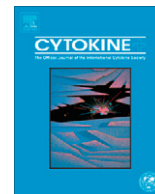


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Effect of the fungal immunomodulatory protein FIP-*fve* on airway inflammation and cytokine production in mouse asthma model

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ABSTRACT

The allergy is dependent on the balance between Th1 and Th2. The fungal immunomodulatory protein (FIP-*fve*) was isolated from *Flammulina velutipes*. FIP-*fve* has been demonstrated to skew the response to Th1 cytokine production. We investigated whether oral administrations of FIP-*fve* inhibited allergen (OVA)-induced chronic airway inflammation in the mouse asthma model. After intranasal challenge with OVA, the airway inflammation and hyperresponsiveness were determined by bronchoalveolar lavage fluid (BALF) analysis and ELISA assay. Both pre-treated and post-treated with FIP-*fve* suppressed the airway hyperresponsiveness by methacholine challenge and significantly decreased the number of infiltrating inflammatory cells and Th2 cytokines in bronchoalveolar lavage fluid (BALF) and serum compared with the OVA sensitized mice. In addition, FIP-*fve* reduced OVA-specific IgE levels in serum. FIP-*fve* markedly alleviated the OVA-induced airway hyperresponsiveness (AHR) to inhaled methacholine. Based on lung histopathological studies using hematoxylin and Liu's staining, FIP-*fve* inhibited inflammatory cell infiltration compared with the OVA-sensitized mice. Oral FIP-*fve* had an anti-inflammatory effect on OVA-induced airway inflammations and might possess the potential for alternative therapy for allergic airway diseases.

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1. Introduction

Asthma is one of the most common respiratory diseases in adults and chronic diseases in children. The incidence and severity of asthma, a chronic disease, have greatly increased in the United States over the past three decades [1]. In Taiwan, asthma, bronchitis, emphysema, and COPD are ranked the 7th among the leading causes of death [2,3]. The inhalation of allergens stimulates both bone marrow-derived cells and non-bone marrow-derived cells in the innate immune system in order to secrete cytokines that promote Ag expressions on CD4⁺ T cells, and activate the APCs

and the T cells to produce Th2 responses [4]. Th2 cytokines, such as IL-4, IL-5, IL-9, and IL-13 [5] then induce the changes in the airways and lung parenchyma that are associated with asthma, airway eosinophilia, pulmonary lymphocytosis, mastocytosis, alternative macrophage activation, epithelial cell proliferation with goblet cell hyperplasia (GCH), increased mucus secretion, smooth muscle hyperplasia, hypertrophy, hypercontractility, subepithelial fibrosis, IgE secretion, increased production of chemokines that attract T cells, eosinophils, neutrophils, mast cells, or their precursors to the lungs, and airway hyperresponsiveness (AHR). Hyperresponsiveness is defined as increased sensitivity to agents, such as cholinergic agents and other stimuli that cause smooth contractions and increase airway resistance by narrowing the airways [5–7]. IFN- γ produced by Th1 cells is known to have the ability to suppress the proliferation of Th2 cells, inhibit IgE production, and further promote Th1 differentiation by inducing IL-12 receptor β 2-chain expressions on T cells and IL-12 production by macrophages [8]. In contrast, IL-4, IL-6, and IL-10 produced by Th2 cells can further promote Th2 differentiation and inhibit macrophage

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antigen expression and IL-12 production. However, recent studies suggest that an array of proinflammatory mediators contributes to both the acute and chronic inflammations associated with asthma [6–9].

An inhaled corticosteroid is the principle treatment for asthma. However, this therapy is not applicable for a considerable percentage of patients with asthma, and only about one third of patients actually benefit from the addition of the leukotriene inhibitors [10]. Severe and corticosteroid-resistant forms of asthma actually lead to life-threatening attacks, and the mortality rate of this disease is showing an increase trend. There is thus an urgent need to devise treatments for asthma that are more effective, but have fewer undesirable side effects [1]. Due to the cytokines involvement in chronic inflammation and airway remodeling [1], they are the considered main targets in novel allergy and asthma therapies.

Earlier studies show that the increased levels of TGF- β and IL-10 produced by Treg cells can suppress IgE production and simultaneously increase the production of non-inflammatory antibodies IgG4 and IgA [7]. Treg cells can suppress Th2 responses to allergens, airway eosinophils, mucous hypersecretion, and airway hyperresponsiveness [8]. In addition, the studies also show that the regulatory T cells play an important role in protecting against asthma [11].

Golden needle mushrooms are a popular edible mushroom in Asia. A new fungal immunomodulatory protein (FIP-*fve*) was isolated and purified from the edible golden needle mushroom (*Flammulina velutipes*). It is a simple protein that consists of 114 amino acid residues with a molecular weight of 13 kDa, while its cDNA has been cloned [12–16]. A previous study had showed that FIP-*fve* stimulated the proliferation of human peripheral blood mononuclear cells (PBMCs) in vitro with increased transcripts of IL-2 and IFN- γ . Intraperitoneal (i.p.) injection of FIP-*fve* appeared to have modified the immune responses in mice and have inhibited the anaphylactic responses [13,15]. FIP-*fve* induced the production of IFN- γ through a p38 MAPK pathway and calcium-dependent PKC pathway [12,17]. Both FIP-*fve* and Dp2 peptide could relieve the rDp2 induced airway inflammation by local nasal immunotherapy [12]. Moreover, Hsieh et al., reported that orally administrated FIP-*fve* inhibits food-allergic reactions in vivo and reduces liver tumor [18].

We hypothesized that orally administrated FIP-*fve*, should be able to switch the immune response toward Th1 and Treg response. To test this hypothesis, oral FIP-*fve* treatment during the OVA-sensitization/challenge was evaluated for the effect of FIP-*fve* in pre-established Th2 response mice.

2. Materials and methods

2.1. Mice

Female BALB/c mice at 5–8 weeks of age and with body weights of 20–25 g were purchased from the National Laboratory Animal Center. All animal care and housing requirements of the Chung-Shan Medical University and all procedures were performed in accordance with the Chung Shan Medical University Animal Care and Use Committee. Animals known to be high IgE responders were used, and the mice were maintained on an ovalbumin (OVA)-free diet and were individually housed in rack-mounted stainless cages with access to food and water. Experiments are grouped into four groups as follows: (1) normal control group used normal saline plus Alum with intraperitoneally and used normal saline in intranasal; (2) positive group used 50 μ g OVA plus Alum with intraperitoneally and used 5% OVA in intranasal; in (3) pre- and (4) post-FIP group used 50 μ g OVA plus Alum with intraperitoneally and used 5% OVA in intranasal, and feed 200 μ g of FIP-*fve*.

2.2. The FIP-*fve* purification

The FIP-*fve* was purified from the crude extract of *F. velutipes* through the previously described ion-exchange column chromatography [13,14]. The fruiting bodies of golden needle mushroom (300 g) were homogenized with 1000 mL of ice-cold 5% acetic acid in the 0.05 M 2-mercaptoethanol, and the soluble proteins in the supernatant were precipitated by the addition of ammonium sulfate to 95% saturation and centrifuge (8000 rpm, 20 min, 4 °C; centrifugal: Hitachi CR-21 from Chung Shan Medical University). The precipitation samples were collected and then dialysed against 10 mM Tris/HCl (pH 8.0) at 4 °C for 48 h with three changes of dialysis solution. The dialysate was applied to a DEAE-cellulose column (DE-52) which was previously equilibrated with 10 mM Tris/HCl pH 8.0. The column was washed with 200 ml equilibration buffer and then eluted with 200 ml 0.5 M NaCl in 10 mM Tris/HCl pH 8.0. Fractions of FIP-*fve* as a single peak on absorbance at 280 nm were collected. The protein was present as a single band on SDS-PAGE at 13 kDa. To further examine the purity, the protein solution was analyzed by SDS-PAGE. The activity of FIP-*fve* was checked with a proliferation assay on human peripheral blood lymphocytes. The active fractions were collected, with about 50 mg purified FIP-*fve* normally being obtained [13,14].

2.3. Sds-page

This was performed according to Laemmli in 12% slab gels on a Bio-Rad mini protein II gel apparatus. The gels were stained with Coomassie brilliant blue R [13].

2.4. The mouse model of allergic asthma

The allergic asthma model is as shown in Fig. 1a. The mice remained normal saline Alum or were sensitized to OVA intraperitoneally with Alum (50 μ g OVA in alum) on day 1, 2, 3 and 14, followed by intranasal challenge with OVA (3% solution, 50 μ l) or normal saline on day 14, 17, 21, 24, and 27. The animals received either vehicle control or FIP-*fve* feed (200 μ g/day FIP-*fve* diluted in ddH₂O) from day 1 to day 14 (pre-treated FIP-*fve* mice, as shown in Fig. 1b), or from day 14 to day 27 (post-treated FIP-*fve* mice, as shown in Fig. 1c). FIP-*fve* dose were used as reported by Hsieh KY and colleagues [12].

2.5. AHR and BALF isolation

AHR was assessed in unrestrained mice through whole-body barometric plethysmography (Model PLY 3211; Buxco Electronic Inc., Sharon, CT) in order to record enhanced pauses (Penh). Penh, a dimensionless parameter, was used to measure the pulmonary resistance, which was calculated by changing the chamber pressures through methacholine challenged during inspiration and expiration. After a brief acclimatization at the chamber, the mice received an initial baseline challenge of saline, followed by increasing doses of nebulized methacholine (0, 5, 10, and 20 mg/ml). Exposed parts of the methacholine, each mouse are different dose exposure and doses are as follows: 0 (saline), 5 mg, 10 mg, and 20 mg. Then spray in the part of exposure time, each dose of 3 min, and then enter the breathing frequency of reading time is 3 min. The box pressure waveforms generated from the respiratory cycle were used to calculate the peak expiratory pressure (PEP), the peak inspiratory pressure (PIP), and the time of expiration. Penh was then calculated using the following formula: $\text{Penh} = \text{Pause} \times \text{PEP}/\text{PIP}$. After that, the Penh values were averaged and reported as baseline saline values in percentages [16,19].

Bronchoalveolar lavage fluid (BALF) was isolated in 1 ml of PBS. The BALF cellularity was determined using a hemocytometer. The

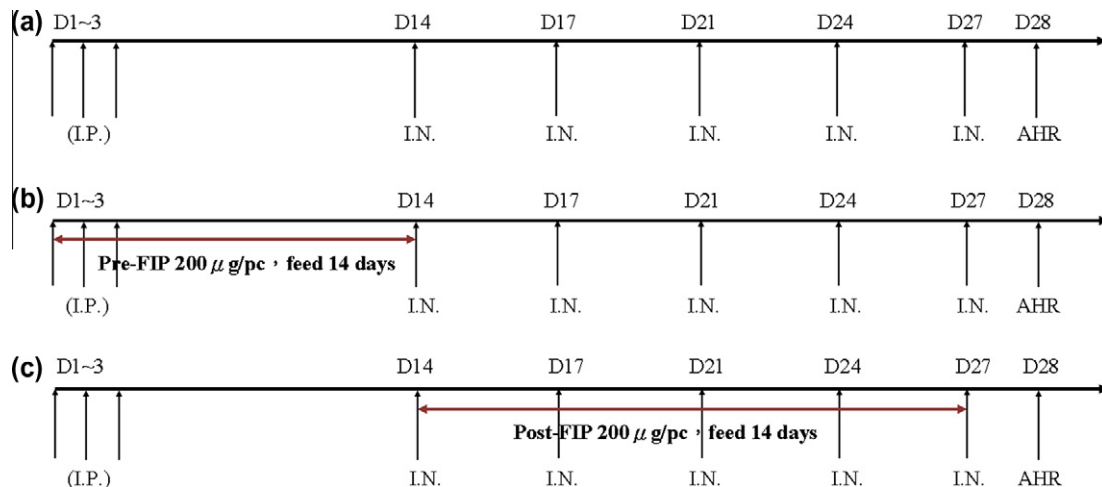


Fig. 1. The protocols used for the mouse models of allergic asthma. (a) The protocols used for the mouse models of allergic asthma. (b) The protocols used for the mouse models of allergic asthma and the pre-treatment with FIP-fve. (c) The protocols used for the mouse models of allergic asthma and the post-treatment with FIP-fve. The protocol mice were challenged with 50 μ g/pc OVA by I.P. and 50 μ l 5% OVA solution by I.N.

cells were centrifuged onto slides and were fixed and stained using the Liu's staining. The observer counted 200–300 cells per slide using the standard morphological criteria to classify the individual leukocyte populations [18,20].

2.6. The measurement of OVA-specific antibodies in serum

The levels of OVA-specific IgE and IgG2a in serum were first determined. In short, the 96-well microtitre plates were coated with 100 mg/mL of OVA in 0.1 M NaHCO₃ at 4 °C, and left unattended overnight. The plates were washed with Tween 20 solution (0.05%) and then blocked with bovine serum albumin (BSA) (3%) for 1 h at 37 °C. After washing, 50 μ l of serially diluted sera in 3% BSA were added and incubated at 4 °C and were left unattended overnight. After washing, HRP conjugated with the anti-mouse isotype-specific antibody (BD Pharmingen™), and optimal dilutions were added. The plates were incubated at 37 °C for 2 h and were then washed. To determine the IgE/IgG2a, p-nitrophenyl phosphate substrate (Sigma Chemical Co., St Louis, MO, USA) was added and the absorbance at 490 nm was measured.

2.7. The measurement of cytokines

The samples of serum and BALF were collected from each group of mice after sacrifice. The samples were assayed for the presence of cytokines using the R&D system per manufacturer's protocol. After that, IFN- γ , IL-4, IL-5, IL-10, IL-13, and TGF- β were assayed according to the manufacturer's kit protocol (Quantikine ELISA Kit., R&D system, USA), and the ELISA plate was read at 450 nm using a Bio-Rad ELISA Reader. The data was then analyzed through the SoftMax Pro software. The unknowns were compared using a standard curve containing at least five to seven dilution points, which were the relevant recombinant cytokines on each assay plate.

2.8. Histological analysis

To assess the pathological changes, samples of the lungs were taken after the sacrifice. The samples were fixed in neutrally buffered 10% formaldehyde and were embedded in paraffin. Five micrometer sections were stained with hematoxylin and eosin (H&E).

2.9. Statistical analyses

All data points represent the mean $\pm$ SEM of the individual mice groups. Analyses were performed using the GraphPad Instat software (San Diego, CA), and the Mann–Whitney nonparametric test was conducted to determine the statistical significance, where appropriate. The result of $p < 0.05$ was considered significant.

3. Results

3.1. The effect of FIP-fve on the AHR

The mice sensitized and challenged with OVA were significantly more sensitive to methacholine exposure than the normal control group ($p < 0.001$). In this study, we investigated whether FIP-fve had a benefit effect during the inflammation. The mice that received the FIP-fve during the challenge phase (post-treated) had significantly lower AHR to methacholine compared with the OVA mice tested at 20 mg/ml level ($p < 0.001$) and comparable responses to the normal control animals were observed at the same dose. Similar results were observed in terms of the significant inhibition of AHR when FIP-fve was administered during sensitization phase (pre-treated) (Fig. 2).

3.2. The effect of FIP-fve treatment on the reversal of allergen-induced airway inflammation and serum ovalbumin-specific antibodies in serum

The significance of certain Immunoglobulin isotypes, including IgE and IgG2a was implicated. The serum IgE and IgG2a elevated significantly after the OVA sensitization/challenge compared with the normal control group, and oral FIP-fve treatment (pre-treated and post-treated) during the sensitization/challenge to OVA the serum IgE were decreased significantly ($p < 0.001$, Table 1, Fig. 3a) and the serum IgG2a on the other hand were increased significantly ($p < 0.001$, Table 1, Fig. 3b).

3.3. The effect of FIP-fve on the infiltrating cells of the lungs

The infiltrating cells of the lungs are the hallmark of asthma. We evaluated the BALF cell differential counts (Fig. 4), it was found that total cells, eosinophils and lymphocytes were significantly increased in the mice sensitized/challenged with OVA than in normal

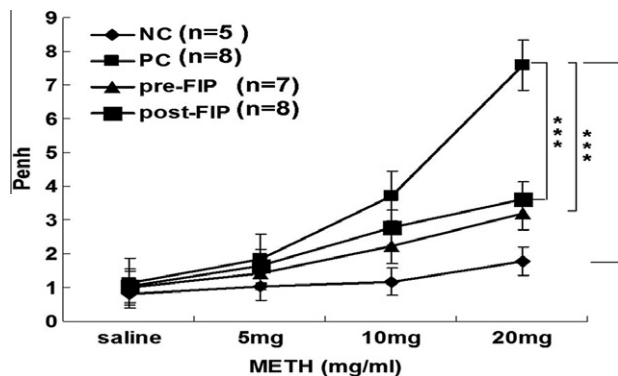


Fig. 2. The effects of FIP-fve on the pre-treated (A) and post-treated (B) mouse models of allergic asthma. The female BALB/c mice remained normal saline (normal control group) or were sensitized/challenged with OVA $\pm$ treatment with FIP-fve (200 μ g/mouse) during the challenge phase. The controlled and OVA-sensitized mice not receiving FIP-fve were treated with vehicle control. The airway hyperactivity (AHR) to methacholine was assessed in the unrestrained mice using whole-body barometric plethysmography (Model PLY 3211; Buxco Electronic Inc., Sharon, CT) to record enhanced pauses (Penh). Each bar represents the mean $\pm$ SEM of the Penh value from individual mice. NC, $n = 5$ for normal control group; PC, $n = 8$ for only OVA treated; pre-FIP, $n = 7$ for pre-treated FIP-fve (challenge). Post-FIP, $n = 8$ for post-treated FIP-fve. Each data represents the mean $\pm$ SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** $p < 0.05$; *** $p < 0.001$.

control group and inhibited significantly through FIP-fve pre- and post-treatment ($p < 0.001$). The monocytes were all elevated in the sensitized/challenged with OVA group, pre-treated and post-treated FIP-fve mice.

3.4. The effect of FIP-fve treatment on cytokines in serum and BALF

In the pre-treated and post-treated FIP-fve protocols, all cytokines in the mice sensitized/challenged with OVA assayed in-

creased, compared with the normal control group. The FIP-fve treatment administered during the challenge phase significantly decreased the Th2 cytokines, including IL-4, IL-5 and IL-13 (Fig. 5), IL-10 also decreased little after FIP-fve treatment in the BALF, however, not significantly. Th1 and Treg cytokines including IFN- γ and TGF- β significantly increased in FIP-fve pre-treated and post-treated groups (Tables 2 and 3 and Fig. 6). IL-12, however, in serum and BALF of each group was not significantly different (data not show).

3.5. The effect of FIP-fve on lung inflammation

The effect of the FIP-fve treatment in the OVA-sensitized/challenged mice on the overall lung inflammation was evaluated through histological staining with H&E (Fig. 7). The mice in both the pre-treated FIP-fve (Fig. 7c) and post-treated FIP-fve (Fig. 7d) models of allergic asthma had severe inflammation, compared with the normal control group (Fig. 7a) that had no inflammation. In the pre-treated and post-treated FIP-fve models (Fig. 7c and d) of allergic asthma, after the FIP-fve treatment was administered, there was significantly less inflammation, compared with the OVA mice (Fig. 7b). The lung tissue sections were also evaluated for other structural changes associated with the airway remodeling. A morphometric examination of the airway sections (Fig. 7) revealed that the OVA exposure (Fig. 7b) significantly increased the epithelial cell thicknesses. In both the pre-FIP (Fig. 7c) and post-FIP (Fig. 7d) models of airway inflammation, however, the FIP-fve treatment during the challenge phase of either asthma model led to significantly decreased epithelial cell thicknesses to those of the OVA mice.

4. Discussions

In the ongoing study, the mice allergen-induced airway inflammatory model was used to explain the role of OVA in mediating

Table 1

The effects of FIP-fve treatment on the serum IgE and IgG2a levels observed in OVA-treated mice.

Serum levels of anti-OVA antibodies	Treatment of mice			
	Normal control group	OVA-treated mice	Pre-treated FIP	Post-treated FIP
IgE (OD/ml)	0.053 $\pm$ 0.015**	0.58 $\pm$ 0.024	0.24 $\pm$ 0.0183**	0.263 $\pm$ 0.0190**
IgG2a (ng/ml)	2023 $\pm$ 135.7**	8888 $\pm$ 262.2	21733 $\pm$ 802.2**	16281 $\pm$ 884.8**

Serums of different concentrations were obtained from the naïve control mice and OVA sensitized/ challenged mice in the absence or presence of the treatment with FIP-fve. The results are represented as the means $\pm$ SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** $p < 0.05$; *** $p < 0.001$.

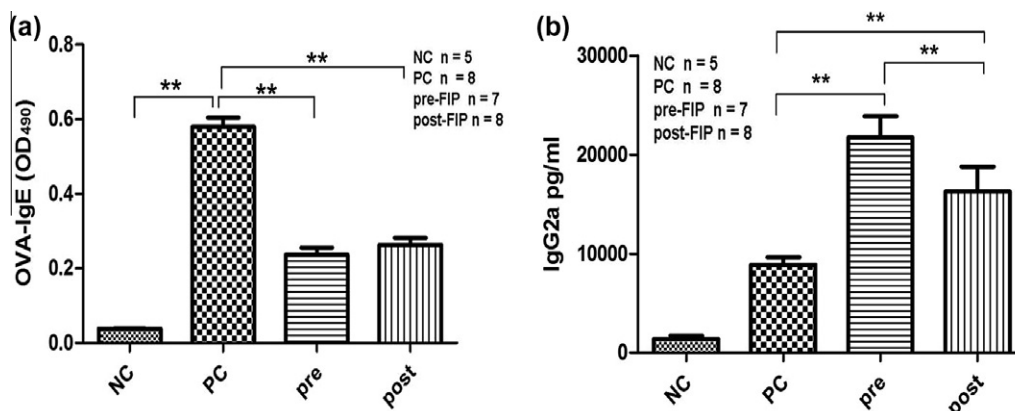


Fig. 3. The effects of the FIP-fve treatment on the specific anti-OVA IgE and IgG2a levels in the OVA-treated mice in serum. Serums in different concentrations were obtained from the normal control group and the OVA sensitized/challenged mice in the absence or presence of the treatment with FIP-fve. The results are represented as the means $\pm$ SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** $p < 0.05$; *** $p < 0.001$.

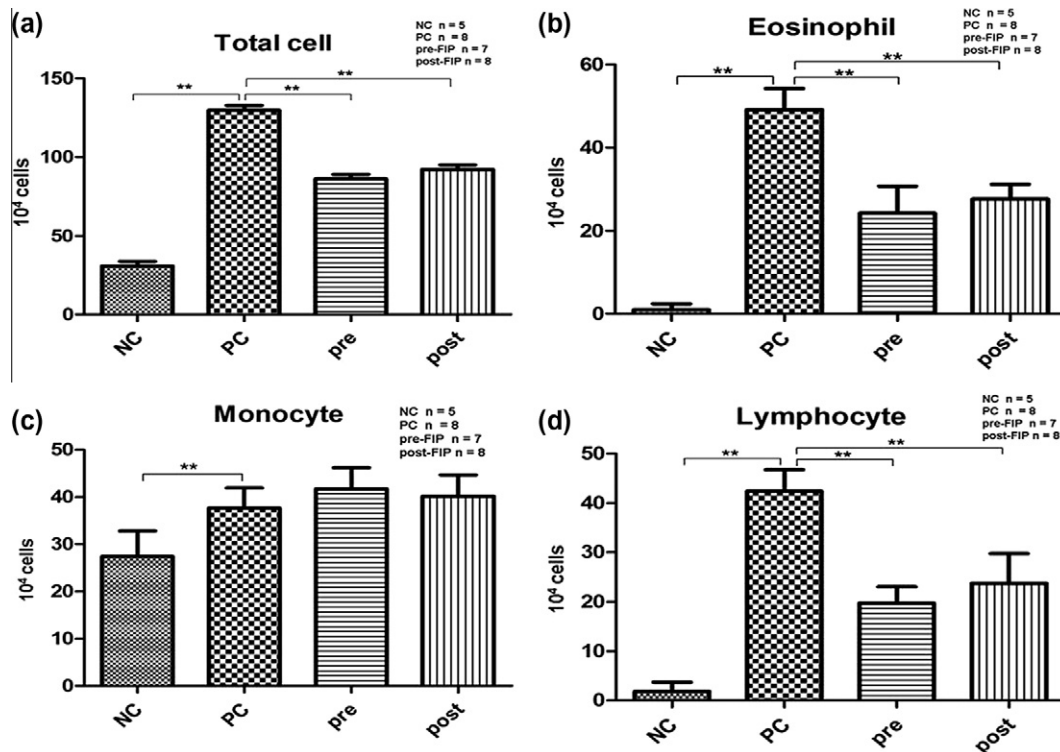


Fig. 4. The effects of the FIP-fve treatment on the inflammatory cell infiltrations in the BALF of the OVA-treated mice. BALF was obtained from the normal control group and OVA sensitized/challenged mice in the absence or presence of the treatment with FIP-fve. The total cells and inflammatory cell were counted ($\times 10^4$) from the BALF in millimeters by the morphometric evaluations of cytopsin preparations. The results are represented as the means $\pm$ SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** $p < 0.05$; *** $p < 0.001$.

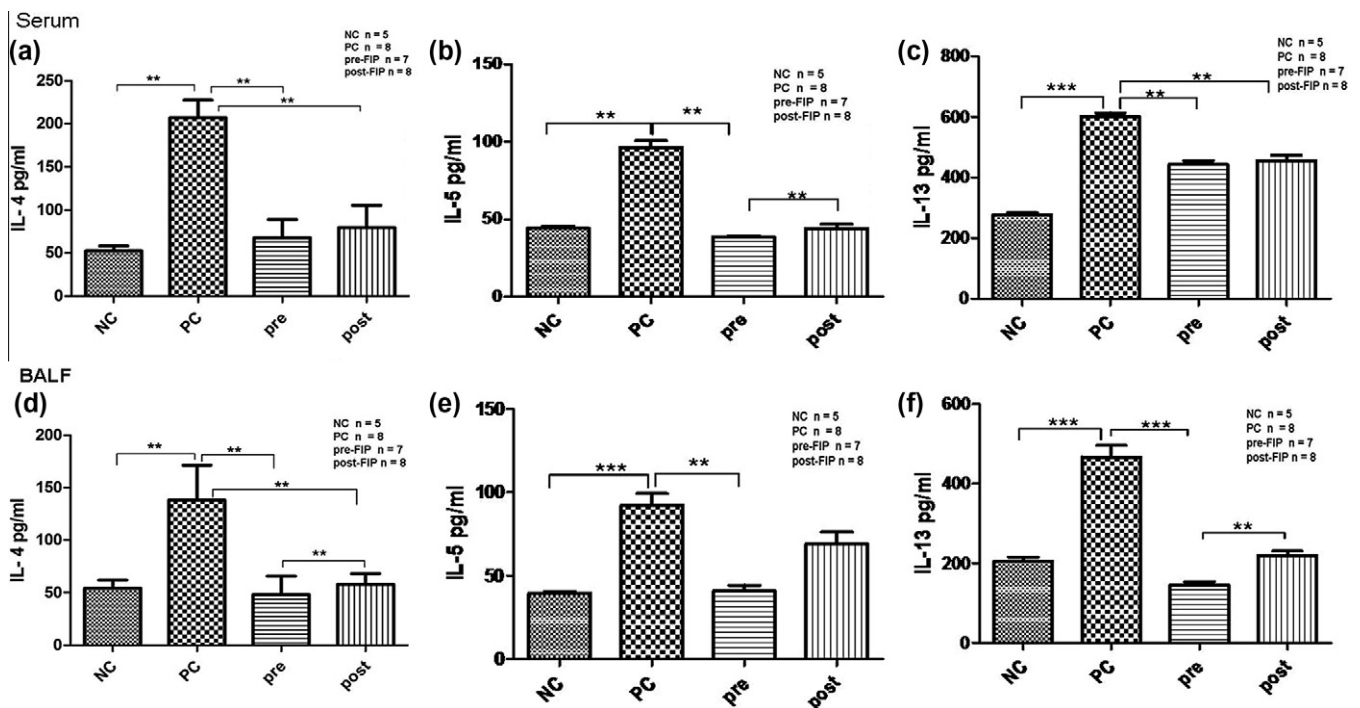


Fig. 5. The effects of the FIP-fve treatment on the Th2 cytokine levels (IL-4, IL-5, IL-13) in the OVA-treated mice in serum and BALF. BALF in different concentrations were obtained from the normal control group and OVA sensitized/challenged mice in the absence or presence of the treatment with FIP-fve. The results are represented as the means $\pm$ SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** $p < 0.05$; *** $p < 0.001$.

pulmonary inflammation, promoting airway remodeling, and promoting airway hyperreactivity to methacholine. The BALB/c mice were found to be susceptible to the IgE mediated allergic responses

[15]. In addition to the responses of the OVA sensitized/challenged mice following an increase in the serum IgE level, the eosinophils also displayed hyperplasia in the mouse asthma model. Early

Table 2The effects of the FIP-*fve* treatment on the cytokine levels in the OVA-treated mice in serum.

Serum levels of cytokine (pg/mL)	Treatment of mice			
	Normal control group	OVA-treated mice	Pre-treated FIP	Post-treated FIP
IL-4	52.21 ± 0.97**	52.21 ± 0.97**	67.90 ± 8.001**	79.75 ± 9.644**
IL-5	47.03 ± 0.26**	96.39 ± 4.415	38.50 ± 0.65**	44.02 ± 2.900**
IL-10	370.64 ± 107.01***	1115 ± 38.36	696.2 ± 31.03***	616.6 ± 32.91***
IL-13	287.04 ± 0.12***	600.6 ± 12.46	443.1 ± 12.32**	455.6 ± 18.13**
IFN- γ	25.37 ± 2.48**	10.69 ± 1.341	116.4 ± 24.49***	97.36 ± 88.64***
TGF- β	42.11 ± 17.06**	82.32 ± 5.142	154.4 ± 8.664**	225.1 ± 12.32**

Serums in different concentrations were obtained from the naïve control mice and OVA sensitized/challenged mice in the absence or presence of the treatment with FIP-*fve*. The results are represented as the means ± SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** p < 0.05; *** p < 0.001.

Table 3The effects of the FIP-*fve* treatment on the cytokine levels in the OVA-treated mice in BALF.

BALF levels of cytokine (pg/mL)	Treatment of mice			
	Normal control group	OVA-treated mice	Pre-treated FIP	Post-treated FIP
IL-4	59.81 ± 8.45**	138.0 ± 33.19	48.09 ± 17.59**	58.14 ± 10.16 **
IL-5	40.88 ± 0.39***	92.33 ± 6.850	40.81 ± 3.321 **	69.05 ± 7.092
IL-10	377.91 ± 104.26**	673.6 ± 55.45	576.9 ± 83.46	568.2 ± 61.62
IL-13	221.03 ± 3.73***	221.03 ± 3.73***	145.9 ± 8.126***	218.6 ± 12.13***
IFN- γ	32.54 ± 2.77**	16.07 ± 1.745	82.61 ± 11.62***	79.80 ± 11.54***
TGF- β	21.21 ± 2.31**	49.18 ± 2.619	62.32 ± 3.273**	60.95 ± 33.12**

BALF in different concentrations were obtained from the naïve control mice and OVA sensitized/challenged mice in the absence or presence of the treatment with FIP-*fve*. The results are represented as the means ± SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** p < 0.05; *** p < 0.001.

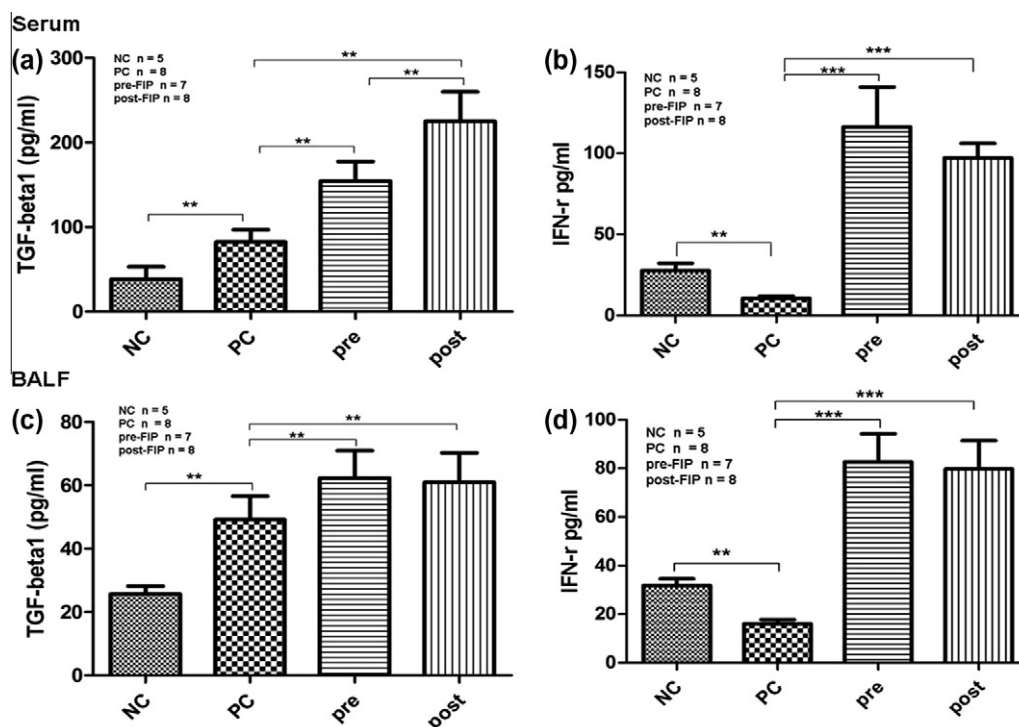


Fig. 6. The effects of the FIP-*fve* treatment on the Treg and Th1 cytokine levels (TGF- β , IFN- γ) in the OVA-treated mice in serum and BALF. Serums in different concentrations were obtained from the normal control group and OVA sensitized/challenged mice in the absence or presence of the treatment with FIP-*fve*. The results are represented as the means ± SEM. The statistical analysis are compared with OVA-treated mice and represented as: ** p < 0.05; *** p < 0.001.

studies show that the oral administration of FIP-*fve* during allergen sensitization induced a Th1-predominant allergen-specific immune response in food-allergic mice [15]. This study suggests that intervention through FIP-*fve* suppresses the airway responsiveness. The consequent development of allergic airway diseases following the structural changes progressed, as was triggered by IgE or other Th2 cytokine. In the present study, the level of IgE in the

serum can be suppressed by oral FIP-*fve* during allergen challenge which persists with the findings of Hsieh and colleagues [15]. FIP-*fve* is a lectin that exhibit hemagglutinating and immunoregulatory activities through binding glycolipids and glycoproteins on the cell surface. Based on our previous study, we think that FIP-*fve* induced the IFN- γ via the activation of signaling like p38 MAPK, calcium release and PKC activation [12]. The anti-allergic

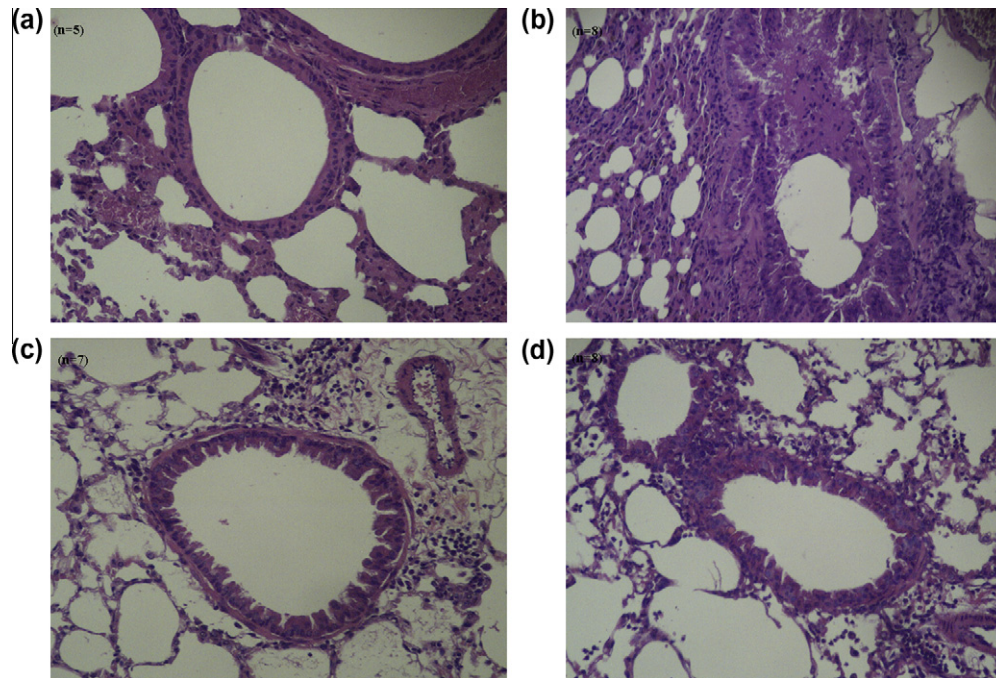


Fig. 7. The effects of the FIP-fve treatment on allergen-induced airway inflammations and remodeling. Lung tissues were obtained on day 28 from the normal control group (a) and the OVA-treated mice in the absence of (b), the pre-fed FIP-fve (c), or the post-fed FIP-fve (d), which were stained with hematoxylin and eosin. (a) There was a scarcity of inflammatory cells around the airway in the saline-treated control. (b) A large number of inflammatory cells were identified through the peribronchial tissues of the OVA-treated mice. In this group, the thickened smooth muscles in the airway were noted. (c and d) Inflammatory cell infiltration and hyperplasia in the smooth muscles of the airway were reduced by the FIP-fve given through repetitive OVA challenges.

effects of FIP-fve in Th2-mediated allergic response are believed to be partly associated with the ability of FIP-fve to enhance activation of IFN- γ releasing Th1 cells.

The Th2 cytokine-IL-4 is known to directly promote the features of asthma, such as eosinophil infiltration, goblet cell metaplasia, airway hyperresponsiveness, IgE production in serum, mastocytosis, airway remodeling, and Th2 induction/maintenance [21,22]. Indeed, our results show that the OVA sensitized mice displayed higher IL-4 in serum and BALF than those of naive mice. In the fed FIP-fve group, the IL-4 levels decreased in the serum and BALF. Moreover, earlier study had shown that FIP-fve could inhibit IL-5-mediated survival of eosinophils through the modulation of IL-5 R expression and apoptotic signal protein production [23]. FIP-fve had modulatory effects on eosinophil apoptosis in vitro and eosinophil-related allergic inflammation in vivo, which correlates with the reduced eosinophil numbers in FIP-fve treated mice in the current study (Tables 2 and 3, Fig. 4).

However, not all cytokines promote allergic airway diseases; IL-12, type I and type II IFNs, and the anti-inflammatory cytokines (IL-10, TGF- β) actually display suppressive effects [24–28]. Earlier studies show that the treatment with large doses of IFN- γ or type I IFN can at least suppress some characteristics of allergic airway inflammation. Besides, LZ-8 is one of fungal immunomodulatory family proteins derived from the fungus *Ganoderma lucidum*. The protein sequence of LZ-8 and FIP-fve are highly homologue. LZ-8 induces IL-12 p40, IL10, IL23 production through MD2 mediated TLR4 and maturation of human monocyte derived Dendritic cells [29]. Earlier studies show that in the experiment models, the role of CD4⁺CD25⁺ Treg cells in mediating oral tolerance were supported. Obtained from the feeding, the CD4⁺CD25⁺ Treg cells controlled the immune responses in the infectious agents, transplants, and cancer [30]. Previous studies also confirmed that the autoimmune or exogenous antigens and allergens' immune tolerance was mediated by several mechanisms, including clonal deletion, immune deviation, and immune suppression by Tregs in the

animal model experiment [15]. Wu et al., reported that Foxp3 Treg cells in CD4⁺CD25⁺ T cells, IL-10⁺ and TGF- β ⁺ Treg cells was also significantly increased in *D. fariniae* allergic patients under FIP-fve treatment [31]. Our results had demonstrated that TGF- β was increased both in lavage fluid and serum with oral administration of FIP-fve in OVA sensitized mice (Tables 2 and 3, Fig. 6a and c). In the presented model it is still unclear whether or not high levels of TGF- β directly work on Treg activation. Therefore, we will further investigate whether the activation of Treg by FIP-fve is involved in the inhibitory effects.

Earlier studies show that the IL-10 and TGF- β productions were associated with T cell tolerance and Tregs, which are induced by mucosal exposure to antigen [32]. In the animal models, the oral tolerance has been used to block the development of both Th1 and Th2 mediated diseases [33]. Furthermore, in the present study, IL-10 was overexpressed both in serum and BALF in OVA-sensitized mice. Similar results were found in humans where plasma IL-10 concentrations were significantly higher in allergic asthmatic patients than in normal control subjects [34]. However, IL-10 was vital for suppression of allergic responses and inhibition of IgE production [35,36]. Interesting, FIP-fve reduced the OVA-mediated IL-10 expression in this study. T regulatory cells may reeducate dendritic cell function in the lung, resulting in suppression of inflammation. FIP-fve stimulate the maturation of splenic dendritic cells in vivo [37]. Suppression of inflammation may be due to dendritic cells activation. IL-10 was induced from various routes such as Th1, Th2 or Treg. However, it must be clarified in the future. In light of this, the prophylactic strategies of immune deviation using various microbial products such as Th1 stimuli have been well investigated recently through the animal models of allergy, and they have been proven to successfully inhibit the development of allergic Th2 responses [15,23,38,39]. However, in comparison with the other strategies that adopt microbial product injections, the oral administration of FIP-fve has its unique advantage that it is easier to administer in practice and is generally more acceptable

for prophylactic uses by infants and young children. Moreover, the oral FIP-fve might be used against all kinds of known and unknown antigens that exist in the environment.

Clinically, the systematic administration of interferon is associated with a number of side effects such as flu-like syndromes and weight loss [15]. In this study, no adverse was observed in the mice fed with FIP-fve. Moreover, earlier study had shown oral administration of golden needle mushroom did not adapt to inhibit the development of asthma. The other content from golden needle mushroom contained the cardiotoxin [40]. Therefore, we think that FIP-fve should be purified to inhibit the allergy disease. For digestibility, FIP-fve was resistant to digestive enzymes in simulated gastric fluid and simulated intestinal fluid [41]. This study demonstrated that oral FIP-fve had an anti-inflammatory effect on OVA-induced airway inflammations and possesses the potential to be used as an alternative therapy for allergic airway diseases. These results suggest that FIP-fve have activities that are stable and have a strong potential of being applied to food or pharmaceutical products for commercial development. The safety of FIP-fve is another requirement for an ideal treatment strategy. Conclusion, asthma is a complicated disease of study interest. It was found that the oral FIP-fve may produce an anti-inflammatory effect on OVA-induced airway inflammations, and FIP-fve may be an alternative therapy for allergic airway diseases.

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References

- [1] Munroe ME, Businga TR, Kline JN, Bishop GA. Anti-inflammatory effects of the neurotransmitter agonist Honokiol in a mouse model of allergic asthma. *J Immunol* 2010;185:5586–97.
- [2] Yeh KW. Allergens and allergic diseases in children. *Acta Paediatr Taiwan* 2006;47:169–74.
- [3] Chuang JG, Su SN, Chiang BL, Lee HJ, Chow LP. Proteome mining for novel IgE-binding proteins from the German cockroach (*Blattella germanica*) and allergen profiling of patients. *Proteomics* 2010;10:3854–67.
- [4] Saenz SA, Taylor BC, Artis D. Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. *Immunological Rev* 2008;226:172–90.
- [5] Finkelman F. Cytokine regulation of type 2 immunity; 2001.
- [6] Chanez P, Wenzel SE, Anderson GP, Bel EH, Boulet LP, Brightling CE, et al. Severe asthma in adults: what are the important questions? *J Allergy Clin Immunol* 2007;119:1337–48.
- [7] Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. *J Allergy Clin Immunol* 2005;116:961–9.
- [8] Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of Treg in immune regulation of allergic diseases. *Eur J Immunol* 2010;40:1232–40.
- [9] Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002;3:715–20.
- [10] Umetsu DT, Akbari O, DeKruyff RH. Regulatory T cells control the development of allergic disease and asthma. *J Allergy Clin Immunol* 2003;112:480–7.
- [11] Umetsu DT, DeKruyff RH. The regulation of allergy and asthma. *Immunol Rev* 2006;212:238–55.
- [12] Wang PH, Hsu CI, Tang SC, Huang YL, Lin JY, Ko JL. Fungal immunomodulatory protein from *Flammulina velutipes* induces interferon-gamma production through p38 mitogen-activated protein kinase signaling pathway. *J Agric Food Chem* 2004;52:2721–5.
- [13] Ko JL, Hsu CI, Lin RH, Kao CL, Lin JY. A new fungal immunomodulatory protein, FIP-fve isolated from the edible mushroom, *Flammulina velutipes* and its complete amino acid sequence. *Eur J Biochem* 1995;228:244–9.
- [14] Ko JL, Lin SJ, Hsu CI, Kao CL, Lin JY. Molecular cloning and expression of a fungal immunomodulatory protein, FIP-fve, from *Flammulina velutipes*. *J Formos Med Assoc* 1997;96:517–24.
- [15] Hsieh KY, Hsu CI, Lin JY, Tsai CC, Lin RH. Oral administration of an edible-mushroom-derived protein inhibits the development of food-allergic reactions in mice. *Clin Exp Allergy* 2003;33:1595–602.
- [16] Gueders MM, Paulissen G, Crahay C, Quesada-Calvo F, Hacha J, Van Hove C, et al. Mouse models of asthma: a comparison between C57BL/6 and BALB/c strains regarding bronchial responsiveness, inflammation, and cytokine production. *Inflamm Res* 2009;58:845–54.
- [17] Ou CC, Hsiao YM, Wu WJ, Tasy GJ, Ko JL, Lin MY. FIP-fve stimulates interferon-gamma production via modulation of calcium release and PKC-alpha activation. *J Agric Food Chem* 2009;57:11008–13.
- [18] Cataldo DD, Tournoy KG, Vermaelen K, Munaut C, Foidart JM, Louis R, et al. Matrix metalloproteinase-9 deficiency impairs cellular infiltration and bronchial hyperresponsiveness during allergen-induced airway inflammation. *Am J Pathol* 2002;161:491–8.
- [19] Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997;156:766–75.
- [20] Gueders MM, Balbin M, Rocks N, Foidart JM, Gosset P, Louis R, et al. Matrix metalloproteinase-8 deficiency promotes granulocytic allergen-induced airway inflammation. *J Immunol* 2005;175:2589–97.
- [21] Oettgen HC, Geha RS. IgE in asthma and atopy: cellular and molecular connections. *J Clin Invest* 1999;104:829–35.
- [22] Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu Rev Immunol* 1999;701–38.
- [23] Hsieh CW, Lan J, Meng Q, Cheng YW, Huang HM, Tsai JJ. Eosinophil apoptosis induced by fungal immunomodulatory peptide-fve via reducing IL-5alpha receptor. *J Formos Med Assoc* 2007;106:36–43.
- [24] Oh JW, Serogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. *J Allergy Clin Immunol* 2002;5:110.
- [25] Fu CL, Ye YL, Lee YL, Chiang BL. Effects of overexpression of IL-10, IL-12, TGF-beta and IL-4 on allergen induced change in bronchial responsiveness. *Respir Res* 2006;7:72–86.
- [26] van Scott MR, Justice JP, Bradfield JF, Enright E, Sigounas A, Sur S. IL-10 reduces Th2 cytokine production and eosinophilia but augments airway reactivity in allergic mice. *Am J Physiol Lung Cell Mol Physiol* 2000;278:L667–74.
- [27] Hansen G, McIntire JJ, Yeung VP, Berry G, Thorbecke GJ, Chen L, et al. CD4(+) T helper cells engineered to produce latent TGF-beta1 reverse allergen-induced airway hyperreactivity and inflammation. *J Clin Invest* 2000;105:61–70.
- [28] Urban JF Jr, Madden KB, Cheever AW, Trotta PP, Katona IM, Finkelman FD. IFN inhibits inflammatory responses and protective immunity in mice infected with the nematode parasite, *Nippostrongylus brasiliensis*. *J Immunol* 1993;151:7086–94.
- [29] Lin YL, Liang YC, Tseng YS, Huang HY, Chou SY, Hsueh RS, et al. An immunomodulatory protein, Ling Zhi-8, induced activation and maturation of human monocyte-derived dendritic cells by the NF-kappaB and MAPK pathways. *J Leukocyte Biol* 2009;86:877–89.
- [30] Karlsson MR, Rugtveit J, Brandtzaeg P. Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. *J Exp Med* 2004;199:1679–88.
- [31] Wu CC, Liao EC, Lee MF, Tsai JJ. Augmentation of regulatory T cells in allergic individuals by recombinant Der f 2 peptide with fungal immunomodulatory peptide fve. *Ann Allergy Asthma Immunol* 2009;102:216–22.
- [32] Lloyd CM, Hawrylowicz CM. Regulatory T cells in asthma. *Immunity* 2009;31:438–49.
- [33] Petrarca C, Lazzarin F, Pannellini T, Iezzi M, Braga M, Mistrello G, et al. Monomeric allergoid intragastric administration induces local and systemic tolerogenic response involving IL-10-producing CD4(+)CD25(+) T regulatory cells in mice. *Int J Immunopathol Pharmacol* 2010;23:1021–31.
- [34] Wong CK, Ho CY, Ko FW, Chan CH, Ho AS, Hui DS, et al. Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. *Clin Exp Immunol* 2001;125:177–83.
- [35] Punnonen J, De Waal Malefyt R, van Vlasselaer P, Gauchat JF, de Vries JE. IL-10 and viral IL-10 prevent IL-4-induced IgE synthesis by inhibiting the accessory cell function of monocytes. *J Immunol* 1993;151:1280–9.
- [36] Akdis CA, Blesken T, Akdis M, Wüthrich B, Blaser K. Role of IL-10 in specific immunotherapy. *J Clin Invest* 1998;102:98–106.
- [37] Ding Y, Seow SV, Huang CH, Liew LM, Lim YC, Kuo IC, et al. Coadministration of the fungal immunomodulatory protein FIP-Fve and a tumour-associated antigen enhanced antitumour immunity. *Immunology* 2009;128:881–94.
- [38] Ngoc PL, Gold DR, Tzianabos AO, Weiss ST, Celedón JC. Cytokines, allergy, and asthma. *Curr Opin Allergy Clin Immunol* 2005;5:161–6.
- [39] Huang TJ, MacAry PA, Eynott P, Moussavi A, Daniel KC, Askenase PW, et al. Allergen-specific Th1 cells counteract effector Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN-gamma. *J Immunol* 2001;166:207–17.
- [40] Lin JY, Lin YJ, Chen CC, Wu HL, Shi GY, Jeng TW. Cardiotoxic protein from edible mushrooms. *Nature* 1974;252:235–7.
- [41] Ou CC, Hsiao YM, Wang WH, Ko JL, Lin MY. Stability of fungal immunomodulatory protein, FIP-gts and FIP-fve, in IFN-γ production. *Food Agric Immunol* 2009;20:319–32.