Original Article

Spleen aminopeptides (FUKETUO) elevate the therapeutic effect of house dust mite desensitization on allergic asthma by inducing interleukin-10 positive regulatory T cells (IL-10$^+$ Tregs) expression

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Background: As a novel immunomodulator, spleen aminopeptides (FUKETUO) can correct the imbalance of immune cells and elevate their functions. Spleen aminopeptides have been used in the treatment of respiratory diseases. However, the regulatory mechanism of it on allergic asthma and desensitization has not been reported, further study is critically needed. This study aimed to investigate the effect and mechanism of spleen aminopeptides on allergic asthma and desensitization. We established an allergic asthma model by house dust mite (HDM) with/without desensitization treatment.

Methods: The allergic asthma mouse model was established with HDM and treated with desensitization and increasing dose of spleen aminopeptides according to different immune phases. Pathological markers such as airway hyper-responsiveness, and cell composition were monitored to determine the effectiveness of treatment.

Results: Spleen aminopeptides can promote the proportion of interleukin-10 positive (IL10$^+$) allergen-specific regulatory T cells (Tregs), and further promote interleukin-10 (IL-10) expression in desensitization. They alleviated the allergic symptoms and elevated desensitization, decreased airway hyper-reaction and lung tissue injury, reduced specific immunoglobulin E (IgE) in serum, eosinophil number and interleukin-4 (IL-4) expression in bronchoalveolar lavage fluid (BALF), therefore, being able to control allergic asthma.

Conclusions: Our results suggested that spleen aminopeptides (FUKETUO) could elevate the expression

of ($CD4^+CD25^+IL10^+$) Tregs, especially when it co-immunized with desensitization. Thereby, FUKETUO improved the efficacy of desensitization, and inhibited the development of allergic asthma.

Keywords: Spleen aminopeptides; allergic asthma; allergic enteritis; interleukin-10 (IL-10); eosinophil

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Introduction

Asthma is a heterogeneous disease characterized by chronic inflammation of the respiratory tract, which is influenced by the interaction between genetic and environmental factors (1). Clinically, asthma can be primarily divided into allergic asthma and non-allergic asthma (2,3). Allergic asthma is caused by an allergic reaction which is a chronic inflammatory disease mediated by immunoglobulin E (IgE) and is characterized by pulmonary eosinophil infiltration and airway hyperresponsiveness (AHR). It is the most common chronic disease in children, with the morbidity rate of 5% to 20% (4). Inhalation allergens [allergens of house dust mites (HDMs), animal dander, fungi and pollen] are the main causes of allergic asthma (2); HDMs are the most common allergens in allergic asthma. Despite the regional differences, 50% to 85% of asthmatic patients are sensitive to HDM (5,6). Therefore, how to effectively control allergic asthma has become a major challenge for public health, clinical research and global economy.

$CD4^+$ T cells play a central role in the immune system (7). Naive $CD4^+$ T (Th0) cells can differentiate into T helper cell 1 (Th1) and T helper cell 2 (Th2). Specifically, Th1 cells are involved in delayed-type hypersensitivity and regulate phagocyte-mediated host defence primarily through interferon-γ (IFN-γ) expression (8,9). On the contrary, Th2 cells are the main triggering cells that participate in the pathology of allergic disease and regulate allergic reactions by producing interleukin-4 (IL-4), IL-5, IL-13 and other cytokines (10-12). The T cell receptor on the surface of naive T cells can specifically recognize the allergen fragment complex and then differentiate into Th2 cells, which produce and release IL-4 or IL-13 and interact with the receptor on B cells. IL-4 or IL-13 causes class switching of immunoglobulin (Ig) from IgM to IgE and then these immunoglobulins are secreted by B cells (13,14). In addition, mast cells and basophils have high-affinity IgE receptors (FcεRI) on the surface. When the body contacts allergens again, allergens combine with IgE and FcεRI to form complexes, leading to the degranulation of mast cells and basophils. During this stage, many soluble mediators, such as histamine, cytokines, chemokines and leukotrienes are released (15), rapidly generating and aggravating the symptoms of allergic reaction.

The development of allergic reactions may also be linked to the reduction or impaired function of allergen-specific regulatory T cells (Tregs) in genetically susceptible individuals, which promotes allergic airway inflammation in asthma (16). Tregs mainly express Foxp3 (except in Tr1 cells) and secret anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) (17,18).

Highlight box

Key findings

- We demonstrated that FUKETUO can participate in desensitization therapy and enhance the effect of the treatment. Additionally, we elucidated the mechanism by which FUKETUO operates, showing that it induces interleukin-10 positive regulatory T cells (IL-10$^+$ Tregs), which accelerate the balance between T helper cell 1 (Th1) and T helper cell 2 (Th2) responses, thereby improving the outcomes of desensitization treatment.

What is known and what is new?

- The spleen aminopeptides can correct children's dysfunctional immune function by regulating the levels of various T cell subsets and also enhance antiviral immunity by inhibiting the secretion of IL-4 by Th2 cells to treat diseases.
- We further showed that the FUKETUO may induce IL-10$^+$ Tregs which accelerate the balance of Th1/Th2 to enhance the treatment of desensitization. This effect may lead to a reduction in airway hyper-reactivity and lung tissue damage, as well as a decrease in serum levels of specific immunoglobulin E, eosinophil count, and inflammatory cytokine expression.

What is the implication, and what should change now?

- The spleen aminopeptide may serve as a novel immunomodulator in the future, in combination with allergen vaccines or other anti-allergic drugs to treat allergic diseases such as allergic asthma. The use of spleen aminopeptide also provides a new approach to clinical treatment of allergic diseases.

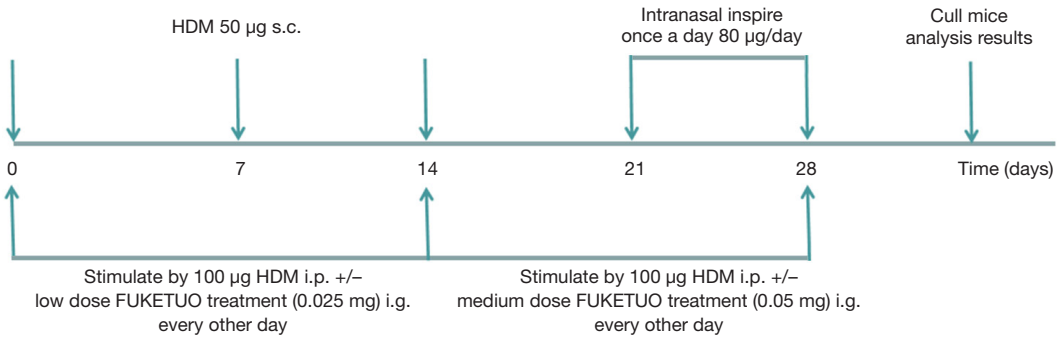

Figure 1 Experimental procedure of spleen aminopeptides in the treatment of dust mite allergic asthma mice. Mice are separated by their treatment: normal saline control group (NC group), asthma model group, desensitization group, spleen aminopeptides group alone, and desensitization and spleen aminopeptides combined group. Six mice per group, 30 mice in total. The whole experiments repeated three times. HDM, house dust mite; i.p., intraperitoneal injections; FUKETUO, a trade name of spleen aminopeptides; i.g., intragastric injection; s.c., subcutaneous injections; NC, negative control.

In recent years, Tregs have been extensively studied in relation to the pathogenesis of allergic diseases. They play a key role in the treatment of allergies by inhibiting Th2 cells and the activation of mast cells and basophils (19).

At present, patients with allergic asthma typically use antihistamine therapy, glucocorticoid therapy and bronchodilator in their clinical treatment. However, the therapeutic effects of these therapies are still not ideal, it is hard to achieve the goal of complete control, and the symptoms are likely to relapse once the drug is discontinued. It is worth mentioning that glucocorticoids are widely used in the clinical treatment of asthma, which reduces host defence in asthma patients and increases the risk of infection, thus aggravating the difficulty of asthma treatment (20). The occurrence of allergic asthma is related to an imbalance of autoimmune regulation (3). Spleen aminopeptides (FUKETUO) are novel oral bidirectional immunomodulators consisting of peptides and nucleotides extracted from fresh pig spleens (Figure S1). The main functions of spleen aminopeptides are correcting the imbalance of immune cells and improving the function of immune system (21). Previous studies support the effectiveness of spleen aminopeptides in the treatment of infectious pneumonia, bronchial asthma and other respiratory diseases and inflammatory diseases (22,23). Studies also show that the spleen aminopeptides can correct children's dysfunctional immune function by regulating the levels of various T cell subsets. In addition, the spleen aminopeptides can also enhance antiviral immunity by inhibiting the secretion of IL-4 by Th2 cells, thus alleviating the inhibition of IL-4 on lymphocyte and macrophage phagocytosis (24,25). However, the regulation mechanism of spleen aminopeptides on allergic asthma has not been reported so far. Further studies are necessary to determine its therapeutic effect, to provide more evidence and find a new direction for the treatment of allergic diseases. We present this article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-24-398/rc).

Methods

Asthma mouse model

Healthy female BALB/C mice, aged 4 to 6 weeks, were purchased from Guangdong Provincial Animal Centers and kept in specific-pathogen free (SPF) animal centers, with access to food and water *ad libitum*. The asthma model was constructed with HDM and treated with desensitization and spleen aminopeptides (FUKETUO, Zhejiang Feng'an Bio-Pharmaceutical Co., Ltd., Taizhou, China). This model was established according to the existing experimental model (*Figure 1*) (26). The animal experiments were approved by the Animal Care and Use Committees of Shenzhen University, China (No. A20230063), granted by institutional ethics committee of Shenzhen University, in compliance with Regulations on the Administration of Laboratory Animals in China 1999 national guidelines for the care and use of animals. The experimental protocol was prepared before the experiment according to the existing protocol on *Bio-protocol Journal*: https://bio-protocol.org/cn (27).

AHR measurement

The mice were tested for AHR 24 hours after the last stimulation. Mice in each group were placed in a small animal test container and then inhaled a gradient concentration of acetylcholine solution (0, 3.125, 6.25, 12.5, 25, 50 mg/mL) by the way of atomization excitation. The Penh values (expiratory interval, enhanced expiratory pause) were recorded. A change curve of the ratio of Penh values to the control was plotted to reflect the lung function levels of the mice.

Detection of serum specific IgE, IgG1 and IgG2a levels

Mice were placed in a CO_2 chamber and culled following ethical procedures. Blood was collected, centrifuged and the supernatant was isolated for further analysis. HDM was coated as antigen, and the serum-specific IgE, IgG1, and IgG2a levels were detected by enzyme-linked immunosorbent assay (ELISA). Secondary antibodies (goat anti-mouse IGE-hrp, goat anti-mouse IGG1-hrp and goat anti-mouse IGG2A-HRP, BD, CA, USA) were used for detection. After incubation, absorbance was measured at 450 nm on a microplate reader (Multskan Go, Thermo Scientific, Waltham, USA), readings for each group were recorded and analyzed.

ELISA

Mice IL-4, IL-10 and IFN-γ in serum, bronchoalveolar lavage fluid (BALF) and splenocyte supernatants were quantified using DuoSet ELISA Development Kits (R&D systems, Minnoeapolis, USA) according to the manufacturer's instructions. The mouse IL-4 ELISA kit has a detection range of 20–1,000 pg/mL, while the mouse IL-10 and IFN-γ kits have a detection range of 10–2,000 pg/mL.

Mice BALF cell measurement

After sacrificing the mice, lung BALF was collected by washing with phosphate buffered saline (PBS). A similar volume of precipitated cells was resuspended and further separated by cytocentrifuge (Cytopro, ELITechGroup, USA). After centrifugation, cells were fixed on the glass slide for Wright-Giemsa staining (Solarbio, Beijing, China). The numbers of different types of immune cells (eosinophils, neutrophils, lymphocytes and mononuclear cells) were counted using a microscope.

Preparation and culture of mouse splenocyte suspension

After the mice were sacrificed, the spleen was removed and grounded into a single-cell suspension. The supernatant was removed by centrifugation, and red blood cell lysate was added. The cells were resuspended and washed with sterile PBS. The cells were resuspended in complete Roswell Park Memorial Institute (RPMI) medium, and the cell concentration was adjusted to 1×10^6/mL in 24-well cell culture plates.

- Flow cytometry: the splenocytes were stimulated by adding 50 ng phorbol 12-myristate 13-acetate (PMA), 1 μg ionomycin and 40 μg HDM per mL for 12 h. On the second day, 1 μL of brefeldin A (BFA) was added to each well and incubate for 6 h. The cell suspension was collected in a flow tube for flow analysis.
- ELISA detection of splenocyte supernatant: add 40 μg HDM per mL of 1640 complete culture medium to prepare culture medium for resuspended splenic cells (without adding blank wells), incubate for 48 h. Collect cell suspension, centrifuge at 1,200 rpm for 5 min. The supernatant was used for ELISA.

Flow cytometry

After sacrificing the mice, lung BALF or splenocytes were collected by washing with PBS. The precipitated cells were resuspended after centrifugation, and the types of cells and their number in BALF were determined by flow cytometry (Cytoflex, Beckman Coulter, Miami, USA): these cells were cultured with an Fc receptor blocker and then stained with fluorescent-dye conjugated antibodies fluorescein isothiocyanate (FITC) anti-mouse CD4 and APC anti-mouse CD25 (BD), along with their appropriate isotype-matched antibody controls. Gating of cell types is shown in Figure S2. The detection of intracellular IL-4, IFN-γ, IL-10, TGF-β, and Foxp3 expression in these cells was performed with antibodies [APC anti-mouse IL-4, PE anti-mouse IFN-γ, PE anti-mouse IL-10 and PerCP/Cy5.5 anti-mouse LAP (TGF-β1) and PE anti-mouse Foxp3, or isotype control] according to the manufacturer's instructions (BD Bioscience). All samples were pre-incubated with mice IgG (Sigma, St. Louis, USA) for 30 min to block FcγR. The cells were analyzed by flow cytometry (Cytoflex, Beckman Coulter). All data analysed by the software Flowjo.

Hematoxylin-eosin (H&E) and periodic acid-schiff (PAS) staining

H&E staining: after the mice were sacrificed, the middle lobe of the right lung was isolated and soaked in 4% paraformaldehyde for 24 h. The lung tissue was taken out and embedded in paraffin for fixation. After complete coagulation, the lung tissue was sectioned and stained with H&E after dewaxing. Slices were covered and sealed with neutral gum, the morphology of tissue was observed using microscope.

PAS staining: Schiff's reagent and hematoxylin were used for staining. Slices were dry at room temperature and sealed with neutral gum. Tissue morphology was observed by microscope.

Western blot

After the mice were sacrificed, the left lung was removed, and 100 mg of lung tissue was added to radio immunoprecipitation assay (RIPA) lysate to obtain a lung tissue homogenate. The supernatant was collected after centrifugation. The protein concentration was determined using the bicinchoninic acid (BCA) protein assay. After denaturing the protein, Western blot was used to detect the expression of IL-4 and IL-10 in lung tissue protein. Enhanced chemiluminescence (ECL) luminance solution was used and exposed in a chemiluminescence imager and photographed for preservation. The images were analyzed in grayscale by ImageJ, β-actin was used as the internal reference.

Statistical analysis

The experimental data were statistically analyzed by GraphPad Prism 7 software, and the values were expressed as mean ± standard error of the mean (SEM). The *t*-test was used to analyze the significance between two samples and non-parametric Kruskal-Wallis test for group comparison; the P value was used to reflect the significant difference between the data. ****, P<0.0001; ***, P<0.001; **, P<0.01; *, P<0.05; ns means no significant difference between groups.

Results

Spleen aminopeptides combined with desensitization therapy can significantly alleviate symptoms in the allergic asthma mice

To analyze the effect of these treatments, several disease factors that can directly reflect asthma severity were measured after treatments. The following were assessed: mice AHR, BALF cell number and subtype count; lung tissue staining and antibodies level measurements. These analyses were conducted to explore the function of spleen aminopeptides (FUKETUO) in allergic asthma.

AHR of mice was tested 24 hours after the last challenge. The mice inhaled a gradient concentration of acetylcholine solution, from low to high, using atomization excitation. The Penh value was observed and recorded at each concentration to evaluate the severity of allergic asthma. Compared with the normal group, the AHR of the dust mite protein sensitized asthma model group increased with the increasing concentration of methacholine, which was significantly higher than that of the control group; compared with the asthma model group, the AHR of mice was reduced after the desensitization treatment with HDM and desensitization combined with spleen amino peptide. Moreover, compare with the asthma model group, the AHR of the spleen aminopeptides alone group also shown a slightly decrease. Results indicating that the desensitization treatment of HDM combined with spleen aminopeptides can effectively inhibit the AHR of mice with asthma (*Figure 2A*).

The levels of HDM-specific IgE, IgG1, and IgG2a in mouse serum were detected by ELISA. As shown in *Figure 2B*, serum HDM specific IgE and IgG1 expressions in asthma model group were significantly higher than those in the normal group (P<0.0001). Moreover, after three immunotherapy treatments, HDM-specific IgE levels were lower than that in asthma group (P<0.01), the spleen aminopeptides combined with desensitization group has the most significant decrease in IgE levels (P<0.001). There was no significant difference in HDM-specific IgG1 expression among all the treatment groups and the asthma model group. In addition, serum IgG2a levels were significantly increased after three treatments, and the desensitization treatment was the most significant increase group (P<0.0001). These results suggest that the spleen aminopeptides may have the effect of inhibiting specific IgE, which is an efficient antibody causes allergic reaction. Furthermore, the spleen aminopeptides also promote the production of IgG2a, which works as a blocking antibody to allergic reaction. The therapeutic effect of spleen aminopeptides is more obvious when it is combined with dust mite desensitization treatment.

To observe the inflammatory condition in mouse lung tissues, lung tissues of test mice were stained with H&E and PAS, as shown in *Figure 2C,2D*. There was obvious

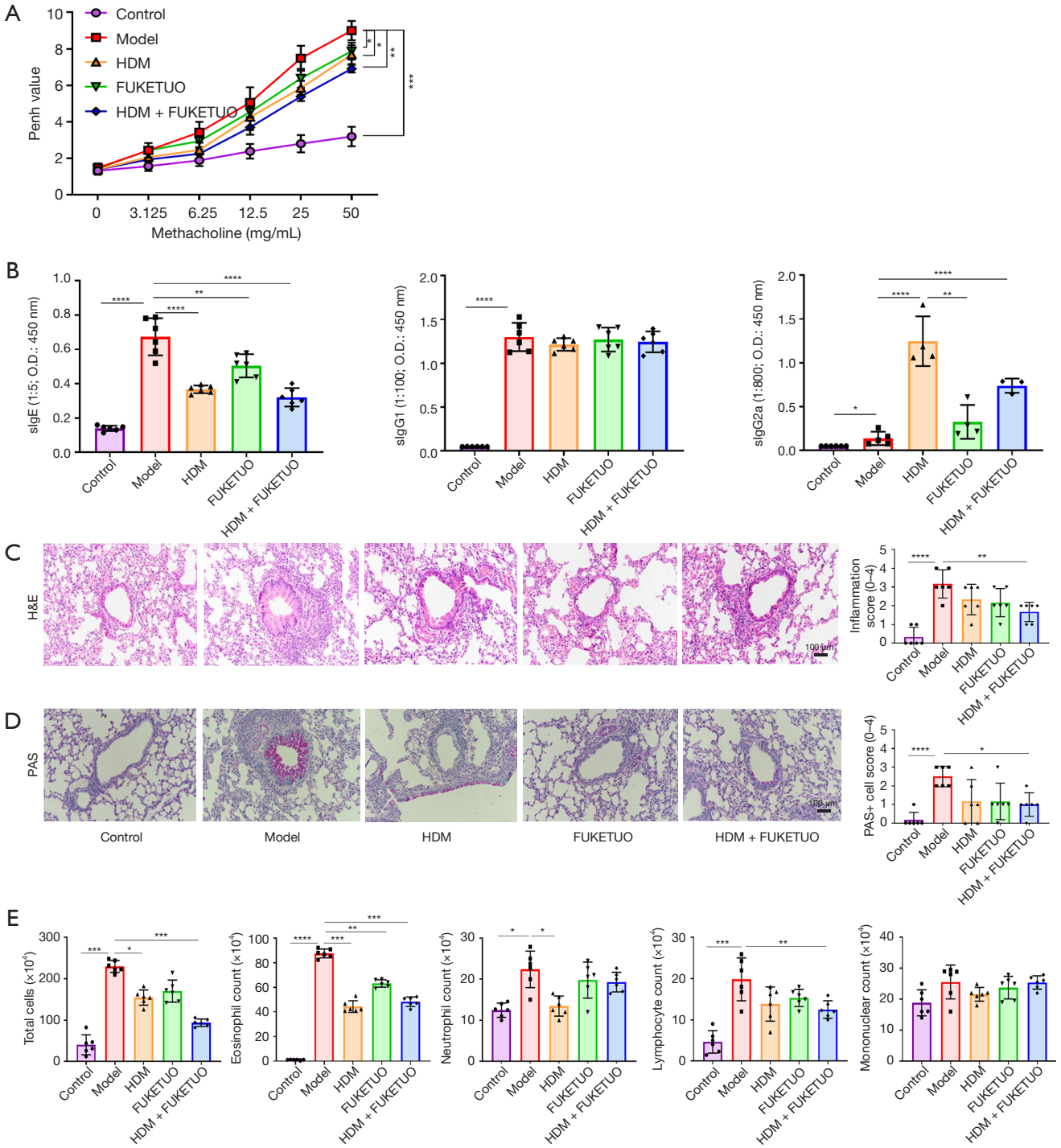

Figure 2 Spleen aminopeptides combined with desensitization therapy in allergic mice model. Several disease factors were measured to check the function of spleen aminopeptides in allergic asthma mice model. (A) The AHR measurement. (B) The expression of HDM specific IgE, IgG1 and IgG2a in mouse serum detected by ELISA. (C) H&E and (D) PAS staining of mouse lung tissues. Pictures were displayed in ×100. (E) The number of cells in mouse BALFs (×10⁴). Data are representative of three independent experiments (n=6). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. HDM, house dust mite; FUKETUO, a trade name of spleen aminopeptides; sIgE, specific immunoglobulin E; OD, optical density; H&E, hematoxylin-eosin; PAS, periodic acid-schiff; AHR, airway hyperresponsiveness; ELISA, enzyme-linked immunosorbent assay; BALF, bronchoalveolar lavage fluid.

bronchial inflammatory cells infiltration, alveolar structure damage, thickening of the alveolar septa and vascular walls, marked mucus hypersecretion and goblet cell proliferation in asthma group. All symptoms were alleviated in the desensitization group, spleen aminopeptides group and desensitization combined with the spleen aminopeptides group compared with the asthma group. The results of H&E and PAS showed that spleen aminopeptides could reduce the lung inflammation of allergic asthma.

A typical feature of allergic asthma is the recruitment and infiltration of lung eosinophils and lymphocytes, and a large number of eosinophils gathering in BALFs. Wright-Giemsa staining was used to identify the cell types in BALFs from the allergic asthma mouse model (Figure S2), with results shown in *Figure 2E*. Compared to the control group, the total number of cells in asthma model group increased ($P<0.01$), especially eosinophils ($P<0.0001$). Further compared to the asthma model group, the total number of inflammatory cells in BALF was significantly decreased after spleen aminopeptides treatment and after spleen aminopeptides combined desensitization treatment, especially the eosinophils ($P<0.001$). However, dust mite desensitization treatment had no significant inhibitory effect on inflammatory cell differentiation in BALF.

Spleen aminopeptides combined with desensitization therapy reduce inflammation in the lung of allergic asthma mice

The protein levels of IL-4 and IL-10 in mouse lung tissue were detected by Western blot, results are shown in *Figure 3A*. The level of IL-4 in the asthma group was significantly higher than in the control group ($P<0.0001$). Due to negative feedback regulation, the IL-10 protein level was also elevated. Compared to the asthma group, IL-4 levels in HDM desensitization treatment group ($P<0.001$), spleen aminopeptides group ($P<0.001$) and desensitization combined with spleen aminopeptides group ($P<0.0001$) were decreased, especially in the combined group. Moreover, the level of IL-10 in the desensitization treatment group was slightly lower than that in the asthma model group, while the levels of IL-10 in spleen aminopeptides group and combined group was significantly higher ($P<0.01$).

Furthermore, the levels of IL-4, IFN-γ, and IL-10 level in the BALFs were detected by ELISA (*Figure 3B*). Similar to the Western blot result, IL-4 levels in the asthma group significantly increased compared to the control group ($P<0.001$). Additionally, IFN-γ ($P<0.0001$) and IL-10 ($P<0.01$) also increased due to negative feedback regulation. However, IL-4 levels in desensitization group decreased significantly ($P<0.001$), and IFN-γ levels also decreased ($P<0.05$), while IL-10 increased ($P<0.01$). In the spleen aminopeptides group IL-4 was also decreased ($P<0.01$), the expression of IL-10 was higher than asthma group ($P<0.01$). In desensitization combined with the spleen aminopeptides group, IL-4 levels decreased ($P<0.01$), the IFN-γ levels decreased ($P<0.05$), and IL-10 increased significantly ($P<0.001$). We also provide the Facs data for BALF, but the cell number is limited (*Figure 3C*). These results indicate that spleen aminopeptides can promote the secretion of IL-10 to inhibit allergic inflammation, especially in combination with HDM desensitization treatment, which can promote the secretion of IL-10 better, thereby inhibiting allergic Th2 inflammatory reaction for the long term.

Spleen aminopeptides combined with desensitization therapy reduce inflammation in the spleen of allergic asthma mice

IL-4 and IFN-γ levels in mouse spleen cells were detected by flow cytometry, and the results are shown in *Figure 4A*. IL-4 in the asthma group increased significantly ($P<0.0001$) compared with the control group, and IFN-γ also increased (due to the negative feedback regulation). The levels of IL-4 and IFN-γ in the desensitization group were decreased compare with the asthma group ($P<0.001$). Moreover, the IL-4 and IFN-γ levels in the spleen aminopeptides group were also decreased ($P<0.05$). In the group with desensitization combined with spleen aminopeptides, IL-4 and INF-γ levels were much lower than in the asthma group ($P<0.01$). These results suggest that spleen aminopeptides can reduce Th2 type cytokine (IL-4) levels and down-regulate the negative feedback regulation of IFN-γ.

The IL-4, IFN-γ, and IL-10 protein levels in the spleen were further detected by ELISA, with results shown in *Figure 4B*. IL-4 in asthma group increased significantly ($P<0.0001$), while IFN-γ ($P<0.0001$) and IL-10 ($P<0.01$) also increased compared with the control group. Compared with the asthma group, IL-4 in desensitization group decreased significantly ($P<0.001$), and IFN-γ also decreased ($P<0.01$), while IL-10 increased ($P<0.01$); the levels of IL-4 and IFN-γ in the spleen aminopeptides group and the desensitization combined with spleen aminopeptides group were decreased compared with the control group, but the IL-10 level was significantly increased ($P<0.001$). These

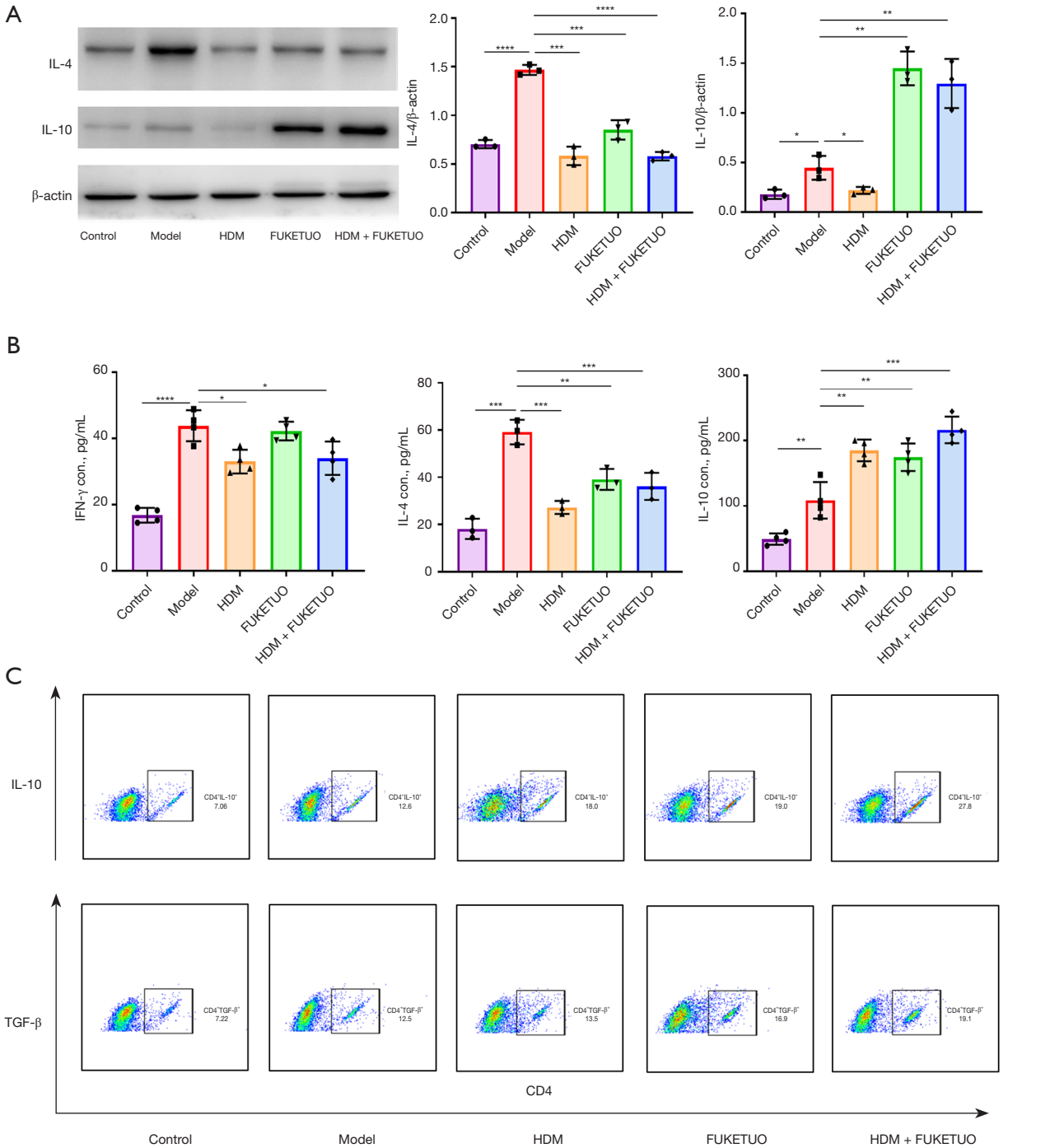

Figure 3 Inflammation related protein expression in lung and BALF. (A) The protein levels of IL-4 and IL-10 in mouse lung tissue Western blot (pg/mL). β-actin was used as an internal control protein. (B) IL-4, IFN-γ and IL-10 expression in mouse BALFs. (C) The expression of IL-10 and TGF-β in mouse BALFs. ImageJ was used to calculate the gray value, and the result displayed by the ratio of the gray results of target protein and β-actin. Data are representative of three independent experiments (n=3). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. IL, interleukin; con., concentration; HDM, house dust mite; FUKETUO, a trade name of spleen aminopeptides; IFN-γ, interferon-γ; TGF-β, transforming growth factor-β; BALF, bronchoalveolar lavage fluid.

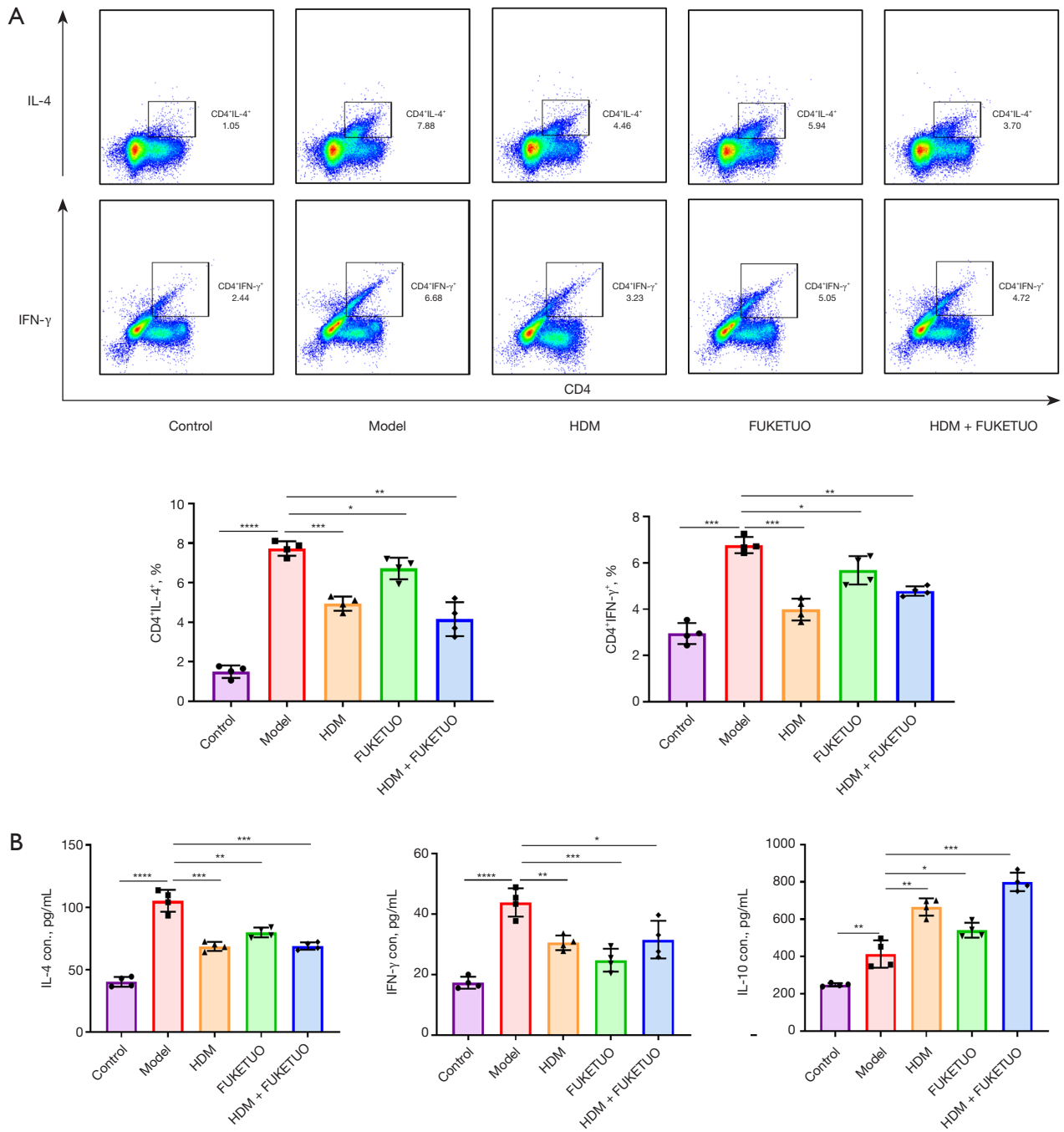

Figure 4 The cytokine expression in allergic asthma mice spleen. (A) The expression of IL-4 and IFN-γ in mouse spleen cells. IL-4 and IFN-γ in spleen cells were detected by flow cytometry. Using $CD4^+$ and $IL\text{-}4^+/IFN\text{-}\gamma^+$ to set gating to analyse the proportion of $CD4^+IL4^+$ and $CD4^+IFN\text{-}\gamma^+$ (shown as histograms). (B) The expression of IL-4, IFN-γ and IL-10 in mouse spleen cell supernatant (pg/mL). Data are representative of three independent experiments (n=4). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. IL, interleukin; IFN-γ, interferon-γ; HDM, house dust mite; FUKETUO, a trade name of spleen aminopeptides; con., concentration.

results further suggest that the spleen aminopeptides, either alone or combined with desensitization can increase IL-10 expression and thus inhibit allergic inflammation.

Spleen aminopeptides combined with desensitization therapy enhance the expressions of IL-10+ Treg levels in allergic asthma mice

The expression of mouse immunomodulatory-relate molecules was detected by flow cytometry, as shown in *Figure 5*. In the asthma group, the proportion of $CD4^+CD25^+IL10^+$ cells ($P<0.01$), $CD4^+CD25^+TGF\text{-}\beta^+$ cells ($P<0.001$) and $CD4^+CD25^+Foxp3^+$ cells ($P<0.01$) in the spleen increased due to feedback regulation. Compared with the asthma group, the proportion of $CD4^+CD25^+IL10^+$ cells in the desensitization group ($P<0.001$), the spleen aminopeptides treatment group ($P<0.01$) and the desensitization combined with spleen aminopeptides treatment group ($P<0.0001$) all increased. Among them, the increase in the desensitization combined with spleen aminopeptides treatment group is more obvious.

Compared with the asthma model group, the proportion of $CD4^+CD25^+TGF\text{-}\beta^+$ cells increased in the desensitization group ($P<0.05$), and slightly decreased in the spleen aminopeptides treatment group ($P<0.05$), however, no significant difference observed between the desensitization combined with spleen aminopeptides treatment group and the asthma model group.

Moreover, the percentage of $CD4^+CD25^+Foxp3^+$ cells in the desensitization group was significantly lower than in the asthma model group ($P<0.01$) and was almost the same level as in the control group. In addition, the proportion of $CD4^+CD25^+Foxp3^+$ cells in the spleen aminopeptides treatment group was slightly decreased, but there was no significant difference compared with the asthma model group. The proportion of $CD4^+CD25^+Foxp3^+$ cells in the desensitization combined spleen aminopeptides treatment was also decreased ($P<0.05$). Therefore, the spleen aminopeptides can promote the differentiation and proliferation of Treg secreting IL-10, especially when it is immunized with desensitization therapy. The proportion of $IL\text{-}10^+$ Treg increased, thus inhibiting TH2 cell polarization, further inhibiting the occurrence and development of allergic asthma.

Discussion

Allergic asthma is a chronic inflammatory disease of the heterogeneous respiratory tract, characterized by AHR, eosinophilic airway inflammation, increased specific IgE levels, increased mucus production, and reversible airway obstruction and remodeling (2). Spleen aminopeptides (FUKETUO) are a new class of oral bidirectional immunomodulators, which are peptides and nucleotides complex extracted from the spleens of healthy pigs. The average molecular weight of spleen aminopeptides is 3500 Dalton, and they contain more than 10 essential trace elements along with a variety of immune regulatory factors. A study has shown that spleen aminopeptides can correct the dysregulated immune function of children by regulating the levels of various T cell subsets (21).

Bronchial AHR is a classic manifestation of allergic asthma, presenting with chest tightness, wheezing, and coughing. The degree of AHR is related to the severity of asthma and is associated with chronic inflammation of the respiratory system (22), making AHR a useful measurement for evaluating the severity of allergic asthma. AHR can be detected through the bronchial provocation test, which uses methacholine to stimulate airway smooth muscle to observe the changes in airway hyper-responsiveness. In all spleen aminopeptides groups, the AHR levels were lower than those in the asthma positive group, indicating that spleen aminopeptides (FUKETUO) may reduce the AHR levels in allergic asthma.

In allergic asthma, Th2 cells produce IL-4, which induces B cells to produce allergen-specific IgE and IgG1 antibodies, which are markers of allergic symptoms. Th1 cells produce IFN-γ, which further promote the production of allergen-specific IgG2a antibodies to antagonize IgE and IgG1 (11). IgE binds to FcεR1α on mast cells and basophils. When contacting with allergen again, they are activated, releasing a large number of cytokines to produce allergic symptoms. Spleen aminopeptides (FUKETUO) can decrease the level of antigen-specific IgE and IgG1 in allergic asthma. Moreover, the lung structure change was checked by H&E and PAS staining. Results suggest that spleen aminopeptides (FUKETUO) help to maintain the alveolar structure and avoid tissue remodeling in the allergic asthma mouse model. Spleen aminopeptides therapy significantly reduced the level of AHR in allergic asthma, and alleviated the symptoms of airway wall thickening,

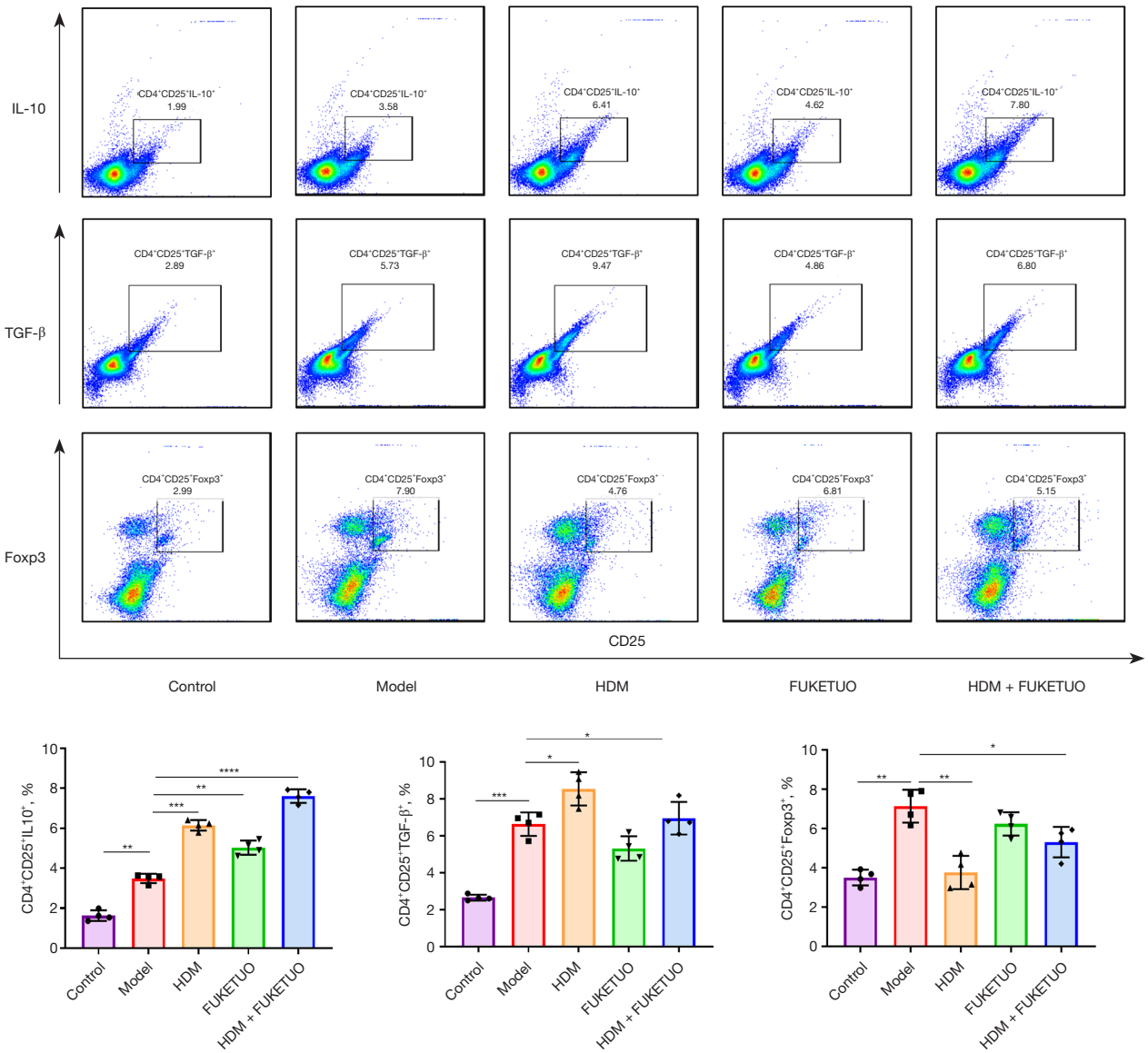

Figure 5 $CD4^+CD25^+$ cells expression in mouse spleen. The expressions of IL-10, TGF-β and Foxp3 in mouse spleen cells were detected by flow cytometry. $CD4^+$ cells were gated by CD4 and FSH, and then $CD25^+$ and $IL\text{-}10^+$/$TGF\text{-}\beta^+$/$Foxp3^+$ cells were isolated from $CD4^+$cells to analyse the proportion of $IL10^+$ Tregs, $TGF\text{-}\beta^+$ Tregs and $Foxp3^+$ Tregs cells in splenocytes. Data are representative of three independent experiments (n=4). *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. IL, interleukin; TGF-β, transforming growth factor-β; HDM, house dust mite; FUKETUO, a trade name of spleen aminopeptides; FSH, forward scatter-height; Tregs, regulatory T cells.

mucus hypersecretion, and the eosinophil ratio induced by HDM. In the combined treatment of desensitization and spleen aminopeptides (FUKETUO), spleen aminopeptides (FUKETUO) can significantly relieve the symptoms of increased eosinophil proportion caused by allergic asthma.

Furthermore, another important factor in allergic asthma is the inflammatory response. In allergic asthma, aberrant inflammation is often attributed to an imbalance between Th1 and Th2. Specifically, Th2 cells initiate the allergic immune response and produce cytokines that promote allergic responses, such as IgE class switching (IL-4) in B cells, and Th1 cells secreting IFN-γ to antagonize Th2 inflammatory damage, Treg cells also secrete IL-10 to inhibit Th2 cell function. The imbalance will lead to mass antibody

production and tissue damage. To alleviate allergic airway inflammation and lymphocyte infiltration, Tregs regulate the immune response to allergens by maintaining tolerance (16). In a mouse model of allergic asthma treated with spleen aminopeptides, the Th2 cytokine IL-4 in BALF and splenocyte supernatant were decreased, while IL-10 levels were significantly up-regulated. In particular, the expression of IL-4 and IL-10 in the combined therapy group was more obvious. The abnormal activation of Th2 cells in allergic reaction may be caused by Treg injury mediated by antigen specific T cells, which can reduce eosinophilia and mucus secretion in the airway. In the allergic asthmatic mouse model treated with spleen aminopeptides, the production and function of IL-10$^+$ Tregs were promoted by spleen aminopeptides due to the significant up-regulation of IL-10$^+$ Tregs.

Conclusions

In summary, spleen aminopeptide combined with desensitization therapy can further reduce the level of AHR and the eosinophil ratio, thereby enhancing the overall effect of desensitization therapy. The spleen aminopeptide may serve as a novel immunomodulator in the future, in combination with allergen vaccines or other anti-allergic drugs to treat allergic diseases such as allergic asthma. The use of spleen aminopeptide also provides new approach for clinical treatment of allergic diseases.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Experiments were performed under a project license (No. A20230063), granted by institutional ethics committee of Shenzhen University, in compliance with Regulations on the Administration of Laboratory Animals in China 1999 national guidelines for the care and use of animals.

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