

Effect of the Fungal Immunomodulatory Protein FIP-*fve* in the Neutrophilic Asthma Animal Model

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Keywords

Ovalbumin · Neutrophilic asthma · Asthma model · Fungal immunomodulatory peptide-*fve* · Airway inflammation

Abstract

Background: Asthma animal models provide valuable information about the pathogenesis and the treatment of asthma. An ovalbumin (OVA)/complete Freund's adjuvant (CFA)-sensitized model was developed to induce neutrophil-dominant asthma and to investigate whether fungal immunomodulatory peptide-*fve* (FIP-*fve*) could improve asthma features in the OVA/CFA-sensitized model. **Methods:** We used female BALB/c mice and sensitized them intraperitoneally with OVA/CFA on days 1, 2, and 3. On days 14, 17, 21, 24, and 27, they were challenged with intranasal OVA. The airway hyper-responsiveness (AHR) was detected by BUXCO, inflammatory cells were stained with Liu's stain, the cytokines were detected using ELISA, and the airway inflammation was analyzed with hematoxylin and eosin stain. **Results:** According to the results, OVA/CFA sensitization could induce AHR, high levels of IgE, and inflammatory cells espe-

cially neutrophils infiltration in the lung and airway inflammation. IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-25, IL-33, and transforming growth factor- β (TGF- β) increased in the OVA/CFA-sensitized mice. OVA/CFA-sensitized mice treated with FIP-*fve* not only increased IL-12 and IFN- γ but also decreased IL-4, IL-5, IL-6, IL-8, IL-13, IL-17, IL-25, IL-33, and TGF- β in the bronchoalveolar lavage fluid. Moreover, FIP-*fve* significantly decreased neutrophil infiltration in the lung. **Conclusion:** The OVA/CFA model induced neutrophilic asthma successfully, and FIP-*fve* improved neutrophil-dominant asthma.

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Introduction

Asthma, a chronic inflammatory disease of the airway that affects 235 million people worldwide, is associated with a significant health and economic burden [1, 2]. The prevalence continues to increase [3]. Asthma is classical-

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ly characterized as a T-helper (Th)-2-mediated process, which involves inflammatory cell infiltration, airway hyper-responsiveness (AHR), and mucus hypersecretion [4].

In most asthmatic subjects, Th2 bias and eosinophil-dominated bronchial inflammation have been found; however, some patients are characterized by neutrophil-predominant asthma sub-phenotypes along with Th1 and Th17 cells [5]. Due to the insufficiency of appropriate experimental models, little is known about the etiology of many neutrophil-dominated asthma sub-phenotypes [5]. In order to understand the distinct immune-pathological features of eosinophilic asthma and neutrophilic asthma, an ovalbumin (OVA)-based mouse model of neutrophil-dominated allergic pulmonary inflammation was developed [5, 6]. This neutrophil-dominated model was sensitized with OVA and complete Freund's adjuvants (CFAs) and then challenged by OVA aerosol. In contrast to the Th-2-dominated phenotype provoked by OVA/alum, OVA/CFA adjuvant-based sensitization elicited a neutrophil-dominated pulmonary inflammation that was poorly controlled by dexamethasone [5–7].

Previous studies have indicated that the immunomodulatory protein fungal immunomodulatory peptide-*fve* (FIP-*fve*) extracted from the mushroom, which is often consumed in our daily life, can make HPBMCs and produce a significant amount of IFN- γ [8, 9]. From the previous studies of FIP-*fve* on asthma and allergic diseases, we based our study on the following findings [10]. In the experimental model of OVA/alum-sensitized mice, FIP-*fve* reduced AHR, decreased the level of IgE in the serum, and the levels of IL-4, IL-5, and IL-13 in the bronchoalveolar lavage fluid (BALF). Otherwise, the OVA/alum-sensitized mouse models with chronic allergic pulmonary inflammation found that FIP-*fve* decreased not only eosinophils but also neutrophils, reduced the levels of IL-4, IL-5, IL-13, and IL-17, and improved airway remodeling [11], which drew our attention to FIP-*fve* as a possible choice of treatment in neutrophilic asthma. In order to parallel neutrophil-dominated allergic pulmonary inflammation, we used an asthma model with OVA/CFA sensitization and evaluated the efficacy of FIP-*fve* in this model.

Materials and Methods

Mice

Female BALB/c mice (6 weeks old; National Laboratory Animal Center, Taiwan) known to be high IgE responders were used. The mice were individually housed in rack-mounted stainless steel

cages with free access to food and water. There were 5 mice in each of the normal control groups and 8 mice in each of the experiment groups, and the experiment was repeated 3 times in this study.

Fungal Immunomodulatory Protein-*fve*

We extracted FIP-*fve* from *Flammulina velutipes*. The isolation protocol was based on the previous studies [8, 12], and we gave each treated mouse 200 μ g FIP-*fve* orally.

Protocol for Mice Allergen Sensitization and Challenge

There were 2 asthma animal models in this study (Fig. 1).

Protocol 1-OVA/Alum

The sensitization protocol was as follows: mice received an intraperitoneal injection of 50 μ g of OVA complexed with 5 $\times$ diluted alum (total volume in 2 mL) on days 1, 2, and 3 and an intranasal dose of 5% OVA 50 μ L on days 14, 17, 21, 24, and 27. The control group received normal saline with alum intraperitoneally. On day 28, following the methacholine challenge, AHR was determined in all the experimental groups, and the mice were then sacrificed.

Protocol 2-OVA/CFA

The sensitization protocol was as follows: mice received an intraperitoneal injection of 50 μ g of OVA emulsified in 75 μ L of CFA on days 1, 2, and 3 and an intranasal dose of 5% OVA 50 μ L on days 14, 17, 21, 24, and 27.

Regarding the OVA/CFA-treated groups, the pre-group was fed FIP-*fve* (200 μ g/pc) on days 1–14, and the post-groups were fed FIP-*fve* on days 14–27. Both of them were still sensitized with the above protocol 2. The negative control group received an intraperitoneal injection of 75 μ L CFA on days 1, 2, and 3, and the positive control group received an intraperitoneal injection of 50 μ g of OVA emulsified in 75 μ L of CFA on days 1, 2, and 3 and an intranasal dose of 5% OVA 50 μ L on days 14, 17, 21, 24, and 27. On day 28, following the methacholine challenge, AHR was determined in all the experimental groups, and the mice were then sacrificed.

Airway Resistance Assessment

Airway resistance measurements were performed using a Fine-Pointe RC system (Buxco Research Systems, Wilmington, NC, USA). The mice were subsequently challenged with aerosolized normal saline (baseline) before being treated with methacholine at the following ascending doses: 0, 5, 10, and 20 mg/mL. Average compliance values were recorded during a 3-min period following each challenge.

BALF Preparation and Inflammatory Cell Count

Bronchoalveolar lavage was performed immediately after the last measurement of airway responsiveness. Lung lavage was performed 3 times with 1.0 mL of normal saline. The BALF was centrifuged at 5,000 rpm for 8 min. A cytospin centrifuge of 100 μ L was used, and differential cell counts were performed using the centrifuged preparations and stained with Liu's stain, counting 300 or more cells for each animal at a magnification of $\times$ 1,000 (oil immersion).

The Measurement of OVA-Specific Antibodies in Serum

The serum levels of OVA-specific IgE, IgG1, and IgG2a were determined first. In short, the 96-well microtitre plates were coat-

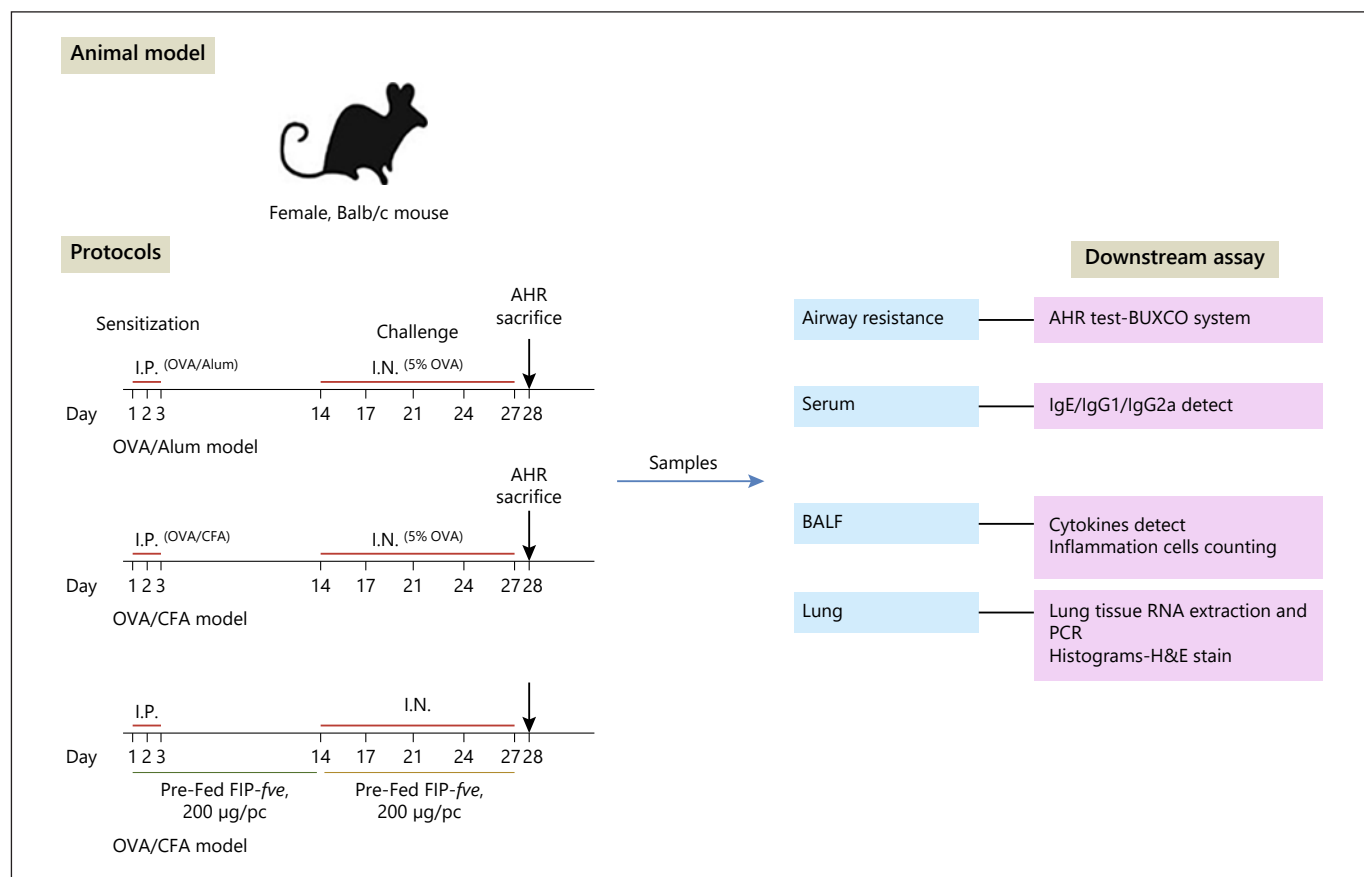


Fig. 1. Asthma sensitized/challenged protocols and the schedules for treatment or evaluation. The protocols used for the mouse models of allergic asthma. (1) The protocols used for the OVA/alum asthma models. (2) The protocols used for the OVA/CFA asthma models. (3) The protocols used for the OVA/CFA asthma models and the pre- or post-treatment with FIP-fve. OVA, ovalbumin; CFA, complete Freund's adjuvant; FIP, fungal immunomodulatory peptide; H&E, hematoxylin and eosin.

ed with 100 mg/mL of OVA in 0.1 M NaHCO₃ at 4°C and left overnight. The plates were washed with Tween 20 solution (0.05%) and then blocked with bovine serum albumin (3%) for 1 h at 37°C. After washing, 50 µL of serially diluted sera (5× sample diluted) in 3% bovine serum albumin was added and incubated at 4°C, and the plates were left unattended overnight. After washing, HRP conjugated with 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/IgG1/IgG2a, p-nitrophenyl phosphate substrate (Sigma Chemical Co., St Louis, MO, USA) was added, and the absorbance at 490 nm was measured. The detection antibody was alkaline phosphatase-labeled.

Cytokine Measurement

We measured BALF cytokine levels of IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IL-25, IL-33, IFN-γ, transforming growth factor-β (TGF-β), and matrix metalloproteinase 9 (MMP9) with detecting enzyme immunoassays according to the manufacturer's protocol (R&D Systems, Inc.).

All reagents and samples were brought to room temperature before use. The cytokine kit was used according to the manufacturer's instructions.

Quantitative Real-Time PCR Analysis

We isolated the total RNA from the lung homogenates with Trizol (Invitrogen; Grand Island, NY, USA) and reverse-transcribed them. We performed the real-time PCR with a ABI StepOne™ System (Applied Biosystems, Foster City, CA, USA). We used the primers as follows: β-actin forward 5'-TGG AAT CCT GTG GCA TCC ATG AAAC-3' and reverse 5'-TAA AAC GCA GCT CAG TAA CAG TCCG-3'; T-bet forward 5'-GCC AGG GAA CCG CTT ATA TG-3' and reverse 5'-GAC GAT CAT CTG GGT CAC ATT GT-3'; GATA3 forward 5'-TTT ACC CTC CGG CTT CAT CCT CCT 3' and reverse 5'-TGC ACC TGA TAC TTG AGG CAC TCT-3'; RORrt forward 5'-TCT ACA CGG CCC TGG TTCT-3' and reverse 5'-ATG TTC CAC TCT CCT CTT CTC TTG-3'; and FOXP3 forward 5'-TCA TCC GCT GGG CCA TCC TG-3' and reverse 5'-GTG GAA ACC TCA CTT CTT GGT C-3'.

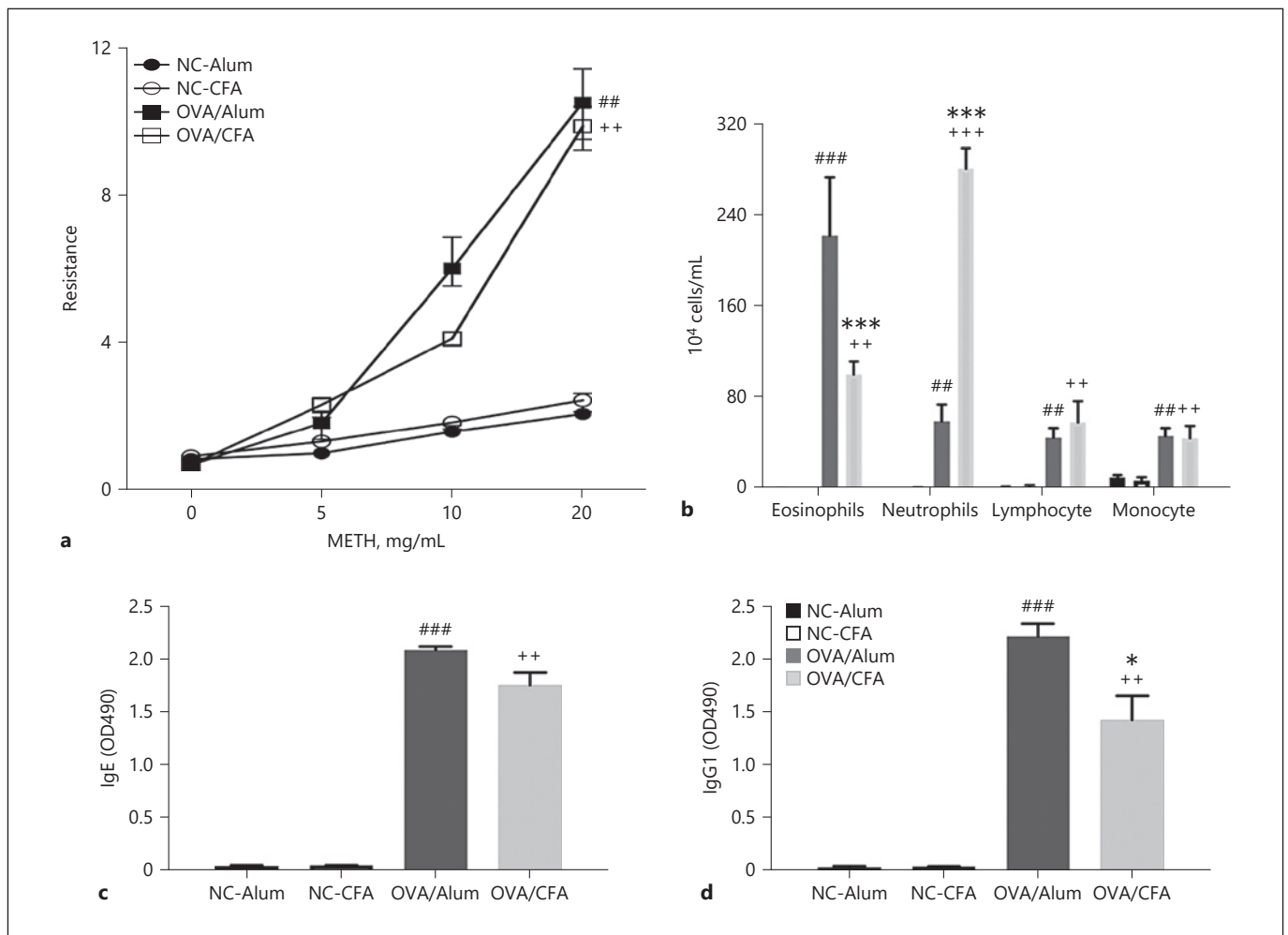


Fig. 2. Asthmatic features in the 2 asthma animal models. The features of asthma models with OVA/alum or OVA/CFA sensitization. **a** The airway resistance values. **b** The inflammatory cell infiltration in the lung. **c, d** The OVA-specific IgE/IgG1 expression of the 2 asthma animal models. The statistical analysis comparing the OVA/alum-sensitized mice is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The statistical analysis comparing the normal

group (NC-alum) of mice is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The statistical analysis comparing the normal group (NC-CFA) of mice is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The numbers of animals were NC-alum (5), NC-CFA (5), OVA/alum (8) and OVA/CFA (8). OVA, ovalbumin; CFA, complete Freund's adjuvant; NC, negative control.

Histograms

The mice were euthanized after the final exposure, and the right lung and trachea were filled intratracheally with a fixative (0.8% formalin and 4% acetic acid) using a ligature around the trachea. The lung tissue was then embedded in paraffin, cut into 5- μ m sections, and stained with hematoxylin and eosin.

Statistical Analysis

All data were analyzed using GraphPad Prism 6 for Windows. The data were expressed as median $\pm$ interquartile range. For variables that did not have a normal distribution, the Kruskal-Wallis test was used for comparisons. Differences with p values $< 5\%$ were regarded as statistically significant.

Results

Asthmatic Features in the Two Asthma Animal Models

The results of AHR, inflammation cells, OVA-specific IgE/IgG1, and several cytokines of the 2 asthma animal models are shown in Figures 2 and 3. AHR had rising values in both asthma and animal models. The neutrophils increased more in the OVA/CFA-sensitized group than in the OVA/alum-sensitized group. The serum IgE increased in both asthma animal models, and the serum IgG1 increased more in the OVA/alum-sen-

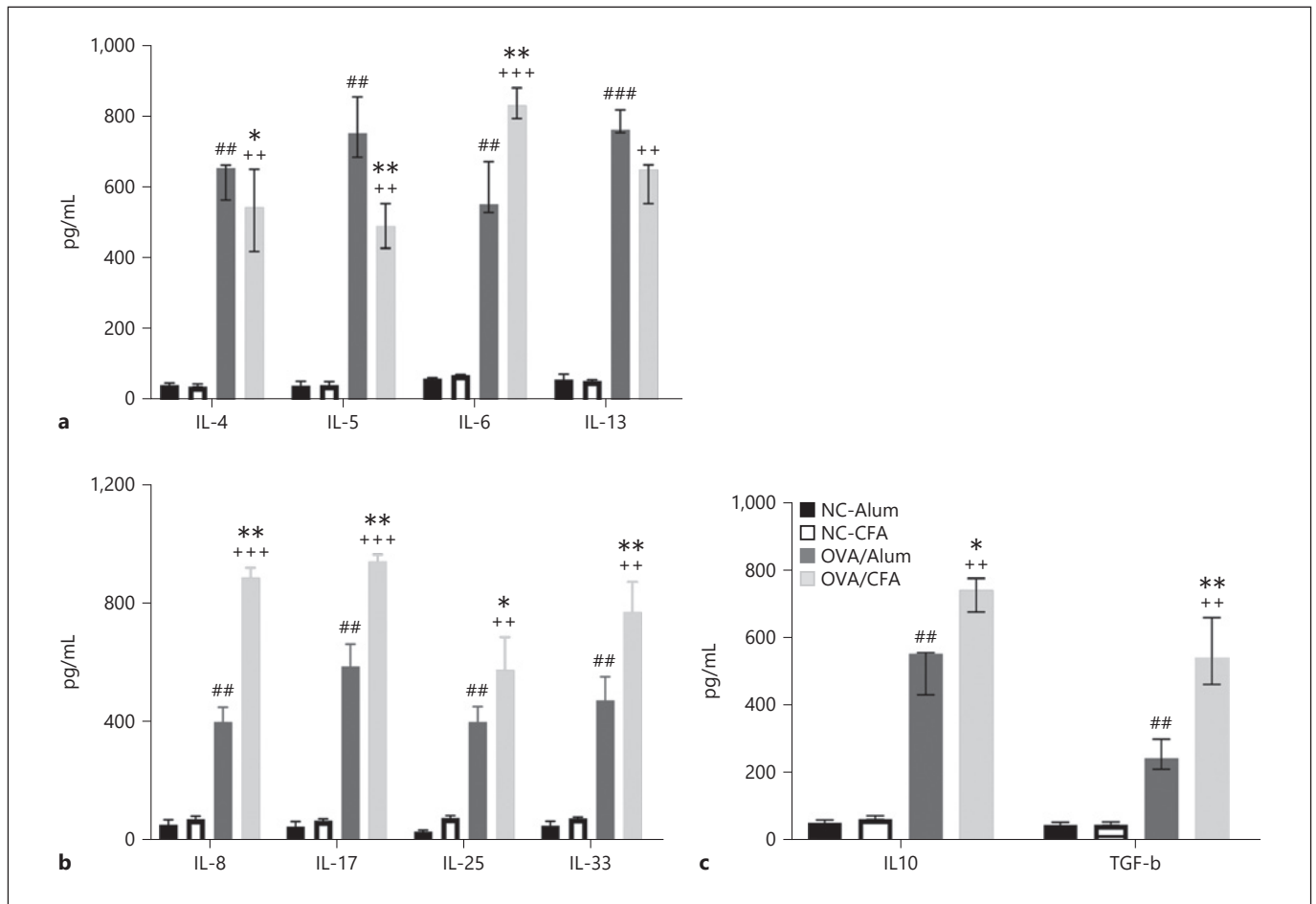


Fig. 3. Cytokines expressed in BALF in the 2 asthma animal models. Cytokines in the BALF were obtained from the 2 asthma models sensitized/challenged with OVA/alum or OVA/CFA. **a–c** The concentration of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-25, IL-33, and TGF- β in the BALF in each group. The statistical analysis comparing the OVA/alum-sensitized mice is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The statistical analysis comparing the normal group (NC-alum) of mice is represented as:

$p < 0.05$; ## $p < 0.01$; and ### $p < 0.001$. The statistical analysis comparing the normal group (NC-CFA) of mice is represented as: + $p < 0.05$; ++ $p < 0.01$; and +++ $p < 0.001$. The numbers of animals were NC-alum (5), NC-CFA (5), OVA/alum (8) and OVA/CFA (8). OVA, ovalbumin; CFA, complete Freund's adjuvant; NC, normal group; BALF, bronchoalveolar lavage fluid; NC, negative control; TGF- β , transforming growth factor- β .

sitized group than in the OVA/CFA-sensitized group. We found that Th2 associated cytokines, such as IL-4, IL-5, and IL-13, showed similar results in 2 models. However, the OVA/CFA-sensitized group expressed higher levels of cytokines (IL-6, IL-8, IL-10, IL-17, IL-25, and IL-33) and TGF- β than those in the OVA/alum-sensitized group.

According to our previous studies [10, 11, 13, 14], FIP-*fve* improved AHR and inflammatory cell infiltration and decreased Th2 associated cytokines in the OVA/alum-sensitized asthma animal model. The effect of FIP-*fve* in the OVA/CFA-sensitized mice was as follows.

The Effect of FIP-fve on AHR in the OVA/CFA-Sensitized Mice

Airway function was assessed by measuring the changes in lung resistance and compliance elicited by methacholine inhalation. The airway resistance and compliance are measured in 4 groups and analyzed in Figure 4a. The results were recorded for the OVA/CFA-sensitized mice which were treated with or without FIP-*fve*. The values of airway resistance showed no significant difference in the control group, the OVA/CFA-sensitized group and the FIP-*fve*-treated groups while challenged with the methacholine doses at 0 (saline), 5 mg, and 10 mg. However, the

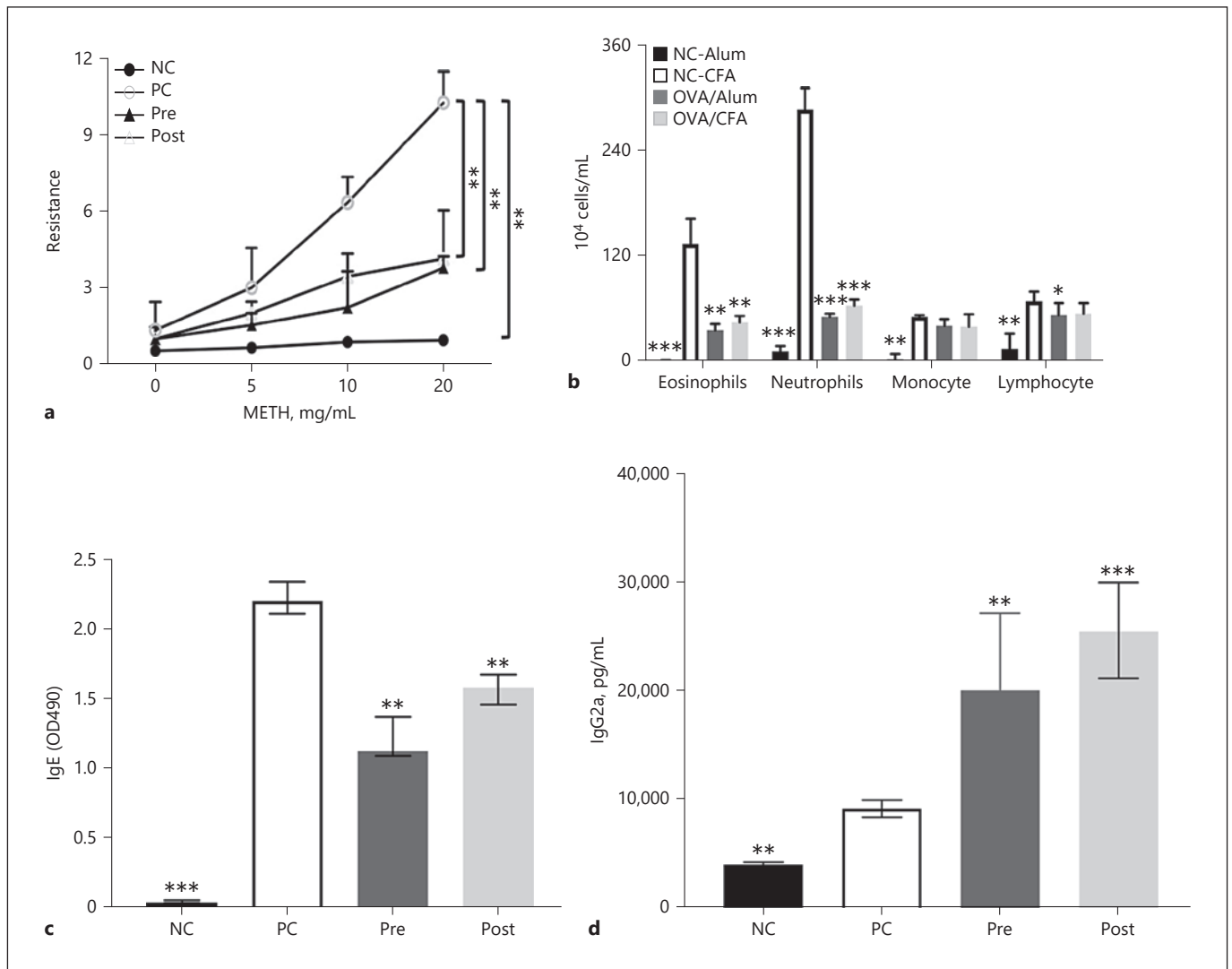


Fig. 4. The effect of FIP-*fve* on the asthmatic features in OVA/CFA-sensitized mice. The features of asthma models in the OVA/CFA-sensitized/challenged mice and the FIP-*fve*-treated groups. **a** The airway resistance values. **b** The inflammatory cell infiltration in the lung. **c, d** The OVA-specific IgE/IgG2a expression in the OVA/CFA-sensitized and the FIP-*fve*-treated groups. The NC group was

sensitized with CFA, while the PC group was OVA/CFA-sensitized/challenged. The statistical analysis comparing the PC group is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The numbers of animals were NC (5), PC (8), pre (8), and post (8). NC, negative control; PC, positive control; OVA, ovalbumin; CFA, complete Freund's adjuvant; FIP, fungal immunomodulatory peptide.

values of airway resistance while challenged with the methacholine dose at 20 mg were higher in the OVA/CFA-sensitized group than in the FIP-*fve*-treated group and the control group.

The Effect of FIP-fve on the Inflammatory Cells in the Lung of the OVA/CFA-Sensitized Mice

We evaluated the inflammatory cells by counting the eosinophils, neutrophils, lymphocytes, and monocytes in the BALF. Figure 4b shows that the inflammatory cells,

especially the neutrophils, are higher in the OVA/CFA-sensitized group than in the control group. Moreover, FIP-*fve* treatment decreased the inflammatory cells, especially the neutrophils.

The Effect of FIP-fve on the OVA-Specific Antibodies in the Serum of the OVA/CFA-Sensitized Mice

We found that the serum IgE and IgG2a elevated after the OVA/CFA sensitization/challenge compared with the normal control group. After the treatment with FIP-

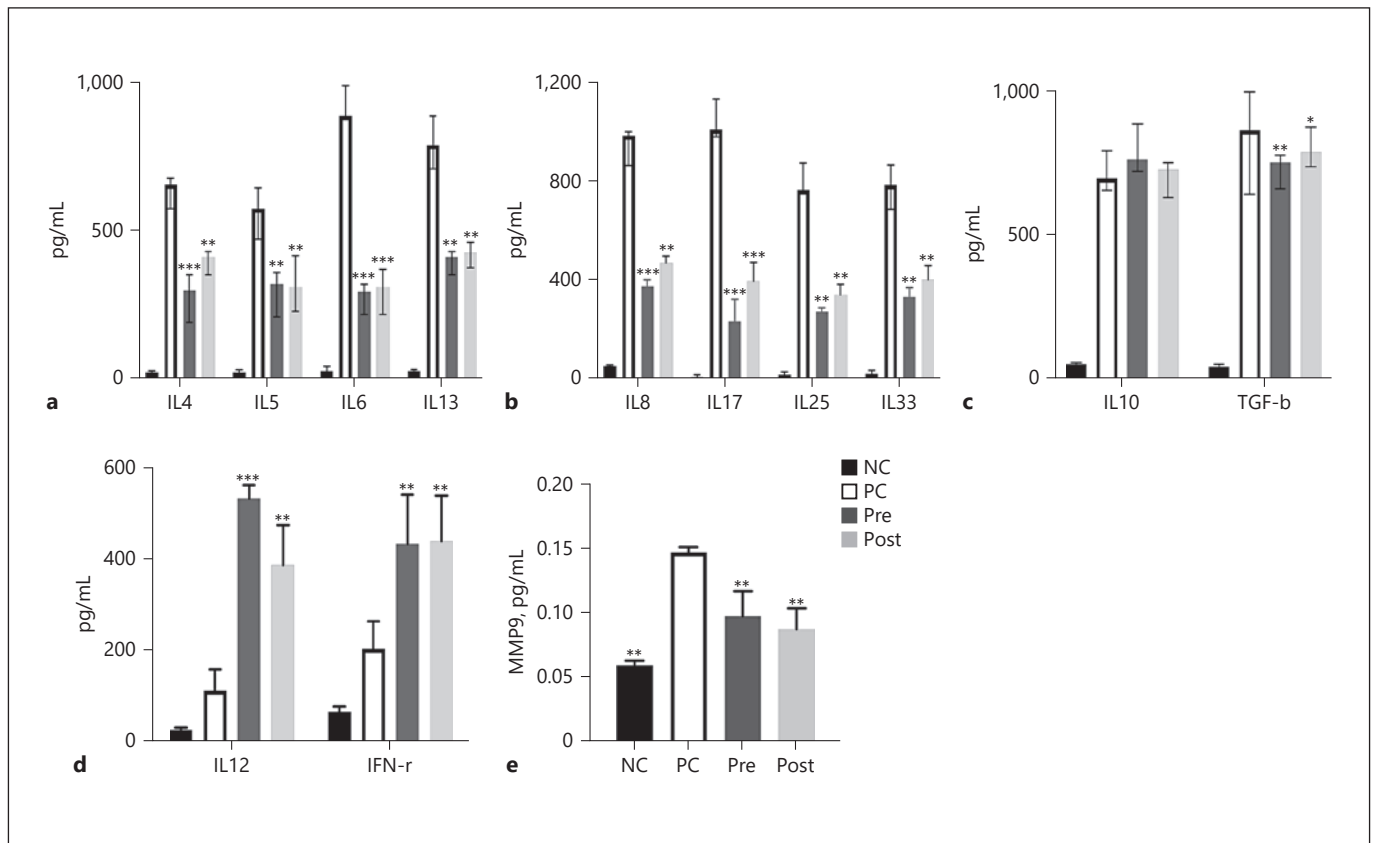


Fig. 5. The effect of FIP-*fve* on the cytokines expressed in BALF in OVA/CFA-sensitized mice. Cytokines in the BALF were obtained from the OVA/CFA-sensitized/challenged mice and the FIP-*fve*-treated groups which were sensitized with OVA/CFA. **a–e** The concentration of IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17, IL-25, IL-33, IFN- γ , and TGF- β in the BALF in each group. The NC group was sensitized with CFA, while the PC group was OVA/

CFA-sensitized/challenged. The statistical analysis comparing the PC group is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The numbers of animals were NC (5), PC (8), pre (8), and post (8). NC, negative control; PC, positive control; OVA, ovalbumin; CFA, complete Freund's adjuvant; FIP, fungal immunomodulatory peptide; BALF, bronchoalveolar lavage fluid; TGF- β , transforming growth factor- β .

fve, in both the pre-treated and the post-treated, the serum IgE decreased (Fig. 4c) and the serum IgG2a increased, especially in the post-treated group ($p < 0.001$, Fig. 4d).

*The Effect of FIP-*fve* on the Cytokines and MMP9 Expressed in BALF of the OVA/CFA-Sensitized Mice*

The concentration of the cytokines in the BALF is shown in Figure 5. In the OVA/CFA-sensitized group, the expression of IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IL-17, IL-25, IL-33, and TGF- β (Fig. 5a) was higher than in the control group, and IL-4, IL-5, IL-6, IL-8, IL-13, IL-17, IL-25, IL-33, and TGF- β decreased after prescribing FIP-*fve*. However, the expression of IL-12 and IFN- γ (Fig. 5b) was mildly higher in the OVA/CFA group than in the control group. After treating with FIP-*fve*, the expression

of IL-12 and IFN- γ became higher. The expression of MMP9 (Fig. 5e) showed a mild increase in the OVA/CFA groups compared to the control group but no statistical decrease after giving FIP-*fve*.

*The Effect of FIP-*fve* on the RNA Levels of the Transcription Factor in the Th1, Th2, Th17, and Treg Cells Expressed in the OVA/CFA-Sensitized Mice*

The total RNA was isolated from the lung homogenates and reverse-transcribed (Fig. 6). In the OVA/CFA-sensitized group, Th1, Th2, Th17, and regulatory T cells (Treg) cells were expressed more than those in the control groups. Besides, FIP-*fve* increased the expression of Th1 and Treg but decreased the expression of Th2 and Th17. With the only FIP-*fve* prescription group, the expression of Th1, Th2, and Treg mildly increased.

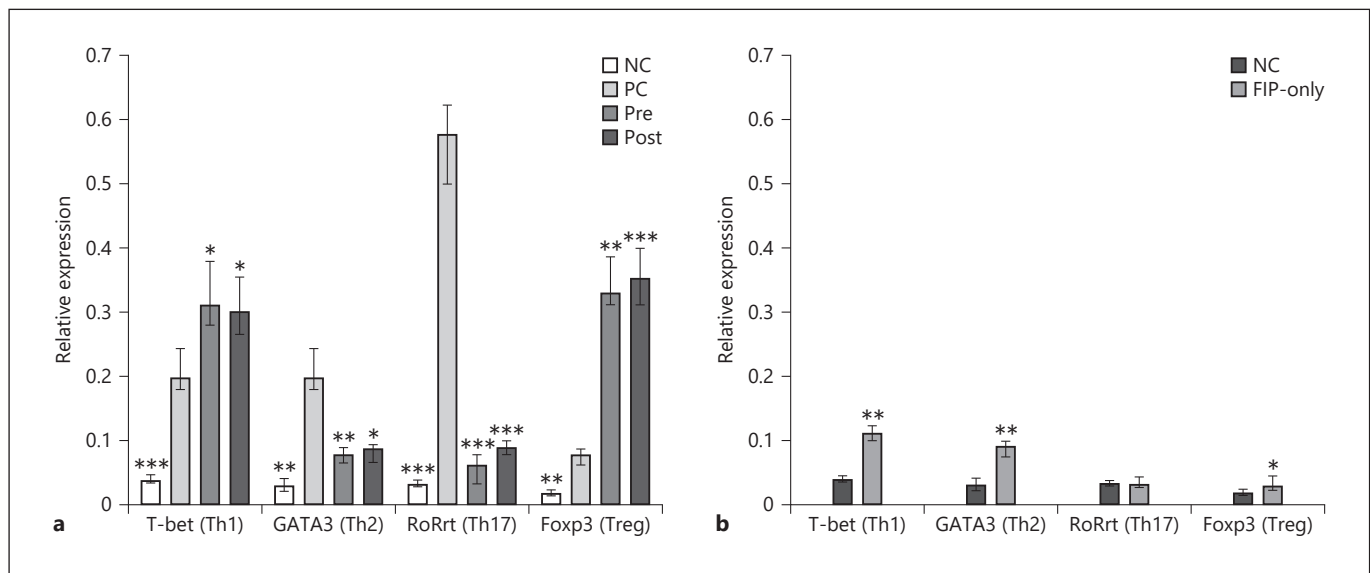


Fig. 6. The effect of FIP-*fve* on the expression of the transcription factors in Th1, Th2, Th17, and Treg cells expressed in the OVA/CFA-sensitized mice or original BALB/c mice. The RNA levels of the transcription factor in the Th1, Th2, Th17, and Treg cells were obtained from the lung tissue. **a** Transcription factor as T-bet, GATA3, RORγT, and FoxP3 expressed in lung tissue from the CFA-sensitized (NC) group, the OVA/CFA-sensitized/challenged mice (PC) or the FIP-*fve*-treated groups which were sensitized

with OVA/CFA. **b** Transcription factor as T-bet, GATA3, RORγT, and FoxP3 expressed in lung tissue from the FIP-*fve*-treated original BALB/c mice. The statistical analysis comparing the PC group is represented as: * $p < 0.05$; ** $p < 0.01$; and *** $p < 0.001$. The numbers of animals were NC (5), PC (8), pre (8), post (8), and FIP-only (8). NC, negative control; PC, positive control; OVA, ovalbumin; CFA, complete Freund's adjuvant; FIP, fungal immunomodulatory peptide.

The Effect of FIP-*fve* on Lung Inflammation in the OVA/CFA-Sensitized Mice

The lung inflammation was evaluated through histologic stain with hematoxylin and eosin (Fig. 7). In the OVA/CFA-sensitized group, airway inflammation was more serious than in the control groups. Besides, both FIP-*fve*-treated groups showed significant improvement in lung inflammation.

Discussion

The results of the present study revealed the fundamentals of an asthma mouse model using OVA sensitization with CFAs. In this neutrophilic asthma animal model, AHR, IgE, and IgG1 expression in the serum, inflammatory cells, and cytokines in the BALF and airway inflammation could be induced successfully. We also found the immunomodulatory protein FIP-*fve* improved the AHR, serum IgE, inflammatory cells, and cytokines IL-4, IL-5, IL-6, IL-8, IL-13, IL-17, IL-25, IL-33, and TGF-β in the BALF and airway inflammation in the OVA/CFA model.

Epithelial cells, the sentinels of the innate immune system, released IL-33, thymic stromal lymphopoietin, IL-25, granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, TGF-β, and IL-1α [15]. Neutralization of either one of the cytokines had the potential to suppress the asthma features such as bronchial hyper-reactivity and goblet-cell metaplasia [16]. The epithelial cytokines IL-33, thymic stromal lymphopoietin, and IL-25 induced activation of Th2 cells, type 2 innate lymphoid cells (ILC2s), and other innate lymphocytes, and IL-33 and IL-25 were activators of ILC2 proliferation and cytokine production [17]. Our study showed FIP-*fve* decreased elevated IL-33 and IL-25, which might be associated with ILC2 activation and further cytokine production.

Animal models of typical asthma mimic features of the human disease including AHR, eosinophilic inflammation, and airway remodeling [18]. Many studies have described procedures to generate acute models of eosinophilic asthma in mice, with OVA as the allergen [18, 19]. Bogaert et al. [5] developed an OVA/CFA mouse model with neutrophil-dominated allergic pulmonary inflammation. The OVA/CFA model elicited neutrophilic asthma which was poorly controlled by dexamethasone [5].

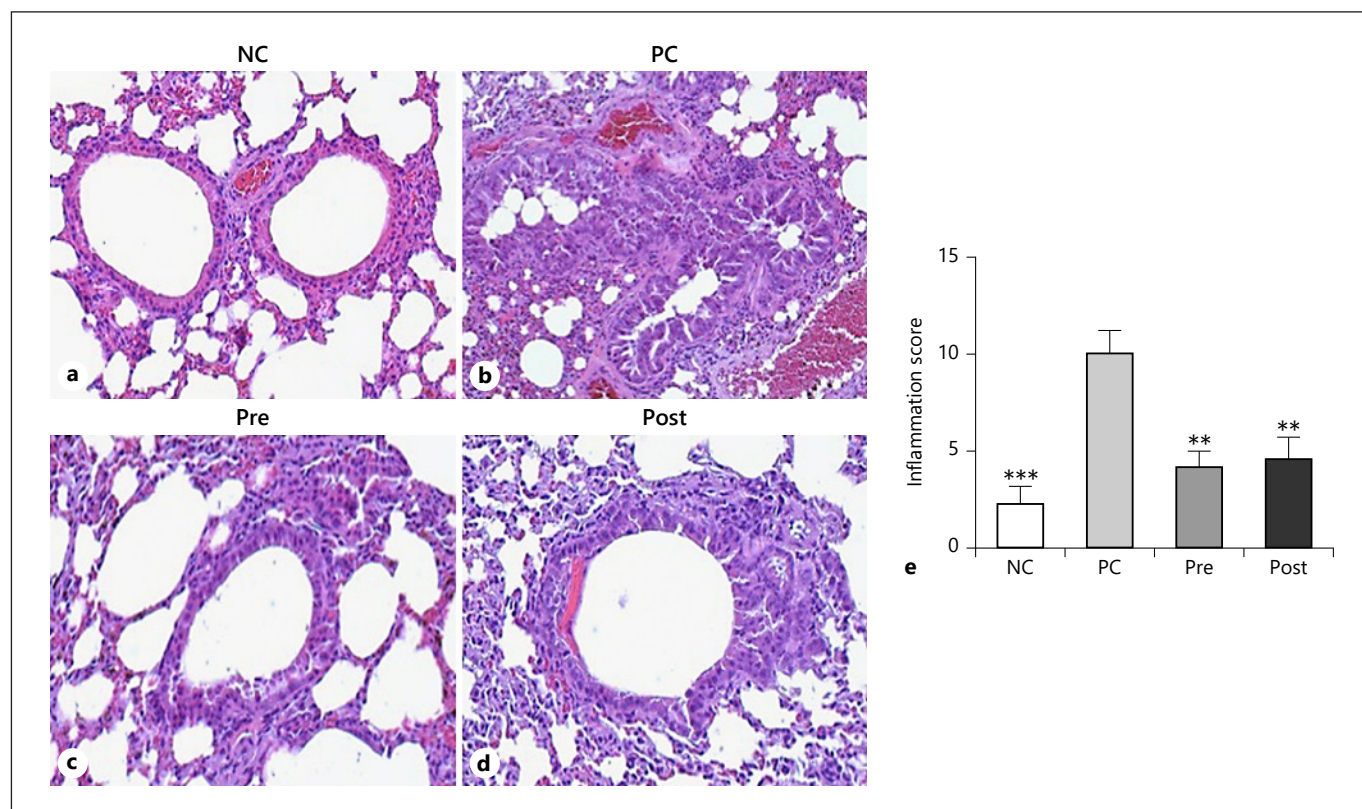


Fig. 7. The effect of FIP-*fve* on lung inflammation in OVA/CFA-sensitized mice. The samples of lung tissue were obtained on day 28 to determine airway inflammation. Figure 4 NC group (a), PC group (b), the OVA/CFA-sensitized/challenged mice and pre- (c) and post- (d) FIP-*fve*-treated groups were sensitized with OVA/CFA. All the lung tissues were stained with H&E. We used Image-

plus pro software for the analysis (e). The statistical analysis comparing the PC group is represented as: ** $p < 0.01$, and *** $p < 0.001$. The numbers of animals were NC (5), PC (8), pre (8), and post (8). NC, negative control; PC, positive control; OVA, ovalbumin; CFA, complete Freund's adjuvant; FIP, fungal immunomodulatory peptide; H&E, hematoxylin and eosin.

Initially, mouse protocols rely on being sensitized against *S. rectivirgula* with CFA as the immunogenic co-factor, leading to a hypersensitivity pneumonitis (HP) [20]. HP is an allergic airway disease, which shows neutrophilic, Th1-driven pulmonary inflammation. Both allergic diseases differ in the nature of their causative agents, while large proteins exert enzymatic activity in asthma, small proteins of microbial origin are present in HP. Further, Bogaert et al. [5] revealed that systemic sensitization to OVA with CFA induced Th1-driven responses with neutrophilic airway inflammation [21]. The same for alum, in which CFA emulsified antigens with mineral oil slow antigen release. Muramyl dipeptide is the minimal mycobacterial cell wall component required for adjuvant activity of CFA [22]. With the same allergen as OVA, OVA/CFA established a neutrophilic asthma animal model while OVA/alum made an eosinophilic asthma animal model. In the OVA/CFA asthma animal model, we

found airway resistance rising with the increased concentration of methacoline, preference neutrophils, and increased IgE and IgG1.

Our study showed increased IL-6, IL-8, and IL-17, especially in the neutrophilic asthma animal model, and decreased after prescribing FIP-*fve*. A previous study showed sputum IL-17A and IL-8 were significantly elevated and correlated with sputum neutrophil counts in asthma patients compared to healthy controls [23]. T lymphocytes, epithelial cells, smooth muscle cells, and macrophages in the airways of asthmatics secrete IL-8, which is related to neutrophilia [24]. IL-17A might recruit neutrophils and stimulate neutrophil maturation, migration, and function [25]. In addition to induction of IL-8 by epithelial cells, IL-17A signaling also induces CXCL8/IL8, IL-6, and IL-19 proteins. Primary bronchial fibroblasts and human smooth muscle cells producing IL-6, IL-8, and IL-11 is another response to IL-17A sig-

naling [26]. However, not all data have shown a link between IL-17 and neutrophilia. Doe et al. [27] revealed IL-17 in bronchial biopsies from severe asthmatic patients but no correlation with airway neutrophilia. Besides, sputum IL-17A mRNA expression is associated with sputum IL-5 mRNA expression in severe asthmatics, which explains why neutrophilia and eosinophilia both increase in some severe patients [28]. Targeting the IL-17a-neutrophil axis is achieved with clinical trials evaluating compounds, such as anti-IL17RA, anti-TNF mAb, CXCR2 antagonist, and macrolides [29]. A previous study suggests that macrolides play an anti-inflammatory role by suppressing CXCL-8 release [30]. Gibson et al. [31] found that azithromycin reduced the rate of exacerbations experienced by those with persistent uncontrolled asthma.

Tregs are important in controlling asthma, and the cytokines most implicated in Treg-mediated suppression of allergic asthma are TGF- β and IL-10 [32]. Previous studies have also revealed that many kinds of structural immune cells, such as fibroblasts, endothelial cells and vascular smooth muscle cells, and inflammatory cells are recruited to the airways and express TGF- β [33, 34]. Dehzad et al. [35] revealed that Treg less effectively suppresses Th2-induced airway inflammation compared with Th1, which is mostly related to neutrophilic asthma. In our study, we found the levels of IL-10 and TGF- β were higher in the OVA/CFA group than in the OVA/alum group. Besides, we also found FIP-*fve* mildly increased IL-10 and decreased TGF- β . In a previous study, FIP-*fve* reduced the OVA-mediated IL-10 and TGF- β expression in the OVA/alum asthma animal models, which might be due to decreased dendritic cell activation [36]. However, in Der f 2 allergic individuals, IL-10⁺ and TGF⁺ cells could be up-regulated by Der f 2 in conjunction with FIP-*fve* [37].

Mice immunized via the peritoneal route with OVA/alum promoted OVA-specific Th2 cells producing IL-4, IL-5, and IL-13 and production of antigen-specific IgE and IgG1. With natural allergens, such as a house dust mite inhalation animal model, we found both adaptive immunity and the innate immune cell influx in the airways. Type 2 cytokines could be produced by ILC2s [38]. ILC2s produce IL-5, IL-9, and IL-13 to control eosinophils, goblet cell metaplasia, and bronchial hyper-responsiveness [39]. In immunocompetent organisms, Th2 cells and ILC2s work complementarily. ILC2s work more in lung repair [40]. It is probably an oversimplification to view eosinophilic asthma as an exclusive Th2 disorder and neutrophilic asthma as an exclusive Th17 disorder. A considerable overlap in the types of cytokines found in an asthma endotype is found in many cases [22]. Our study

revealed increased IL-4, IL-5, IL-6, IL-8, IL-13, and IL-17 in the OVA/CFA neutrophilic asthma animal model, which has an overlap in types of cytokines.

There are some medicine-targeted Th2 associated cytokines, such as mepolizumab, benralizumab, and reslizumab associated with IL-5, dupilumab associated with IL-4 and IL-13, and lebrikzumab and tralokinumab associated with IL-13. However, the clinical benefits do not persist when the treatment is discontinued, which requires longer term treatment for patients. Type-2 immunity is important in anti-helminth defense, tissue repair, metabolism, and cancer, and concerns about increased morbidity from infections and risk of malignancy are not totally realized. Therapeutic strategies interfering with airway neutrophilia, might cause neutropenia, which also increases severe infections. Therefore, a novel and safe drug for steroid-resistant asthma is still being pursued.

FIP-*fve*, an immunomodulatory protein, is extracted from mushrooms, a common food in our daily life. Previous studies have revealed FIP-*fve* treatment improves airway inflammation in the acute [10] and chronic stages [11] of OVA/alum asthma animal models. FIP-*fve* decreases Th2 and Treg-associated cytokines, IL-17, and MMP9. Moreover, FIP-*fve* decreases eosinophils and neutrophils in the acute and chronic stages of the OVA/alum asthma animal model. In our OVA/CFA neutrophil-dominant asthma animal model, FIP-*fve* improved AHR, decreased IL-4, IL-5, IL-6, IL-8, IL-13, IL-17, IL-25, IL-33, and TGF- β , and increased IL-12, IFN- γ in the BALF. The high level of neutrophils in the lung also decreased after prescribing FIP-*fve*. In the neutrophilic asthma animal model, FIP-*fve* not only improved the neutrophil-dominant inflammation but also preserved the epithelial function with decreased cytokines IL-8, IL-25, and IL-33.

Conclusion

Based on our results, FIP-*fve* improved several clinical features of neutrophilic asthma, which demonstrates that FIP-*fve* should be considered as a novel supplementary drug for asthma or allergies.

Statement of Ethics

All animal care and housing requirements and all procedures were performed in accordance with the Chung Shan Medical University Institutional Animal Care and Use Committee (Animal Experiment Approval code: 1767).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

H.-H.P., J.-L.K. conceived and designed the analysis; C.-T.W. and Y.-W.Q. contributed to data or analysis tools; H.-L.S., M.-S.K., and K.-H.L. performed the analysis; H.-H.P. wrote the manuscript; J.-L.K., M.-S.K., and K.-H.L. revised the manuscript.

Data Availability Statement

The data used to support the findings of this study are included within the article.

References

- 1 WHO. Asthma Fact Sheet 2017.
- 2 GINA. GINA Main Report. 2019.
- 3 Akinbami LJ, Moorman JE, Liu X. Asthma prevalence, health care use, and mortality: United States, 2005–2009. *Natl Health Stat Rep*. 2011;32:1–14.
- 4 Boulet LP, Gauvreau G, Boulay ME, O'Byrne P, Cockcroft DW. The allergen bronchoprovocation model: an important tool for the investigation of new asthma anti-inflammatory therapies. *Allergy*. 2007;62:1101–10.
- 5 Bogaert P, Naessens T, De Koker S, Hennuy B, Hacha J, Smet M, et al. Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints. *Am J Physiol Lung Cell Mol Physiol*. 2011 May;300(5):L679–90.
- 6 Tian BP, Xia LX, Bao ZQ, Zhang H, Xu ZW, Mao YY, et al. Bcl-2 inhibitors reduce steroid-insensitive airway inflammation. *J Allergy Clin Immunol*. 2017;140(2):418–30.
- 7 Nakada EM, Shan J, Kinyanjui MW, Fixman ED. Adjuvant-dependent regulation of interleukin-17 expressing $\gamma\delta$ T cells and inhibition of Th2 responses in allergic airways disease. *Respir Res*. 2014;15(1):90.
- 8 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 Mar 1;228(2):244–9.
- 9 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- α activation. *J Agric Food Chem*. 2009;57(22):11008–13.
- 10 Lee YT, Lee SS, Sun HL, Lu KH, Ku MS, Sheu JN, et al. Effect of the fungal immunomodulatory protein FIP-fve on airway inflammation and cytokine production in mouse asthma model. *Cytokine*. 2013;61(1):237–44.
- 11 Lee YT, Wu CT, Sun HL, Ko JL, Lue KH. Fungal immunomodulatory protein-fve could modulate airway remodel through by affect IL17 cytokine. *J Microbiol Immunol Infect*. 2017;50(3):297–306.
- 12 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 Jul;96(7):517–24.
- 13 Wu CT, Chen PJ, Lee YT, Ko JL, Lue KH. Effects of immunomodulatory supplementation with *Lactobacillus rhamnosus* on airway inflammation in a mouse asthma model. *J Microbiol Immunol Infect*. 2016;49(5):625–35.
- 14 Wu CT, Lin FH, Lee YT, Ku MS, Lue KH. Effect of *Lactobacillus rhamnosus* GG immunopathologic changes in chronic mouse asthma model. *J Microbiol Immunol Infect*. 2019;52(6):911–9.
- 15 Hammad H, Lambrecht BN. Barrier epithelial cells and the control of Type 2 immunity. *Immunity*. 2015 Jul 21;43(1):29–40.
- 16 Lambrecht BN, Hammad H, Fahy JV. The cytokines of asthma. *Immunity*. 2019 Apr 16;50(4):975–91.
- 17 Van Dyken SJ, Nussbaum JC, Lee J, Molofsky AB, Liang HE, Pollack JL, et al. A tissue checkpoint regulates type 2 immunity. *Nat Immunol*. 2016 Dec;17(12):1381–7.
- 18 Daubeuf F, Frossard N. Eosinophils and the ovalbumin mouse model of asthma. *Methods Mol Biol*. 2014;1178:283–93.
- 19 Nials AT, Uddin S. Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech*. 2008;1(4–5):213–20.
- 20 Schuyler M, Gott K, Haley P. Experimental murine hypersensitivity pneumonitis. *Cell Immunol*. 1991 Sep;136(2):303–17.
- 21 Salek-Ardakani S, So T, Halteman BS, Altman A, Croft M. Differential regulation of Th2 and Th1 lung inflammatory responses by protein kinase C θ . *J Immunol*. 2004 Nov 15;173(10):6440–7.
- 22 Mountford AP, Fisher A, Wilson RA. The profile of IgG1 and IgG2a antibody responses in mice exposed to *Schistosoma mansoni*. *Parasite Immunol*. 1994 Oct;16(10):521–7.
- 23 Bullens DM, Truyen E, Coteur L, Dilissen E, Hellings PW, Dupont LJ, et al. IL-17 mRNA in sputum of asthmatic patients: linking T cell driven inflammation and granulocytic influx? *Respir Res*. 2006 Nov 3;7:135.
- 24 Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest*. 2001 May;119(5):1329–36.
- 25 Schwarzenberger P, La Russa V, Miller A, Ye P, Huang W, Zieske A, et al. IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. *J Immunol*. 1998 Dec 1;161(11):6383–9.
- 26 Chesne J, Braza F, Mahay G, Brouard S, Aronica M, Magnan A. IL-17 in severe asthma. Where do we stand? *Am J Respir Crit Care Med*. 2014 Nov 15;190(10):1094–101.
- 27 Doe C, Bafadhel M, Siddiqui S, Desai D, Mistry V, Rugman P, et al. Expression of the T helper 17-associated cytokines IL-17A and IL-17F in asthma and COPD. *Chest*. 2010 Nov;138(5):1140–7.
- 28 Seys SF, Grabowski M, Adriaenssens W, Decraene A, Dilissen E, Vanoirbeek JA, et al. Sputum cytokine mapping reveals an “IL-5, IL-17A, IL-25-high” pattern associated with poorly controlled asthma. *Clin Exp Allergy*. 2013 Sep;43(9):1009–17.
- 29 Seys SF, Lokwani R, Simpson JL, Bullens DMA. New insights in neutrophilic asthma. *Curr Opin Pulm Med*. 2019 Jan;25(1):113–20.
- 30 Kobayashi Y, Wada H, Rossios C, Takagi D, Higaki M, Mikura S, et al. A novel macrolide solithromycin exerts superior anti-inflammatory effect via NF- κ B inhibition. *J Pharmacol Exp Ther*. 2013 Apr;345(1):76–84.
- 31 Gibson PG, Yang IA, Upham JW, Reynolds PN, Hodge S, James AL, et al. Effect of azithromycin on asthma exacerbations and quality of life in adults with persistent uncontrolled asthma (AMAZES): a randomised, double-blind, placebo-controlled trial. *Lancet*. 2017;390(10095):659–68.
- 32 Ray A, Khare A, Krishnamoorthy N, Qi Z, Ray P. Regulatory T cells in many flavors control asthma. *Mucosal Immunol*. 2010 May;3(3):216–29.

- 33 Kelley J, Kovacs EJ, Nicholson K, Fabisiak JP. Transforming growth factor-beta production by lung macrophages and fibroblasts. [Chest](#). 1991;99(3):85S–6S.
- 34 Lee KY, Ho SC, Lin HC, Lin SM, Liu CY, Huang CD, et al. Neutrophil-derived elastase induces TGF-beta1 secretion in human airway smooth muscle via NF-kappaB pathway. [Am J Respir Cell Mol Biol](#). 2006 Oct;35(4):407–14.
- 35 Dehzad N, Bopp T, Reuter S, Klein M, Martin H, Ulges A, et al. Regulatory T cells more effectively suppress Th1-induced airway inflammation compared with Th2. [J Immunol](#). 2011 Feb 15;186(4):2238–44.
- 36 Lee YT, Lee SS, Sun HL, Lu KH, Ku MS, Sheu JN, et al. Effect of the fungal immunomodulatory protein FIP-fve on airway inflammation and cytokine production in mouse asthma model. [Cytokine](#). 2013 Jan;61(1):237–44.
- 37 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 Mar;102(3):216–22.
- 38 Haspeslagh E, Debeuf N, Hammad H, Lambrecht BN. Murine models of allergic asthma. [Methods Mol Biol](#). 2017;1559:121–36.
- 39 Verma M, Liu S, Michalec L, Sripada A, Gorska MM, Alam R. Experimental asthma persists in IL-33 receptor knockout mice because of the emergence of thymic stromal lymphopoietin-driven IL-9+ and IL-13+ type 2 innate lymphoid cell subpopulations. [J Allergy Clin Immunol](#). 2018;142(3):793–803.e8.
- 40 Drake LY, Iijima K, Kita H. Group 2 innate lymphoid cells and CD4+ T cells cooperate to mediate type 2 immune response in mice. [Allergy](#). 2014;69(10):1300–7.