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Original Article

Anti-allergic Effect of *Fructus amomi* on Ovalbumin-induced Asthma Mice Model

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Abstract : Despite studies on the anti-inflammatory effect of *Fructus amomi*, mature fruit of *Amomum villosum* Lour, have been getting increasing extensively, it remains unknown about the detailed effects of *F. amomi* on ovalbumin (OVA)-induced mouse asthma model. In this study, we examined the effect of *F. amomi* on OVA-induced asthma by analyzing Th1/Th2 cytokine production, histopathologic changes, and focusing on the NF-κB signaling. Oral administration of *F. amomi* reduced the number of inflammatory cells especially eosinophils and improved airway and pulmonary inflammation in the lungs of OVA-challenged mice. In addition, *F. amomi* significantly downregulated the overproduction of tumor necrosis factor-α, IL-1β, IL-4, IL-5 and the levels of OVA-specific both IgE and IgG1 and increased the secretion of interferon-γ and OVA-specific IgG2a. Moreover, *F. amomi* suppressed the increase of total NF-κB level and the phosphorylation of IκB-α and NF-κB by OVA. *F. amomi* may have therapeutic effect for allergic asthma by modulating Th1/Th2 cytokine imbalance and inhibiting NF-κB signaling activation.

Keywords : *Frutus amomi*, Asthma, Th1/Th2 cytokine imbalance, OVA-specific IgE/IgG1/IgG2, NF-κB signaling

INTRODUCTION

The prevalence of type I hypersensitivity including allergic rhinitis, food allergy, and allergic asthma, has increased in recent decades and allergic diseases are a global health problem, which is associated with life quality [1]. Allergic asthma is severe disease characterized by chronic airway inflammation in response to an allergen, airway eosinophilic inflammation, and airway hyperresponsiveness [1]. Airway inflammation in asthma is initiated by T cell immune response in which helper T cell type (Th) 2 cell-secreted cytokines, such as interleukins (IL)-1β, IL-4, IL-5, IL-6 and tumor necrosis factor (TNF)-α, contribute to eosinophil recruitment, mucus overproduction, and airway hyperresponsiveness [2,3] by regulating the principal process of immunoglobulin (Ig) E production, the growth of mast cells and the

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differentiation and activation of mast cells and eosinophils [4,5]. Mast cells are well-known to associate with the allergic airway disease. Mast cells are found preferentially around host-environment interface and blood vessels [6]. Mast cells are main effector which mediate various inflammatory reactions and can be activated to degranulate and release potent mediators by a variety of stimuli including antibody-antigen reactions, and can respond to a very low dose of specific antigen [7,8]. The drugs which ameliorate allergic diseases have shown to have therapeutic activity either via suppression of histamine release or via inhibition of mast cell degranulation [9]. In spite of many treatments, prolonged use often leads to unexpected side effects. Therefore, the development of drugs which improve allergic disease without any adverse effects is needed.

In contrast to the synthesized chemicals, natural products such as purified substances or extract formula from traditional herbal medicines are safe and represent pools for the development of chronic human pathological conditions because they have been used and applied to medicated food or diet for long times [10]. *Fructus amomi* is the mature fruit of *Amomum villosum* Lour, family Zingiberaceae, and is a tropical medicinal plant [11]. It has been reported that *F. amomi* inhibits free radical formation through the inhibition of NF-κB activation [12] and attenuates heart inflammation in a CVB3-induced myocarditis mouse model [13]. Also, *F. amomi* has been reported to suppress mast cell activity and inhibit TNF-α and IL-6 cytokine expression induced by compound 48/80, phorbol 12-myristate 13-acetate or calcium ionophore A23187 [11,14]. However, despite studies on the anti-inflammatory effects of *F. amomi* have been getting increasing extensively, the knowledge about the detailed anti-asthmatic effects of *F. amomi* on the ovalbumin (OVA)-induced asthma model *in vivo* is still poorly studied. To reveal the anti-asthmatic effect of *F. amomi*, we examined the levels of OVA-specific IgE, IgG1, and IgG2a and the level of Th1/Th2 cytokines of OVA-induced asthma mouse model in this study.

MATERIALS AND METHODS

1. Animals

Balb/c mice (male, 5~6 weeks) for asthma mouse model were purchased from Damool Science (Daejeon, Korea) and kept in standard laboratory environment with a 12/12-h light/dark cycle. All animal treated in accordance with the Institutional Animal Care and Use Committee of Jeonbuk National University Laboratory Animal Center (CBN 2019-071) under the guidelines for Animal Care and Use.

2. Preparation of *F. amomi* extract

The *F. amomi* ethanol extract (KFRI-SL-2119) was provided from the Korea Food Research Institute. The 200 g of dried *F. amomi* underwent reflux extraction twice in 1 L of 95% ethanol. The ethanol extract was dried under a vacuum in a rotary evaporator. The concentrated extract was finally lyophilized to obtain 7.5 g of dried powder (yield, 3.75%) that was kept at 4°C until used. The dried ethanol extract was dissolved in saline and DMSO (Sigma-Aldrich, St. Louis, MO) prior to use.

3. Development of OVA-induced asthma model

As shown in Fig. 1A, OVA-induced asthma mouse model was performed according to our previous studies [15,16]. Mice were randomly divided into 5 groups (n=6) according to treatment and animal experiments were conducted at least twice: control group, OVA (grade VI; Sigma-Aldrich, St. Louis, MO, USA) group, *F. amomi* group (200 mg/kg/day), and dexamethasone (Dex; Sigma-Aldrich, St. Louis, MO, USA) group (2.5 mg/kg/day). Mice were intraperitoneally sensitized by 50 μg OVA with 1 mg Imject Alum Adjuvant (Thermo Scientific, Rockford, MD, USA) in a total volume of 200 μL on day 1 and by only 50 μg OVA without Alum on day 14. On days 27, 28, and 29, these mice were challenged with an aerosol of 5% (wt/vol) OVA using ultrasonic nebulizer (NE-U12; Omron Crop., Tokyo, Japan) for 30 min. And then mice were also orally administered once daily with saline, *F. amomi* or Dex on days from 15 to 26, respectively. Control group was received only saline. On day 30, mice were killed 24 h after the last challenge to evaluate the effect of *F. amomi* on OVA-induced asthma mouse model.

4. Collection and analysis of bronchoalveolar fluid (BALF)

Twenty-four hours after the final OVA challenge, mice were sacrificed after blood collection. BALF was collected by cannulating trachea and lavaging lungs, as described previously [15,16]. The total BALF cell number was counted by trypan blue exclusion method. Cell density (mL) = Total

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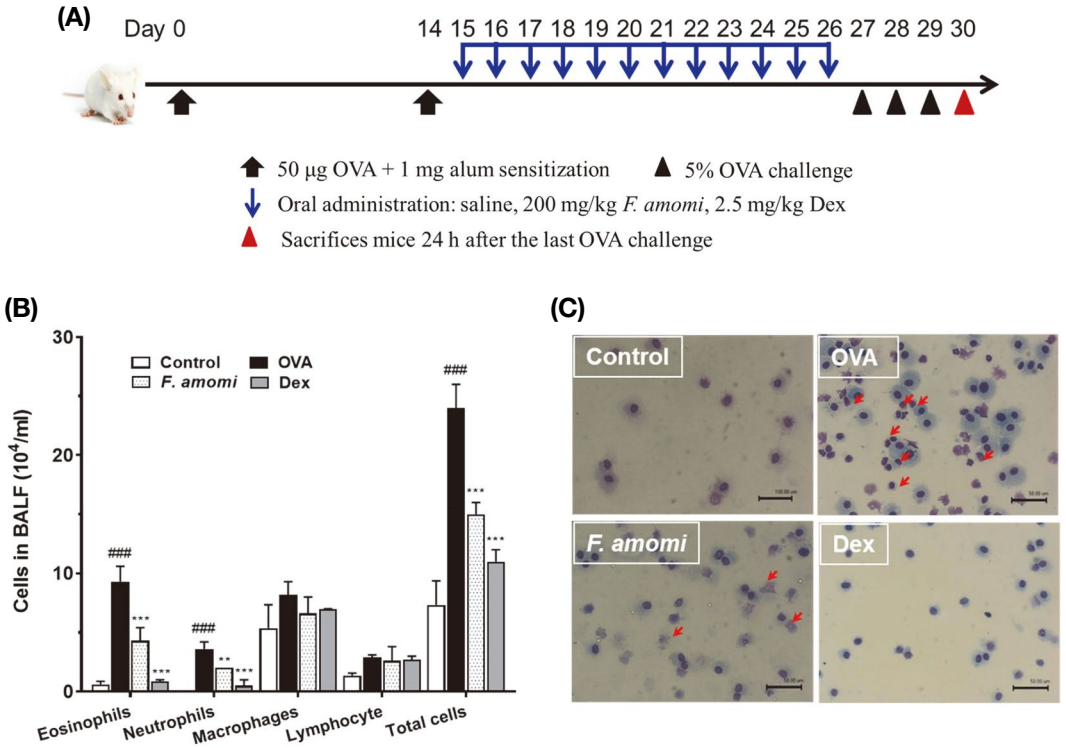

Fig. 1. Effects of *F. amomi* on ovalbumin (OVA)-induced airway inflammation. (A) Experimental protocol for OVA-induced allergic asthma mouse model, (B) Differential cellular components and total cells and (C) Infiltration of inflammatory cells including eosinophils (red arrows) in BALF of asthmatic mice. Bars = 50 μm. Data are the mean ± SD (n = 6 per group). $^{###}P < 0.001$, *vs* Control group. $^{**}P < 0.01$, $^{***}P < 0.001$, *vs* OVA group.

cell count/number of square × Trypan blue dilution factor × 10^4. Total cell number = Cell density × Cell suspension volume (mL). The BALF was centrifuged (Gyro 1730 MR, GYROZEN Co., Gimpo, Korea) at 1,000 g, 4°C for 10 min, and the supernatant was collected for cytokine assay. Differential cell counts were determined with cytospin (Shandon Cytospin 4, Thermo Fisher Scientific, WA71TA, UK) preparation, and then stained with Diff Quik solution (Sysmex Co., Kobe, Japan).

5. Histopathologic analysis

Histopathologic analysis of lung was carried out as previously described [15,16]. Lung was excised and fixed in 10% formalin solution and embedded in paraffin. Serial sections were stained with hematoxylin-eosin (H-E; Sigma, St. Louis, MO, USA) for evaluating inflammatory responses, congo red (Sigma) for identifying eosinophil infiltration, periodic acid-Schiff (PAS; Sigma) for showing the goblet cells and mucus secretion, and Masson trichrome (MT; Sigma) for estimating collagen fiber deposition.

6. Measurement of OVA-specific immunoglobulin (Ig) E, IgG1, and IgG2a and Th1, Th2 cytokines

The plasma levels of OVA-specific IgE (BioLegend, San Diego, CA, USA), OVA-specific IgG1 (Cayman, Ann Arbor, MI, USA), and OVA-specific IgG2a (Chondrex, Redmond, WA, USA) were measured using microplate reader (Bio-Rad 680, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Also, the levels of Th1 cytokines [IL-12 and IFN-γ (R&D System, St. Paul, MI, USA)], Th2-related cytokines [TNF-α, IL-4 and IL-6 (R&D System, Minneapolis, MN, USA)] in the BALF from each mouse by using enzyme-linked immunosorbent assay (ELISA) were measured as described earlier [15,16].

7. Measurement of NF-κB p65, phosphorylated NF-κB p65 (p-NF-κB p65), phosphorylated I-κB (p-I-κB)

To detect NF-κB p65, p-NF-κB p65, and p-I-κB, lung tissues were homogenized on the ice, and then their nuclear

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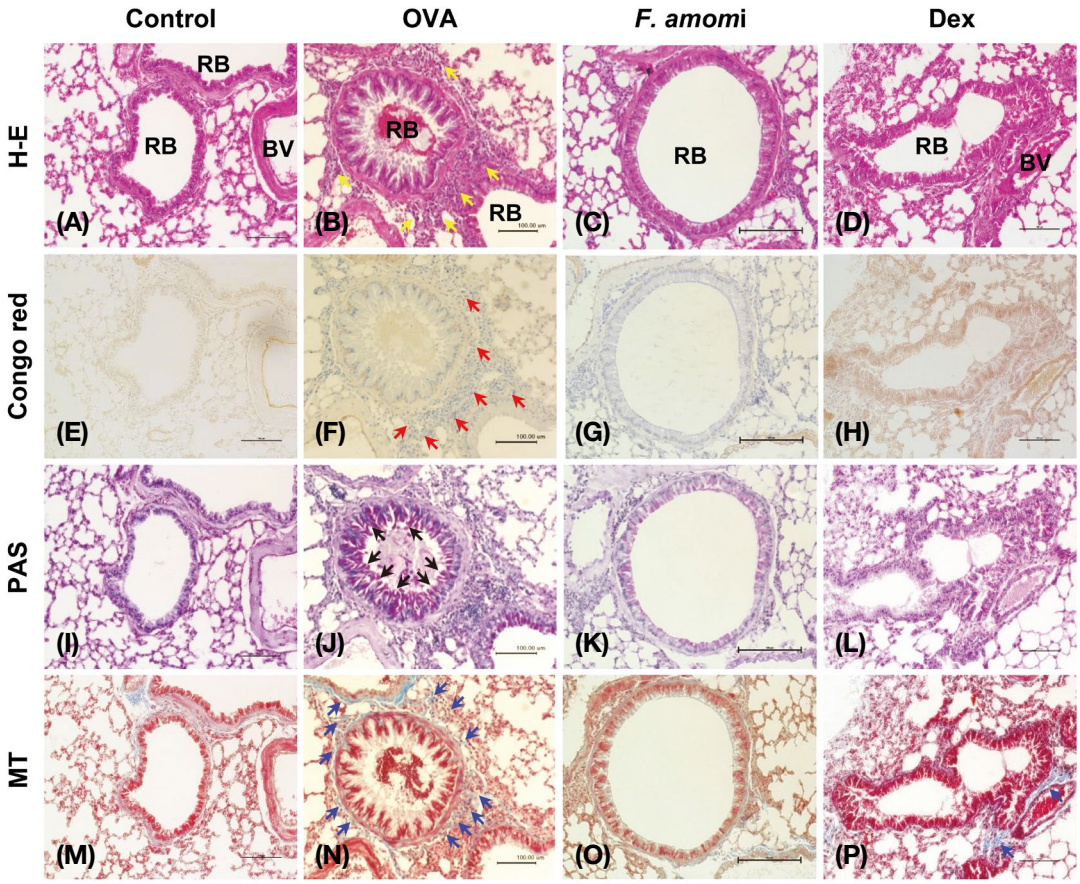

Fig. 2. Effects of *F. amomi* on histopathological changes in the lungs of asthmatic mice. Lung tissues from each group were stained with hematoxylin-eosin (H-E) for general inflammation (yellow arrows), congo-red for eosinophil infiltration (red arrows), periodic acid-Schiff (PAS) for goblet cell hyperplasia (black arrows) and mucus deposit, and Masson trichrome (MT) for collagen fiber deposits (blue arrows). Only a representative picture is shown for each group. (A~D) H-E stain, (E~H) Congo red stain, (I~L) PAS stain, and (M~P) MT stain. RB; respiratory bronchiole, BV; blood vessel. Bars = 100 μm.

fractions were extracted by NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Fischer Scientific, Waltham, MA, USA). Total protein concentration of the lung homogenate was determined with spectrophotometer (BioDrop μLits, Cambridge, England) according to the manufacturer's instructions. NF-κB p65, p-NF-κB p65, and p-I-κB (eBioscience Inc., San Diego, CA, USA) in the lung homogenate from each mouse by ELISA were measured.

8. Statistical analysis

Statistical analysis was performed using Prism 5.0 (GraphPad Software, San Diego, CA, USA). Results were expressed as a mean ± SD. The data were analyzed using Student's *t*-test and one-way ANOVA followed by Tukey's test. Differences were considered statistically significant if $P < 0.05$.

RESULTS

1. Effect of *F. amomi* on eosinophilic inflammation

The hallmark of human asthma is eosinophilic inflammation [1]. As shown in Fig. 1, the number of total cells and differential inflammatory cells (eosinophils, lymphocytes, macrophages) in BALF of OVA group was considerably increased compared with those of mice in control group. However, the administration of *F. amomi* notably inhibited these increases in the numbers of eosinophils and total cells (Fig. 1B, C). Also, Dex, positive control, markedly suppressed the infiltration of eosinophils and inflammatory cells in BALF (Fig. 1B, C).

2. Effect of *F. amomi* on histopathological changes

Compared to control group, the thickness of airway epithe-

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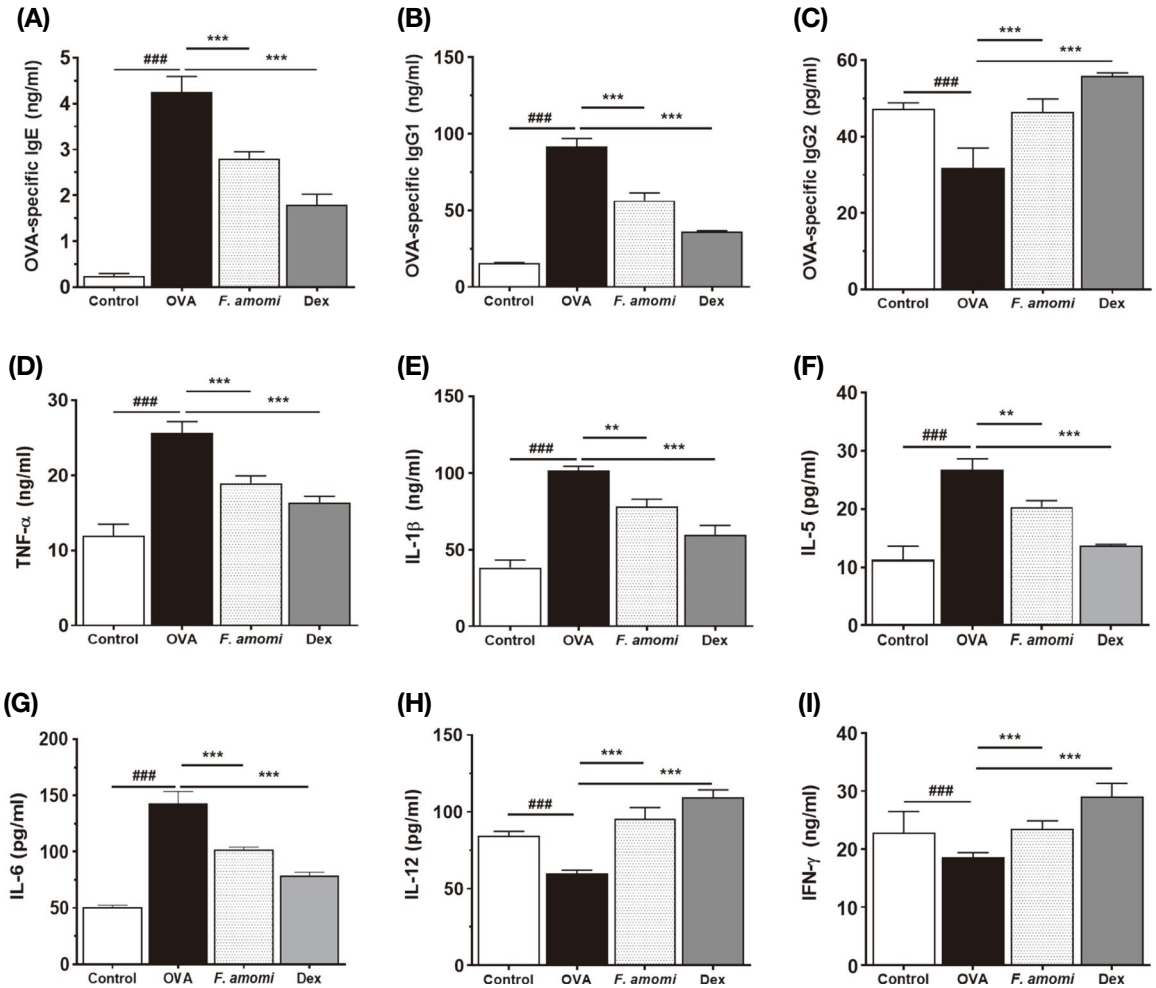

Fig. 3. Effects of *F. amomi* on the levels of OVA-specific IgE/ IgG1/ IgG2 in serum and Th1/Th2 cytokines in bronchoalveolar fluid (BALF). Blood and BALF was collected at 24 h after last OVA challenge. (A) OVA-specific IgE, (B) OVA-specific IgG1, (C) OVA-specific IgG2, (D) TNF-α, (E) IL-1β, (F) IL-5, (G) IL-6, (H) IL-12, and (I) IFN-γ. Data are the mean ± SD (n = 6 per group). $^{###}P < 0.001$, *vs* Control group. $^{**}P < 0.01$, $^{***}P < 0.001$, *vs* OVA group.

lium, the infiltration of inflammatory cells like eosinophils around bronchioles and in proximity to blood vessels, the overproduction of mucus and debris in the bronchial cavity are increased in OVA-challenged mice (Fig. 2B, F, J, N). Also, in OVA-challenged mice (Fig. 2J, N), hyperplasia of goblet cells in the bronchiole epithelium and collagen deposit around bronchiole significantly increased compared to control mice (Fig. 2I, M). However, *F. amomi* significantly relieved the thickness of the airway epithelium, the infiltration of eosinophils into peribronchial and perivascular regions, the secretion of mucus in the airway lumen, and the deposit of collagen fiber between inflammatory cells and bronchioles (Fig. 2C, G, K, O). *F. amomi* generally improved the pulmonary inflammation of OVA-induced asthma mouse model. Dex also significantly decreased pulmonary inflammation (Fig. 2D, H, L, P).

3. Effect of *F. amomi* on the plasma levels of the OVA-specific IgE, OVA-specific IgG1, and OVA-specific IgG2a

To probe into the underlying immunoregulatory mechanism of *F. amomi* on OVA-induced asthma, we estimated plasma levels of the Th2-related Igs (OVA-specific IgE and IgG1), and Th1-related Igs (OVA-specific IgG2a). As shown in Fig. 3, the plasma levels of OVA-specific both IgE and IgG1 in OVA-challenged mice were significantly higher than control mice (Fig. 3A, B). The administration of *F. amomi* dropped down the levels of OVA-specific both IgE and IgG1

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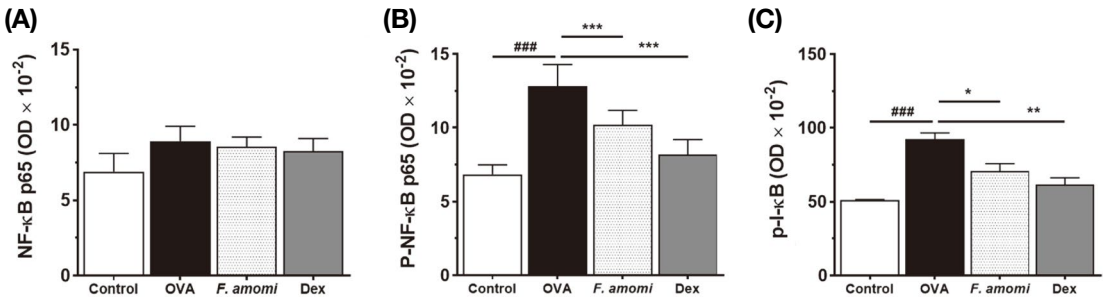

Fig. 4. Effects of *F. amomi* on the activation of NF-κB signaling in lung homogenates. Lung tissues were collected at 24 h after last OVA challenge. (A) NF-κB p65, (B) p-NF-κB p65, and (C) p-I-κB. Data are the mean ± SD (n = 6 per group). $^{###}P < 0.001$, *vs* Control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, *vs* OVA group.

in serum (Fig. 3A, B). In addition, the oral treatment of *F. amomi* significantly increased OVA-specific IgG2a (Fig. 3C).

4. Effect of *F. amomi* on the levels of Th1/Th2 cytokines in BALF

To moreover delineate the anti-asthmatic effects of *F. amomi*, we determined the levels of Th1/Th2 cytokines including interferon (IFN)-γ, TNF-α, IL-5, IL-6 and IL-12 that modulate allergy and asthma mediated by IgE [15,16] in the BALF using appropriate ELISA. The levels of Th2 cytokines TNF-α and IL-1β, IL-5, and IL-6 in BALF were markedly increased (Fig. 3D∼G) and the levels of Th1 cytokines such as IL-12 and IFN-γ in BALF were decreased in OVA-challenged compared with control mice (Fig. 3H, I). The decreased levels of IL-12 and IFN-γ were notably increased (Fig. 3H, I) and the increased levels of TNF-α, IL-1β, IL-5, and IL-6 were reduced (Fig. 3D∼G) by the administration of *F. amomi*, which were similar to the increment of IL-12 and IFN-γ and the reduction of TNF-α, IL-1β, IL-5, and IL-6 in BALF by Dex (Fig. 3D∼I).

5. Effect of *F. amomi* on the activation of NF-κB signaling

It has been reported that a master transcription factor NF-κB regulates Th2 cell differentiation and cytokines TNF-α, IL-1β, IL-5, and IL-6 expressions [17]. To explore the inhibitory effect of *F. amomi* on allergic asthma, NF-κB signaling activation that regulates Th2 cytokines expression in allergic asthma was measured. The levels of total NF-κB, p-NF-κB, and p-IκBα in the lung homogenates of OVA-induced allergic asthma mice were considerably higher than those in the lung homogenates of control mice (Fig. 4A∼C). However, *F. amomi* treatment remarkably reduced the OVA-induced p-NF-κB and p-IκBα levels (Fig. 4B, C). In addition, Dex showed similar inhibitory effects with *F. amomi* on the NF-κB signaling (Fig. 4A∼C).

DISCUSSION

Allergic asthma is increasingly prevalent and one of the most common chronic remitting/relapsing disorders of the airways in children and adults. However, it is not completely curable yet. It is characterized by variable airway obstruction, airway inflammation that recruits various inflammatory cells, cytokines and inflammatory mediators [15,16,18]. Many studies have shown the anti-inflammatory and the anti-oxidative activities of *F. amomi* in several experimental disease models [10-14]. In this study, we revealed the inhibitory effect of *F. amomi* on pulmonary inflammation using a classical OVA-induced asthma mouse model. Allergic asthma induced by OVA is a chronic pulmonary inflammatory disease that typically associated with the infiltration of pro-inflammatory cells into the bronchial lumen [15,16]. Infiltration of eosinophils is marker of allergic airway inflammation in asthma and plays a pivot role in the development of asthma [18,19]. In addition to interrelationship between pulmonary eosinophilia and asthma, the correlation with the level of eosinophils in the BALF is also known [16,18,19]. *F. amomi* significantly improved OVA-induced lung inflammation, such as inflammation around bronchi and blood vessels, hyperplasia of goblet cells, deposit of collagen fibers, infiltration of eosinophils. Moreover, *F. amomi* reduced the number of eosinophils and total cells in BALF.

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In asthma mouse model, OVA significantly increased the serum OVA-specific IgG1and IgE [20]. In our study, oral administration of *F. amomi* significantly reduced serum OVA-specific IgE and IgG1 in OVA treated mice, similar to the reduction in Dex-treated mice. These results suggest that *F. amomi* may have treatment potential for IgE-mediated allergic asthma.

Airway inflammation in asthma is associated with Th2 cell-derived cytokines, IL-1β, IL-5, IL-6, and TNF-α are thought to contribute to airway hyperresponsiveness, mucus hypersecretion, and eosinophil recruitment [2,3] by regulating the critical process of IgE production, the differentiation, the activation, and the infiltration of mast cells and eosinophil [4,5]. Asthma and inflammation are linked with enhanced production of IL-4, IL-5 and decreased production of IFN-γ. Compared with Th2 cytokines (IL-1β, IL-5, IL-6), Th1 cytokines, such as IFN-γ, inhibit the progression of asthma by blocking Th2 cell responses and IgE synthesis. Under physiological conditions, the immune responses of Th1 and Th2 cytokines remains dynamically balanced. Whenever this balance is broken, diseases will occur [21]. Several studies have reported administration of Th1 cytokines, such as IFN-γ, at sensitization, may inhibit the induction of airway hyperresponsiveness and Th2-driven inflammation [22]. Among Th2 cytokines, IL-5 is critically involved in some events leading to the eosinophilic inflammation and airway hyperresponsiveness in asthma. IL-6 induces recruitment and activation of inflammatory cells into the inflammatory site [23]. Moreover, there is a close relationship between eosinophils, IL-4, and IL-5 in the mechanism regulating airway hyperresponsiveness in the asthmatic lung [24]. Because IFN-γ, a kind of Th1 cytokines, has been known to suppress the synthesis of IgE and the differentiation of Th0 cells to Th2 cells, increased IFN-γ has been used to describe the anti-allergic effects of the treatment [25]. Therefore, testing for levels of Th1/Th2 cytokines is an important indicator in the evaluation of asthma. To confirm the effects of *F. amomi* on Th1/Th2 cytokines, we determined the production of Th2 cytokines like TNF-α, IL-1β, IL-5 and IL-6, and Th1 cytokines, such as IFN-γ and IL-12. We showed here that administration of *F. amomi* markedly reduced the production of IL-5 and IL-6, and also decreased the OVA-induced production of pro-inflammatory cytokines like IL-1β and TNF-α. The infiltration of inflammatory cells especially eosinophils were notably inhibited in the BALF and lung tissues by *F. amomi*, consistent with the results of Th2 cytokine levels. However, Oral administration of *F. amomi* promoted Th1 cytokines production. These results confirmed the imbalance of Th1/Th2 cytokines is the main cause of asthma, which increases airway inflammation. These results suggest that *F. amomi* could inhibit the development of airway inflammation by keeping the balance of Th1/Th2 cytokines via shifting from a Th2 to Th1 response in OVA-induced asthma.

It should be noted that a master transcription factor NF-κB plays a key factor in asthma development and Th2 cell differentiation [21,26]. Also, it has been known that inhibition of NF-κB could ameliorate OVA-induced allergic airway inflammation [27]. To find out the underlying mechanism of *F. amomi* in the improvement of allergic asthma, the effects of *F. amomi* on total NF-κB, p-NF-κB, and p-IκBα levels were checked. Oral treatment of *F. amomi* decreased OVA-induced total NF-κB, p-NF-κB and p-IκBα levels which induced Th2 cytokine production. These results suggest that *F. amomi* inhibit OVA-induced asthma by down-regulating NF-κB signaling pathway and then Th2 cytokine production.

In conclusion, *F. amomi* improves OVA-induced airway inflammation by reducing OVA-specific IgE and IgG1 levels and by increasing OVA-specific IgG2a levels. Also, *F. amomi* restored Th1/Th2 balance through the increase in Th1 cytokines and the decrease in Th2 cytokines and NF-κB signaling pathway. Our results suggest that *F. amomi* may have the therapeutic potential of Th2 cell-mediated allergic diseases.

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