



An inhibitor peptide of toll-like receptor 2 shows therapeutic potential for allergic conjunctivitis



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ABSTRACT

Allergic conjunctivitis (AC) is an inflammatory disease of the conjunctiva, which is characterized by antigen challenge and toll-like receptor 2 (TLR2) activation. Here, a designed small peptide ZY12 was found to contain therapeutic potential in staphylococcal enterotoxin B (SEB)-induced AC model. ZY12 treatment showed the remission of clinical signs, plasma total IgE levels, number of mast cells and the proportion of degranulated mast cell in AC mice. Levels of Th2 cytokines (IL-4, IL-5, IL-13) in the lymph nodes or spleen were significantly decreased while those of Th1 cytokine (IFN- γ) were increased in ZY12 treated group, suggesting a protective role of ZY12 in AC by mediating the balance of Th1/Th2 cytokines. Importantly, ZY12 significantly inhibited TLR2 expression in conjunctival tissue. Combined its therapeutic effects with TLR2 inhibitory function, ZY12 might be an ideal candidate for the development of new therapeutic agent for allergic disease.

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1. Introduction

Allergic conjunctivitis (AC) is a common form of ocular allergy, as it affects nearly one-third of the world population [1]. Due to the prevalence of AC, it is a heavy burden on society and a great challenge to the healthcare resources [2]. AC affects the ocular surface, including the eyelid, conjunctiva and/or cornea, leading to the signs and symptoms that most commonly accompany with eye redness and itching and affecting visual function [3]. These clinical symptoms, if not treated properly, impair patients' quality of life.

As an inflammation condition of the conjunctiva tissue, AC is triggered by IgE-mediated and/or non-IgE-mediated allergic responses to allergens interacting with TLR2 [4,5]. Furthermore, AC is mediated by Th2-type cytokines and the role of *Staphylococcus aureus* colonization in the pathophysiology of chronic allergic conjunctivitis has also been suggested [6,7]. Patients with atopic dermatitis frequently experience

colonization with *S. aureus* that has been identified as a trigger factor [8–10]. The products of *S. aureus*, such as staphylococcal enterotoxin (SE), toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxin, are correlated with allergic diseases. Bunikowski et al. reported that 80% patients with AC had SEA- or SEB-specific IgE antibodies in the plasma [11]. Staphylococcal enterotoxin B (SEB) reportedly increases eosinophilic cell number, which is the hallmark of allergic disease [12]. Activation of TLR2 signal by *S. aureus* induced Th2-type immune responses and accelerated experimental allergic conjunctivitis [6], suggesting that antagonism of TLR2 signal is a target to treat AC. More than 20 small antimicrobial peptides (AMP) have been designed in our previous work [13]. Among them, only ZY12 was found to exert inhibitory effect on TLR2 expression. In this study, the small peptide ZY12 was found to exert therapeutic potential for allergic conjunctivitis by inhibiting TLR2 expression.

2. Materials and methods

2.1. Peptide design and synthesis

ZY12 (VKRWKWKWKWKWKV-NH₂) was synthesized by solid phase synthesis on an Applied Biosystems model 433A peptide synthesizer by GL Biochem (Shanghai) Ltd. (Shanghai, China) and analyzed by

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reversed phase high performance liquid chromatography (RP-HPLC) and mass spectrometry to confirm the purity >98%.

2.2. Assays of hemolysis and cytotoxicity

Hemolysis assay was performed using human red blood cells in liquid medium according to our previous reports [14,15]. Briefly, serial dilutions of ZY12 were incubated with human red cells at 37 °C. After incubation for 30 min, the cells were centrifuged and the supernatant was removed. The released hemoglobin was then measured at 540 nm using spectrophotometer. Maximum hemolysis was determined by substituting the sample with 1% Triton X-100, which completely lyses the cells instantaneously. Hemolysis of ZY12 was calculated as the percentage of that of Triton X-100.

Human HEK293T cell and mouse RAW264.7 cell (cultured in Dulbecco's modified Eagle's medium, DMEM, Gibco) were used to evaluate the cytotoxicity of ZY12 in vitro. Two hundred microliter of cell suspension (1×10^5 cells/ml) was seeded in a 96-well plate (Nunc, Wiesbaden, Germany) and then cultured for 12 h. Series dilutions of ZY12 were added to the wells and the cells were then cultured for 24 h. After treatment by testing sample, 20 μ l of 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) (5 mg/ml) was added to each well. The MTT solution was then removed after a 4-h incubation and 200 μ l of dimethyl sulfoxide (DMSO) was added to solubilize the MTT-formazan crystals in living cells. The absorbance was recorded at 570 nm by using the microplate spectrophotometer system (Epoch, BioTek, USA). The experiments were performed in triplicate.

2.3. Allergic conjunctivitis model and treatment protocols

Eight-week-old female Balb/c mice were purchased from the Laboratory Animal Center of Kunming Medical University. The animals were maintained in specific pathogen-free conditions on a 12-h light-dark cycle with controlled temperature (25 °C) and humidity (55%).

Mice were immunized by intraperitoneal injection of 1 μ g of ovalbumin (OVA, Sigma) and 1.5% aluminium hydroxide adjuvant diluted in 200 μ l of sterile saline twice a week according to the reported model with modifications [16,17]. Mice were then challenged with direct instillation of 250 ng of SEB (Sigma) in 2.5 μ l of sterile saline or vehicle (sterile saline) three times daily from post-immunization days 15 to 18. The mice were divided into 6 groups ($n = 8$): (1) normal group (untreated); (2) vehicle group (SEB challenge); (3) positive group (eye drops with 10 μ l of 0.1% olopatadine (Sigma) after SEB challenge); (4)–(6) ZY12-treated groups (eye drops with 2.5, 5 and 10 mg/kg ZY12 in 10 μ l of sterile saline after SEB challenge, respectively). All treatments were with direct instillation and were started 24 h after the last SEB challenge. Treatment was performed twice a day (morning and night) from days 19 to 25. Twenty four hours after the last treatment was administered, mice were anaesthetized to obtain whole blood from the eyeball using a 1.5-ml tapered plastic centrifuge tube for analysis of total serum IgE and anti-OVA IgE levels. The animals were then euthanized and the eyes, cervical lymph nodes and spleen were harvested. All procedures were carried out in accordance with the Animal Care and Use Committee at Kunming Institute of Zoology, Chinese Academy of Sciences (Permit Number: 33-2397).

2.4. Clinical score in AC

To assess the therapeutic efficacy of ZY12, mice were examined for the occurrence and severity of conjunctivitis before the mice were euthanized. Physical and slit lamp biomicroscopic ocular examinations were performed. Three clinical signs were observed, conjunctival hyperemia, conjunctival edema and tearing. According to the methods reported by Magone et al. [18], each symptom was scored on a scale of 0–3 (0 = absence, 1 = mild, 2 = moderate, and 3 = severe symptoms). The

sum of the individual scores range from 0 to 9, and the data were expressed as the mean $\pm$ SD for each group.

2.5. Determination of total serum IgE and anti-OVA IgE levels

Clotted blood was centrifuged at 3000 g for 10 min to obtain the serum. Total IgE and anti-OVA IgE in the serum in different experimental groups were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits (Dakewe, Beijing, China) according to the manufacturer's instructions. Each determination was made in triplicate and the data were reported as mean $\pm$ SD.

2.6. Histopathological assessment

The eyeballs together with the conjunctiva tissue from each group were resected and fixed in 10% buffered formalin. Tissues were dehydrated, fixed, embedded in paraffin wax, sliced into 6 μ m-thick section, and then the mast cells was stained with aldehyde fuchsin (Maixin, Bio, China). For histopathological assessment, six random sections in each slice were examined and the number of mast cells and degranulated mast cells was counted under a light microscope with a magnification of 400. The percentage of degranulated mast cells was calculated according to the following formula: (the number of degranulated mast cells / the number of mast cells) $\times$ 100%.

2.7. Analysis of Th1/Th2 cytokines in cervical lymph nodes and spleen

To demonstrate the effect of ZY12 on allergic responses in the AC model, the mice in each group were soaked for 3 to 5 min in 75% ethanol after sacrifice by cervical dislocation, and then the cervical lymph nodes and spleen were removed and washed three times using sterile saline. The cervical lymph nodes and spleen were pulverized using a rubber syringe plunger and the cell suspension was then obtained by pushing through a 200-mesh sterile trap valve. Subsequently, the cell suspension was centrifuged at 3000 rpm for 20 min at room temperature to discard supernatant. Resuspension was performed by applying 100 μ l of sterile saline to the precipitated cells. After washing the cells again by centrifugation, the cells were ultimately re-suspended with RPMI-1640 medium (containing 10% fetal bovine serum, 100 μ g/ml streptomycin, and 100 U/ml penicillin, Gibco Life Technologies). The cells (5×10^6 cells/well in 1 ml) were then seeded in a 24-well plate and cultured at 37 °C in a humidified 5%-CO₂ atmosphere for 72 h. The supernatants were then collected and the cytokine levels in the supernatants were determined by using ELISA kits (Dekewe, Beijing, China) for IL-4, IL-10, IL-17 and IFN- γ .

2.8. THP-1 cells culture and stimulation

Human monocytic THP-1 cells (1×10^6 cells/ml, Shanghai Caoyan Biotechnology Co. Ltd., China) were cultured in 6-well plates containing serum-free medium (RPMI 1640 medium, Gibco Life Technologies). For stimulation, THP-1 cells were pre-challenged with SEB (2 μ g/ml) for 2 h, and then incubated with ZY12 (0–200 μ g/ml) for 36 h to assess TLR2 expression.

2.9. Western blot analysis

Conjunctival tissue pellets from mice were treated for 10 min on ice with 50 μ l of RIPA lysis buffer (1% Triton X-100 (v/v), 0.5% deoxycholic acid (w/v), 10 mM EDTA in PBS) containing protease inhibitor cocktail. After brief sonication, the samples were centrifuged at 13,000 rpm for 10 min at 4 °C, and the supernatants were stored at –80 °C. Protein concentration was determined by Bradford assay. To detect TLR2 and mitogen-activated protein kinases (MAPKs), 40 μ g of protein extracts were firstly separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene

difluoride membrane. The immunoblots were incubated with 10 ml of PBS with 0.1% Tween 20 (PBST) containing 5% skim milk at room temperature for 1 h, and then incubated overnight with a primary antibody against the phosphorylated and total Erk1/2, SAPK/JNK and p38 MAPK (Abcam, Cambridge, USA) at 4 °C, respectively. A primary antibody against TLR2 (Abcam, Cambridge, USA) was used to detect the total expression of TLR2. Followed by 15 min of washing with Tween 20/Tris-buffered saline (TBST), the blots were incubated at room temperature for 1 h with horseradish peroxidase conjugated secondary antibody (1:1000, Abcam, Cambridge, USA). Blots were developed by enhanced chemiluminescence (Tiangen Biotech, Beijing, China) after washing for three times with TBST.

2.10. Statistical analysis

Statistical analysis was performed using Graph pad prism 5 statistical software. Statistical differences between the means of various groups were evaluated using one way analysis of variance (ANOVA) followed by turkey's test. Data were considered statistically significant at $p < 0.05$.

3. Results

3.1. ZY12 shows no hemolytic and cytotoxic activities

Hemolysis is one of toxic effects of some antimicrobial peptides (AMPs) and it greatly hinders the application of them. Initially, the hemolytic and cytotoxic activities of ZY12 were tested. As shown in Fig. S1A, ZY12 showed no hemolytic activity on human red blood cells at serious concentrations from 50 to 320 mg/ml. In addition, no significant cytotoxicities against human HEK293T embryonic kidney cells (Fig. S1B) and mouse RAW264.7 cells (Fig. S1C) were observed after treatment by ZY12 at various concentrations from 12.5 to 100 μ g/ml.

3.2. Clinical scores of AC are reduced by ZY12 treatment

The total scores for symptoms of AC were measured before the mice were euthanized on the day 26. As illustrated in Fig. 1A, the mice sensitized with OVA followed by SEB challenge significantly displayed more severe symptoms than that of normal mice, suggesting a successful AC model was constructed. After a 7-day treatment with ZY12, the clinical signs of AC were significantly attenuated ($p < 0.01$) in a dose-dependent manner. The clinical scores were reduced to 6.13 ± 2.38 , 5.13 ± 0.71 and 3.25 ± 0.72 by 2.5, 5 and 10 mg/kg of ZY12, respectively, while that in vehicle group is ~ 7.5 . The clinical score of AC (4.75 ± 1.41) was also reduced by olopatadine, which is a well-known histamine release inhibitor and has been proved to be an effective drug for AC.

3.3. Effects of ZY12 on total IgE and OVA-specific IgE levels in serum of the AC model

To further investigate the therapeutic effect of ZY12, total IgE and OVA-specific IgE levels in serum of the AC model were evaluated. As shown in Fig. 1B, the total serum IgE level of SEB challenged group (vehicle group) was markedly increased (2.01 ± 0.11 μ g/ml) compared with control group (0.34 ± 0.04 μ g/ml), also supporting the success of the experimental model. Pharmacological treatment by different doses of ZY12 significantly decreased the level of IgE (1.92 ± 0.05 , 1.55 ± 0.08 and 0.98 ± 0.07 μ g/ml, respectively) in the plasma of mice. The olopatadine administration showed no significant effect on the serum total IgE level. There was no significant change in the serum OVA-specific IgE level in all the 6 groups (Fig. 1C).

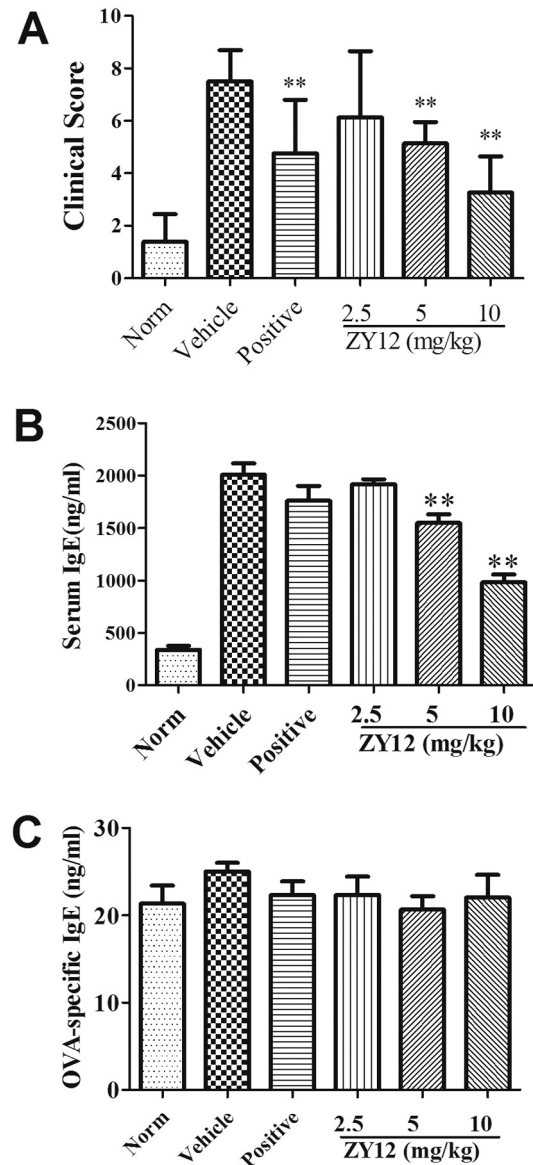


Fig. 1. Effects of ZY12 on clinical score and serum IgE levels in AC mice. (A) Effect of ZY12 on clinical score of AC. (B) Total serum IgE level. (C) OVA-specific IgE level in serum. Norm: without OVA; Vehicle: OVA + SEB; Positive: OVA + SEB + olopatadine; ZY12 group: OVA + SEB + ZY12 (2.5, 5, or 10 mg/kg). The results are given as the mean $\pm$ SE for eight mice in each group. * $p < 0.05$, ** $p < 0.01$.

3.4. The number of mast cells and mast cell degranulation are reduced by ZY12 administration

Since AC is characterized by allergen-specific IgE in the serum and infiltration of mast cells into the conjunctiva, the number of mast cells and mast cell degranulation was determined. The number of mast cells in the vehicle group was higher than that in normal group, and after ZY12 or olopatadine treatment, the number of mast cells was decreased (data not shown). The histological analysis revealed that mast cell degranulation was exacerbated in SEB-treated sensitized mice by comparing with the normal mice, and ZY12 or olopatadine treatment ameliorated the mast cell degranulation (Fig. 2A–2F). Counts of the degranulated mast cells also demonstrated the therapeutic effect of ZY12 treatment and olopatadine treatment (Fig. 2G). The percentage of degranulated mast cells in ZY12-treated group at dose of 5 (39.66 ± 8.02) or 10 mg/kg (32 ± 6.24) was much smaller than that

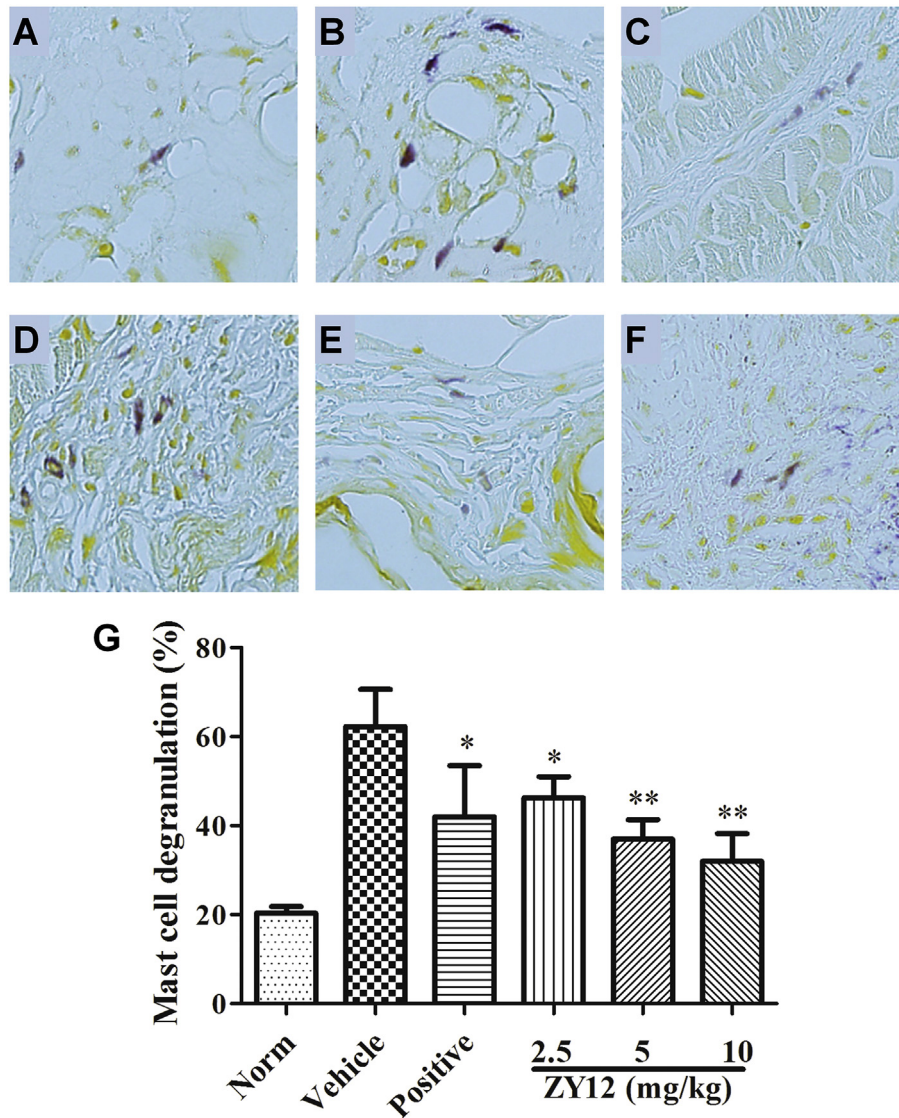


Fig. 2. Effects of ZY12 on mast cell degranulation in conjunctival tissue. Mast cell degranulation in conjunctival tissue from different group was determined by using aldehyde fuchsin staining. (A): Norm; (B): Vehicle; (C): Positive; (D): ZY12 (2.5 mg/kg); (E): ZY12 (5 mg/kg); (F): ZY12 (10 mg/kg). (G) The percentage of mast cell degranulation in conjunctival tissue was compared with those observed in vehicle group. (A)–(F) Magnification: 400 $\times$. The data are presented as the mean $\pm$ SE for eight mice in each group (* p < 0.05, ** p < 0.01).

in olopatadine treated group (42 ± 11.53) (Fig. 2G), suggesting a better curative effect of ZY12.

3.5. ZY12 regulates Th1/Th2 cytokines production during allergic conjunctivitis

T-helper lymphocytes (Th cells) are main T cell type in inflamed conjunctiva, and an altered balance between Th1 and Th2 cells, and between Th1 and Th2 cytokines are thought to play an important role in the development of allergic disorders [19,20]. After challenged by SEB, the levels of IL-4, IL-5 and IL-13 in cervical lymph nodes (Fig. 3) and spleen (Fig. 4) of AC mice were markedly increased by 2–4 times compared with the normal group. However, the IFN- γ level was decreased by 40–60%. ZY12 treatment suppressed the upregulation of IL-4, IL-5 and IL-13 in cervical lymph nodes and spleen, but increased IFN- γ expression in a dose-dependent manner. Olopatadine also inhibited IL-4 and IL-5 secretion in cervical lymph nodes and spleen, but no significant alteration was detected in the IL-13 and IFN- γ levels.

3.6. ZY12 inhibits TLR2 expression and MAPKs activation

To further investigate the mechanisms underlying the effects of ZY12 treatment in AC, Western blot analysis was performed to assess the regulation of ZY12 on TLR2 expression and phosphorylation of ERK, JNK and P38 MAPK using pooled extracts of conjunctival tissue. As illustrated in Fig. 5A, ZY12 treatment markedly down-regulated the TLR2 expression induced by SEB in conjunctival tissue in a concentration-dependent manner. The TLR2 expression was decreased by ~41.6, 88.2, and 95.1% with 2.5, 5, and 10 mg/kg ZY12 treatment, respectively (Fig. 5C). ZY12 treatment also decreased phosphorylated-ERK in a dose-dependent manner (Fig. 5A and E) while it showed no significant influence on the p-JNK expression and p-p38 expression. In contrast, olopatadine had no effect on TLR2 expression and the phosphorylation of ERK, JNK and P38 MAPK (Fig. 5).

To further clarify the cell types the peptide may target and how does it interact with the cells, human leukemic cell line (THP-1) was chosen to investigate the effects of ZY12 on TLR2 expression. In line with a decreasing effect of ZY12 on TLR2 expression in AC, significant decrease of TLR2 was observed by Western blot after administration of ZY12 in a

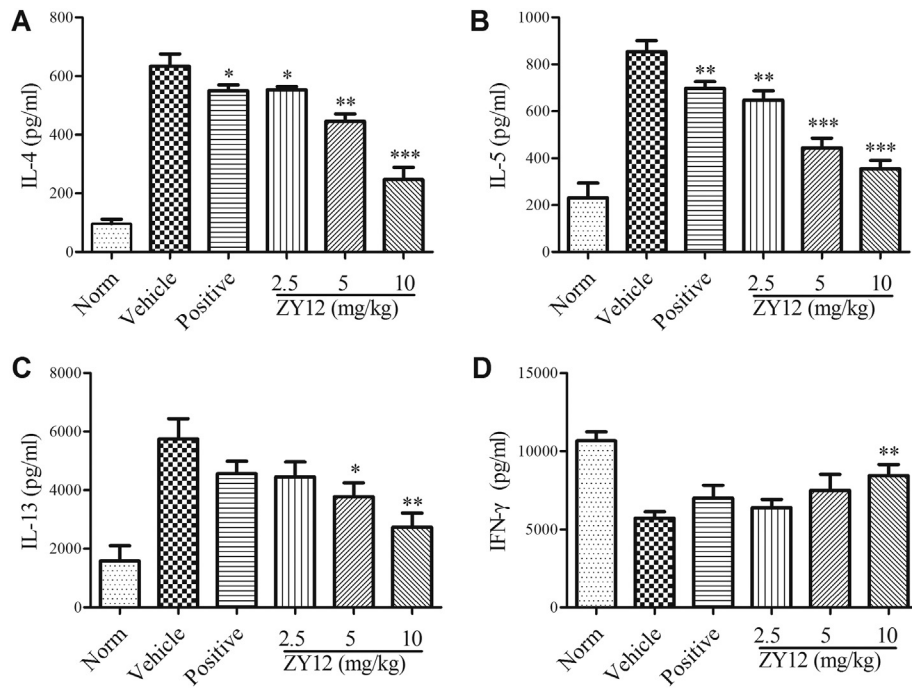


Fig. 3. Effects of ZY12 on IL-4 (A), IL-5 (B), IL-13 (C) and IFN-γ (D) production in mouse cervical lymph node. The graphs present mean value $\pm$ SE ($n = 8$). * $p < 0.05$, ** $p < 0.01$ versus vehicle group.

dose-dependent manner in THP-1 (Fig. 6). These data suggest that ZY12 directly affects TLR2 expression, and the inflammatory mononuclear cell is the main cell type the peptide may target.

4. Discussion

Almost all of therapeutic treatments of AC have side effects, such as antihistamines, mast cell stabilizers, corticosteroids, nonsteroidal anti-inflammatory drugs and immunomodulatory agents. Some allergen-specific immunotherapies could be rendered ineffective due to more

or less the side effects [21]. Thus, the discovery of new pharmacological agents that have high efficacy in controlling the inflammatory response is urgently needed for AC treatment. Considering the important role of TLR2 activation in AC [6], the inhibition of TLR2 function might be a promising selection to treat AC.

We identified a peptide inhibitor (ZY12) of TLR2 expression and evaluated its therapeutic potential and the underlying mechanism of action in mouse AC model induced by SEB. As reported in previous literatures [18,22], current AC model showed significant clinical signs of conjunctivitis as well as increased plasma levels of total IgE and OVA-

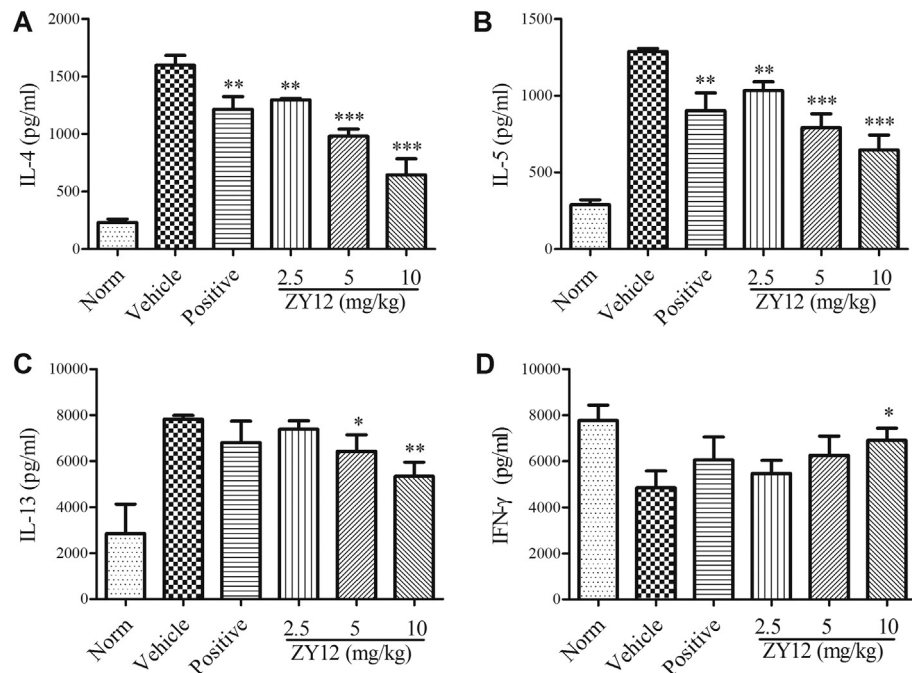


Fig. 4. Effects of ZY12 on IL-4 (A), IL-5 (B), IL-13 (C) and IFN-γ (D) production in mouse spleen. The graphs present mean value $\pm$ SE ($n = 8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus vehicle group.

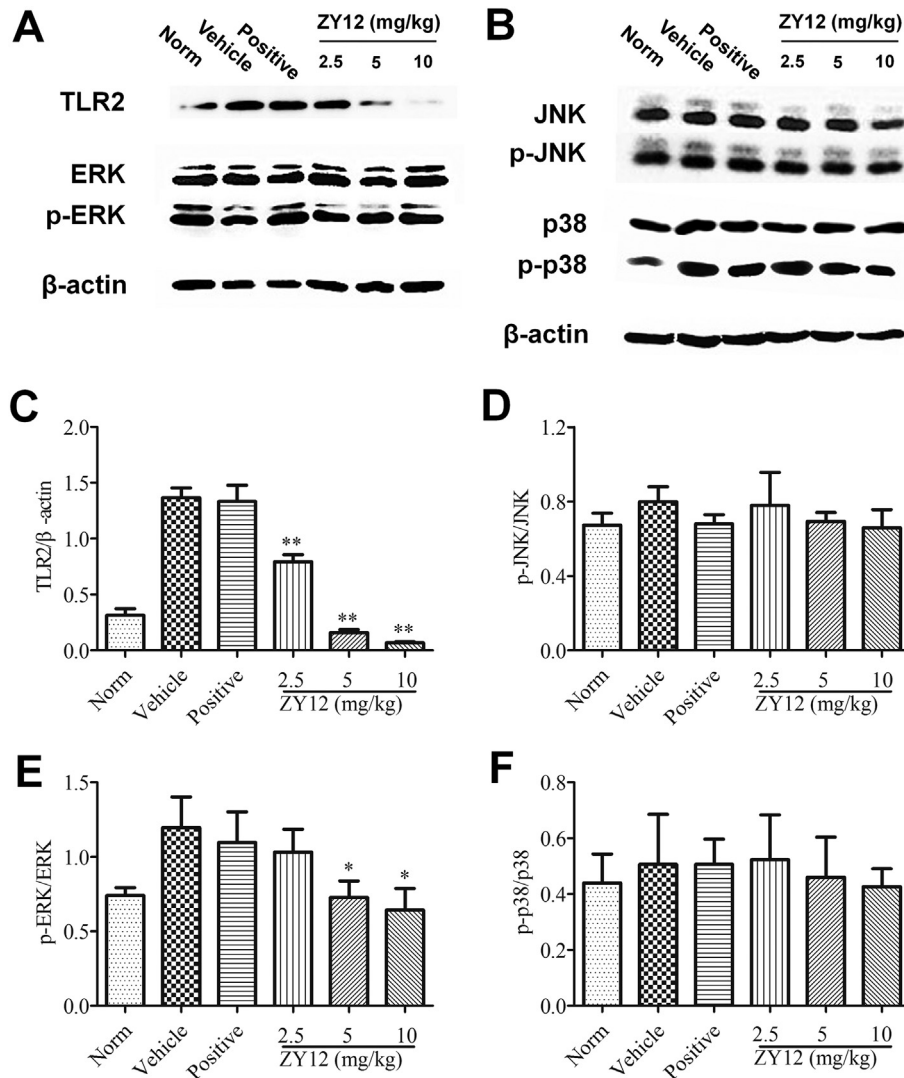


Fig. 5. Effects of ZY12 on TLR2 expression, phosphorylation of ERK, JNK and p38 in mouse conjunctival tissue. (A) Western blot showed effects of ZY12 on TLR2 expression and ERK phosphorylation. (B) Phosphorylation levels of JNK and p38 were measured by Western blot. Densitometry data were then measured using Image J. (C). Relative expression of TLR2 was normalized to β -actin. Densitometry of phosphorylated JNK (D), ERK (E) and p38 (F) were normalized to total JNK, ERK and p38, respectively. Results of a representative experiment out of three are shown. β -Actin was used as loading control for Western blot analysis. Data are presented as mean $\pm$ SE of three independent experiments. * $p < 0.05$, ** $p < 0.01$ versus vehicle group.

specific IgE (Fig. 1). IgE-mediated conjunctival allergic reaction induces an early reaction and degranulation of ocular mast cells, leading to release of inflammatory mediators in allergic conjunctivitis [23–25]. ZY12 exhibited effective anti-allergic activity with a significant decrease in physical score of AC associated with decreased plasma level of total IgE in a concentration-dependent manner. The ocular allergic response checked by histopathology in the conjunctiva tissue of SEB challenged mice was associated with a significant increase in the number of mast cells and the proportion of degranulated mast cells (Fig. 2). This is consistent with other AC models induced by different antigens [26–29]. ZY12 treatment markedly decreased the number of mast cells and the proportion of degranulated mast cells (Fig. 2), suggesting ZY12 was effective on inhibition of mast cell infiltration and activation.

Chronic pathologic processes of allergic conjunctivitis are thought to be a Th2-mediated disease due to abundant systemic levels of IgE and cytokines such as IL-4, IL-5 and IL-13 [23,24,30]. The levels of cytokines (IL-4, IL-5 and IL-13) in the lymph nodes and spleen were markedly increased in SEB treated mice (Figs. 3 and 4). ZY12 exhibited anti-allergic effects by reducing the levels of IL-4, IL-5 and IL-13 in both lymph nodes and spleen (Figs. 3 and 4), which was consistent with the negative regulation of total IgE level and significant improvement in clinical scoring

(Fig. 1). On the other hand, counterbalance in the production of Th1 cytokine (IFN- γ) is crucial in the regulation of allergic responses [31]. Indeed, ZY12 treatment increased IFN- γ production in mouse lymph nodes and spleen. The results suggest a protective role of ZY12 in AC by regulating the balance of Th1/Th2.

Traditionally, TLRs recognize conserved microbial products as ligands or agonists such as lipoproteins, PGN (peptidoglycan) and LTAs [32]. TLR2 found in conjunctival epithelial cells playing a role in the recognition of Gram-positive bacteria at human ocular surface, which recognizes bacterial products in combination with TLR1 or TLR6. Western blot analysis revealed that ZY12 significantly reduced the expression of TLR2 in conjunctivitis tissue (Fig. 5A and C). This result is consistent with previous reports that the decreased level of TLR2 attenuates allergy inflammation [33,34]. MAPKs are well-conserved signaling pathways that include JNK, ERK and p38 proteins, and they have been implicated in allergic responses [35]. ZY12 decreased ERK phosphorylation in conjunctivitis tissue (Fig. 5A and E). Recent studies suggest that the reduction of phosphorylation of ERK is correlation with an alleviation of allergic inflammatory response [36,37]. ZY12 may mitigate inflammation by inhibiting the MAPKs through down-regulation of phosphorylation of ERK.

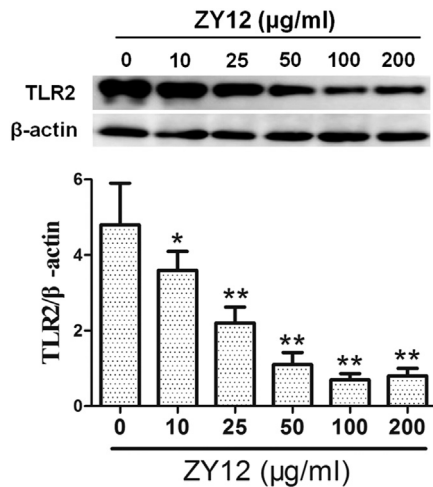


Fig. 6. Effect of ZY12 on TLR2 expression in THP-1. THP-1 was firstly treated by SEB (2 μg/ml) and then incubated with ZY12 (0–200 μg/ml). TLR2 expression was measured by Western blot (top) and relative expression of TLR2 was normalized to β-actin (bottom). * $p < 0.05$, ** $p < 0.01$.

Collectively, the present study suggests a potential immunoregulatory activity of ZY12 by acting on TLR2 in SEB-induced mouse AC model. Treatment with ZY12 negatively regulated the development of clinical signs of conjunctivitis by inhibiting TLR2 expression and ERK phosphorylation in AC model. The present study identifies ZY12 as an ideal candidate and/or template for the development of new therapeutic agents for AC.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.intimp.2017.02.024>.

Conflict of interest

The authors have no conflicts of interest.

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