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Original Article

Role of biomarkers and effect of FIP-fve in acute and chronic animal asthma models



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KEYWORDS

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Abstract *Background:* Asthma is a consequence of complex gene–environment interactions. Exploring the heterogeneity of asthma in different stages is contributing to our understanding of its pathogenesis and the development of new therapeutic strategies, especially in severe cases.

Objective: This study aimed to further understand the relationship between manifestations of acute and chronic asthma and various endotypes, and explore the severity of lung inflammation, cell types, cytokine/chemokine differences, and the effects of FIP-fve.

Materials and methods: Acute and chronic OVA-sensitization mouse asthma models, based on our previously published method, were used and FIP-fve was used to evaluate the effect on these two models. BALF cytokines/chemokines were detected according to the manufacturer's protocol.

Results: Seventeen cytokine/chemokine secretions were higher in the chronic stage than in the acute stage. Whether in acute stage or chronic stage, the FIP-fve treatment groups had reduced airway hyperresponsiveness, infiltration of airway inflammatory cells, secretion of cytokines, chemokines by Th2 cells, and TNF- α , IL-8, IL-17, CXCL-1, CXCL-10, CCL-17, and CCL-22, and it was also found that the Treg cell cytokine IL-10 had increased significantly. PCA (Principal Component Analysis) was also used to compare statistics and laboratory data to find the important biomarkers in different stages and after treatment with FIP-fve.

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Conclusions: There are many different immune responses in the different stages of the asthma process. Drug treatment at the appropriate times might help reduce the worsening of asthma. Copyright © 2020, Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Introduction

Asthma is a chronic inflammatory airway disease, which is fatal when a serious asthma attack occurs. It is a significant public health issue all over the world. Since the 1970s, numerous reports have pointed out that the incidence of asthma continues to increase around the world.¹ The symptoms have become progressively serious along with increased hospitalizations and mortality rates. In addition, according to literature about forty percent of junior high and elementary school students in Taiwan are atopic. Taiwan is located in a subtropical region with a warm and humid climate, which results in the prevalence of allergic diseases. The most common are allergic rhinitis and asthma. In 2016, chronic respiratory diseases (including asthma and other diseases) ranked 7th in Taiwan's Top 10 causes of death as reported by the Ministry of Health and Welfare.²

Asthma is a complex respiratory disease with chronic inflammation. Pathological changes include pulmonary eosinophilic leukocyte (Eosinophil; Eos) infiltration, mucosal edema, hypersecretion of mucus and airway overreaction in acute inflammatory stage. In the long term state of asthma or severe asthma, airway goblet cells, smooth muscle tissue, neutrophil infiltration and angiogenesis are accelerated. The proliferation of airway remodeling has increased the difficulty in controlling asthma.³ Earlier studies have found that cytokines and chemokines in bronchoalveolar lavage fluid (BALF) and lung secretions can regulate inflammation.³ Asthma patients have a lot of T-cell infiltration in their respiratory tracts.^{3,4} Moreover, previous studies have also shown that IL-12 (interleukine-12) contributes CD4 + T cells to differentiate Th1 cells,⁵ and IL-4 could induce differentiation of Th2 cells.⁶ Th1 cells could produce IL-2 and IFN- γ (interferon- γ), which is of great importance for defense, while Th2 cells could produce IL-4, IL-5, IL-6, IL-9, IL-13, which are associated with allergic inflammation mechanisms. Some studies have indicated that such cytokines produced by Th1 cells and Th2 cells are antagonistic.⁷ These reactions are normally suppressed by regulatory T (Treg) cells which secrete IL10 and likely play a central role in maintaining airway tolerance, but the role of transforming growth factor β (TGF- β) is more complex with evidence for immune suppression and remodeling in the airways.

The latest studies point out that asthma is a consequence of complex gene–environment interactions, with heterogeneity in clinical presentation and the type and intensity of airway inflammation and remodeling.⁸ To explore the heterogeneity of asthma in different stages is helping in our understanding of disease pathogenesis and development of new therapeutic strategies, especially in cases of severe disease. According to recent studies,^{8,9} the heterogeneity of immunology in asthma can be divided into eosinophilic (allergic and non-allergic), non-eosinophilic

(neutrophilic type 1 and type 17 and paucigranulocytic), and mixed granulocytic inflammation.

In eosinophilic-allergic asthma, type 2-high airway inflammation, after allergen sensitization the adaptive Th2 cells produce IL-5, IL-4, and IL-13. In non-allergic eosinophilic asthma, innate lymphoid cells produce IL-5 and IL-13 in response to prostaglandin D2 and epithelium-derived IL-33, IL-25, and thymic stromal lymphopoietin (TSLP) is released after epithelial damage by pollutants and microbes.⁸ Non-eosinophilic asthma with neutrophil-predominant disease releases cytokines from Th1 cells, Th17 cells, or type 3 innate lymphoid cells, with activation of macrophages and release of neutrophil chemokines. However, a neutrophilic response could reflect effects of corticosteroids on promotion of neutrophil survival and suppression of type 2 immunity, leading to an increase of type 1 or type 17 immunity.^{8,10}

Moreover, the allergic-dependent and allergic-independent mechanisms that drive eosinophilic inflammation and non-eosinophilic asthma might occur in concert, leading to mixed granulocytic inflammation or changes in the inflammatory profile over time.⁸ There are many clinical and experimental studies over the past years which have implied that allergic asthma is much more heterogeneous and complex than previously thought.

In previous studies of Th17 and Th22 cytokines of asthma, cytokines IL-17 and IL-22 secreted from an emerging group of Th22 immune cells had certain effects on chronic asthma and inflammatory diseases.^{11,12} When Th22 cells do not function, chronic and allergic inflammatory diseases are worsened by excessive inflammation.¹³ Based on the findings, Th22 cells and their secreted cytokines IL-22 and TGF- β are stimulated by immune cells to fix damage resulting from the inflammation. However, when inflammation becomes chronic, Th22 cells, IL-22 and TGF- β cytokines continue to be secreted abnormally. Excessive secretion of Th22 and IL-22 make allergic inflammatory disease more severe. This has been demonstrated in blood tests of patients with psoriasis and atopic eczema.^{11,12} However, a more recent study in 2013 by Davide Pennino et al. revealed that IL-22 could suppress IFN- γ -mediated lung inflammation,¹⁴ and a previous animal study also showed that^{14–16} IL-22 can act as an anti-inflammatory cytokine in the absence of IL-17, whereas in the presence of IL-17, IL-22 contributes to the recruitment of inflammatory cells.

Another important cytokine, TGF- β , is one of the main mediators involved in tissue remodeling in the asthmatic lung. In asthmatic airways, depending on the pathway, TGF- β can induce an antiapoptotic or apoptotic effect in airway epithelial cells, and when epithelial cells come under stress because of continuous exposure to allergens and proinflammatory mediators, detachment of epithelial cells will occur.¹⁷ Although high expression of TGF- β in patients with asthma has been reported,¹⁷ the role of TGF- β in the acute and chronic phases of asthma is unclear. Many studies have suggested that TGF- β plays an important role in most of the

cellular biological processes leading to airway remodeling, especially in severe asthma. When chronic inflammation occurs, incremental secretion of TGF- β worsens the phenomenon of airway remodeling.^{13,17–20} The role of TGF- β , IL-22, IFN- γ and IL-17 in acute and chronic airway inflammation requires further study. Meanwhile the treatment of airway remodeling is an imperative research topic.

Anti-inflammatories and bronchodilators are the most widely used in asthma therapy. Asthma is classified as severe when high-intensity treatment is required to keep it under control or if it remains uncontrolled despite treatment. New biological therapies for treatment of severe asthma, together with developments in biomarkers, present opportunities for phenotype-specific interventions and realization of more personalized treatment. Therefore, another focus of this study is fungal immunomodulatory peptide-fve (FIP-fve) which is an immunomodulatory protein. Some studies have reported²¹ that FIP-fve extracted from the mushroom can cause HPBMCs (human peripheral blood mononuclear cells) to produce IFN- γ . FIP-fve might play a key role in modulating asthma.

Asthma has different endotypes and differences in lung inflammation depending upon the dose and duration of allergen stimulation. In this study we want to further understand the relationship between acute asthma and chronic asthma manifestations and various endotypes, and explore the severity of lung inflammation, cell types, cytokine/chemokine differences, and the effects of FIP-fve.

Materials and methods

Mice

Female BALB/c mice, aged 6 weeks and known to be high IgE responders, were used. They were obtained from the National Laboratory Animal Center (Taipei, Taiwan). The mice were maintained on an ovalbumin (OVA)-free diet and were individually housed in rack mounted stainless steel cages with free access to food and water.

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).

Allergen immunization/challenge protocol

The experimental design in acute stage (Fig. 1a)

The sensitization protocol was as follows: mice received an intraperitoneal injection of 50 μ g of OVA complexed with 5x dilute alum on days 1, 2, 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, airway hyperresponsiveness (AHR) was determined in all the experimental groups, and the mice were sacrificed as well.

Treatment with FIP-fve in acute stage

To assess the effect of FIP-fve on decreased lung inflammation and changed immune microenvironment, the sensitized mice were given FIP-fve. On days 14–27, randomized treatment groups of mice were given FIP-fve

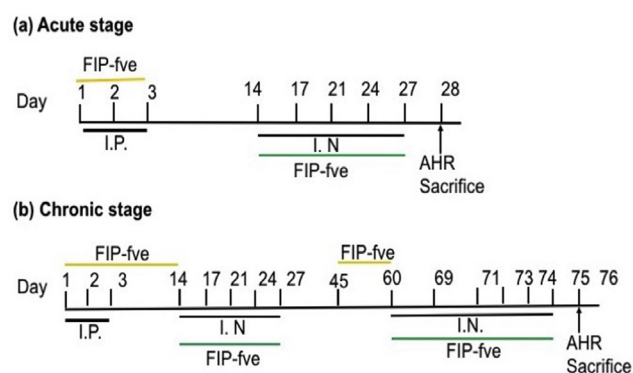


Figure 1. The protocol of OVA sensitization in acute and chronic stage. The female mice received an intraperitoneal injection of 50 μ g of OVA complexed with alum and an intranasal dose of 5% OVA 100 μ l in the acute (Figure 1a) or chronic (Figure 1b) stage. On day 28 or day 76 following the methacholine challenge, airway AHR was determined in all the experimental groups. To assess the effect of FIP-fve on decreased lung inflammation and changed immune microenvironment, the sensitized mice were given FIP-fve. Treatment groups were given FIP-fve (200 μ g/mouse/day) via intragastric feeding.

(200 μ g/mouse/day) via intragastric feeding every day. As a means of prevention the mice were also given FIP-fve on days 1–14.

The experimental design in chronic stage (Fig. 1b)

The sensitization protocol was as follows: mice received an intraperitoneal injection of 50 μ g of ovalbumin (OVA) complexes with alum on days 1, 2, 3 and an intranasal dose of 5% OVA 50 μ l on days 14, 17, 21, 24, 27, 60, 69, 71, 73, 74, and 75. The control group received normal saline with Alum intraperitoneally.

Treatment with FIP-fve in chronic stage

FIP-fve groups of OVA-treated mice were given FIP-fve. The pre-groups were treated with FIP-fve on days 1–14 and days 45–60. The post-groups were treated with FIP-fve on days 14–28 and days 60–75. On day 76 following the methacholine challenge, AHR was determined in all the experimental groups, and the mice were sacrificed as well.

Determination of AHR- airway resistance

Airway responsiveness was measured by FinePointe RC system (Buxco Research Systems, Wilmington, NC) after 24 h after the last I.N., while the animals were awake and breathing spontaneously as a modification of the method described by Hamelmann. Enhanced pause (Penh) to methacholine as measured using barometric plethysmography is a valid indicator of bronchoconstriction in mice and can be used to measure AHR. Aerosolized methacholine in increasing concentrations (2.5–50 mg/ml) was nebulized through an inlet of the main chamber for 3 min.

Bronchoconstriction alters breathing patterns, and changes in the timing of early and late expirations (Pause) and in Penh are the results of alterations in the timing of

breathing, as well as the prolongation of the expiratory time. Furthermore, airway constriction increases the thoracic flow asynchronously with the nasal flow, resulting in an increase in the box pressure signal. Penh is an empiric parameter that reflects changes in the waveform of the measured box pressure signal that are a consequence of bronchoconstriction. Before taking readings, the box was calibrated with a rapid injection of 150 μ L of air into the main chamber. The difference between the pressure in the main chamber of the WBP containing the animal and that in a reference chamber was measured as the box pressure signal, which is caused by the pressure change in the main chamber during the respiratory cycle of the animal. A pneumotachograph with defined resistance in the wall of the main chamber acted as a low-pass filter and allowed thermal compensation. The time constant of the box was determined to be approximately 0.02 s. Mice were placed in the main chamber, and baseline readings were taken and averaged for 3 min.

Serum assay for specific IgE/IgG2a/IgG1 levels

After plethysmography, the mice were sacrificed; each mouse was then exsanguinated by cardiac puncture. The blood was transferred to a 2 ml microcentrifuge tube which contained gel and then centrifuged at 4 °C, 6000 rpm for 5 min. The serum was removed and red blood cells were discarded.

The serum was divided into 3 aliquots and stored at –70 °C until cytokines and antibodies level analyses were performed. Indirect ELISA was employed to determine IgE/IgG2a/IgG1 serum antibody titers. ELISA plates with 96 wells were coated with anti-mouse IgE/IgG2a/IgG1. All reagents in the procedure were allowed to reach room temperature. The required number of strips were arranged and labeled. Standards and samples (20 μ L) were pipetted into appropriate wells. Sample buffer (80 μ L) was dispensed into each well. The plate was covered and incubated at room temperature for 60 min. Following aspiration and washing the plate five times, 100 μ L of HRP (horseradish peroxidase)-labeled anti-mouse IgE (Bio-Rad Laboratories, Inc.)/IgG2a (BD Pharmingen™)/IgG1 (BD Pharmingen™) conjugate was added. After mixing gently, covering the wells, and incubating at room temperature for 30 min, the plate was aspirated and washed again five times. 100 μ L of TMB substrate solution was then added and mixed gently. The wells were covered and incubated at room temperature for 15 min. After that, 100 μ L of stop solution was added into each well and mixed gently (blue color in the wells then turns yellow). Absorbance at 450 nm was measured using ELISA reader and values of the unknown samples were calculated.

Bronchoalveolar lavage fluid collection and cell counting

After measurement of AHR, the mice were anatomized after CO₂ euthanasia. Lung lavage fluid was collected via the trachea with 1 ml of normal saline for the BALF. The cellularity of the BALF was investigated using a hemocytometer. The cells were centrifuged onto slides, fixed, and

then stained with Liu's staining. The remaining BALF was stored at –70 °C until used in the assay.

The BAL fluid was filtered through wet gauze (4 × 4 inches). Trypan blue exclusion for viability and total cell count was performed. The BAL fluid was centrifuged at 150×g for 10 min. The obtained pellet was immediately suspended in 4 ml of physiological saline, and total cell numbers in the BAL fluid were counted in duplicate with a hemocytometer (improved Neubauer counting chamber). A 100- μ L aliquot was centrifuged in a cytocentrifuge (model 2 Cytospin; Shandon Scientific Co., Pittsburgh, PA), and differential cell counts were performed using the centrifuged preparations stained with Diff-quick, counting 500 or more cells for each animal at a magnification of × 1000 (oil immersion).

Measurement of mouse cytokines and chemokines

BALF was collected via the trachea with 1 ml of normal saline lavation. Airway inflammation was evaluated by IFN- γ , TGF- β , TNF- α , IL-4, IL-5, IL-8, IL-10, IL-13, IL-17, IL-22, IL-25, IL-33, CXCL-1, CXCL-10, CCL11, CCL-17 and CCL-22 in the BALF and assessed using an ELISA kit (R&D systems, inc.) according to the manufacturer's protocol. The ELISA plate was read at 450 nm using a Bio-Rad ELISA Reader. Samples were compared using a standard curve containing seven dilution points, which were the relevant recombinant cytokines on each assay plate.

Histograms

To assess pathological changes, the lungs of the mice were soaked in 10% formaldehyde for fixation and embedded in paraffin immediately after being sacrificed with CO₂. Lung tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome stain (to analyze collagen precipitation with airway remodeling) after being cut from the paraffin blocks.

Statistical analysis

Analysis of variance was used for statistical analysis. All data were analyzed using GraphPad Prism 6 for Windows. All values were given as median $\pm$ IQR. The Kruskal-Wallis test (Tnon-parametric version of ANOVA) was used for comparisons of differences with p-values less than 5% which were regarded as statistically significant. PCA (Principal Component Analysis) was performed using R.

Results

The difference in acute and chronic stages of AHR and FIP-*fve* effect

The allergen induced acute and chronic asthmatic animal models were successful. The results are shown in Fig. 2. However, the mice sensitized and challenged with OVA in acute and chronic stages had no significant difference with the methacholine dose of 0 mg, 5 mg and 10 mg. The methacholine dosage of 20 mg, had a significant difference

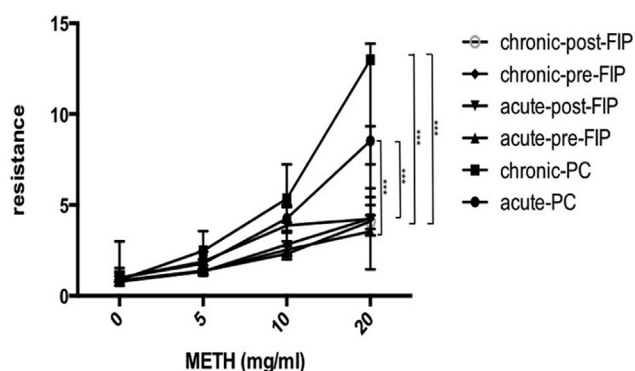


Figure 2. The difference in acute and chronic stage of AHR and FIP-fve effect. The female BALB/c mice were sensitized/challenged with OVA $\pm$ treatment with FIP-fve during the acute and chronic stages. The airway hyperresponsiveness (AHR) to methacholine was assessed with the Buxco system. Acute-OVA (acute-PC) group is shown as ($\bullet$), chronic-OVA (chronic-PC) group is shown as ($\blacksquare$), acute-pre- and post-FIP group are shown as ($\blacktriangle$, $\triangle$) and chronic-pre- and post-FIP group are shown as ($\blacklozenge$, $\lozenge$).

in acute and chronic stages. The AHR value was higher in the chronic stage. Moreover, sensitized mice treated with FIP-fve showed improvement in AHR not only in the acute stage, but also in the chronic stage.

The difference in acute and chronic stages of OVA-specific antibodies in serum and FIP-fve effect

A characteristic of allergic diseases is high concentrations of IgE in serum. According to our results (Table 1), higher IgE expression in serum appeared in these two models which were sensitized with OVA and not given FIP-fve. Moreover, the IgE levels were highest in the chronic stage. However, the sensitized mice not only in the acute, but also in the chronic stage treated with FIP-fve decreased IgE levels.

In addition, IgG1 and IgG2a levels confirm the Th2 and Th1 response.²² In our results (Table 1), the groups that received FIP-fve treatment during the sensitization/challenge to OVA, had significantly increased levels of serum IgG2a in the two stages, and IgG1 decreased in the FIP-fve groups.

The difference in acute and chronic stages of infiltrating cells of the lungs and FIP-fve effect

The hallmark of acute and chronic asthma stages is the infiltration of inflammatory cells, such as eosinophils and neutrophils into the lung. BALF was used to evaluate cell infiltration of the lung, and eosinophils and neutrophils were counted. According to our results (Fig. 3), infiltrating inflammatory cells in the lungs of OVA-sensitized mice in acute and chronic stages increased significantly. In the acute stage, eosinophils were significantly higher than other cells. However, in the chronic stage neutrophils were higher than others. Moreover, the infiltrating cells in the lungs of treated FIP-fve groups decreased significantly, not only eosinophils, but also neutrophils in acute and chronic stages which were OVA-sensitized.

The difference in acute and chronic stages of cytokines and chemokines and FIP-fve effect

A cytokine array kit was used to screen the BALF in different stages first (data not shown). Second, we detected seventeen kinds of cytokines and chemokines in the BALF including IFN- γ , TGF- β , TNF- α , IL-4, IL-5, IL-8, IL-10, IL-13, IL-17, IL-22, IL-25, IL33, CXCL-1, CXCL-10, CCL11, CCL-17 and CCL-22. According to the results (Table 2), IL-4, IL-5 and IL-13 presented in the same rising trend both in acute and chronic stages. Moreover, the TGF- β , TNF- α , IL-8, IL10, IL-17, IL-25, IL33, CXCL-1, CXCL-10, CCL-17 and CCL-22 were higher in the chronic stage compared with the acute stage. In FIP-fve groups, Th2 cytokines and TNF- α , IL-8, IL-17, IL-25, IL33, CXCL-1, CXCL-10, CCL-17 and CCL-22 decreased and it was also found that the Treg cell cytokine IL10 has increased significantly in acute and chronic stages. Interestingly, IL-22 increased significantly in FIP-fve groups in the chronic stage.

The difference in acute and chronic stages of lung inflammation and FIP-fve effect

The lung inflammation was evaluated through histological staining with H&E (Fig. 4). In the OVA-sensitized mice groups, airway inflammation was very serious, especially in the chronic stage. After the FIP-fve treatment, there was

Table 1 The difference in acute and chronic stage of OVA-specific antibodies in serum and FIP-fve affected. Data points represent the median $\pm$ IQR of the individual mouse groups.

samples	groups					
	Acute-OVA	Chronic-OVA	Acute-pre-FIP	Acute-post-FIP	Chronic-pre-FIP	Chronic-post-FIP
IgE (ng/ml)	387.25 $\pm$ 39.55	279.81 $\pm$ 47.22#	89.82 $\pm$ 34.11***	101.53 $\pm$ 47.42***	114.52 $\pm$ 19.85++	94.74 $\pm$ 28.33++
IgG1 (IU/ml)	337.92 $\pm$ 13.72	298.37 $\pm$ 7.24#	104.32 $\pm$ 6.63***	123.52 $\pm$ 8.21***	149.52 $\pm$ 11.63++	162.46 $\pm$ 25.21++
IgG2a (IU/ml)	37.55 $\pm$ 6.42	65.72 $\pm$ 13.59###	89.67 $\pm$ 15.79***	94.15 $\pm$ 5.34***	97.21 $\pm$ 25.47+	113.32 $\pm$ 24.63+

Serum was obtained from the acute-/chronic-OVA, acute-pre-/post-FIP or chronic-pre-/post-FIP groups. The statistical analyses compared acute-OVA or chronic-OVA group Represented as: * or + or # which p value $\leq$ 0.05, ** or ++ or ## which p value $\leq$ 0.01, *** or +++ or ### which p value $\leq$ 0.001.

* acute groups compared with acute-OVA group.

+ chronic groups compared with chronic-OVA group.

acute-OVA compared with chronic-OVA groups.

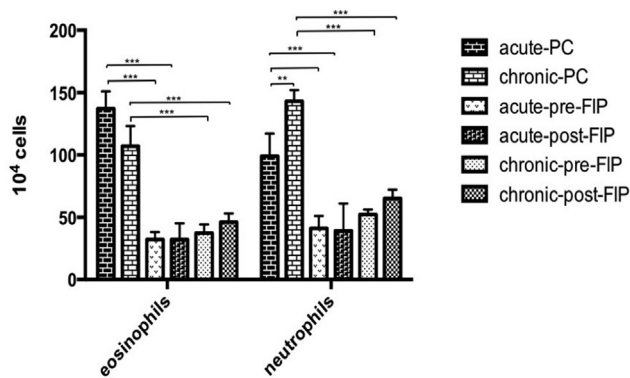


Figure 3. The difference in acute and chronic stage of infiltrating cells of the lungs and FIP-fve effect. All cell counts were obtained from the acute-OVA (acute-PC) group and chronic-OVA (chronic-PC) sensitized/challenged mice or mice treated with FIP-fve. The inflammatory cells were counted ($\times 10^4$) from the BALF in millimeters by morphometric evaluations of cytospin preparations. The statistical analysis compared OVA-treated mice and is represented as: ** $p < 0.05$; *** $p < 0.001$.

significantly less inflammation not only in the acute, but also in the chronic stage. Airway remodeling also improved.

Comparison of PCA in different groups

PCA of cytokines and chemokines expressed in BALF was also used to assess the major sources of variation among samples. Most of the samples were classified into five groups: normal control, acute-OVA, chronic-OVA, acute-FIP and chronic-FIP groups. The results indicated that the constituents of the samples from sensitization groups, treated FIP groups and controls were significantly different (Fig. 5).

Discussion

Previous studies have reported that asthma is a chronic airway hyperresponsive disease associated with complex etiology and unclear pathogenesis. Symptoms of bronchial asthma may manifest due to limited ventilation, airway constriction, airway remodeling, and other pathological changes. Multiple factors contribute to the pathogenesis of this disease, but recent research indicates it may exhibit a polygenetic inheritance. Consequently, research on the genetic susceptibility and gene regulatory factors of asthma has become prevalent in the research community.^{23–26}

The heterogeneity of immunological responses in asthma can be classified into 3 major types: eosinophilic, non-eosinophilic and mixed granulocytic inflammation. Eosinophilic asthma has two subtypes: allergic and non-allergic; while neutrophilic type 1 and type 17 and paucigranulocytic are the subtypes of non-eosinophilic asthma.^{8,9,27}

According to previous studies,^{8,9,23–27} the underlying pathophysiological mechanisms of asthma are diverse depending on the dose and duration of allergen stimulation, overlapping, and endotypes. Acute or chronic stages may also account for differences in the immune environment.

In this study, PCA analysis of cytokine and chemokine expression was also used to assess the major sources of variation among samples. Most of the samples were classified into five groups, indicating that the constituents of the samples from the normal control, acute-OVA group, chronic-OVA group, acute-FIP group and chronic-FIP group were significantly different (Fig. 5). According to PCA results, IL-13, IL-4, IL5, IL-8, IFN- γ , IL-25 and TGF- β were the important predictor cytokines in the acute and chronic asthma models. However, in laboratory results, IL-4 and IL-5 had higher expressions in the acute stage than in the chronic stage. Moreover, IFN- γ , TGF- β , TNF- α , IL-8, IL-10, IL-17, IL-25, IL-33, CXCL-1, CXCL-10, CCL-11, CCL17 and CCL22 were higher in the chronic stage than in the acute stage.

According to previous studies,^{8,9,27} the diagnostic biomarkers of type 2 inflammation are eosinophils, specific-IgE, and Th2 cytokines (IL-4, IL5 and IL-13); whereas the biomarkers of non-type 2 inflammation are neutrophils, IL-1, IL6, IL8, IL-17, IL-22, TNF- α and IFN- γ . However, asthma is a complex disease with mixed pathophysiology, thus, a phenotype-driven treatment is not always sufficient to obtain optimal control. Endotype classification based on thorough investigations of the underlying pathophysiological mechanisms is therefore gaining more attention. Endotyping will provide more insight into the inter-individual variability of clinical presentation and treatment response in patients with identical phenotypes. Therefore, this study provides more information on biomarkers in different stages of asthma.

In this study, the use of OVA at different times stimulated mice to induce asthma models at different periods to mimic human asthma such as acute asthma and chronic asthma. Nids et al.,²⁸ detected and compared only Th2 cytokines, AHR and inflammatory cells in the lung was in acute and chronic asthma mouse models. However, in order to find the difference between acute and chronic asthma, we screened many kinds of cytokines and chemokines, not limiting screening to just Th2 cytokines.

An overview of the results showed that the two mouse models of asthma were successful. Both the acute stage and the chronic stage presented symptoms of asthma, such as AHR, higher IgE in serum, higher Th2 cytokines, inflammatory cell infiltration in the lungs (especially eosinophils and neutrophils) and airway inflammation.

According to our results, there are notable differences in the acute and chronic stage of asthma. The first, AHR and airway inflammation in the chronic stage was more serious than in the acute stage. Second, the numbers of inflammatory cells in the BALF were also different, such as eosinophils and neutrophils. Moreover, we used cytokine and chemokine arrays to screen the differences. Then, we also found the expression of seventeen kinds of cytokines and chemokines in the BALF were different in the acute and chronic stages.

Previous studies have reported that high IL-8 and CXCL-8 expression occurs in severe or chronic asthma or COPD patients^{29,30} and those markers might be used to distinguish between asthma and uncontrolled asthma^{31–33}. Coincidentally, our acute and chronic animal models showed the same results. Moreover, in our study, not only these two markers, but TGF- β , TNF- α , IL-17, CCL-22, CXCL-1 and

Table 2 The difference in acute and chronic stage of cytokines and chemokines and FIP-fve affected in BALF. Data points represent the median $\pm$ IQR of the individual mouse groups.

Samples	Group						
	NC (n = 5)	Acute-OVA (n = 8)	Chronic-OVA (n = 8)	Acute-pre-FIP (n = 8)	Acute-post-FIP (n = 8)	Chronic-pre-FIP (n = 8)	Chronic-post-FIP (n = 8)
IFN-r (pg/ml)	72.31 $\pm$ 3.58	88.6 $\pm$ 3.9	241.8 $\pm$ 4.2###	497.6 $\pm$ 26.56***	511.2 $\pm$ 26.90***	334.0 $\pm$ 35.7+	359.8 $\pm$ 36.24+
TGF- β (pg/ml)	55.32 $\pm$ 14.86	159.4 $\pm$ 11.4	852.2 $\pm$ 22.44###	304.2 $\pm$ 24.4**	325.8 $\pm$ 25.16**	289.2 $\pm$ 24.4+++	360.2 $\pm$ 24.67+++
TNF- α (pg/ml)	72.53 $\pm$ 47.7	357.98 $\pm$ 21.45	696.75 $\pm$ 42.56##	142.56 $\pm$ 3.42**	162.64 $\pm$ 53.32**	267.42 $\pm$ 55.32+++	313.53 $\pm$ 62.42++
IL-4 (pg/ml)	43.01 $\pm$ 11.36	699.6 $\pm$ 63.22	802.4 $\pm$ 51.15#	287.8 $\pm$ 28.8***	406.8 $\pm$ 14.60**	255.6 $\pm$ 15.8+++	310.6 $\pm$ 52.25++
IL-5 (pg/ml)	57.86 $\pm$ 5.76	685.8 $\pm$ 51.13	788.2 $\pm$ 37.54#	312.8 $\pm$ 26.67***	331.4 $\pm$ 34.03**	295.0 $\pm$ 35.79+++	299.4 $\pm$ 10.48+++
IL-8 (pg/ml)	69.85 $\pm$ 7.88	101.21 $\pm$ 32.64	575.89 $\pm$ 46.57###	88.37 $\pm$ 14.33*	74.54 $\pm$ 26.32*	123.54 $\pm$ 23.6++	159.32 $\pm$ 34.72++
IL-10 (pg/ml)	92.34 $\pm$ 11.32	257.32 $\pm$ 54.23	346.53 $\pm$ 65.67#	567.11 $\pm$ 52.63**	432.32 $\pm$ 26.32**	696.24 $\pm$ 77.42++	500.31 $\pm$ 92.43+
IL-13 (pg/ml)	44.61 $\pm$ 27.93	873.2 $\pm$ 58.20	902.0 $\pm$ 58.67	405.8 $\pm$ 14.59***	416.8 $\pm$ 16.70**	486.5 $\pm$ 36.48++	474.0 $\pm$ 37.33++
IL-17 (pg/ml)	54.52 $\pm$ 3.79	174.37 $\pm$ 35.55	478.42 $\pm$ 52.63##	83.71 $\pm$ 12.02**	100.21 $\pm$ 7.84**	213.0 $\pm$ 42.63++	244.75 $\pm$ 31.67++
IL-22 (pg/ml)	47.22 $\pm$ 1.13	162.32 $\pm$ 72.73	243.64 $\pm$ 88.24#	178.53 $\pm$ 58.0.35	138.53 $\pm$ 94.88	494.82 $\pm$ 99.88++	472.35 $\pm$ 66.59++
IL-25 (pg/ml)	40.76 $\pm$ 9.57	105.37 $\pm$ 4.232	559.32 $\pm$ 64.52##	174.67 $\pm$ 52.34**	134.65 $\pm$ 25.32**	234.63 $\pm$ 1.13+++	208.64 $\pm$ 34.21++
IL-33 (pg/ml)	57.75 $\pm$ 27.8	278.43 $\pm$ 13.53	463.64 $\pm$ 78.53#	101.33 $\pm$ 29.81**	157.31 $\pm$ 47.32*	222.53 $\pm$ 33.64++	198.53 $\pm$ 72.53+++
CXCL-1 (pg/ml)	33.86 $\pm$ 2.65	243.57 $\pm$ 41.35	737.8 $\pm$ 59.65##	111.456 $\pm$ 64.24**	99.42 $\pm$ 35.3**	189.678 $\pm$ 7.32+++	202.36 $\pm$ 15.61+++
CXCL-10 (pg/ml)	29.64 $\pm$ 4.82	365.73 $\pm$ 25.32	1231.34 $\pm$ 152.42###	233.52 $\pm$ 12.42*	202.42 $\pm$ 42.42*	343.53 $\pm$ 25.53+++	456.32 $\pm$ 14.52+++
CCL11 (pg/ml)	62.64 $\pm$ 19.96	588.3 $\pm$ 73.97	1445.23 $\pm$ 85.32###	135.29 $\pm$ 1.22**	147.63 $\pm$ 25.23**	321.56 $\pm$ 24.63+++	377.64 $\pm$ 89.31+++
CCL-17 (pg/ml)	22.29 $\pm$ 11.34	288.46 $\pm$ 43.63	472.73 $\pm$ 56.32##	149.24 $\pm$ 53.77*	201.63 $\pm$ 45.11	227.43 $\pm$ 17.31++	199.32 $\pm$ 103.53++
CCL-22 (pg/ml)	37.75 $\pm$ 9.92	241.63 $\pm$ 36.11	505.24 $\pm$ 96.22##	99.62 $\pm$ 32.2**	104.53 $\pm$ 36.1*	214.63 $\pm$ 62.67++	295.53 $\pm$ 46.16++

Bronchoalveolar lavage fluid (BALF) was obtained from the acute-/chronic-OVA, acute-pre-/post-FIP or chronic-pre-/post-FIP groups. The statistical analyses compared acute-OVA or chronic-OVA group Represented as: * or + or # which p value $\leq$ 0.05, ** or ++ or ## which p value $\leq$ 0.01, *** or +++ or ### which p value $\leq$ 0.001. * acute groups compared with acute-OVA group. + chronic groups compared with chronic-OVA group. # acute-OVA compared with chronic-OVA groups.

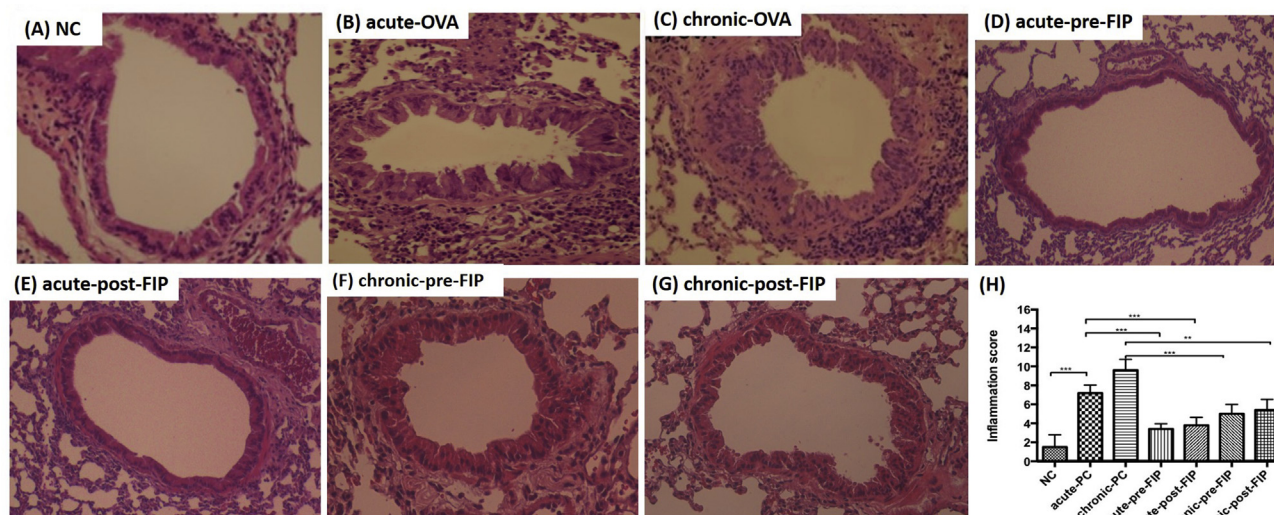


Figure 4. The difference in acute and chronic stage of lung inflammation and FIP-fve effect. Lung tissue samples were obtained on day 28 (acute) and 76 (chronic) to determine airway inflammation. Fig. 4(A) the NC group 4(B) the OVA sensitized/challenged mice in acute stage, 4(C) the OVA sensitized/challenged mice in chronic stage and the groups acute-pre- and post-treated with FIP-fve respectively 4(D) and 4(E), the groups chronic-pre- and post-treated with a FIP-fve respectively 4(F) and 4(G) which were stained with hematoxylin and eosin. The image analysis was performed with Image-plus pro software and the statistical analysis compared OVA-treated mice 4(H) and is represented as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CXCL-10 also had different expressions during the acute and chronic stages.

Chemokine are a large family of small secreted protein that regulation migration of white blood cells to effect allergic disease, such as CXCL-1 induced neutrophil chemotaxis,³⁴ CXCL-10 induced T cell and monocyte chemotaxis,³⁵ CCL-11 selectively recruit eosinophil,³⁶ CCL-17 and CCL-22 developed and main allergic disease.³⁷ Recent studies have implicated that many mediators including pro-inflammatory cytokines IL-1, IL-6, IL-17, and TNF- α and various chemokines such as CXCL-8, CXCL-10, CCL-2, CCL-4, CCL-11, and others may also participate in the pathogenesis of asthma by acting directly or indirectly on the target cells.^{38–40}

Studies have shown TNF- α mRNA-positive cells were significantly elevated in the BALF of asthmatics compared with controls⁴¹ and TNF- α might contribute to local recruitment of neutrophils and eosinophils, promoting the activation of T-cells, inducing chronic airway remodeling and mediating steroid resistance.^{42,43}

The chemokine CXCL-10 and its receptor, CXCR-3, have been implicated in the pathogenesis of chronic obstructive pulmonary disease.⁴⁴ Hardaker et al., obtained lung tissue from patients diagnosed with COPD. ELISA assays were performed on whole lung homogenates and it was discovered that CXCL-10 levels were elevated eightfold in the COPD patients compared with unaffected subjects.⁴⁴ Our result was similar in our chronic stage model in which CXCL-10 was also higher than in the acute stage.

There are many biomarkers related to asthma and identifying them in a timely manner can aid in asthma diagnosis or enable better prognostic predictions for emergency treatment of severe acute asthma attacks and to facilitate improved outcomes.

FIP-fve, another focus in this study, can improve not only acute stage, but also chronic stage of OVA-sensitization airway inflammation and other symptoms as demonstrated in our previous studies.^{45,46} Moreover, FIP-fve can also decrease cytokines and chemokines expression in the BALF, such as TNF- α , TGF- β , IL-8, IL-17, CXCL-10, CCL-11 and CCL-22. Our previous studies revealed that FIP-fve can regulate Th1/Th2 immune system balance and improve airway inflammation in the acute stage.⁴⁵ FIP-fve can also affect IL-17 and IL-22 to improve airway remodeling in the chronic stage sensitized with OVA.⁴⁶

A prior study⁴⁷ reported that IL-22 is required for the sensitization phase of allergic inflammation and IL-22 acts to strengthen epithelial barrier functions and is involved in tissue homeostasis as well as in tissue repair and wound healing. Moreover, IL-22 inhibits IFN- γ -induced expression of proinflammatory chemokines and adhesion molecules in human bronchial epithelial cells and the levels of IL-22 in the BALF of asthma patients are inversely correlated with the levels of proinflammatory chemokines (such as CXCL-10), suggesting protective roles of IL-22 in asthma patients.^{48,49} We found the same results that increased levels of IL-22 can improve airway inflammation. However, some studies^{48–50} have also reported that increased IL-22 levels in asthma patients are positively correlated with disease severity. IL-22R1 is expressed in airway smooth muscle cells and IL-22 enhances their proliferation and migration, suggesting that IL-22 may be involved in smooth muscle cell hyperplasia. Therefore, IL-22 seems to have a dual role in allergic airway inflammation and airway remodeling. The mechanisms by which IL-22 regulates allergic airway inflammation remain largely unknown and further clarification is required.

TGF- β plays a central role in wound healing, in tissue repair and is also one of the main mediators involved in

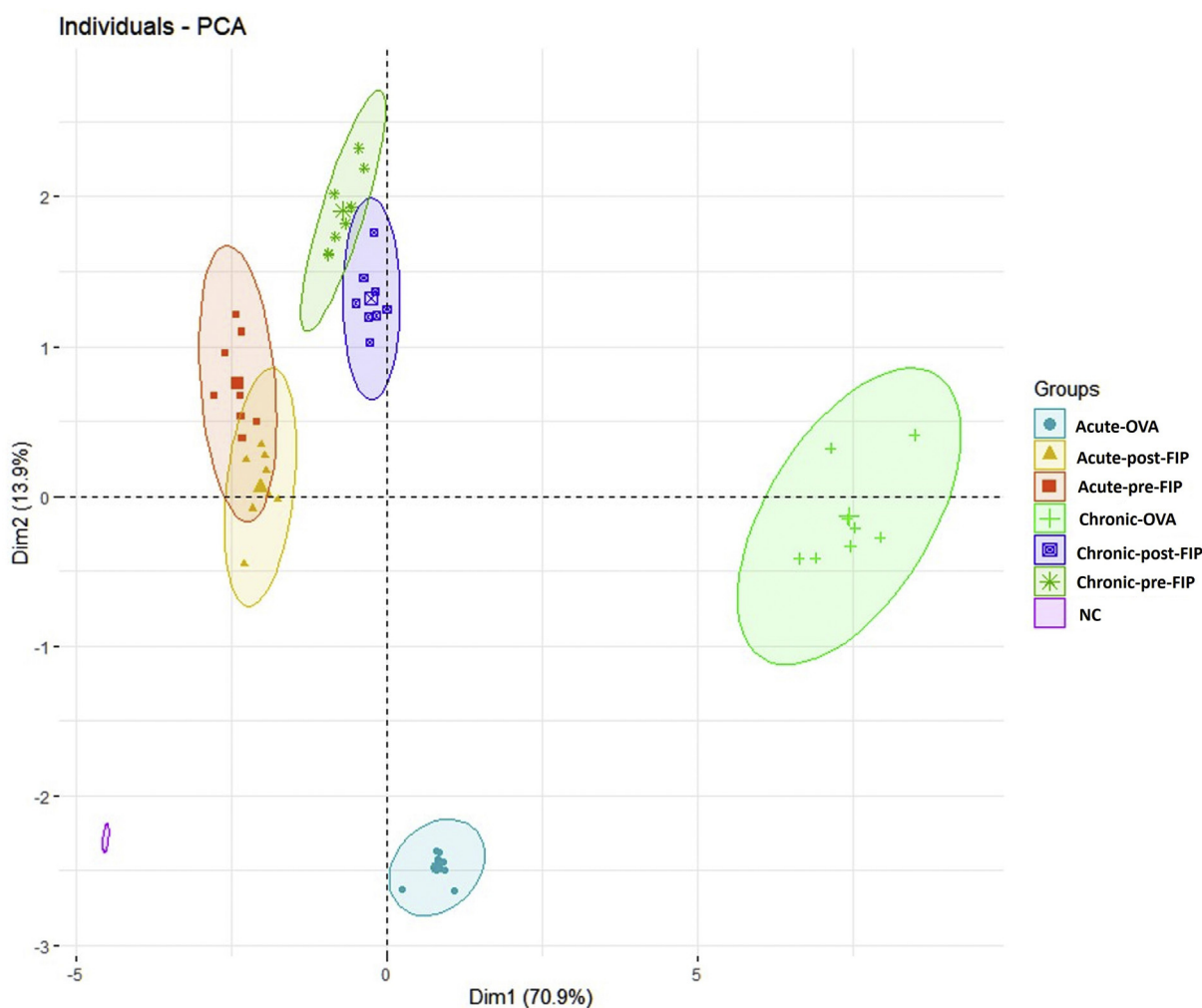


Figure 5. The PCA (Principal Component Analysis) in acute and chronic stage. BALF data from normal controls, OVA sensitization groups and OVA sensitization with FIP-fve fed groups. Heat map of expression of selected cytokines and chemokines in BALF of normal controls, OVA sensitization groups and OVA sensitization with FIP-fve fed groups. The markers are shown as: NC: pink; acute-OVA: blue; chronic-OVA: light green; acute-pre-FIP: red; acute-post-FIP: yellow; chronic-pre-FIP: purple; chronic-post-FIP: green.

tissue remodeling in the asthmatic lung. It is produced by a number of cells, including macrophages, epithelial cells, fibroblasts, and eosinophils.¹⁷ Many studies have reported that during exacerbation of asthma, all kinds of structural cells, as well as inflammatory cells are recruited to the airway and are able to express TGF- β .^{51–54} Although high expression of TGF- β is apparent in asthmatic patients, it still remains controversial as to whether the concentration of TGF- β is correlated with disease severity. The TGF- β complex functions as a molecular sensor which responds to environmental perturbations by releasing an active TGF- β ligand, to promote or inhibit cell proliferation in a context-dependent manner. In fact, the overexpression of TGF- β in T cells resulted in the suppression of allergic asthma in a murine model of asthma.⁵⁵ Moreover, accumulating evidence supports the important role of TGF- β in promoting the suppressive function of regulatory T cells and in the inhibition of effector Th1 and Th2 cells.⁵⁶ However, TGF- β is one of the main mediators involved in tissue remodeling, especially in chronic inflammation;

incremental secretion of TGF- β worsens the phenomenon of airway remodeling.

In previous studies⁵⁷ of Th22 cytokines in asthma, IL-22 secreted from an emerging group of Th22 immune cells had certain effects on chronic asthma and inflammatory diseases. When Th22 cells do not function, chronic and allergic inflammatory diseases are worsened by excessive inflammation. Based on the findings, Th22 cells and their secreted cytokines IL-22 and TGF- β are stimulated by immune cells to fix damage resulting from the inflammation. However, when inflammation becomes chronic, Th22 cells, IL-22 and TGF- β cytokines continue to be secreted abnormally.

In our study, TGF- β had a small increase in the acute stage but was highest in the chronic stage in the OVA sensitization mice which might be due to the auto-modulation of tissue repair and remodeling. However, FIP-fve modulated the immune system as TGF- β increased significantly in the acute stage, which might promote the suppressive function of Treg cells, inhibit effector Th1 and

Th2 cells,⁵⁸ and repair tissue in the lung. Moreover, in the chronic stage, there was airway remodeling caused by many inflammatory cells and tissue cells secreting TGF- β . The FIP-fve groups could not only enhance Treg cells but also significantly reduce TGF- β secretion, and there might be other processes to improve airway remodeling. However, IL-10 cytokines increased not only in acute, but also chronic stages, in the FIP-fve groups. Therefore, FIP-fve in the acute stage, could increase TGF- β to repair airways. In chronic stage, FIP-fve could down-regulate many kinds of inflammatory cells and decrease TGF- β to improve airway remodeling.

Some studies have reported that IL-25 and IL-33 are the epidermal derived cytokines that up-regulate in the bronchial epithelium of asthma patients when exposed to allergens.^{59–61} In our study, IL-25 and IL-33 had the highest expression in the chronic stage compared to the acute stage in the OVA sensitization group.

A previous study⁶² has shown that IL-25 not only promotes Th2-type immune responses, but also inhibits immune responses mediated by Th1/Th17. However, IL-25 knockout mice are highly susceptible to autoimmune encephalomyelitis and rapid deterioration.⁶³ In our study, IL-25 rose in the oral FIP-fve groups in the acute stages which may be due to high expression of Th1. Furthermore, IL-25 was over-expressed in the chronic OVA sensitization group, but in the oral FIP-fve groups, IL-25 and IL-33 were lower than the chronic OVA sensitization group. The pathology results in our study showed that FIP-fve could also prevent the destruction of the bronchial epithelium. Therefore, we believe that FIP-fve might also regulate IL-25 and IL-33 to protect the epithelium and decrease damage at the same time in the chronic stage.

Moreover, we used PCA to analyze the cytokines and chemokines. However, according to PCA results in the FIP-fve groups, IL-10, IL-22 and IFN- γ were the important predictor cytokines in FIP-fve fed groups (acute or chronic). We believe that FIP-fve might modulate the Th1, Treg and Th22 immune environment to improve airway inflammation and remodel in different stages. From laboratory results, we also believe that FIP-fve could down-regulate IL-17, IL-25 and IL-33 to protect the epithelium and reduce damage.

FIP-fve could improve asthma through immune regulation (such as Th1/Th2/Treg and Th17) and preserve epithelial function both in acute and chronic stages. In acute stage, FIP-fve could significantly regulate Th1 and Th2 cytokines. Moreover, FIP-fve could also regulate Treg cells (TGF- β) to increase tissue repair.

FIP-fve could reduce eosinophil infiltration to improve the airway by regulating cytokines. Moreover, according to our previous study, epithelial cell damage may be caused by chronic allergen sensitization and IL-8, IL-25 and IL-33 increase significantly in the chronic stage.⁶⁴ According to our study, we believe that FIP-fve could also regulate Th17, Treg cytokines and protect/repair epithelial cells to improve airway inflammation and remodeling. Therefore, FIP-fve could also reduce neutrophils in chronic airway inflammation.

In conclusion, there are many different immune responses in the different stages of asthma. Drug treatment at the appropriate time, might prevent worsening asthma symptoms. Moreover, the change of cytokine

concentrations at different time points requires further study. However, FIP-fve could improve asthma through immune regulation not only in the acute, but also in the chronic stage. Therefore, FIP-fve might be an alternative therapy that could be used in the treatment of allergic airway diseases such as asthma.

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