International Immunopharmacology 24 (2015) 256–266

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

A novel pentapeptide originated from calf thymus named TIPP shows an inhibitory effect on lung allergic inflammation

Qianqian Lian [a], Wenjie Jiang [a], Yanna Cheng [b], Hongzhi Cao [c], Maoxuan Liu [a], Jinfeng Wang [a], Yan Li [a], Xinlei Song [a], Fengshan Wang [a,c,*]

[a] Key Laboratory of Chemical Biology of Natural Products (Ministry of Education), Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China
[b] Department of Pharmacology, School of Pharmaceutical Sciences, Shandong University, Jinan 250012, China
[c] National Glycoengineering Research Center, Shandong University, Jinan 250012, China

ARTICLE INFO

Article history:
Received 28 October 2014
Received in revised form 2 December 2014
Accepted 9 December 2014
Available online 19 December 2014

Keywords:
Allergy
Inflammation
Cytokine
MAP kinases
NF-κB
Thymus

ABSTRACT

Thymic immunosuppressive pentapeptide (TIPP) is a novel pentapeptide originally obtained from calf thymic immunosuppressive extract. In this study we aimed to investigate the anti-inflammatory effect and mechanisms of TIPP *in vivo* with an ovalbumin-induced mouse allergic asthma model. We investigated the effects of TIPP on the infiltration of inflammation cells, immune cell subtypes, Th2 cytokines in BALF and IgE in serum, mRNA levels of IL-4, IL-10, TNF-α and eotaxin-1, expression of MCP-1, VCAM-1 and COX-2, and activation of MAP kinases and NF-κB. Our results showed that TIPP significantly inhibited the increase in Th2 cytokines and OVA-specific IgE production, mRNA levels of IL-4, TNF-α and eotaxin-1 and the expression of MCP-1, VCAM-1 and COX-2 in lung tissues, as well effectively resisting the balance changes of cells in BALF. In addition, it was found that the administration of TIPP attenuated the activation of MAP kinases and NF-κB in the lung tissues of the allergic mice. Our data suggest that TIPP effectively suppresses the allergic and inflammatory responses in allergic mice *via* blocking MAP kinases/NF-κB signalling pathway. The investigation indicated that TIPP may become an anti-allergic and anti-inflammatory drug.

1. Introduction

Asthma, an allergic inflammatory disease, is prevalent worldwide both in adults and children [1,2]. Airway inflammation, resulting from interactions between T lymphocytes, B lymphocytes, mast cells, granulocytes, dendritic cells and macrophages, is fundamental to asthma pathogenesis [3,4]. A series of cytokines and chemokines are involved in this disorder process. Th2 cytokines (IL-4, 5, 13) are thought to be in a dominant position. IL-4 and IL-13 are crucial for IgE production by B cells and mucus hypersecretion. In addition, they can induce the expression of macrophage chemoattractant proteins (MCPs), eotaxins and vascular cell adhesion molecule 1 (VCAM-1), which promote the infiltration of inflammatory cells into lung tissues [5,6]. IL-5 plays an essential role in eosinophil differentiation and accumulation, and enhances eosinophil cytotoxicity and the release of pro-inflammatory mediators [7]. Tumour necrosis factor-α (TNF-α), a pleiotropic inflammatory cytokine secreted from T cells and mast cells, presents higher levels in bronchoalveolar fluid from asthma patients [8,9]. As an immunosuppressive cytokine, IL-10, along with its secreting cells, $CD4^+CD25^+$ regulatory cells, is reported to play an inhibitory effect in pulmonary inflammation and asthma [10].

The transcription factor, nuclear factor-κB (NF-κB), which exists in almost all cell types, plays an essential role in immunity and inflammation. Evidence has shown that there was a close connection between NF-κB activation and allergic asthma [11,12]. The activation of NF-κB induces the expression of cytokines (*e.g.*, IL-4, IL-5, and TNF-α), chemokines (*e.g.*, eotaxin and MCP-1), VCAM-1 and cyclooxygenase-2 (COX-2), which facilitates the development of asthma [13]. Therefore, a lot of studies have focused on the NF-κB signalling pathway in asthma, and the results have demonstrated that this pathway is a target for the treatment of asthma.

In the 1970s, thymic extracts containing non-cytotoxic-specific inhibitors termed as 'thymic chalones' were isolated from the thymus of animals [14–16]. Experimental results showed that these crude extracts had a negative regulating function in the synthesis of DNA and the proliferation of lymphocytes [17,18]. Our lab began to study thymic immunosuppressive extract (TISE) in the 1980s and developed a new method for TISE preparation. Our prior research has shown that TISE inhibits the immune and allergic responses effectively both *in vitro* and *in vivo* [19–21]. For further investigation of the immunosuppressive activity and mechanism, low molecular weight immunosuppressive factors

* Corresponding author at: Institute of Biochemical and Biotechnological Drugs, School of Pharmaceutical Sciences, Jinan 250012, Shandong, China. Tel.: +86 531 88382589; fax: +86 531 88382548.
E-mail addresses: fswang@sdu.edu.cn, wangfengshansdu@hotmail.com (F. Wang).

http://dx.doi.org/10.1016/j.intimp.2014.12.019
1567-5769/

derived from calf thymus were fractionated with micrococcal nuclease digestion, acid hydrolysis and RP-HPLC separation, through tracking the immunosuppressive activity. After further isolation and purification, a novel pentapeptide with the sequence of Ala-Glu-Trp-Cys-Pro was obtained from TISE. We found that this pentapeptide had an inhibitory effect on splenocyte proliferation caused by Con A stimulation and named it thymic immunosuppressive pentapeptide (TIPP, Fig. 1). In view of the apparent effects of TISE against allergic responses, we speculated that TIPP may have therapeutic actions in allergic inflammation. In order to confirm our speculation, we investigated the anti-inflammatory effects and molecular mechanism of TIPP on lung allergic inflammation in an ovalbumin (OVA)-induced mouse model of allergic asthma.

2. Materials and methods

2.1. Materials

TIPP (>95% purity, endotoxin free) was synthesised by ChinaPeptides Co., Ltd. (Shanghai, China). RPMI 1640 Medium and foetal bovine serum (FBS) were purchased from Gibco (Paisley, UK). Ovalbumin (OVA, Grade V), concanavalin A (Con A), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharides (LPS) and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, USA). Alu-Gel-S suspension was provided by SERVA Electrophoresis GmbH (Heidelberg, Germany). Enzyme-linked

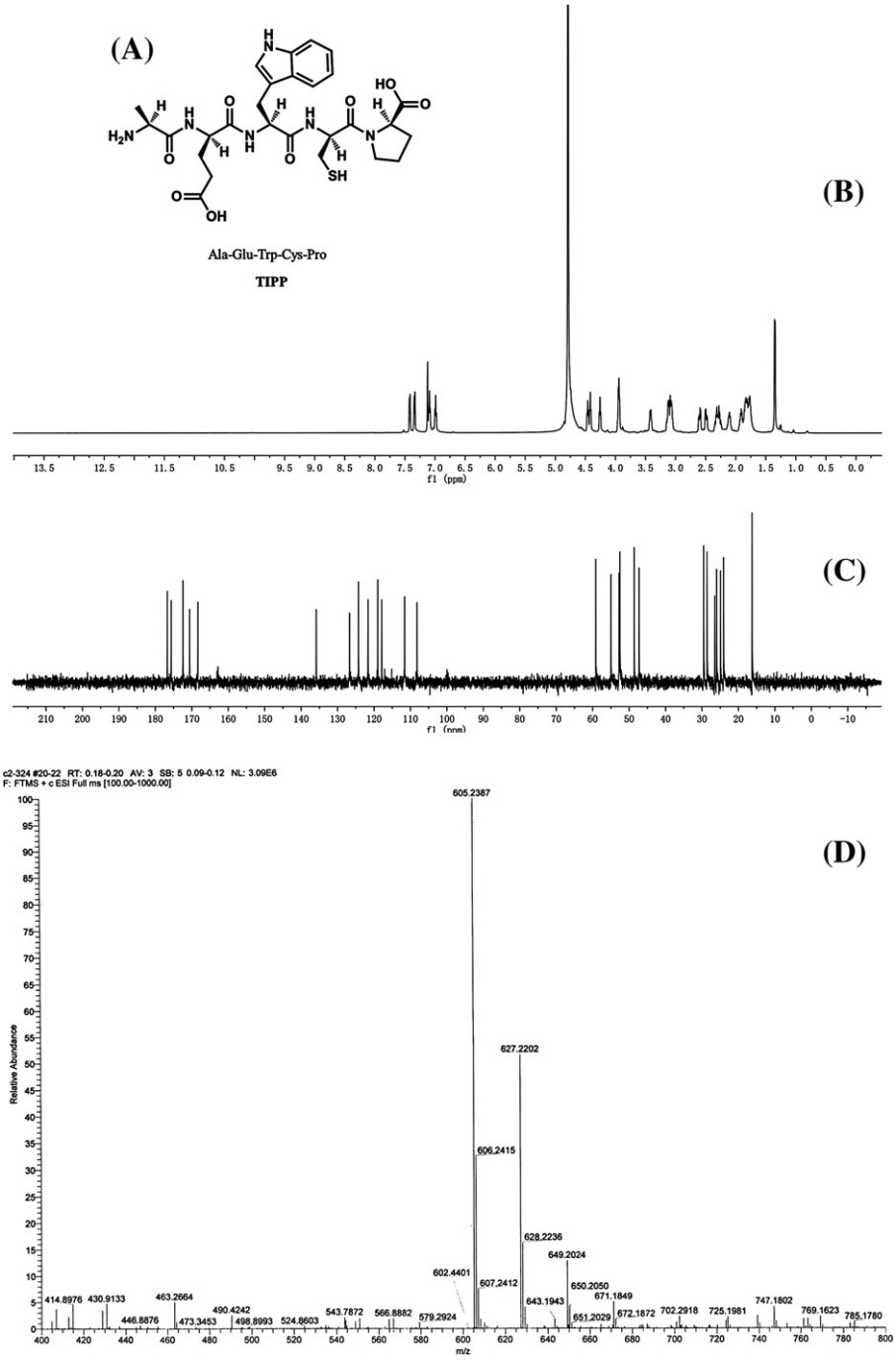

Fig. 1. (A) Chemical structure of TIPP; (B) 600 MHz ^{1}H NMR spectra of TIPP in D_2O; (C) 150 MHz ^{13}C NMR spectra of TIPP in D_2O; (D) HRMS spectra of TIPP.

immunosorbent assay (ELISA) kits were purchased from BioLegend (San Diego, CA, for IgE) or eBioscience (Vienna, Austria, for IL-4, IL-5, IL-13 and IFN-γ). All antibodies for flow cytometric analysis were obtained from eBioscience. TRIzol reagent from Invitrogen (Carlsbad, CA, USA), FastQuant RT Kit from Tiangen Biotech Co., Ltd. (Beijing, China) and SYBR® Green Real-time PCR Master Mix from Toyobo Co., Ltd. (Osaka, Japan) were used for quantification PCR analysis. Total protein extraction kit was purchased from BestBio (Shanghai, China). Anti-MCP-1 and anti-VCAM-1 antibodies were obtained from Abcam (Hong Kong) Ltd. Anti-COX-2 and anti-phospho-NF-κB p65, anti-phospho-ERK, anti-phospho-JNK, anti-phospho-p38, anti-NF-κB p65, anti-ERK, anti-JNK, anti-p38, anti-β-actin antibodies and secondary antibodies (anti-rabbit or anti-mouse) were obtained from Cell Signaling Technology (Danvers, MA, USA). Immobilon™ Western chemiluminescent HRP substrate was produced by Millipore Corporation (Billerica, MA, USA). Dexamethasone sodium phosphate was produced by Shandong Xinhua Pharmaceutical Co., Ltd. (Shandong, China).

2.2. Effects of TIPP on splenocytes

Splenocytes were prepared from BALB/c mice and cultured in RPMI 1640 with 10% heat inactivated FBS. For cytotoxicity detection, splenocytes were transferred into 96-well microplates (8×10^5 cells/well) and were incubated with different concentrations of TIPP for 68 h at 37 °C in a humidified incubator (5% CO_2, 95% air). In splenocyte proliferation experiments, splenocytes (2×10^5 cells/well) were cultured with 5 μg/mL of Con A or 10 μg/mL of LPS under different concentrations of TIPP for 68 h at 37 °C in a humidified incubator (5% CO_2, 95% air). Then 20 μL of 5 mg/mL of MTT was added and the cells were incubated for another 4 h at 37 °C. The precipitate was dissolved in DMSO and the absorbance was measured at 570 nm with a microplate reader (Bio-Rad 680, USA).

2.3. Animals

Five-week-old female BALB/c mice (specific pathogen free) were obtained from Beijing HFK Bioscience Co. Ltd. (Beijing, China). The animal room was maintained at 22–24 °C with a 12-h light/dark cycle. In addition, all mice took OVA-free food and water freely during the experiment. The studies involving animals were conducted according to the strict guiding principles of the National Institution of Health for Experimental Care and Use of Animals. The experimental design and procedures were approved by the Institutional Ethical Committee for Animal Care and Use of Shandong University, People's Republic of China.

2.4. OVA sensitisation and challenge protocol

Mice were sensitised with 50 μg of OVA adsorbed in 2.25 mg of aluminium hydroxide (Alu-Gel-S suspension), by intraperitoneal injection on days 0, 7 and 14 in all mice except those in the normal group which was sensitised with saline. The mice were challenged with 50 μL 2 mg/mL OVA in saline intranasally once daily for 6 consecutive days from days 28 to 33 as the reference described [22]. For the treatment groups, the mice were injected with 2, 10, 50 mg/kg TIPP or 3 mg/kg dexamethasone sodium phosphate (Dex) subcutaneously 1 h before OVA challenge. Normal group mice were injected and challenged with saline, and control group mice were challenged with OVA while injected with saline. All mice were analysed 24 h after the last OVA challenge.

2.5. Peripheral white blood cell (WBC) differential counting

Peripheral blood was collected *via* the tail vein with EDTA·2K as an anticoagulant [23]. WBC differential counting was performed on Hemavet 950 New, Fast, Veterinary Multi-species Haematology System.

2.6. Measurement of OVA-specific serum IgE

Immediately after anaesthesia, blood samples were collected *via* the tail vein. The blood was allowed to clot at room temperature, and then centrifuged at 1000 ×*g* for 10 min. Aliquots of serum were stored at −72 °C until analysis for OVA-specific serum IgE using an ELISA kit.

2.7. Bronchoalveolar lavage fluid (BALF) preparation

Immediately after blood collection, thoracic cavities of the mice were carefully opened. Tracheas were exposed, and BALF was collected by cannulating the upper part of the trachea with a 20-G angiocatheter and lavaging with three 0.6 mL aliquots of cold PBS (>85% of the volume inputted was recovered). BALFs were centrifuged at 650 ×*g* for 5 min at 4 °C to collect the cells in BALFs. The supernatants of BALFs were stored at −72 °C for the determination of cytokine levels. The collected cells were resuspended in cold PBS containing 0.1% BSA and the total cell counts were determined using a haemacytometer. Various immune cell subtypes in BALF were analysed by flow cytometry.

2.8. Flow cytometric analysis of cell subtypes in BALF

Immune cell subtypes in BALF were analysed by flow cytometry using different fluorescence-labelled monoclonal antibodies, such as anti-CD3, CD4, CD8, CD25, CD19, CD69, CD14, CCR3, CD11b, and Gr-1. The cells were stained with the indicated antibodies for 30 min at 4 °C and were analysed by flow cytometry on a FACSCalibur using CellQuest software (BD Biosciences).

2.9. Histology analysis

Unmanipulated left lungs were removed and fixed in a 4% paraformaldehyde solution. According to the standard processes, tissue sections were stained with haematoxylin–eosin (HE) for inflammation and Periodic acid–Schiff (PAS) for mucus detection. The degrees of inflammatory cell infiltration and goblet cell hyperplasia were scored in a double-blind screen by two independent pathologists in accordance with previous studies [24,25].

2.10. Determination of cytokines in BALF and OVA-specific IgE in serum

Cytokines (IL-4, IL-5, IL-13 and IFN-γ) in BALF and OVA-specific IgE in serum of mice were measured by ELISA according to the manufacturer's instructions with ELISA kits.

2.11. Quantification PCR of cytokines and eotaxin-1 mRNA in mouse lung tissues

Total RNA from mouse right lung tissue samples was extracted using TRIzol reagent. Every 50 mg of lung tissue sample was extracted with 1 mL of TRIzol reagent and was homogenised using an ultra Turrax T8 homogeniser (IKA Labortechnik, Staufen, Germany) on ice and operated according to manufacturer's protocol. One microgramme of total RNA was used for the synthesis of cDNA using FastQuant RT Kit and target genes were assayed using SYBR® Green Real-time PCR Master Mix (*via* Roche Light Cycler™) with their respective primers (Table 1). The PCR conditions were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s, 57 °C for 10 s, and 72 °C for 15 s. Transcription levels of β-actin were used as an internal control to calculate fold induction and the fold changes in transcription levels were calculated using the $2^{-\triangle\triangle Ct}$ method $\triangle\triangle Ct = (Ct_{\text{target gene}} - Ct_{\beta\text{-actin}})_{\text{treated groups}} - (Ct_{\text{target gene}} - Ct_{\beta\text{-actin}})_{\text{Normal}}$.

2.12. Western blot analysis

Lung tissue samples were added to cell lysis buffer combined with protease and phosphatase inhibitors (BestBio, Shanghai, China) and homogenised on ice. After centrifugation at 10,000 rpm for 5 min at 4 °C, the supernatants were collected and the protein contents were quantified with the BCA method. Equal amounts (30 μg) of total lung proteins were loaded into 10% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) and were incubated with primary antibodies overnight at 4 °C. The membranes were washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse) for 1 h at room temperature. After another three washes with TBST, the target proteins were determined using chemiluminescent HRP substrate.

2.13. Statistic analysis

Data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post-test using SPSS 18.0 software. Results were presented as means ± SD or SEM and differences were considered significant when $p < 0.05$.

3. Results

3.1. Effects of TIPP on splenocytes

The cytotoxicity of TIPP on splenocytes and the inhibitory activity of TIPP on splenocyte proliferation were evaluated with MTT assays. The cytotoxicity assay indicated that TIPP (0–1000 μg/mL) did not show cytotoxic effect on splenocytes (Fig. 2A) and the proliferation assay showed that TIPP apparently inhibited splenocyte proliferation stimulated by Con A in a dose-dependent manner while only had weak inhibitory effect on LPS stimulated splenocyte proliferation at high doses (200 and 800 μg/mL) (Fig. 2B). The results suggested that TIPP had an inhibitory effect on the proliferation of splenocytes, especially on the proliferation of spleen T lymphocytes, and this inhibitory activity was not relevant to the cytotoxicity.

3.2. Peripheral WBC differential counting

To evaluate the *in vivo* toxicity of TIPP, the effect of TIPP on peripheral WBCs in an OVA-induced mouse allergic asthma model was examined. The results showed that Dex treatment caused a significant decrease of WBC counts in allergic mice, which was almost 69.5% lower than that of the control group ($p < 0.001$), while the TIPP-treated group showed a mild decrease in WBC counts compared with the Dex-treated group (only 37% decrease when administrated with 50 mg/kg TIPP, $p < 0.01$ *vs.* control group) (Fig. 3A). In addition, Dex administration significantly changed the ratios of lymphocytes, neutrophils and monocytes in peripheral WBCs, while TIPP had a slight effect on the cell percentage changes (Fig. 3C).

3.3. Histological analysis

In order to evaluate the effect of TIPP on the lung inflammation in the OVA-induced mouse asthma model, mouse lung tissue sections were stained with HE (Fig. 4A). The lung sections from the control group showed marked infiltration of inflammation cells into peribronchial areas, and the TIPP- (50 mg/kg) and Dex-treated groups showed weakened inflammation compared with the control group (2.0 ± 0.6 and 1.8 ± 0.2 *vs.* 3.8 ± 0.3, $p < 0.05$). To determine the effect of TIPP on mucus production caused by goblet cell hyperplasia, lung sections were stained with PAS (Fig. 4A). The results showed that there were significant increases in PAS-stained goblet cells in allergic mice compared with the normal group, and marked decreases in the numbers of goblet cells in the TIPP-treated (50 mg/kg) and Dex-treated allergic mice (1.7 ± 0.3 and 1.3 ± 0.3 *vs.* 3.7 ± 0.3, $p < 0.05$).

3.4. Inhibitory effect of TIPP on the OVA-specific IgE levels in serum

As shown in Fig. 4B, the concentration of OVA-specific IgE in serum was apparently increased in control mice at 24 h after the final OVA challenge (889.9 pg/mL). TIPP notably suppressed the level of OVA-specific IgE at the tested concentration of 2 mg/kg (483.6 pg/mL, $p < 0.05$ *vs.* control group), and 10, 50 mg/mL (421.6 pg/mL, 338.8 pg/mL, $p < 0.01$ *vs.* control group), as well as Dex (413.3 pg/mL, $p < 0.01$ *vs.* control group).

3.5. Inhibitory effect of TIPP on the accumulation of inflammation cells into BALF

The effects of TIPP on the infiltration of inflammation cells into BALF in OVA-challenged mice were examined (Fig. 4C). A significant increase in BALF cells was observed in control mice *versus* normal mice ($p < 0.001$), which indicated inflammation in the lungs of allergic mice. The total number of BALF cells was significantly decreased in Dex-treated mice ($p < 0.001$); in addition, TIPP treatment also obviously decreased the total number of BALF cells ($p < 0.05$ at 2 mg/kg, $p < 0.01$ at 10 and 50 mg/kg).

Flow cytometric analysis was used to detect the effect of TIPP on cell subtypes of asthma mice (Table 2). The proportions of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+$ regulatory T cells, $CD3^+CD69^+$ early activated T cells, $CD3^-CCR3^+$ eosinophils, $CD3^+CCR3^+$ Th2 cells and $CD11b^+Gr\text{-}1^+$ granulocytes presented apparent uptrends in mice of the control group ($p < 0.01$), and treatment with TIPP and Dex resulted in significant reductions in the proportions of $CD3^+CD4^+$ T cells ($p < 0.05$, $p < 0.001$), $CD3^+CD8^+$ T cells ($p < 0.05$, $p < 0.001$), $CD3^+CD69^+$ early activated T cells ($p < 0.05$, $p < 0.01$), $CD3^-CCR3^+$ eosinophils ($p < 0.01$, $p < 0.001$), and $CD11b^+Gr\text{-}1^+$ granulocytes ($p < 0.01$, $p < 0.001$). Nevertheless, different to these cells, the proportion of $CD14^+$ monocytes/macrophages showed a significant reduction in OVA-challenged mice ($p < 0.001$), while Dex- and TIPP-treatment weakened this change ($p < 0.001$, $p < 0.05$).

3.6. Inhibitory effect of TIPP on cytokine levels in BALF

Th2 cytokines (IL-4, 5 and 13) are the major mediators in lung inflammation. As illustrated in Fig. 5, marked increases of IL-4, 5 and 13 in BALF were detected in OVA-challenged mice ($p < 0.01$). The administration of TIPP significantly decreased the levels of IL-4, 5 and 13 ($p < 0.05$). Dex showed a similar inhibitory effect ($p < 0.05$). In addition, the level of IFN-γ, a Th1 cytokine, was also examined in our test. We found that IFN-γ concentration was also increased in OVA-challenged mice ($p < 0.001$) and was significantly decreased by TIPP ($p < 0.001$) in a dose-dependent manner.

Table 1
Primers for quantification PCR of cytokines and eotaxin-1 mRNA in mouse lung tissues.

Genes	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
IL-10	TCAGGGGCTAGACATACTGAAG	CCAAGGAACTCTTGCAGGTAAT
TNF-α	TGTCCCTTTCACTCACTGGC	CATCTTTTGGGGGAGTGCCT
Eotaxin-1	TGAGGAACACAATGGGACGAG	TCGTGCAAAACAATAGACATTTTC
β-Actin	GGCTGTATTCCCCTCCATCG	GGGGTACTTCAGGGTCAGGA

3.7. Inhibitory effects of TIPP on the mRNA levels of IL-4, IL-10, TNF-α, and eotaxin-1 in lung tissues

The mRNA levels of IL-4, IL-10, TNF-α, and eotaxin-1 were detected by fluorescent quantitative PCR and were assayed with β-actin as an internal control. The experimental results (Fig. 6) indicated that TIPP treatment significantly decreased the up-regulation of the mRNA levels of IL-4, eotaxin-1 and TNF-α ($p < 0.05$), while showed no influence on the mRNA level of IL-10. Dex administration also inhibited the increase in the mRNA levels of IL-4, TNF-α and eotaxin-1 ($p < 0.05$), but showed no apparent effect on IL-10 mRNA level in the lung tissues of allergic mice.

3.8. Effects of TIPP on the expression levels of MCP-1, VCAM-1, and COX-2 in lung tissues

As MCP-1 (CCL2) and VCAM-1 are important factors for the accumulation of inflammation cells in allergic asthma, we examined their levels in lung tissues of asthma mice (Fig. 7A, B, C). There was enhanced expression of MCP-1 and VCAM-1 in lung tissues of the control group compared with the normal group ($p < 0.001$). TIPP administration had inhibitory effects on their levels in lung tissues ($p < 0.001$).

COX-2, a responder to inflammation stimulation, was also determined by Western blot (Fig. 7D). Similarly, TIPP administration reduced the protein level, which is usually increased in the lung tissues of the allergic mouse model.

3.9. Effects of TIPP on the activation of mitogen-activated protein (MAP) kinases and NF-κB in lung tissues

NF-κB pathway and three MAP kinase (ERK, JNK and p38) pathways have been identified to reflect immune responses and inflammation. Thus, the levels of phosphorylation of NF-κB p65 and these three MAP kinases were examined in lung tissues. The results showed that TIPP reduced the up-regulation of phosphorylation of NF-κB p65 ($p < 0.001$) and three MAP kinases ($p < 0.05$) in lung tissues (Fig. 8).

4. Discussion

Previous research has shown that there were non-cytotoxic-specific inhibitors in thymic extracts which had inhibitory effect in the synthesis of DNA and proliferation of lymphocytes [18,26]. Our lab found that TISE extracted from porcine thymus significantly inhibited the delayed type hypersensitivity (DTH) reaction induced by sheep red blood cells in mice, decreased the diameter of blue spot in passive cutaneous anaphylaxis of rats and suppressed Arthus reaction in rabbits [20,21]. As TIPP is a pentapeptide isolated from TISE and shows an inhibitory activity in splenocyte proliferation stimulated by Con A, we speculated that TIPP might have anti-allergic and anti-inflammatory activities.

An OVA-induced mouse model of allergic asthma was used to evaluate the *in vivo* anti-inflammatory effects and mechanisms of TIPP. OVA-induced mouse model of allergic asthma is a 'classic' animal model for understanding the pathogenetic mechanisms and finding potential therapeutic agents for asthma [27]. After OVA challenge to sensitised mice, infiltration of inflammation cells occurred following the increases of IgE, cytokines and chemokines. Th2 cells and Th2 cytokines play pivotal roles in asthma pathogenesis (Fig. 9A).

As previously described, IL-4, IL-5 and IL-13 are recognised as asthma-related factors. TNF-α, one of the most important eosinophil chemoattractant cytokines, is also closely related to pulmonary inflammation. In our study, there were obvious increases in the levels of IL-4, IL-5 and IL-13 in BALF and IgE in the serum of OVA challenged mice. Furthermore, the mRNA levels of IL-4 and TNF-α in lung tissues were multiplied. TIPP treatment clearly reduced concentrations of IL-4, IL-5, IL-13 and IgE, while suppressing the transcription of IL-4 and TNF-α mRNA in lung tissues. Although asthma is a Th2-polarised disease and the increase of Th2 cytokine production is often accompanied by the decreased secretion of IFN-γ [28,29], we found that IFN-γ was elevated in the BALF of asthmatic mice and decreased after TIPP administration. The up-regulation of IFN-γ in BALF of asthmatic mice also occurred in many studies [30,31]. The evidence has shown that IFN-γ secretion may aggravate disease severity [32,33].

IL-10 induces T cell tolerance and is an effective inhibitor of allergen-specific IgE and inflammatory cytokines. Recent studies have shown an increased expression of IL-10 in both serum and BALF of asthma patients [34,35], which may be the function of self-adjusting to an abnormal immune challenge. In our study, IL-10 mRNA was indeed excessively transcribed in OVA-challenged mice, but the administration of TIPP had little effect on the mRNA level of IL-10.

Airway inflammation is a disorder involving various cell types. Lots of studies have focused on the changes in the number of different cell subtypes. There was a significant increase in BALF cells in the asthma mouse model, and TIPP indeed decreased the total number of inflammation cells in BALF. However, a new question emerged in our minds. We doubted whether the changes in the proportions of cells in airway occurred and were related to asthma development. TIPP could have a positive effect in asthma due to its negative effects in these changes. In order to determine this, we analysed the proportions of different cell subtypes by flow cytometry. As previous results have shown, TIPP treatment weakened the changes in the proportion of BALF cells, which meant that it had an immunoregulatory effect on the immune disorders in the lung.

$CD4^+$ T lymphocytes are the dominant cells in asthma and there is often an increase in their number in the airways of asthmatic patients

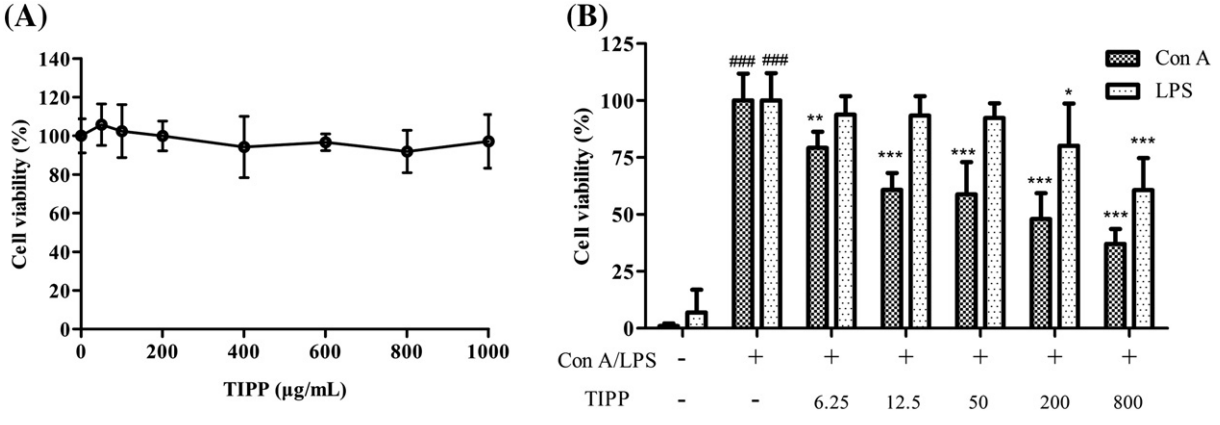

Fig. 2. (A) Cytotoxicity of TIPP on splenocytes; (B) effect of TIPP on splenocyte proliferation stimulated by 5 μg/mL of Con A or 10 μg/mL of LPS, respectively. Unit of TIPP concentration: μg/mL. Results are expressed as mean ± SD (n = 5). Compared to normal, $^{###}p < 0.001$; compared to control (only stimulated with Con A or LPS), $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

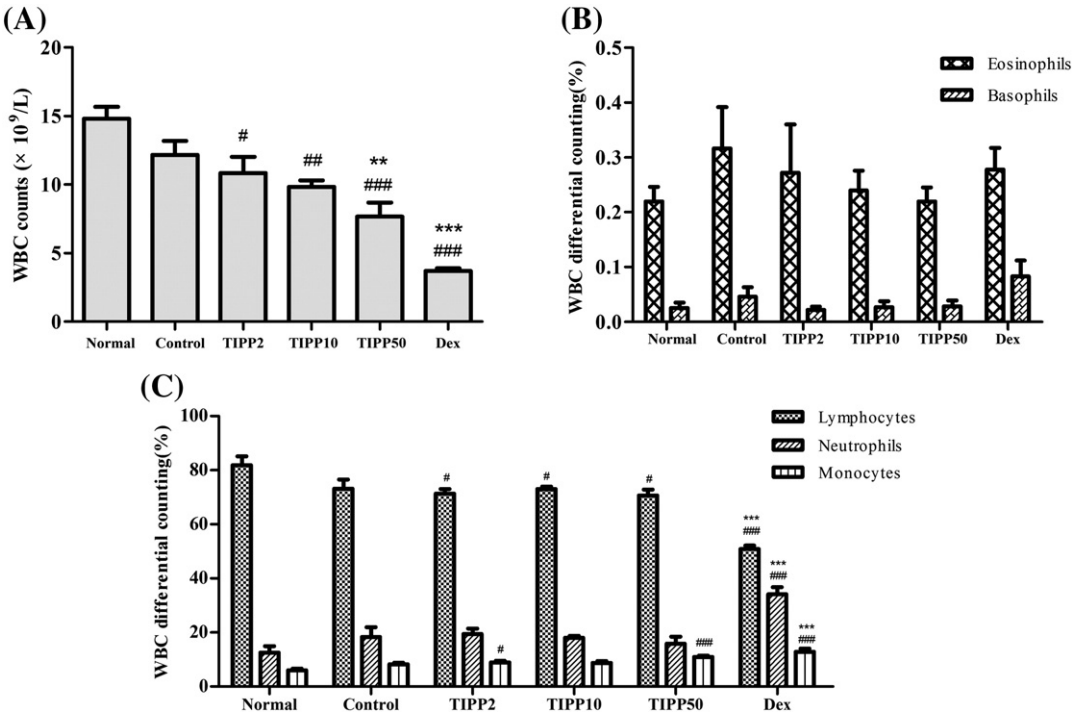

Fig. 3. Effects of TIPP on WBC counts (A) and percentages of cell subtypes of WBC (B, C). Results are expressed as mean ± SEM (n = 7). Compared with normal group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with control group, $^{**}p < 0.01$, $^{***}p < 0.001$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP, respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

[36]. $CD4^+$ T cells can differentiate into Th2 cells secreting Th2 cytokines and $CD4^+CD25^+$ regulatory T cells (T_{reg}) producing IL-10 and transforming growth factor-β (TGF-β). $CD8^+$ T cells possess pro-inflammatory functions in asthma when they develop into cytotoxic T lymphocytes (CTLs) with the increased expression of IFN-γ and granzyme B [37]. As a very early cell activation antigen of lymphocytes, CD69 is important for T cell differentiation and inflammatory responses [38]. In our OVA-induced mouse asthma model, we found that there were significant increases in the percentages of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells, $CD4^+CD25^+$ regulatory T cells, and $CD3^+CD69^+$ early activated T cells. TIPP administration decreased the percentages of $CD3^+CD4^+$ T cells, $CD3^+CD8^+$ T cells and $CD3^+CD69^+$ early activated T cells apparently, and showed an insignificant effect on $CD4^+CD25^+$ cell percentage, which was in accordance with mRNA level changes of IL-10.

CCR3 maintains a high level of expression in eosinophils and acts as the primary chemokine receptor that is responsible for eosinophil trafficking. In addition, CCR3 is a marker of Th2 cells [39,40]. Prior research used CCR3 as an identification marker for eosinophils and Th2 cells in an OVA-induced mouse allergic asthma model [41,42]. Moreover, eosinophils are important parts of granulocytes, and are known to express Gr-1 on their surface. Percentages of $CD3^-CCR3^+$ eosinophils, $CD3^+CCR3^+$ Th2 cells and $CD11b^+Gr\text{-}1^+$ granulocytes were up-regulated in the allergic mice of the control group, and treatment with TIPP or Dex decreased their percentages *versus* the control group. The results indicated the accumulation of two prominent cells in asthma, eosinophils and Th2 cells, and the homeostatic maintenance activity of TIPP and Dex.

Monocytes/macrophages play an important role in asthma pathogenesis. Interestingly, we found that the number of $CD14^+$ monocytes/macrophages in BALF was increased in asthmatic mice; however, the percentage decreased significantly in the control group and there were recoveries in the treated groups on different levels. This may be the result of significant eosinophil accumulation. TIPP and Dex resist the down-regulation through inhibiting the infiltration and accumulation of eosinophils.

Eotaxin-1 is a selective chemoattractant and activator of eosinophils *via* interaction with CCR3 [43]. MCP-1 and VCAM-1 are also involved in the infiltration and accumulation of inflammatory cells. The enhanced expressions of eotaxin-1, MCP-1 and VCAM-1 were observed in asthmatic lung tissues [44,45]. Therefore, the reduction in the abnormal expression of eotaxin-1, MCP-1 and VCAM-1 is an effective treatment strategy for lung inflammation. Our results were consistent with that. The mRNA levels of eotaxin-1 and the expressions of MCP-1 and VCAM-1 significantly increased in asthmatic lung tissues, and TIPP treatment could regulate their abnormal levels. Thus, our data demonstrated that the inflammation suppression roles of TIPP may be related to the reduction of MCP-1 and VCAM-1 levels caused by the down-regulation of Th2 cytokines.

The reduced expression of COX-2 was also observed in the lung tissues of TIPP-treated asthma mice. COX-2 is an inducible enzyme of cyclooxygenases associated with inflammation for its high response to pro-inflammatory cytokines and mediators. Studies have demonstrated that the expression of COX-2 is enhanced in asthmatic patients and COX-2 inhibitors have therapeutic potential in asthma [46,47]. This result also indicated the possible therapeutic effects of TIPP on allergic asthma.

A number of studies have proven the pivotal role of the NF-κB pathway in the development of immune and inflammatory responses. The persistent activation of NF-κB was observed in severe asthma and the exaggerated NF-κB activation accelerated the production of inflammatory mediators [48]. Transcription of the genes for IL-4, IL-5, IL-13, TNF-α, eotaxin-1, MCP-1, VCAM-1 and COX-2 can all be regulated by the NF-κB signalling pathway [49–51]. In our study, it was found that TIPP could decrease the concentrations of Th2 cytokines in BALF, and the mRNA levels of IL-4, TNF-α and eotaxin-1 and the protein levels of MCP-1, VCAM-1 and COX-2 in lung tissues of the asthma mouse

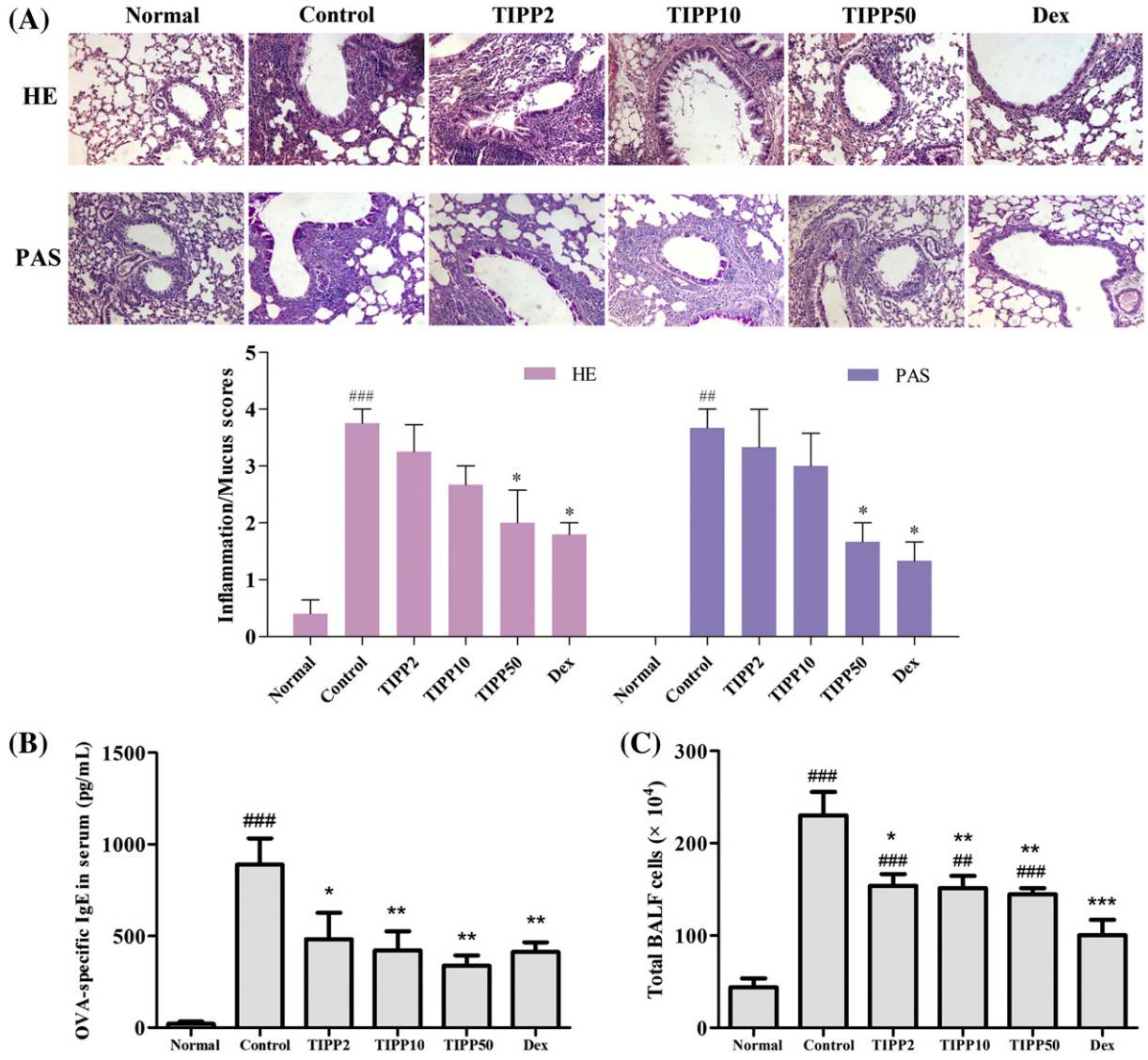

Fig. 4. (A) Effect of TIPP on the histology of lung tissues (HE and PAS staining, magnification: 200×). HE: haematoxylin–eosin stain; PAS: Periodic acid–Schiff stain. Results are expressed as mean ± SEM (n = 3–5); (B) effect of TIPP on OVA-specific IgE in serum. Results are expressed as mean ± SEM (n = 6); (C) effect of TIPP on total cell numbers in BALF. Results are expressed as mean ± SEM (n = 10). Compared with normal group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP, respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

model. In addition, the decreased expression of phospho-NF-κB p65 was observed. These results demonstrated that TIPP plays an anti-inflammatory role in allergic asthma through its inhibitory effect on activation of the NF-κB signalling pathway.

MAP kinases regulate many kinds of physiological processes and lots of studies have suggested that MAP kinases play an important role in immune responses. The phosphorylation levels of MAP kinases including ERK1/2, JNK and p38 are up-regulated in asthma patients

Table 2
Percentages of various cell subtypes in BALF by FACS analysis.

Cell phenotypes in BALF	Normal (%)	Control (%)	TIPP2 (%)	TIPP10 (%)	TIPP50 (%)	Dex (%)
$CD3^+CD4^+$	1.22 ± 0.12	6.42 ± 1.15$^{\#\#\#}$	5.90 ± 0.65$^{\#\#\#}$	3.93 ± 0.31$^{\#,*}$	4.38 ± 0.13$^{\#\#}$	1.98 ± 0.16***
$CD3^+CD8^+$	0.62 ± 0.09	3.49 ± 0.81$^{\#\#\#}$	2.09 ± 0.12	1.77 ± 0.10*	1.51 ± 0.19*	0.63 ± 0.17***
$CD4^+CD25^+$	0.80 ± 0.09	2.20 ± 0.20$^{\#\#}$	1.51 ± 0.22	1.34 ± 0.08	1.41 ± 0.17	1.08 ± 0.23**
$CD3^+CD69^+$	1.08 ± 0.26	3.89 ± 0.62$^{\#\#}$	2.85 ± 0.39$^{\#}$	1.93 ± 0.18*	1.91 ± 0.18*	1.15 ± 0.17**
$CD3^-CD19^+$	0.32 ± 0.10	1.21 ± 0.29	1.23 ± 0.73	0.18 ± 0.04	1.14 ± 0.33	0.37 ± 0.13
$CD19^+CD69^+$	0.11 ± 0.02	0.54 ± 0.05	0.56 ± 0.27	0.14 ± 0.03	0.38 ± 0.06	0.16 ± 0.03
$CD3^-CCR3^+$	1.10 ± 0.62	46.04 ± 0.23$^{\#\#\#}$	43.42 ± 2.69$^{\#\#\#}$	37.77 ± 2.29$^{\#\#\#}$	28.31 ± 4.73$^{\#\#\#,**}$	12.79 ± 1.91$^{\#,***}$
$CD3^+CCR3^+$	0.26 ± 0.11	6.14 ± 0.78$^{\#\#\#}$	3.16 ± 0.29$^{\#\#,**}$	4.20 ± 0.60$^{\#\#}$	3.39 ± 0.46$^{\#\#,**}$	4.24 ± 0.32$^{\#\#}$
$CD11b^+Gr\text{-}1^+$	1.62 ± 0.48	52.53 ± 3.18$^{\#\#\#}$	36.68 ± 3.15$^{\#\#\#,*}$	35.90 ± 1.44$^{\#\#\#,*}$	26.83 ± 5.52$^{\#\#,**}$	21.93 ± 3.11$^{\#\#,***}$
$CD14^+$	71.68 ± 3.47	28.78 ± 1.88$^{\#\#\#}$	22.96 ± 2.23$^{\#\#\#}$	34.05 ± 0.24$^{\#\#\#}$	40.11 ± 2.67$^{\#\#\#,*}$	65.21 ± 2.51***

Results are expressed as mean ± SEM (n = 3). Statistical significance was assessed by ANOVA. Compared with normal group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

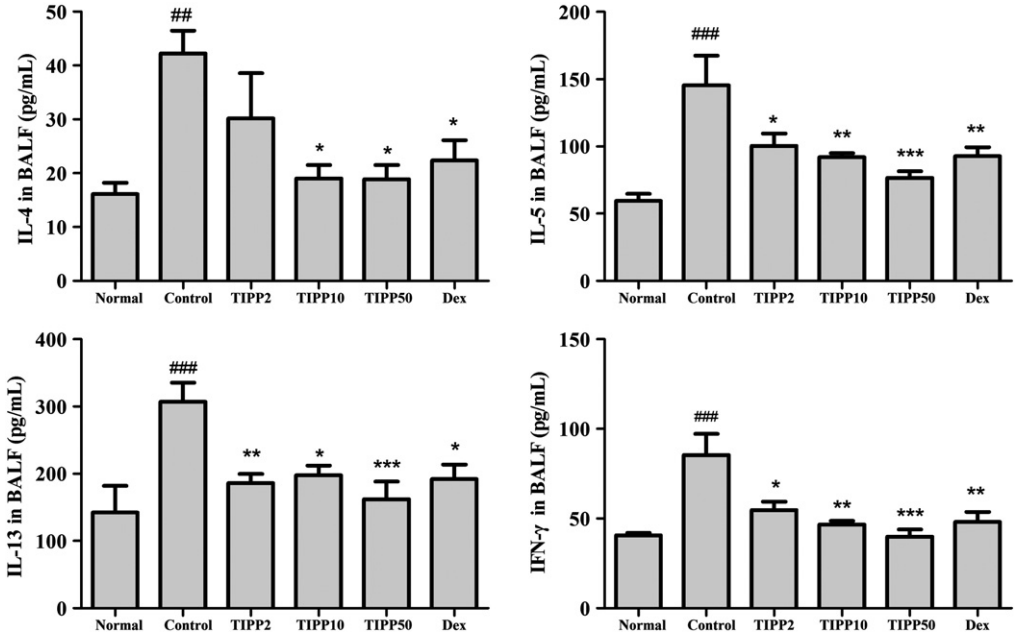

Fig. 5. Effects of TIPP on IL-4, IL-5, IL-13 and IFN-γ in BALF. Results are expressed as mean ± SEM (n = 7). Compared with normal group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP, respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

and animal models [52,53]. In addition, MAP kinases have been suggested to participate in the processes of NF-κB phosphorylation [54,55]. As TIPP administration decreased the activation of ERK1/2, JNK, and p38, we suggest that TIPP has anti-inflammatory effects in asthma *via* regulating MAP kinases/NF-κB pathways.

Our study demonstrates that TIPP is an effective anti-inflammation agent. TIPP was able to inhibit the expression of pro-inflammatory cytokines and mediators to suppress airway inflammation in an OVA-induced mouse allergic asthma model. The anti-inflammatory effect may be triggered *via* MAP kinases/NF-κB pathways. Based on the present results, we hypothesise that the anti-inflammation activity of TIPP in asthma may be the result of its inhibitory activity on the activation of mast cells and T cells *via* MAP kinases/NF-κB pathways. TIPP inhibits the activation of T cells and mast cells, which will cause decreases in the levels of cytokines involved in inflammation. The decrease in IL-4 and IL-13 causes lower IgE production and lower expression of eotaxin-1, MCP-1, VCAM-1; the decrease in IL-5 causes less eosinophil mature and infiltration. In addition, the decreased levels of IL-4 can further

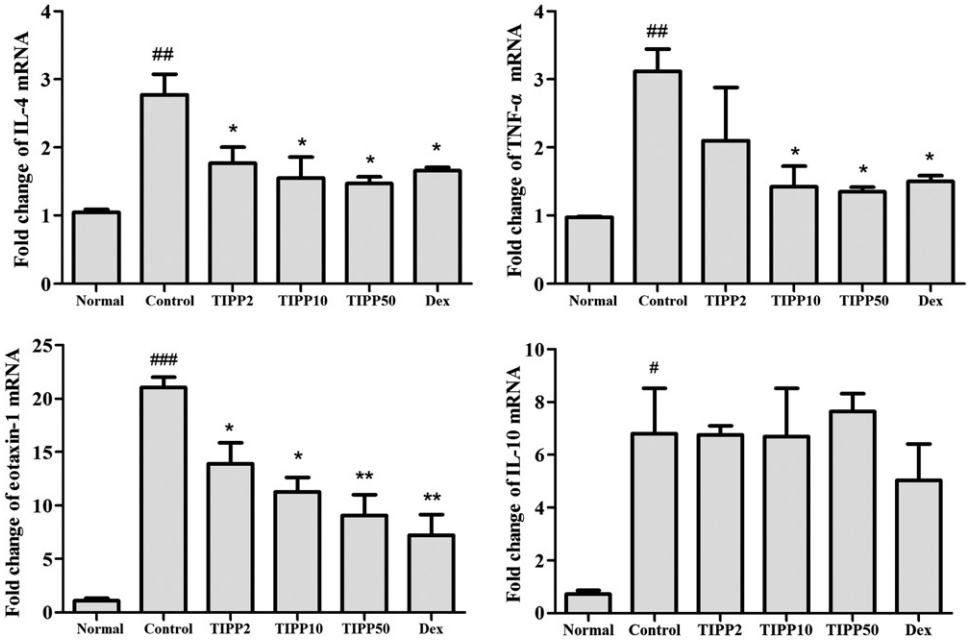

Fig. 6. Effects of TIPP on the mRNA levels of IL-4, TNF-α, eotaxin-1 and IL-10 in lung tissues. mRNA levels of β-actin were used as an internal control. Results are expressed as mean ± SEM (n = 3). Compared with normal group, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP, respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

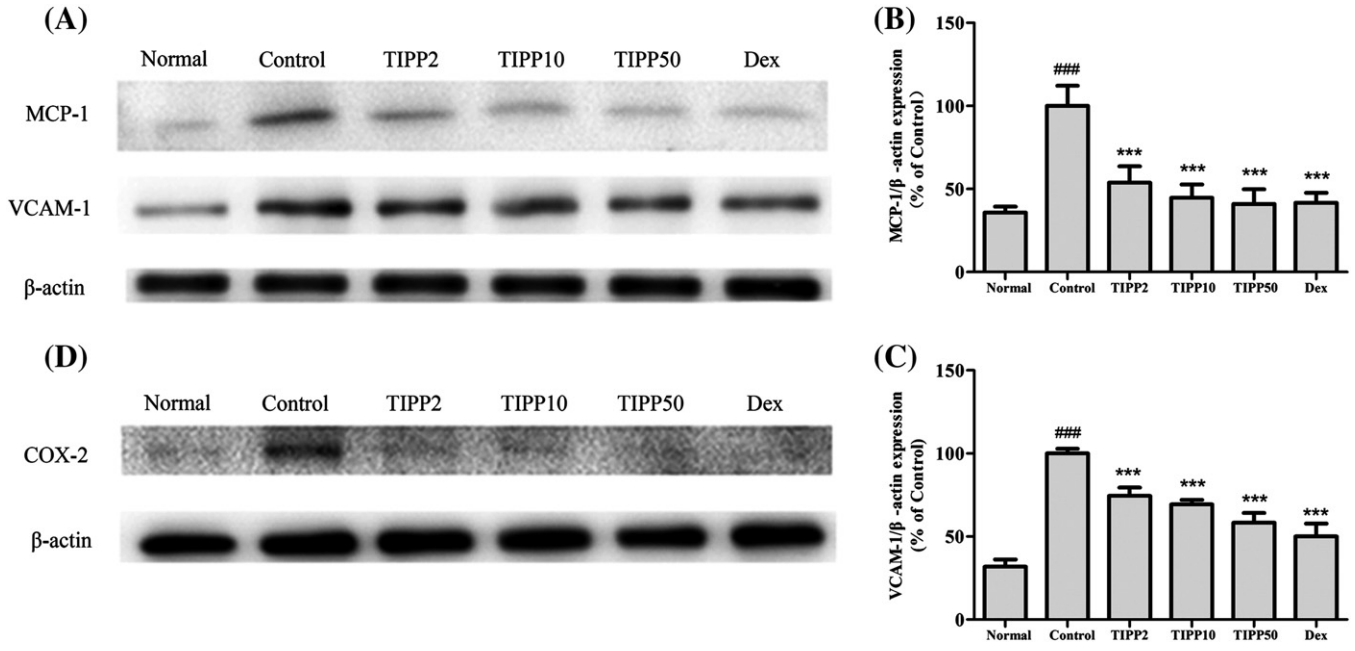

Fig. 7. Western blot analysis of MCP-1, VCAM-1 (A, B, C) and COX-2 (D) expressions in lung tissues. This Western blot diagram is a representative of three independent experiment diagrams with similar results. Results are expressed as mean ± SEM (n = 3). Compared with normal group, $^{###}p < 0.001$; compared to control group, $^{***}p < 0.001$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP, respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

reduce T cell activation and cytokine production, amplifying the inhibitory activity of TIPP. All of these can result in the decreased infiltration of inflammation cells (Fig. 9). In addition, the results of peripheral WBC differential counting showed that Dex not only caused a significant decrease in WBC number but also created a serious disorder in cell subtypes in peripheral WBC for its side effects, while TIPP treatment showed a slight effect on the peripheral WBC differential count. This indicated that TIPP had lower toxicity *in vivo*.

TIPP is a pentapeptide originated from calf thymus with immunosuppressive activity, and its activity is different from the factors with immune enhancing activity which have been purified from thymus (*e.g.*, thymopoietin and its active site fragment TP5). Further studies are needed to evaluate the regulatory effects of TIPP in immunity, find out its target of action and confirm its regulatory mechanisms. In addition, TIPP can be considered as a lead for the development of a new series of derivatives which have anti-inflammatory and immunosuppressive activities. We believe that our study into the anti-inflammatory effects of TIPP may contribute to the development of new anti-inflammatory drugs and may provide new insight into immunodepressant development.

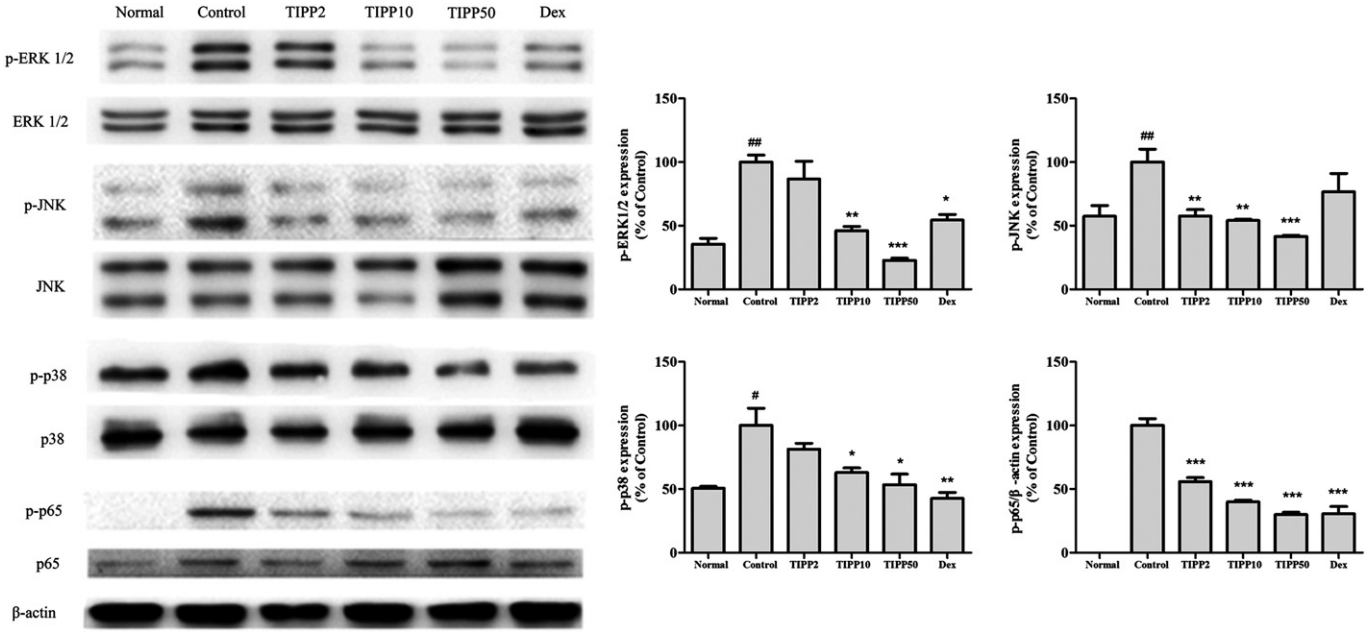

Fig. 8. Effects of TIPP on the activation of MAP kinases and NF-κB in lung tissues. This Western blot diagram is a representative of three independent experiment diagrams with similar results. Results are expressed as mean ± SEM (n = 3). Compared with normal group, $^{#}p < 0.05$, $^{##}p < 0.01$; compared with control group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Normal, normal mice treated with saline only; Control, OVA-sensitised and challenged mice; TIPP2, TIPP10 and TIPP50, OVA-sensitised and challenged mice treated with 2, 10 and 50 mg/kg TIPP, respectively; Dex, OVA-sensitised and challenged mice treated with 3 mg/kg Dex.

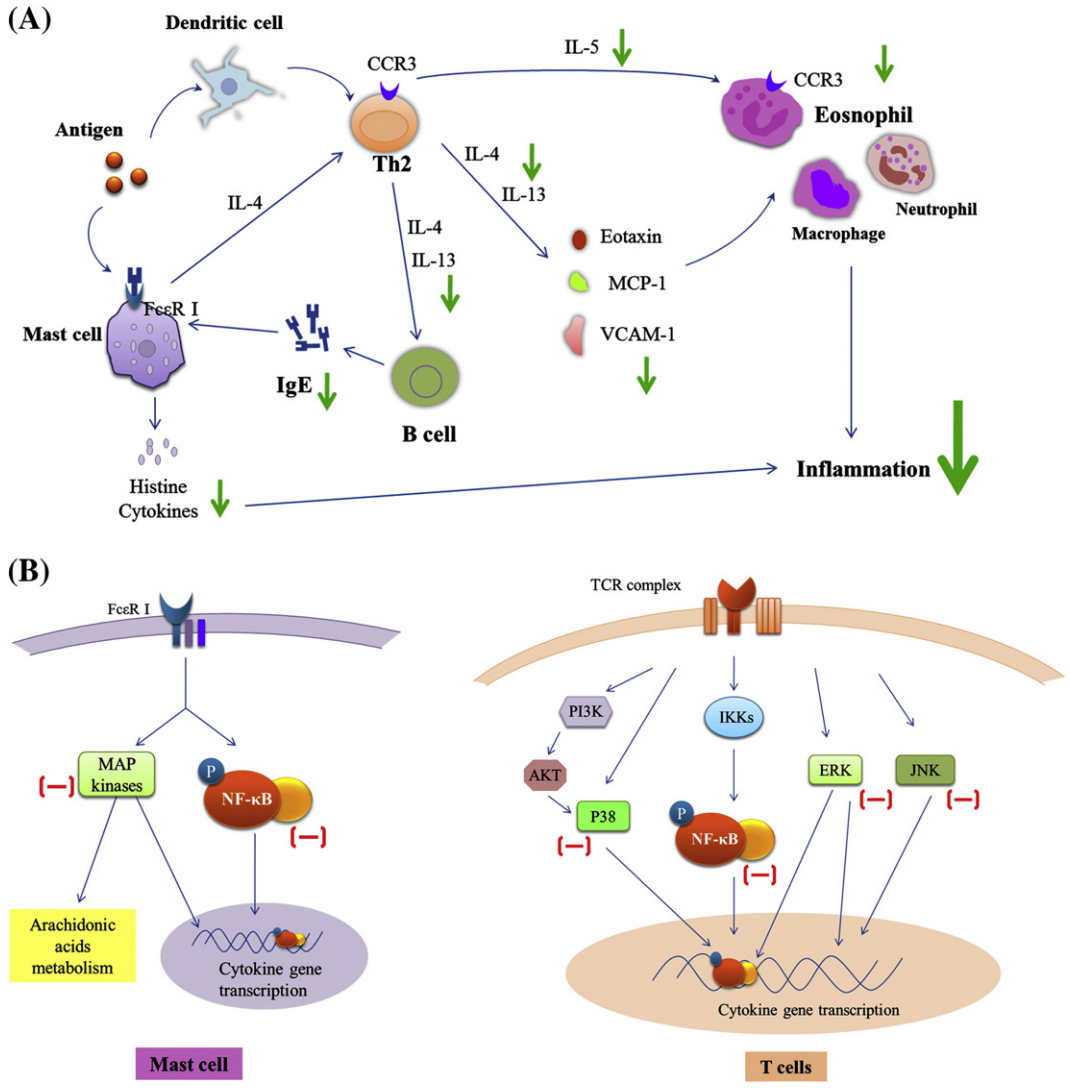

Fig. 9. The detected effects of TIPP on inflammation development in this study (A) and the hypothesised mechanisms (B). Inhaled antigens are presented by dendritic cells and thus promote Th2 differentiation and Th2 cytokines (IL-4, 5 and 13) production. The secreted Th2 cytokines contribute to IgE and a series of inflammatory mediator expressions and result in the infiltration of inflammation cells. Antigens can also activate the mast cells with IgE bound to FcεRI on cell surface, and cause degranulation to promote the pathological process. The green arrows represent the inhibitory effects of TIPP in our study and the red '(−)' represent the hypothesised mechanisms of TIPP in mast cells and T cells.

Acknowledgements

This work was supported by the "973" grant from the Ministry of Science and Technology of China (2012CB822102) and Taishan Scholar Scheme (Special Term Expert for Pharmacy) (2012GSF30022).

References

[1] Lee MY, Ahn KS, Lim HS, Yuk JE, Kwon OK, Lee KY, et al. Tiarellic acid attenuates airway hyperresponsiveness and inflammation in a murine model of allergic asthma. Int Immunopharmacol 2012;12(1):117.
[2] Gaffin JM, Kanchongkittiphon W, Phipatanakul W. Perinatal and early childhood environmental factors influencing allergic asthma immunopathogenesis. Int Immunopharmacol 2014;22(1):21.
[3] Holgate ST. Pathogenesis of asthma. Clin Exp Allergy 2008;38(6):872.
[4] Wills-Karp M, Karp CL. Biomedicine. Eosinophils in asthma: remodeling a tangled taleScience 2004;305(5691):1726.
[5] Rosenwasser LJ, Zimmermann N, Hershey GK, Foster PS, Rothenberg ME. Chemokines in asthma: cooperative interaction between chemokines and IL-13. J Allergy Clin Immunol 2003;111(2):227.
[6] Wills-Karp M. Interleukin-13 in asthma pathogenesis. Immunol Rev 2004;202(1):175.
[7] Hamelmann E, Gelfand EW. IL-5-induced airway eosinophilia—the key to asthma? Immunol Rev 2001;179(1):182.
[8] Akdis M, Blaser K, Akdis CA. T regulatory cells in allergy: novel concepts in the pathogenesis, prevention, and treatment of allergic diseases. J Allergy Clin Immunol 2005;116(5):961.
[9] Berry MA, Hargadon B, Shelley M, Parker D, Shaw DE, Green RH, et al. Evidence of a role of tumor necrosis factor α in refractory asthma. N Engl J Med 2006;354(7):697.
[10] Oh JW, Seroogy CM, Meyer EH, Akbari O, Berry G, Fathman CG, et al. CD4 T-helper cells engineered to produce IL-10 prevent allergen-induced airway hyperreactivity and inflammation. J Allergy Clin Immunol 2002;110(3):460.
[11] Song MY, Jeong GS, Lee HS, Kwon KS, Lee SM, Park JW, et al. Sulfuretin attenuates allergic airway inflammation in mice. Biochem Biophys Res Commun 2010;400(1):83.
[12] Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-κ B in pulmonary diseases. Chest 2000;117(5):1482.
[13] Gagliardo R, Chanez P, Mathieu M, Bruno A, Costanzo G, Gougat C, et al. Persistent activation of nuclear factor-κB signaling pathway in severe uncontrolled asthma. Am J Respir Crit Care Med 2003;168(10):1190.
[14] Houck JC, Hennings H. Chalones specific endogenous mitotic inhibitors. FEBS Lett 1973;32(1):1.
[15] Allen JC, Smith CJ, Curry MC, Gauqas JM. Identification of a thymic inhibitor ('chalone') of lymphocyte transformation as a spermine complex. Science 1977;267(5612):623.
[16] Kiger N, Florentin I, Lorans G, Mathe G. Further purification and chemical characterization of the lymphocyte-inhibiting-factor extracted from thymus (LIFT). Immunology 1977;33(4):439.
[17] Houck JC, Irausquin H, Leikin S. Lymphocyte DNA synthesis inhibition. Science 1971;173(4002):1139.

[18] Blazsek I. Inhibition of lymphocyte production via thymic factors. Blut 1981;42(4):235.
[19] Zhao SM, Zhang TG, Yue QA, Zang HC, Wang FS, Wang HY. The effect of U2 fraction of porcine thymus immunosuppressive extract on lymphocyte proliferation. Chin J Biochem Pharm 2003;24(3):133 [in Chinese].
[20] Shang XY, Zhang SL, Jiao B, Lu FY, Tian G, Li FC. Anti-allergic effects of thymic immunosuppressive fraction. Chin Pharmacol Bull 1996;21(4):360 [in Chinese].
[21] Xin XL, Zhang SL, Wang FS. Effects of thymic immunosuppressive extract on mouse immune functions. Chin J Biochem Pharm 1996;17(5):185 [in Chinese].
[22] Pichavant M, Goya S, Hamelmann E, Gelfand EW, Umetsu DT. Animal models of airway sensitization. Curr Protoc Immunol 2007;79 15.18. 1.
[23] Nemzek JA, Bolgos GL, Williams BA, Remick DG. Differences in normal values for murine white blood cell counts and other hematological parameters based on sampling site. Inflamm Res 2001;50(10):523.
[24] Deng Y, Guan M, Xie X, Yang X, Xiang H, Li H, et al. Geniposide inhibits airway inflammation and hyperresponsiveness in a mouse model of asthma. Int Immunopharmacol 2013;17(3):561.
[25] Ford JG, Rennick D, Donaldson DD, Venkayya R, McArthur C, Hansell E, et al. IL-13 and IFN-γ: interactions in lung inflammation. J Immunol 2001;167(3):1769.
[26] Patt LM, Gleisner JM, Barrantes DM, Houck JC. Low molecular weight inhibitors of lymphocyte transformation. Pharmacology 1981;23(3):117.
[27] Kumar RK, Herbert C, Foster PS. The classical ovalbumin challenge model of asthma in mice. Curr Drug Targets 2008;9(6):485.
[28] Reber LL, Daubeuf F, Plantinga M, De Cauwer L, Gerlo S, Waelput W, et al. A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma. J Immunol 2012;188(7):3478.
[29] Bao Z, Guan S, Cheng C, Wu S, Wong SH, Kemeny DM, et al. A novel antiinflammatory role for andrographolide in asthma via inhibition of the nuclear factor-κB pathway. Am J Respir Crit Care Med 2009;179(8):657.
[30] Kim DY, Park JW, Jeoung D, Ro JY. Celastrol suppresses allergen-induced airway inflammation in a mouse allergic asthma model. Eur J Pharmacol 2009;612(1):98.
[31] McKay A, Leung BP, McInnes IB, Thomson NC, Liew FY. A novel anti-inflammatory role of simvastatin in a murine model of allergic asthma. J Immunol 2004;172(5):2903.
[32] Cho S-H, Stanciu LA, Holgate ST, Johnston SL. Increased interleukin-4, interleukin-5, and interferon-γ in airway CD4+ and CD8+ T cells in atopic asthma. Am J Respir Crit Care Med 2005;171(3):224.
[33] Lampinen M, Carlson M, Håkansson LD, Venge P. Cytokine-regulated accumulation of eosinophils in inflammatory disease. Allergy 2004;59(8):793.
[34] 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.
[35] Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, et al. The levels of CD4+ CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. Clin Exp Immunol 2007;148(1):53.
[36] Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2008;8(3):183.
[37] McNamara JT, Schramm CM, Singh A, Secor Jr ER, Guernsey LA, Lefrancois L, et al. Phenotypic changes to the endogenous antigen-specific CD8+ T cell response correlates with the development and resolution of allergic airway disease. Am J Pathol 2012;180(5):1991.
[38] Borges O, Borchard G, de Sousa A, Junginger HE, Cordeiro-da-Silva A. Induction of lymphocytes activated marker CD69 following exposure to chitosan and alginate biopolymers. Int J Pharm 2007;337(1–2):254.
[39] Uhm TG, Kim BS, Chung IY. Eosinophil development, regulation of eosinophil-specific genes, and role of eosinophils in the pathogenesis of asthma. Allergy Asthma Immunol Res 2012;4(2):68.
[40] Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev 2000;14(14):1693.
[41] Kim SH, Hong JH, Lee YC. Ursolic acid, a potential PPARγ agonist, suppresses ovalbumin-induced airway inflammation and Penh by down-regulating IL-5, IL-13, and IL-17 in a mouse model of allergic asthma. Eur J Pharmacol 2012;701(1–3):131.
[42] Kim SH, Kim BK, Lee YC. Antiasthmatic effects of hesperidin, a potential Th2 cytokine antagonist, in a mouse model of allergic asthma. Mediators Inflamm 2011:485402.
[43] Kitaura M, Nakajima T, Imai T, Harada S, Combadiere C, Tiffany HL, et al. Molecular cloning of human eotaxin, an eosinophil-selective CC chemokine, and identification of a specific eosinophil eotaxin receptor, CC chemokine receptor 3. J Biol Chem 1996;271(13):7725.
[44] Gong JH, Shin D, Han SY, Kim JL, Kang YH. Kaempferol suppresses eosionphil infiltration and airway inflammation in airway epithelial cells and in mice with allergic asthma. J Nutr 2012;142(1):47.
[45] Lee MY, Shin IS, Lim HS, Seo CS, Ha H, Shin HK. Kochia scoparia fruit attenuates allergic airway inflammation in ovalbumin (OVA)-induced murine asthma model. Inhal Toxicol 2011;23(14):938.
[46] Sousa AR, Pfister R, Christie PE, Lane SJ, Nasser SM, Schmitz-Schumann M, et al. Enhanced expression of cyclo-oxygenase isoenzyme 2 (COX-2) in asthmatic airways and its cellular distribution in aspirin-sensitive asthma. Thorax 1997;52(11):940.
[47] Ishiura Y, Fujimura M, Ishiguro T, Ohkura N, Myou S. COX-2 inhibition attenuates cough reflex sensitivity to inhaled capsaicin in patients with asthma. J Investig Allergol Clin Immunol 2009;19(5):370.
[48] Cheng DS, Han W, Chen SM, Sherrill TP, Chont M, Park GY, et al. Airway epithelium controls lung inflammation and injury through the NF-κB pathway. J Immunol 2007;178(10):6504.
[49] Blackwell TS, Christman JW. The role of nuclear factor-κ B in cytokine gene regulation. Am J Respir Cell Mol Biol 1997;17(1):3.
[50] Brasier AR. The NF-κB regulatory network. Cardiovasc Toxicol 2006;6(2):111.
[51] Lee JI, Burckart GJ. Nuclear factor kappa B: important transcription factor and therapeutic target. J Clin Pharmacol 1998;38(11):981.
[52] Bhavsar P, Hew M, Khorasani N, Torrego A, Barnes PJ, Adcock I, et al. Relative corticosteroid insensitivity of alveolar macrophages in severe asthma compared with non-severe asthma. Thorax 2008;63(9):784.
[53] Wei M, Chu X, Guan M, Yang X, Xie X, Liu F, et al. Protocatechuic acid suppresses ovalbumin-induced airway inflammation in a mouse allergic asthma model. Int Immunopharmacol 2013;15(4):780.
[54] Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-κB and IκB proteins: implications in cancer and inflammation. Trends Biochem Sci 2005;30(1):43.
[55] Saklatvala J. The p38 MAP kinase pathway as a therapeutic target in inflammatory disease. Curr Opin Pharmacol 2004;4(4):372.