. Both mycelia and fruiting bodies of G. tsugae are studied and used to treat diseases. Water-soluble and alcohol-soluble extracts of G. tsugae mycelia enhance activities of splenic natural killer cells and serum levels of interferon in mice [32]. Anti-tumor active polysaccharides from the fruiting bodies of G. tsugae are characterized [33].

Although G. tsugae is extensively used and studied, anti-inflammatory effects of G. tsugae on bronchoalveolar inflammation in vivo is not evaluated yet. In this study, G. tsugae products, YK01 and YK07, prepared from the fruiting bodies and mycelia of G. tsugae were investigated for their anti-inflammatory effects on bronchoalveolar inflammation in an OVA-sensitized and -challenged airway inflammation murine model.

2. Materials and methods

2.1. Materials and sample preparations

Samples of G. tsugae were kindly provided from Double Crane Group, Yung-Kien Industry Corp, Taiwan, ROC. Two kind products, G. tsugae YK01 and YK07, were used to test their anti-inflammatory effects on bronchoalveolar inflammation in an airway sensitization murine model. Both YK01 and YK07 products were prepared from the same species of G. tsugae. The YK01 was prepared from baby G. tsugae which was harvested at 2–3 weeks after fruiting, whereas the YK07 was prepared from mature G. tsugae which was harvested at 1–2 months after fruiting. The original source of G. tsugae was selected from a wild type growing in Puli, Nantou, Taiwan and then tamed to be G. tsugae YK01. After suitable cultivation, mycelia and fruit bodies of G. tsugae were harvested and extracted with hot water twice, respectively. The hot-water-extracts were respectively spray-dried to obtain dry powder. According to a commercial formula, the dry powders from mycelia and fruit bodies were mixed with a fixed ratio. The G. tsugae products were finally encapsulated and preserved at room temperature. G. tsugae YK01 and YK07 contained similar components and consisted of 0.28% protein, 0% lipid, 55.56% carbohydrate, 1.67% sodium, 12.5% polysaccharides, and 12% triterpenes.

2.2. Experimental animals and feeds

Inbred female BALB/c mice (6 weeks old) were purchased from the Laboratory Animal Center, College of Medicine, National Taiwan University and maintained in the Department of Biochemical Science and Technology, College of Life Science, National Taiwan University. 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) to acclimatize for 2 weeks before feeding the experimental diet. After this equilibrium period, the mice were divided into six groups ($n = 14$) including non-sensitized con-

trol (SC), dietary control (DC), and G. tsugae supplementation groups, YK01-L, YK01-H, YK07-L, YK07-H. Each group was fed with one of the specified experimental diets for 5 consecutive weeks ad libitum. A series of preliminary experiments were conducted to decide the effective dosage and safety evaluation on G. tsugae products. According to the preliminary data, the formulas of G. tsugae diets were used in this study. The specified diets consisted of AIN 76 semi-purified diets that respectively superadded with 2.0 and 4.0 g G. tsugae YK01 to 1000 g AIN 76 feed (feeding for YK01-L and YK01-H group, respectively), and 3.3 and 6.6 g G. tsugae YK07 to 1000 g AIN 76 feed (feeding for YK07-L and YK07-H group, respectively). Non-sensitized control (SC), dietary control (DC) groups were fed with AIN 76 feed. The ingredients and experimental feeds compositions are given in Table 1. Mice food intake and body weight were measured every 3 days during the study period. There were no differences in food intake, feed efficiency and body weight gain between the six diet groups.

2.3. Sensitization of allergic airway inflammation

To test effects of G. tsugae on airway inflammation, the mice (9 weeks old) were sensitized and challenged to induce allergic airway inflammation. The mouse allergic airway inflammation model was manipulated as described by Nagai et al. [21] and slightly modified to enhance the induction of airway inflammation. In brief, mice were sensitized by an intraperitonal injection (i.p.) of 0.5 ml alum-precipitated antigen containing 8 μg of ovalbumin (OVA, albumin chicken egg grade III, Sigma A-5378, MO, USA) and 2 mg Al(OH)$_3$ to induce primary immunity after supplementation of G. tsugae diets for one week. Two booster injections of this alum–OVA mixture were given 7 and 14 days later, respectively. Non-sensitized control mice received alum only. One week later, the mice were then challenged twice at 3 day intervals by aerosolized OVA at a concentration of 50 mg OVA per milliliter PBS for 30 min. The aerosolized OVA was created by an ultrasonic nebulizer (Ultra-Neb99, Devilbiss). Non-sensitized control mice received PBS only. Two days

Table 1
Ingredients and chemical compositions of the experimental feeds

Ingredients (g/kg feed)	AIN 76	YK01-L	YK01-H	YK07-L	YK07-H
Casein	200	200	200	200	200
DL-methionine	3	3	3	3	3
Corn starch	325	325	325	325	325
Sucrose	325	325	325	325	325
Cellulose	50	50	50	50	50
Soybean oil	50	50	50	50	50
AIN 76 mineral mix	35	35	35	35	35
AIN 76 vitamin mix	10	10	10	10	10
Choline bitartrate	2	2	2	2	2
Ganoderma tsugae YK01	–	2.0	–	–	–
Ganoderma tsugae YK01	–	–	4.0	–	–
Ganoderma tsugae YK07	–	–	–	3.3	–
Ganoderma tsugae YK07	–	–	–	–	6.6

later, the animals were anesthetized with diethyl ether, exsanguinated by retro-orbital venous plexus puncture and immediately euthanized by CO_2 inhalation. The bronchoalvelor lavage fluid (BALF) was collected and assayed. The experimental design is given in Fig. 1.

2.4. Collection of BALF and cellular differential counts

The BALF collection and differential cell counts were manipulated as described by Ye et al. [16]. Briefly, the mice were anesthetized with diethyl ether, exsanguinated by retro-orbital venous plexus puncture and immediately euthanized by CO_2 inhalation. After intubation, their lungs and airways were lavaged with 5 aliquots of 1 ml sterile saline (0.9% NaCl) for a total of 5 ml through the trachea. The bronchoalvelor lavage fluid (BALF) was centrifuged at $200 \times g$ for 10 min at 4 °C. The supernatant (BALF) was collected and stored at -70 °C for future assay. The cell pellet was washed and resuspended in 250 µl normal saline containing 10% bovine serum albumin (Sigma A-9418). Total leukocytes were counted with a hemocytometer using trypan blue dye exclusion method. Aliquots of 100 µl total cells were cytocentrifuged and then stained with Liu's stains for differential cell counts. According to the standard morphologic criteria, a minimum of 200 white blood cells were counted. The cells from BALF were classified as monocytes/macrophages, lymphocytes, neutrophils, or eosinophils.

2.5. Assay of BALF

2.5.1. Inflammatory mediators in BALF

2.5.1.1. Histamine.
The histamine level in BALF was determined by the Histamine-ELISA kit (Cat. No. RE 59221, IBL Hamburg). The manipulation was according to manufacturer's instructions for use. In brief, the BALF samples and plasma standards (0, 0.12, 0.33, 2.00, 5.33, 17.33, 45.33 ng/ml) were acylated with acylation reagent first. Then, aliquots of 150 µl acylated samples, acylated standards and acylated control were pipetted into the 96-microplate wells (Nunc), respectively. Aliquots of 50 µl enzyme conjugate and 50 µl antiserum were pipetted into each well. The plate was

shook carefully (500 rpm) for 3 h on an orbital shaker at room temperature. The plate was washed four times. Aliquots of 200 µl tetramethylbenzidine (TMB) substrate solution were pipetted into the 96-microplate wells and the plate was put on an orbital shaker (500 rpm) for 30 min to develop color. After incubation, aliquots of 100 µl TMB stop solution were added to stop the reaction. The plate was measured the optical density at 450 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria) within 60 min. The concentrations of the standards (abscissa, logarithmic) were plotted against their corresponding optical density (ordinate, linear) to obtain a standard curve. Using the standard curve, the histamine concentration for each unknown sample was determined.

2.5.1.2. Prostaglandin E_2 (PGE_2).
The PGE_2 level in BALF was determined by the method of competitive enzyme immunoassay (Prostaglandin E_2 Enzyme Immunoassay kit, Assay Designs, Inc.). The kit uses a monoclonal antibody to PGE_2 to bind, in a competitive manner, the PGE_2 in the sample or a PGE_2 which conjugated with an alkaline phosphatase molecule. After a simultaneous incubation at room temperature, the excess reagents were washed away and the substrate was added. After a proper incubation time, the enzyme reaction was stopped and the yellow color generated read on a microplate reader at 405 nm (ELISA reader, ASYS Hitech GmbH, Austria). The yellow color intensity was inversely proportional to the concentration of PGE_2 in either standards or BALF. Using the standard curve, the PGE_2 concentration for each unknown sample was determined.

2.5.1.3. Nitric oxide (NO).
Aliquots of 80 µl of BALF samples and standards (0–100 µM sodium nitrite (Sigma S-2252) dissolved in double distilled water) were pipetted into the 96-microplate wells (Nunc), respectively. Then, aliquots of 160 µl Griess reagent were added, respectively, into each well to develop color. The Griess reagent was freshly prepared from Reagent A and B at a ratio of 1 : 1 (Reagent A: 2% (w/v) sulfanilamide (Sigma S-9251) dissolved in 2.5% (v/v) phosphoric acid; Reagent B: 0.2% (w/v) N-1-naphthylethylene diamide dihydrochloride (Sigma N-9125) dissolved in 2.5% (v/v) phosphoric acid). After incubation for 10 min, the plate was read on a plate reader (ELISA reader, ASYS Hitech
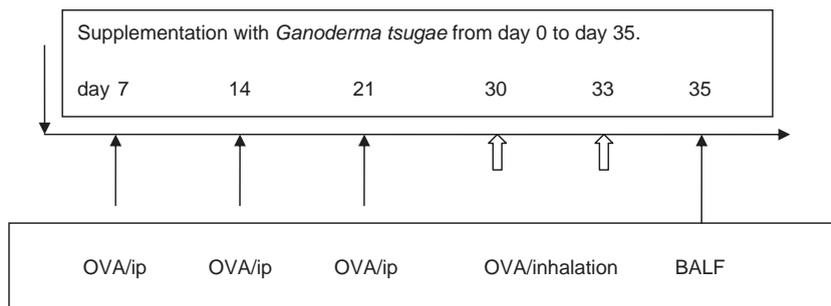

Fig. 1. Sensitization and challenge protocols of allergic airway inflammation. Mice were sensitized with OVA/alum on days 7, 14, 21. The sensitized mice were then challenged twice with aerosolized OVA for 30 min on days 30, 33. All mice were sacrificed on day 35 and bronchoalvelor lavage fluid (BALF) was collected and analyzed.

GmbH, Austria) at 540 nm. Using the standard curve, the NO concentration for each unknown sample was determined.

2.5.2. Protein content analysis

The protein content of the BALF was analyzed using the BCA (bicinchoninic acid) protein assay kit (product No. 23225, Pierce), according to the accompanying instructions, using a 96-well microtitre plate. Briefly, aliquots of 25 µl of each standards (bovine serum albumin, BSA, product No. 23209, Pierce) or unknown samples were pipetted into the appropriate microwell plate wells (Nunc). Aliquots of 200 µl of the working reagent (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide and cupric sulfate) were added to each well and mixed the plate well on a plate shaker for 0.5 min. Then, the plate was covered and incubated at 37 °C for 30 min. After incubation, the plate was cooled to room temperature and measured the absorbance at or near 562 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria). Using the standard curve, the protein concentration for each unknown sample was determined.

2.5.3. Measurement of cytokine and chemokine levels in BALF by an ELISA

2.5.3.1. IL-1β, IL-2, IL-4, IL-5, and IL-6 levels in BALF.
Cytokine (IL-1β, IL-2, IL-4, IL-5, IL-6) levels in BALF were determined by sandwich ELISA kits, respectively. The IL-1β, IL-2, IL-4, IL-5, and IL-6 concentrations were assayed according to the cytokine ELISA protocol of manufacturer's instructions (mouse DuoSet ELISA Development system (R&D Systems). The sensitivity of these cytokine assays was 15.6 pg/ml. Briefly, 100 µl of 1:180 diluted (with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2–7.4, 0.2 µm filtered)) anti-mouse capture antibodies were added to 96-microwell plate wells (Nunc) and incubated overnight at 4 °C. After incubation, plates were washed four times with ELISA wash buffer (0.05% Tween 20 in PBS, pH 7.2–7.4). To block non-specific binding, 200 µl of block buffer (1% bovine serum albumin (BSA), 5% sucrose in PBS with 0.05% NaN_3) were added to each well. The plates were incubated at room temperature for 1 h. After incubation, plates were washed three times with ELISA wash buffer. Volumes of 100 µl of BALF or standard in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris-buffered saline (20 mM Trizma base, 150 mM NaCl), pH 7.2–7.4, 0.2 µm filtered) were added to the 96-microwell plate wells and the plates were incubated at room temperature for 2 h. A seven point (in duplicate) standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 1000 pg/ml were conducted. After incubation, plates were washed four times with ELISA washing solution. Then, 100 µl of detection antibody (biotinylated goat anti-mouse monoclonal antibody at 1:180 dilution in reagent diluent) were added to each well. Plates were incubated at room temperature for 2 h. After incubation, plates were washed six times with ELISA wash buffer. 100 µl of working dilution of streptavidin-HRP (horseradish peroxidase) were added to each well. Plates were incubated at room tem-

perature for 20 min. After incubation, plates were washed six times with ELISA wash buffer. 100 µl of substrate solution (tetramethylbenzidine; TMB) were pipetted into the 96-microwell plate wells. Plates were incubated at room temperature for 20 min to develop color. Then, 50 µl of stop solution (2 N H_2SO_4) were added to each well to stop reaction. The plates were measured the absorbance at 450 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria). Using the seven point standard curves, the levels of IL-1β, IL-2, IL-4, IL-5, and IL-6 in BALF were determined, respectively.

2.5.3.2. Eotaxin level in BALF.
The eotaxin concentration in BALF was determined by mouse eotaxin sandwich ELISA kit (Quantikine M murine, R&D Systems). The eotaxin concentration was assayed according to the manufacturer's instructions. The sensitivity of this assay was 15.6 pg/ml. Briefly, 50 µl of assay diluent RD1-21 (a buffered protein solution) and 50 µl of standard, control or sample were added to 96-microwell plate wells (pre-coated with polyclonal antibody specific for mouse eotaxin) and incubated for 2 h at room temperature. After incubation, plates were washed five times with wash buffer (a buffered surfactant). 100 µl of mouse eotaxin conjugate (containing antibody against mouse eotaxin conjugated to horseradish peroxidase) were added to each well. The plates were incubated at room temperature for 2 h. After incubation, plates were washed five times with wash buffer. 100 µl of substrate solution (tetramethylbenzidine; TMB) were pipetted into the 96-microwell plate wells. Plates were incubated at room temperature for 30 min to develop color. Then, 100 µl of stop solution (2 N H_2SO_4) were added to each well to stop reaction. The plates were measured the absorbance at 450 nm on a plate reader (ELISA reader, ASYS Hitech GmbH, Austria). A seven point (in duplicate) standard curve using 2-fold serial dilutions in reagent diluent, and a high standard of 1000 pg/ml were conducted. Using the standard curve, the eotaxin level in BALF was determined.

2.6. Statistical analysis

Data are expressed as mean ± S.E. and analyzed statistically using Dunnett's test of parametric type. The statistics on *G. tsugae* YK01 and YK07 products were run separately. Differences between the dietary control and other groups were considered statistically significant if $P < 0.05$.

3. Results

3.1. Effects of G. tsugae supplementation on cellularity of BALF from OVA-sensitized and -challenged mice

Sensitization and challenge with OVA significantly increased an influx of total leukocytes (increase from $4.92 \pm 0.51 \times 10^5$ to $32.52 \pm 8.79 \times 10^5$ cells/mouse) into the lungs and airways (Table 2). However, the supplementation of *G. tsugae*, YK01-L, YK01-H, YK07-L, and YK07-H, significantly decreased an influx of total leukocytes (16.43 ± 2.82, 14.75 ± 3.47, 15.59 ± 3.44, $16.23 \pm 1.89 \times 10^5$ cells/mouse,

respectively) into the lungs and airways of OVA-challenged mice compared to dietary control (DC) group. The reduction in total leukocyte numbers in lungs and airways might indicate an alleviation of airway inflammation. Sensitization and challenge with OVA significantly increased infiltrations of eosinophils and lymphocytes (increase from $0.00 \pm 0.00\%$ to $30.57 \pm 5.90\%$, and increase from $2.26 \pm 0.74\%$ to $16.71 \pm 1.82\%$, respectively) into the lungs and airways, however the percentage of monocytes/macrophages in BALF reduced. *G. tsugae* supplementation diets decreased the percentages of eosinophils and lymphocytes in BALF, however the percentages of monocytes/macrophages increased. Supplementation of *G. tsugae*, YK01-L, YK01-H, and YK07-H, significantly decreased influxes of lymphocytes into the lungs and airways compared to DC group. Although the percentage of infiltrating eosinophils in BALF slightly decreased, it did not significantly differ from DC group. The reduction in the frequency of infiltrating eosinophils and lymphocytes into the lungs and airways might reduce the eosinophilia allergic inflammation.

The number of neutrophils in the BALF was also examined. However, the number of neutrophils was too scarce to count. Although chronic asthma inflammation of the airways is characterized by the continued presence of increased numbers of Th2 lymphocytes, eosinophils, neutrophils, and other leukocytes, the number of neutrophils in the BALF was few in this airway sensitization and challenge mouse model. We suppose that the airway sensitization and challenge mouse model in this study induced an acute but not chronic inflammation of the airways. Therefore, few neutrophils infiltrated into the airways in this airway inflammation animal model.

3.2. Effects of G. tsugae supplementation on eotaxin levels in BALF from OVA-sensitized and -challenged mice

Eotaxin levels in BALF from groups of non-sensitized control (SC), dietary control (DC), YK01-L, YK01-H, YK07-L, and YK07-H were 48.44 ± 1.78, 58.36 ± 5.10, 65.80 ± 4.04, 38.15 ± 3.31, 39.49 ± 3.01, and 42.65 ± 4.04 pg/mouse, respectively (Fig. 2). The eotaxin level in BALF slightly increased after OVA challenged (DC group) compared to PBS challenged (SC group). However, *G. tsugae* supplementation, YK01-H, YK07-L, and YK07-H, significantly reduced the secretion of eotaxin into the lungs and airways of
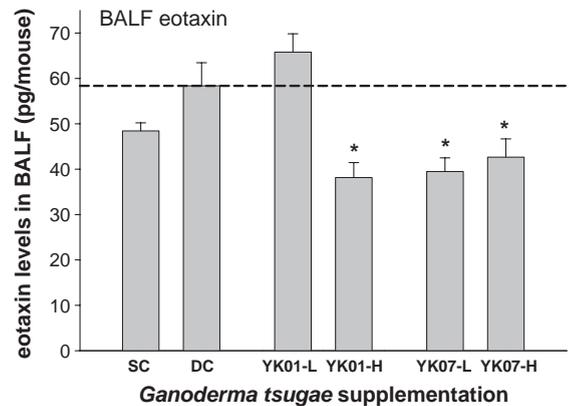

Fig. 2. Effects of *Ganoderma tsugae* supplementation on eotaxin levels in bronchoalvelor lavage fluid (BALF) from ovalbumin-sensitized and -challenged female BALB/c mice through five weeks feeding. Each value represents a mean $\pm$ S.E. ($n = 14$). *$P < 0.05$ vs. dietary control (DC) group.

OVA-challenged mice. Eotaxin can activate eosinophils and influence airway responsiveness. The reduction of eotaxin levels in the BALF might alleviate the airway eosinophilia.

3.3. Effects of G. tsugae supplementation on inflammatory mediator levels in BALF from OVA-sensitized and -challenged mice

Sensitization and challenge with OVA significantly increased the secretion of histamine and PGE_2 (increase from 23.45 ± 1.57 to 33.37 ± 2.20 ng/mouse, and increase from 0.70 ± 0.06 to 34.50 ± 6.16 ng/mouse, respectively) to the lungs and airways (Table 3). *G. tsugae* supplementation, YK01-L, and YK01-H, significantly decreased the levels of histamine and PGE_2 in BALF in dose-dependent manners. *G. tsugae* supplementation of YK07-L and YK07-H also reduced the levels of histamine and PGE_2 in BALF. However YK07-L and YK07-H supplementation diets could not significantly decreased the PGE_2 level in BALF yet. Sensitization and challenge with OVA and *G. tsugae* supplementation could not significantly affect the production of nitric oxide (NO) in BALF. The reduction in histamine and PGE_2 levels in BALF might alleviate the allergic airway inflammation.

Table 2
Effects of *Ganoderma tsugae* supplementation on cellularity in bronchoalvelor lavage fluid (BALF) from ovalbumin-sensitized and -challenged female BALB/c mice through five weeks feeding

	Cell numbers and cell distribution in BALF			
	Total leukocytes ($\times 10^5$ cells/mouse)	eosinophils (%)	monocytes/macrophages (%)	lymphocytes (%)
Non-sensitized control (SC)	4.92 ± 0.51*	0.00 ± 0.00*	97.74 ± 0.74	2.26 ± 0.74*
Dietary control (DC)	32.52 ± 8.79	30.57 ± 5.90	52.72 ± 5.58	16.71 ± 1.82
YK01-L	16.43 ± 2.82*	23.14 ± 4.87	66.02 ± 5.47	10.84 ± 1.81*
YK01-H	14.75 ± 3.47*	18.13 ± 5.03	71.33 ± 5.70*	10.54 ± 1.64*
YK07-L	15.59 ± 3.44*	23.09 ± 6.76	63.34 ± 6.89	13.57 ± 2.15
YK07-H	16.23 ± 1.89*	24.01 ± 5.39	69.22 ± 5.63	6.77 ± 1.14*

Each value represents a mean $\pm$ S.E. ($n = 14$). *$P < 0.05$ vs. dietary control (DC) group in the same column.

Table 3
Effects of *Ganoderma tsugae* supplementation on inflammatory mediators in bronchoalvelor lavage fluid (BALF) from ovalbumin-sensitized and -challenged female BALB/c mice through five weeks feeding

	Inflammatory mediators in BALF		
	Histamine (ng/mouse)	PGE$_2$ (ng/mouse)	NO (nmol/mouse)
Non-sensitized control (SC)	23.45 ± 1.57*	0.70 ± 0.06*	3.62 ± 0.33
Dietary control (DC)	33.37 ± 2.20	34.50 ± 6.16	5.11 ± 0.56
YK01-L	23.32 ± 1.54*	17.17 ± 3.21*	4.36 ± 0.56
YK01-H	10.65 ± 1.02*	15.04 ± 4.56*	3.98 ± 0.66
YK07-L	20.35 ± 2.10*	18.80 ± 4.53	3.23 ± 0.77
YK07-H	26.86 ± 1.98*	20.34 ± 3.98	4.83 ± 0.68

Each value represents a mean ± S.E. ($n = 14$). *$P < 0.05$ vs. dietary control (DC) group in the same column.

3.4. Effects of G. tsugae supplementation on protein levels in BALF from OVA-sensitized and -challenged mice

Protein levels in BALF from groups of non-sensitized control (SC), dietary control (DC), YK01-L, YK01-H, YK07-L, and YK07-H were 185.63 ± 7.14, 611.82 ± 100.08, 465.64 ± 60.18, 286.31 ± 24.44, 339.65 ± 35.96, and 426.95 ± 54.04 μg/mouse, respectively (Fig. 3). The BALF protein level from DC group was significantly higher than that from SC group. However, *G. tsugae* supplementation diets, YH01-L, YK01-H, YK07-L, and YK07-H, significantly decreased the secretion of protein into the lungs and airways of OVA-challenged mice. Excess production of mucus proteins may damage epithelial lining and induce airway inflammation sequentially. The results of protein production in BALF suggest that proper supplementation of *G. tsugae* might alleviate airway inflammation of OVA-challenged mice.

3.5. Effects of G. tsugae supplementation on the levels of pro-inflammatory cytokines in BALF from OVA-sensitized and -challenged mice

Table 4 shows the effects of *G. tsugae* supplementation on the levels of inflammatory cytokine IL-1β and IL-6 in
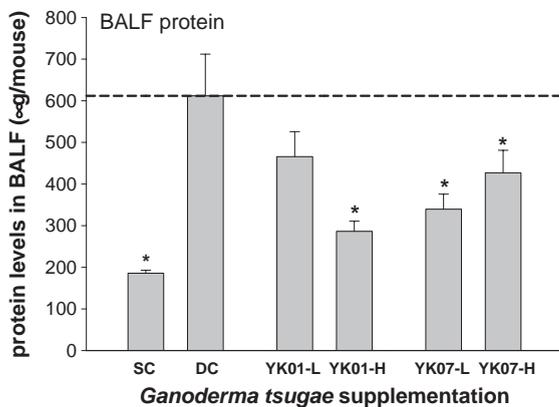
Fig. 3. Effects of *Ganoderma tsugae* supplementation on protein levels in bronchoalvelor lavage fluid (BALF) from ovalbumin-sensitized and -challenged female BALB/c mice through five weeks feeding. Each value represents a mean ± S.E. ($n = 14$). *$P < 0.05$ vs. dietary control (DC) group.

BALF from OVA-challenged mice through five weeks feeding. Sensitization and challenge with OVA significantly increased the secretion of IL-1β in BALF, however IL-6 level in BALF did not significantly change. *G. tsugae* supplementation diets, YK01-L, YK01-H, YK07-L, and YK07-H, did not significantly change the levels of inflammatory cytokines in BALF.

3.6. Effects of G. tsugae supplementation on the levels of Th1/Th2 cytokines in BALF from OVA-sensitized and -challenged mice

To evaluate the effects of *G. tsugae* supplementation on the secretion of Th1/Th2 cytokines in the local tissues of lungs and airways, the amounts of IL-4, IL-5 (Th2 cytokine), and IL-2 (Th1 cytokine) in BALF were measured (Table 5). Sensitization and challenge with OVA significantly increased IL-2, IL-4, and IL-5 cytokine production in BALF, however the increases in IL-4 and IL-5 secretion were much higher than that of IL-2. These results indicate that sensitization and challenge with OVA propelled the Th1/Th2 balance incline to Th2 immune response in the lungs and airways. *G. tsugae* supplementation diets, YK01-L, YK01-H, YK07-L, and YK07-H, did not significantly change, but had a trend of decrease, the levels of IL-2 (Th1 cytokine) in BALF. However, *G. tsugae* supplementation diets significantly increased the amount of Th2 cytokines such as IL-4 (YK01-L, YK01-H, YK07-L, and YK07-H diets) and IL-5 (YK01-L and YK01-H diets) in BALF. The secretion amount of IL-5 (10.59 ± 1.06 to

Table 4
Effects of *Ganoderma tsugae* supplementation on the levels of pro-inflammatory cytokines IL-1β and IL-6 in bronchoalvelor lavage fluid (BALF) from ovalbumin-sensitized and -challenged female BALB/c mice through five weeks feeding

	IL-1β (pg/mouse)	IL-6 (pg/mouse)
Non-sensitized control (SC)	66.81 ± 7.74*	1346 ± 168
Dietary control (DC)	171.88 ± 19.60	1368 ± 93
YK01-L	170.67 ± 18.07	1054 ± 123
YK01-H	176.19 ± 20.41	1471 ± 149
YK07-L	182.00 ± 23.29	1393 ± 221
YK07-H	198.23 ± 21.17	1442 ± 199

Each value represents a mean ± S.E. ($n = 14$). *$P < 0.05$ vs. dietary control (DC) group in the same column.

Table 5
Effects of *Ganoderma tsugae* supplementation on the levels of Th1- and Th2-type cytokines in bronchoalvelor lavage fluid (BALF) from ovalbumin-sensitized and -challenged female BALB/c mice through five weeks feeding

	Th1	Th2 cytokine	
	IL-2 (U/mouse)	IL-4 (pg/mouse)	IL-5 (pg/mouse)
Non-sensitized control (SC)	17.41 ± 2.76*	0.84 ± 0.16*	10.59 ± 1.06*
Dietary control (DC)	28.48 ± 3.02	3.20 ± 0.30	144.93 ± 29.71
YK01-L	27.29 ± 5.60	9.12 ± 1.55*	291.49 ± 48.72*
YK01-H	26.20 ± 3.96	10.92 ± 0.79*	326.76 ± 79.33*
YK07-L	19.26 ± 3.48	8.55 ± 0.67*	251.10 ± 26.39
YK07-H	18.15 ± 3.88	9.37 ± 0.88*	227.63 ± 45.97

Each value represents a mean ± S.E. ($n = 14$). *$P < 0.05$ vs. dietary control (DC) group in the same column.

326.76 ± 79.33 pg/mouse) was much higher than that of IL-4 (0.84 ± 0.16 to 10.92 ± 0.79 pg/mouse) in BALF.

4. Discussion

The objective of this study was to evaluate the effects of *G. tsugae* on airway inflammation in an allergic asthma animal model. To evaluate this, a mouse model for allergic asthma was established. Mice sensitized and challenged with OVA induce the formation of asthma-like symptoms, e.g. allergic airway inflammation, differential cells infiltrating into the local tissues of lungs and airways, eosinophilia, and chemical mediator releases including histamine, thromboxane A_2 (TXA_2), leukotrienes, Th1/Th2-type cytokines, nitric oxide (NO), and eotaxin [1,2,9–17]. Using the established model of this study, we demonstrated that sensitization and challenge with OVA significantly increased an influx of total leukocytes especially eosinophils and lymphocytes, releases of histamine, prostaglandin E_2, protein, IL-1β, IL-2, IL-4, and IL-5 into the bronchoalveolar tissues. Even though monocytes/macrophages infiltration, and nitric oxide, eotaxin, and IL-6 releases did not significantly change, an intermediate airway inflammation mouse model was achieved. This airway inflammation mouse model (Fig. 1) was practicable for the purpose of evaluating the effects of *G. tsugae* supplementation on airway inflammation in vivo.

In this study, we report the therapeutic effects of *G. tsugae* diets on airway inflammation in an induced mouse model. *G. tsugae* supplementation diets can reverse established airway inflammation. Total infiltrating leukocytes, eosinophils, and lymphocytes in BALF were reduced by *G. tsugae* supplementation diets about 49.5% to 54.6%, 24.3% to 40.7%, and 18.8% to 59.5%, respectively (Table 2). Eotaxin levels in BALF were also significantly reduced by YK01-H, YK07-L, and YK07-H about 34.6%, 32.3%, and 26.9%, respectively (Fig. 2). Eotaxin in the lungs and airways may be involved in trafficking and activation of leukocytes

such as eosinophils, Th2 cells, basophils and mast cells [14]. The recruitment of differential leukocytes to the airways and lungs may worsen the inflammation status, however decrease of cells infiltration into BALF may alleviate allergic asthmatic inflammation [15,16]. Our results suggest that *G. tsugae* supplementation inhibits the secretion of eotaxin into local tissues of lungs and airways and subsequently reduces total infiltrating leukocytes into the local tissues.

Our established asthma model in this study emulated the late asthmatic response. With this allergic asthmatic animal model, we found that *G. tsugae* supplementation diets reduced the levels of histamine, PGE_2, and protein in BALF by about 19.3% to 67.9%, 41.0% to 56.4% (Table 3), and 23.9% to 53.2% (Fig. 3), respectively. These results suggest that *G. tsugae* might alleviate the bronchoalveolar inflammation via inhibiting the release of inflammation mediators such as histamine, PGE_2, protein, and eotaxin into the lungs and airways, although *G. tsugae* supplementation diets could not significantly reduce the levels of nitric oxide, and pro-inflammatory cytokine IL-1β and IL-6 in BALF.

Th2 cytokines are generally regarded as a central role in the pathogenesis of allergic asthma [20]. Th2-mediated allergic asthma is defined by eosinophilia, mucus overproduction, and airway hyperresponsiveness. However, Th1 cells associated with lymphocytes and macrophages, but not neutrophils in the airways also can induce strong airway hyperresponsiveness independent of IL-4 and IL-13 [1]. In this study, we demonstrated that sensitization and challenge with OVA significantly induced IL-2 (Th1 cytokine), IL-4, and IL-5 (Th2 cytokines) production in the BALF (Table 5). However, few neutrophils were recruited to the airways in this airway inflammation mouse model. We deduced that both Th1 and Th2 responses were involved in this airway inflammation model. This model induced an acute but not chronic airway inflammation. Morokata et al. [19] have reported that

male BALB/c mice, sensitized by an intraperitoneal injection of aluminum hydroxide gel (alum) (2 mg) and OVA at the dose of 8 μg twice at 5 day intervals and then exposed for 1 h to aerosolized OVA (0.5%) dissolved in 0.9% saline 7 days later, generated Th1-type cytokines in their lungs, including low levels of IL-4, IL-5 and high level of IFN-γ [19]. However, Leung et al. [34] have established a chronic allergen-induced airway asthmatic Brown Norway (BN) rat model by intraperitoneal injections of OVA three times and then exposed to 1% OVA aerosol for 20 min six times at 3 day intervals to evaluate the effects of corticosteroid on allergen-induced airway inflammation. Inhaled glucocorticoids are considered as a standard therapy for asthma, and reduce airway inflammation, bronchial hyperresponsiveness (BHR) and airway remodeling. In the above asthmatic BN rat model study, ciclesonide, a nonhalogenated inhaled corticosteroid, inhibits the allergen-induced increase in airway eosinophils and T cells, reduces goblet cell hyperplasia, and decreases 5-bromo-2′-deoxyuridine-immunoreactive airway smooth muscle (ASM) and epithelial cells. The allergen (OVA) treatment in this study was similar to the asthmatic BN rat model, and induced an acute but not chronic airway inflammation. In our established airway inflammation mouse model, sensitization and challenge with OVA significantly increased an influx of total leukocytes, eosinophils, and lymphcytes, and the secretion of inflammatory mediators. However, the supplementation of G. tsugae potently inhibited the allergic airway inflammation. This allergic airway inflammation model provides an antecedent approach of G. tsugae for the treatment of allergic airway inflammation.

A traditional Japanese medicine, Bu-Zhong-yi-qi-tang (Japanese name: Hochu-ekki-to, HOT), is found to have dichotomous effects on allergic asthma in mice [22]. Oral administration of HOT in the eliciting phase (late asthmatic response), well after the Th2 responses have developed, aggravates the allergic inflammation after OVA inhalation. It is suggested that HOT may be applicable for prophylactic use to prevent allergy responses but must be used carefully for patients with apparent allergic inflammation [22]. Our results demonstrate that supplementation of G. tsugae daily might have prophylactic effects on allergic airway inflammation. However, continuous administration of G. tsugae enhanced Th2 but slightly decreased Th1 cytokine secretion in the lungs and airways (Table 5). As Th2-type cytokines, especially IL-5, are up-regulated in the sites of inflammation, the number of eosinophils and the airway eosinophilia would become exacerbated.

However, some Th2 cytokines, such as IL-4 and IL-10, are regarded as anti-inflammatory cytokines [35,36]. IL-4 and IL-10 cytokines inhibit the production of inflammatory mediators, such as prostaglandins. IL-4 reduces lipopolysaccharide (LPS)-stimulated PGE_2 production in decidual cells and is a potential immunomodulatory agent in decidual inflammation [36]. G. tsugae supplementation significantly enhanced the secretion of IL-4 cytokine in BALF. IL-4 cytokine in the airways might play an antagonistic agent against IL-5 and alleviate bronchoalveolar inflammation. Eotaxins are CC chemokines that act predominantly on eosinophils. However, eosinophils may also be activated by Th2 cytokines, especially IL-5. G. tsugae supplementation decreased eotaxin levels (Fig. 2) but increased IL-5 secretion in the BALF (Table 5). The antagonistic effects between IL-5 and eotaxin might result in little change in the eosinophil level of the airways (Table 2). However, the immunomodulatory mechanisms of G. tsugae on cytokine and chemokine secretions remain to be further clarified.

G. tsugae is the most widely cultivated species of medicinal mushrooms in Taiwan. The mycelia and fruiting bodies of G. tsugae are used as Chinese traditional medicines to treat diseases such as tumors [27,28], wound healing [29], liver fibrosis [30], and autoimmune diseases [31]. Bioactive constituents of G. tsugae are constantly unraveled. As G. tsugae YK01 and YK07 have similar components and consist of 0.28% protein, 0% lipid, 55.56% carbohydrate, 1.67% sodium, 12.5% polysaccharides, and 12% triterpenes, we presume that the polysaccharides and triterpenes in G. tsugae might be involved in the immunomodulatory effects of anti-inflammation and Th1/Th2 balance [37,38]. However, the effective compounds and pharmaceutical effects of G. tsugae on allergic asthma remain to be elucidated. We suppose that both G. tsugae products YK01 (baby G. tsugae) and YK07 (mature G. tsugae) alleviated bronchoalveolar inflammation via decreasing the infiltration of inflammatory cells and the secretion of inflammatory mediators into the local tissues of lungs and airways. These results provide an antecedent approach using G. tsugae for the treatment of allergic asthma.

In summary, supplementation of G. tsugae reduced inflammatory mediators in BALF and alleviated airway inflammation in an allergic airway inflammation murine model. These results are important and useful for the future exploitation of G. tsugae to develop a Chinese immunopharmacological drug or a novel functional food for safeguarding health of allergic asthma patients.

Acknowledgements

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