• Basic Research •

Ragweed pollen induces allergic conjunctivitis immune tolerance in mice *via* regulation of the NF-κB signal pathway

Meng-Tian Bai[1,2,3,4,5,6], *Yun Li*[7], *Zhu-Lin Hu*[1,2,3,4,5]

[1]Department of Ophthalmology, Fourth Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan Province, China
[2]Yunnan Eye Institute, Kunming 650032, Yunnan Province, China
[3]Key Laboratory of Yunnan Province for the Prevention and Treatment of Ophthalmology, Kunming 650032, Yunnan Province, China
[4]Provincial Innovation Team for Cataract and Ocular Fundus Disease, Fourth Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan Province, China
[5]Expert Workstation of Yao Ke, Kunming 650032, Yunnan Province, China
[6]Department of Ophthalmology, Suining Central Hospital, Suining 629000, Sichuan Province, China
[7]Department of Oncology, Suining Central Hospital, Suining 629000, Sichuan Province, China
Co-first authors: Meng-Tian Bai and Yun Li
Correspondence to: Zhu-Lin Hu. Department of Ophthalmology, Fourth Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan Province, China. hzl77@263.net
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Abstract

● **AIM:** To investigate the feasibility and mechanism of immune tolerance in allergic conjunctivitis.

● **METHODS:** The allergic conjunctivitis immune tolerance mice model was established by ragweed pollen (RW) and the related cytokines were detected. The mice were divided into 9 groups and the maslinic acid (MA) or PBS were given for different group after modeling. The expression levels of chemokine ligand 5 (CCL5) and P-65 in the conjunctival tissue were analyzed by immunohistochemistry, quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blot. The percentage of interleukin-17 (IL-17) and CD4+CD25+ in the splenocyte supernatant was analyzed by flow cytometry. Furthermore, the serum and splenocyte supernatant concentration of total-IgE, interleukin-10 (IL-10), and IL-17 was analyzed by enzyme linked immune response (ELISA).

● **RESULTS:** After the model was established, symptoms of conjunctivitis were alleviated, the level of P-65, CCL5, IL-17, and total-IgE was raised, while the expression of IL-10, CD4+CD25+ was decreased. This result fully demonstrated that a typical IL-17/regulatory-T-cells (Treg cells) imbalance and NF-κB activation. When the NF-κB signal pathway was suppressed, it showed that there was a further relief of conjunctivitis in mice. At the same time, the expression of total-IgE, IL-17, and CCL5 was decreased and the expression of anti-inflammatory factor (IL-10, CD4+CD25+) was increased.

● **CONCLUSION:** In the state of immune tolerance, symptoms of conjunctivitis in mice are alleviated, the Th-17 cells of allergic conjunctivitis mice are inhibited, and Treg cells activity is enhanced.

● **KEYWORDS:** allergic conjunctivitis; immune tolerance; TH-17 cell; Treg cell; NF-κB signal pathway

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INTRODUCTION

Allergic conjunctivitis is not only prone to recurrence and could trigger allergic diseases. It can also seriously interfere the lifestyle and custom of patients. At present, it is considered that allergic conjunctivitis is closely related to antigen-specific IgE-mediated I-type hypersensitivity and specific T-cell-mediated IV-type hypersensitivity. After the mastocyte is activated, the antigen binds to the IgE on the FcεRI, resulting in the early phase reaction that mastocyte was degranulated and release a series of cytokines. Subsequently, the inflammatory factors were release by activated eosinophils, the factors include eosinophil negative protein and eosinophil peroxidase. On the other hand, Th-2 cells can secrete interleukin (IL)-4, IL-5, and IL-13, which will aggravate the eye and whole body allergic reaction. This phase was named as late phase reaction, which will cause the damage of

conjunctival tissue and corneal tissue[1]. It has been confirmed that antihistamine, mastocyte stabilizer and glucocorticoid are effective in the treatment of allergic conjunctivitis, but the side effects caused by these drugs are avoidless, which makes the treatment of allergic conjunctivitis mainly free from allergen and remission of symptoms[2].

The whole body mucosa, including conjunctival tissue, respiratory epithelial tissue, intestinal epithelial tissue, and subcutaneous tissue needs to maintain the balance of promoting inflammation and inhibiting inflammation when exposed to antigen, so as to achieve the immune steady state. Mucosal tissue can react strongly to external antigens, but at the expense of tissue damage. However, the self-protection effect of the body can be achieved by raising the threshold of allergic reaction, this effect is called mucosal immune tolerance[3], which is an innate and adaptive peripheral immune tolerance state. That is, a low response or no response state of local and systemic immune responses induced by antigens recognized as "harmless" when it through mucous membranes[4].

Continuous antigen exposure can obstruct the functions of dendritic cells, effector T cells, and reduce the local and systemic reactivity to antigens, thus forming an immune tolerance state. When the body is in this state, Treg cells can migrate to target tissues and release immune regulatory cytokines, such as IL-10, which is a powerful immune and inflammatory inhibitor and can interact with other immune cells. IL-10 plays an important role in reduce adhesion ability of monocytes, inhibit the function of monocytes, and reduce the expression of major histocompatibility complex II and tumor necrosis factor-α (TNF)-α. In recent years, it has been found that Treg's immune tolerance function is also associated with its expression of cytotoxic T lymphocyte-associated antigen 4 (CTLA4) and procedural cell death protein 1 (PD1)[5].

NF-κB belongs to a homodimer composed of Rel. In eukaryotes, its components include NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), c-Rel and RelB. They can be expressed in oligodendrocytes, neuronal cells, lymphoid cells[6]. NF-κB signal pathway is inactive in the cytoplasm when it binds to the inhibitors of NF-κBs and becomes a stable trimer[7]. When the NF-κB signal pathway is activated, Ikk induces the inhibitors of NF-κBs phosphorylation, which leads to trimer dissociation. Then, the nuclear localization sequence of NF-κB dimer was exposed and it rapidly transferred into the nucleus to bind to the specific sequence on the DNA so that it promotes the transcription of the target gene[8]. Previous studies have shown that the expression of interleukin-1β (IL-1β), nterleukin-6 (IL-6), TNF-α and chemokine (CCL1, CCL5, CCL11) in epithelial cells and monocytes of patients with allergic asthma is significantly increased, and the activation of NF-κB is the common mechanism of the high expression of these factors[9]. Although the NF-κB has proved to act on an extremely important role in the fields of autoimmune disease, tumorigenesis and organ transplantation, the function of the NF-κB signal pathway in the immune tolerance mice of allergic conjunctivitis is known less.

In our research, we focused on the function of the NF-κB signal pathway in allergic conjunctivitis tolerance mice. Our data revealed that the suppression of the NF-κB signal pathway could induce by maslinic acid (MA). It may be a one of the candidate locus for the therapy of allergic conjunctivitis in our upcoming research.

MATERIALS AND METHODS

Ethical Approval This research was legally approved by the Animal Care & Welfare Committee of Kunming Medical University (kmmu2019002). The study was conducted adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Animals The 54 Balb/c newborn mice (half male and half female, 3-4 weeks old) were purchased from Kunming Medical University (SCXK k2015-0002) and raised in the individual ventilated cages system.

Grouping and Ragweed Pollen Injection The 54 mice were divided into 9 groups, including negative control (NC) group, positive control (Control) group, immune tolerance (IT) group, NC+PBS group, NC+MA group, Control+PBS group, Control+MA group, IT+PBS group, IT+MA group, 6 in each group. For IT, IT+PBS, and IT+MA group, to induce immune tolerance status in mice, ragweed pollen (RW; 5 mg/kg, Greer Labs, USA) was inject subcutaneously into each mouse once every other day from 1 week of age until 3 weeks of age. For the mice in Control, Control+PBS, Control+MA, IT, IT+PBS, and IT+MA group, when mice were 7 weeks old, RW (25 mg/kg) were dissolved in 10 μL aluminum hydroxide adjuvant (Thermo, USA) and then injected intraperitoneally for each mouse. When mice were 8 weeks old, each mouse was drip into both eyes for 3d with RW (10 mg/kg, Greer Labs, USA) dissolved in 10 μL of aluminum hydroxide adjuvant (Thermo, USA). For the mice in NC, NC+PBS, and NC+MA group, saline with equal mass fraction was given in the same way.

PBS and Maslinic Acid Injection On the day after modeling, the PBS (Thermo, USA) was injected into the abdominal cavity of mice in NC+PBS, Control+PBS, and IT+PBS group, the MA (MCE, China, 200 mg/kg) was injected into the abdominal cavity of mice in NC+MA, Control+MA, and IT+PBS group.

Tissue and Serum Harvest The 20min after the last liquid was drip into eyes, we observed and recorded the change of mice' ocular surface in each group. Twenty-four hours after the last liquid was drip into eyes, mice were completely anesthetized and sterilized. After supine fixation, the mice

Table 1 All primers were synthesized by ThermoFisher

Target	Forward	Reverse
CCL5	5'-TGCCTAAAGTGTCACATTTTGCTCA-3'	5'-CTAAGGAGTGATACACCTCGTAGTTG-3'
P65	5'-GCATTCTG ACCTTGCCT ATCT-3'	5'-CTCCAGTCTCCGAGTGAAGC-3'
β-actin	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGT AACAATGCCATGT-3'.

eyeball was fully exposed to the operation microscope (OPMI Lumera 300, and the magnification rate was 4×). The conjunctival tissue at the limbal of the cornea was gently lifted with toothed forceps, and then the conjunctiva (2.5×3 mm^2) was cut off in parallel along the equator with ophthalmic scissors. Afterward, the chest of mice was opened with sterile scissors to expose the heart and spleen. Needle (1 mL) was inserted into the right heart and the blood was carefully drawn into a 1.5 mL centrifuge tube and placed in a 4℃ refrigerator for 24h. The blood was then centrifuged at 3500 revolutions per minute (rpm) for 15min at 4℃. The spleen was cut off and washed repeatedly by PBS and placed in a 10 mL petri dish. After that, it was ground with a sterile syringe plunger and the mixture was blown and mixed. The mixture then was transferred to the 200-meshes screen and ground again to collect the suspension. The supernatant was discarded after centrifugation (1000 rpm, 10min), and 5 mL of erythrocyte lysate (Biolegend, USA) was added to treat for 3min at room temperature. Subsequently, the tissues were centrifuged at 1000 rpm for 15min following by washing with PBS for 2 times and resuspended in 5 mL PBS (Thermo, USA) for further use.

Immunohistochemical Staining To analyse the level of CCL5 in conjunctival tissue, the conjunctival tissue samples which had been sliced and dewaxed were used for immunohistochemical staining. The sections were heated at 65℃, for 2h, and washing by PBS (Thermo, USA) for 3×5min. Then, repaired with EDTA buffer, and rinsed with PBS (Thermo, USA) for 3×5min after natural cooling. Incubated with the 3% H_2O_2 peroxide for 10min at room temperature followed by washing with PBS (Thermo, USA), 3×5min. After spin-drying, seal with 5% bovine albumin (BSA; Roche, Switzerland) for 20min. The sections were then incubated with CCL5 antibody (CST, USA, 1:300) overnight at 4℃ and washed with PBS (Thermo, USA) for 3×5min. Afterward, the sections were incubated with the secondary antibody (ASPEN, USA, 1:200) at room temperature for 30min. After repeating the process of washing with PBS (Thermo, USA) for 3 times, 50 μL diaminobenzidine (DAB) was used and color development was observed by microscope. These sections were then rinsed by tap water and double steamed water, and counterstained by hematoxylin (ASPEN, USA). The sections were rinsed with hydrochloric acid alcohol for 5min, and flush to return to blue. It was then dehydrated by gradient alcohol, cleared by xylene and mounted by neutral gum. The sections were observed and photographed using an optical microscope at 24h later.

Table 2 PCR was performed using the 12K flex

Step	Temperature (℃)	Time	No. of cycles
1	95	3min	1
2	95	15s	40
	60	34s	
3	95	15s	1
	60	1min	
	95	15s	

Quantitative Reverse Transcription Polymerase Chain Reaction The CCL5 and P65 mRNA relative level was detected through the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method. The experimental steps are as follows. Total RNA was extracted by conjunctiva bulbi through the trizol (TaKaRa, Japan) and its concentration and purity were determined. The RNA was reverse transcribed into cDNA by a reverse transcription kit (TaKaRa, Japan). All primers were synthesized by ThermoFisher (Table 1). mRNA expression levels were analyzed by TB Green QuantiTect RT-PCR Kit (TaKaRa, Japan). qRT-PCR was run on a QuantStudio 12K flex (ABI, USA; Table 2). The data were analyzed by the Ct method, and the relative expression was normalized through $2^{-\Delta\Delta Ct}$ method.

Western Blot Conjunctiva bulbi tissues were cleaned and treated for 30min with protein extraction reagent. Homogenates were then centrifuged at 12 000 rpm 5min at 4℃. Add 5× volume sample buffer and put it at 90℃ for 10min. Prepare the separation gel, shake well, and add a proper amount of water to flatten the rubber surface. After 45min, pour the upper layer of water and dry the residual water. After the concentrated glue is prepared, the mixture is added and inserted into the comb teeth. And adding the electrophoresis buffer solution after the comb teeth are pulled out and adding the sample to be tested. Constant pressure electrophoresis was carried out according to concentrated glue 80 V and separated glue 120 V. Place the membrane sponge, 3-layer filter paper, PVDF membrane, gel, 3-layer filter paper, and membrane sponge in order, and remove the bubbles. Proteins were electrotransferred onto PVDF membranes that were blocked in sealing fluid for 1h at room temperature. The blocking solution was then removed, and the diluted CCL5 rabbit mAb (CST, USA, 1:500) and P65 rabbit mAb (CST, USA) were added and overnight at 4℃. The

next day, the membrane was rinsed with TBST 3 times and incubated with secondary antibody (HRP-rabbit anti-mouse, ASPEN, USA) at 20°C for 20min. Finally, blots were rinsed, and the signal was detected by enhanced chemiluminescence (ECL; ASPEN, USA) using the LumiGlo substrate (Beyotime, Shanghai, China).

Enzyme-Linked Immune Response The detection methods of IgE, IL-10, IL-17 in serum and splenocyte supernatant are enzyme-linked immune response (ELISA). The list of ELISA kits is as follows: mice IgE ELISA kit, mice IL-10 ELISA kit, mice IL-17 ELISA kit. All these kits were purchased from ELK Biotechnology (Wuhan, China).

Flow Cytometry Briefly, 3 mL single-cell suspension of the spleen was added to the cell culture plate. The 3.6 mL Roswell Park Memorial Institute-1640 (RPMI 1640) medium (Thermo, USA), 0.4 mL fetal bovine serum (FBS; Roche, Switzerland), 10 μL cell activator (Biolegend, USA), and the cell activator (Biolegend, USA) were mixed and then placed in a cell incubator (Biolegend, USA). The 24h later, centrifuge it in 1200 rpm for 8min and discard the upper clearance. The mixture was resuspended with 1 mL of PBS (Thermo, USA), and 0.5 mL of the mixed solution was then added to the flow cytometry injection tube (tube 1, tube 2) respectively. And the residual liquid is suspended after centrifuged at 1200 rpm for 8min. Next, 5 μL of CD3-PerCP-CY5.5, CD4-FITC, CD8a-APC-CY7 is added into the tube 1 and placed it in room temperature for 30min. The 1 mL membrane breaking solution (Biolegend, USA) and 2 μL IL-17-PE was then added in turn, stored at room temperature for 30min and keep away from light. And then the PBS is added twice. For the first time, 1 mL was added and well mixed, centrifuged at 1200 rpm, 8min, and the supernatant was discarded, the residue was resuspended. For the second time, 200 μL was added and the detection was performed. For the tube 2, 5 μL CD3-PerCP-CY5.5, CD4-FITC, CD8a-APC-CY7, CD25-APC was added and centrifuged at 1200 rpm for 8min after placed it in room temperature for 30min. Discard the supernatant and resuspend the residue. The 1 mL PBS (Thermo, USA) was then added into and centrifugate as described above, the supernatant was removed and the residual liquid was resuspended. At last, 200 μL of PBS (Thermo, USA) was added and the data was detection. All these flow cytometry antibodies were purchased from Biolegend (USA).

Statistical Analysis The data were represented as the mean±standard deviation. ANOVA was used to compare the means of three or more groups. The comparison between the two groups was used by LSD. Two-way ANOVA was used for the analysis of the variance of the randomized block design between the PBS and the MA treatment group. $P<0.05$ was considered to indicate a statistically significant difference.

RESULTS

Immune Tolerance Mice Model Successfully Established by Ragweed Pollen To investigate the induction mechanism of immune tolerance in allergic conjunctivitis, we established the mice model of allergic conjunctivitis tolerance. Immunohistochemistry analysis showed that the level of CCL5 in the IT was lower than that in the Control (Figure 1A, $P<0.05$). The data of flow cytometry showed that the percentage of IL-17 among CD4+ cells in the IT was lower than that of the Control ($P<0.05$). And the content of CD4+CD25+ among CD4+ cells significantly increased after the modeling (Figure 1B, $P<0.01$). Otherwise, data of qRT-PCR and Western blot showed that the relative level of CCL5 mRNA, P65 mRNA, CCL5 protein and P65 protein in the conjunctiva of the IT was higher than that of Control (Figure 1C, $P<0.05$). The concentration of total-IgE, IL-10, and IL-17 in splenocyte supernatant and serum was detected by ELISA. It was found that compared with the Control, the concentration of total-IgE was lowered both in serum and splenocyte supernatant ($P<0.05$), the concentration of IL-17 also decreased in serum ($P<0.01$) and splenocyte supernatant ($P<0.05$). The concentration of IL-10 in the IT is higher than that in the Control in serum ($P<0.05$) However, there is no contrast in splenocyte supernatant (Figure 1D). These results suggest that after the establishment of the immune tolerance model of allergic conjunctivitis, the level of pro-inflammatory factors is down-regulated, and the level of anti-inflammatory factors is maintained or even increased. At the same time, we found that P65 was down-regulated, suggesting that immune tolerance may be related to NF-κB.

MA Inhibits NF-κB Signal Pathway MA can inhibit the DNA-binding activity of the NF-κB signal pathway and abolish the phosphorylation of IκB-α. To further clarify the role of the NF-κB pathway in the induction of immune tolerance of allergic conjunctivitis, we injected MA and PBS into the abdominal cavity of mice individually.

The mRNA and protein levels of CCL5 and P65 were analyzed by Western blot and qRT-PCR. The data revealed that the level of CCL5 mRNA and protein in the conjunctival tissue of the IT was lower than that of the Control after MA injection (Figure 2A, $P<0.05$). However, there was no significant change in the level of P65 mRNA and protein. Compared with the Control+PBS, the expression of CCL5 and P65 protein in the Control+MA were significantly reduced ($P<0.01$). Compared with the IT+PBS, the levels of CCL5 and P65 protein in the IT+MA were also significantly reduced (Figure 2B and 2C, $P<0.01$). We used ELISA to measure total-IgE concentration in serum and splenocyte supernatant after treated with MA and PBS. The result revealed that compared with the Control+PBS, the relative expression of total-IgE in the Control+MA was reduced (Figure 2D and 2E, $P<0.05$).

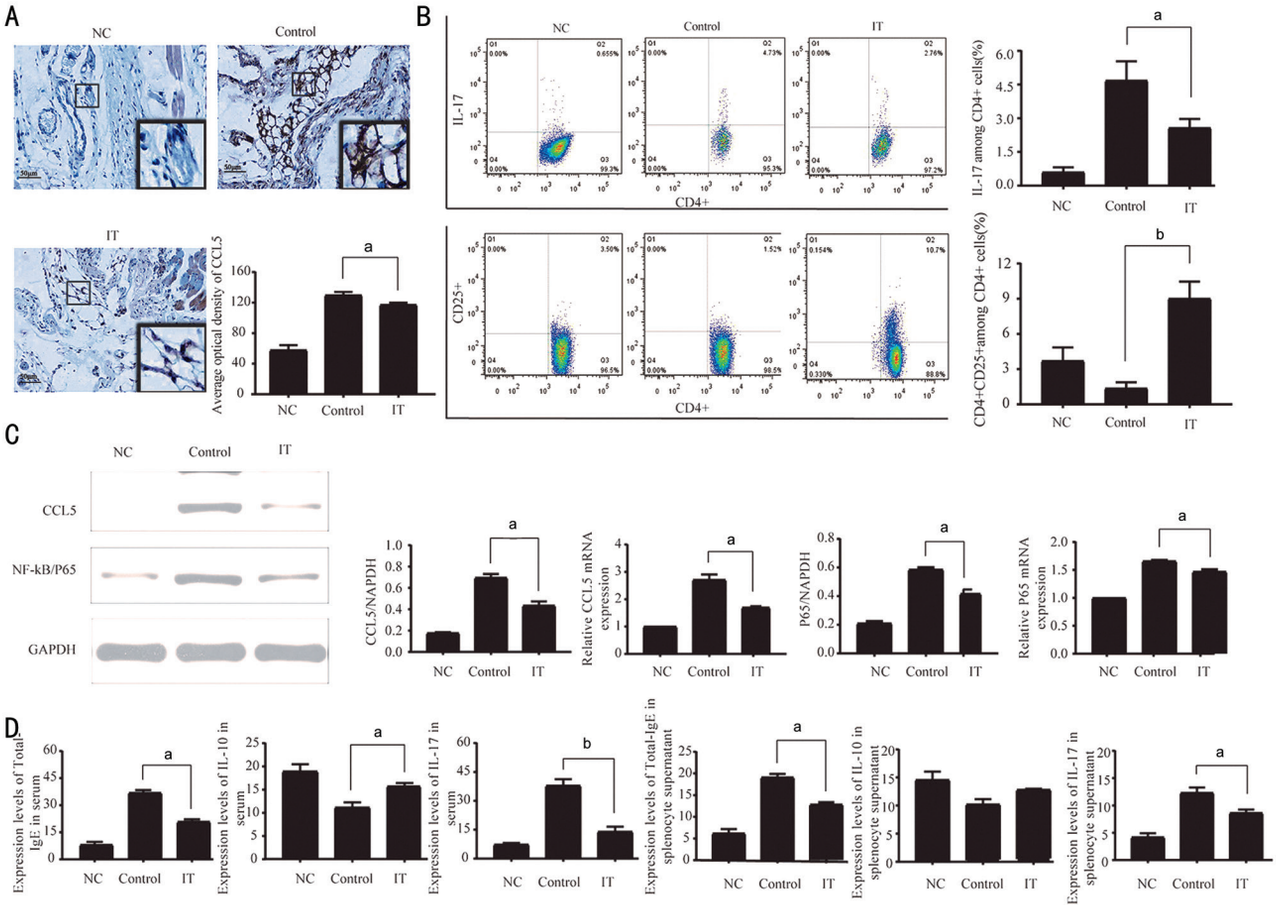

Figure 1 Changes in the expression of inflammatory factors after the establishment of allergic conjunctivitis immune tolerance mice model A: Expression of CCL5 in conjunctival tissue. Scale bar=50 μm. B: Percentage of IL-17 and CD4+CD25+ among splenocyte supernatant; C: Protein and mRNA expression of CCL5 and P65 in conjunctival tissue. D: Concentration of IgE, IL-10, and IL-17 in serum and splenocyte supernatant. NC: Negative control; Control: Positive control; IT: Immune tolerance. n=6/group, ^aP<0.05, ^bP<0.01.

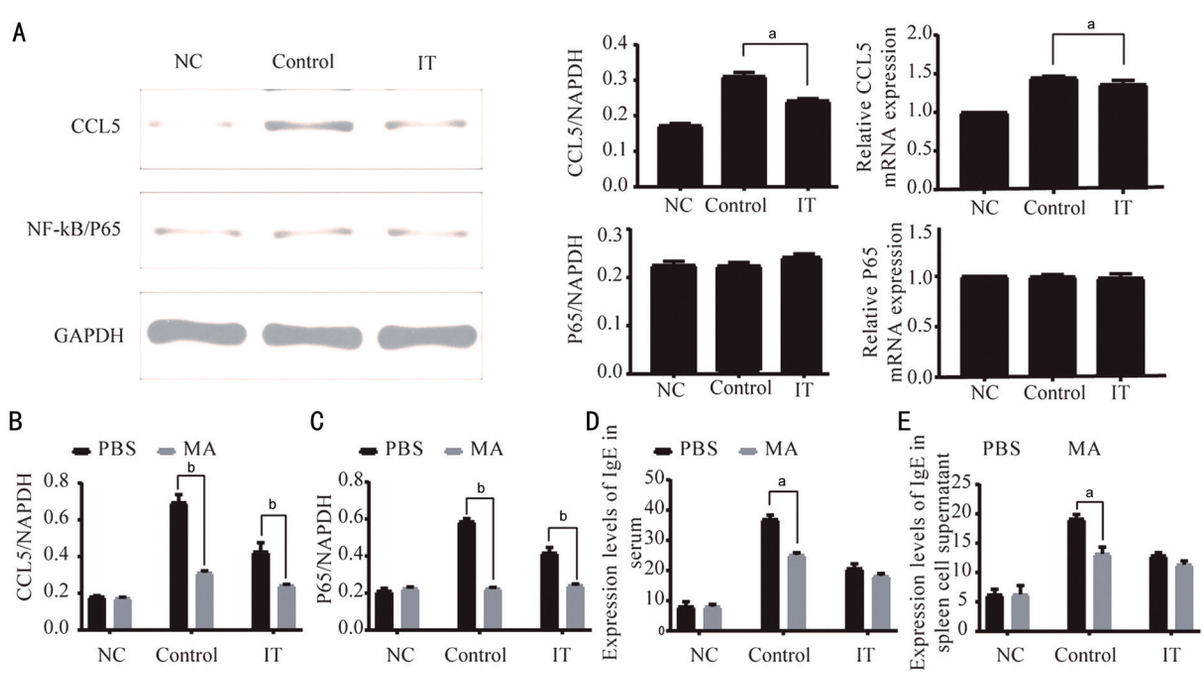

Figure 2 After treatment with PBS and MA, the expression changes of CCL5, P65, and IgE in allergic conjunctivitis immune tolerance mice model A: Protein and mRNA expression of CCL5 and P65 in conjunctival tissue; B, C: Protein expression of CCL5 and P65 in conjunctival tissue; D, E: The expression level of IgE in serum and splenocyte supernatant. NC: Negative control; Control: Positive control; IT: Immune tolerance; MA: Maslinic acid. n=6/group, ^aP<0.05, ^bP<0.01.

Conjunctivitis Symptoms Relived in Mice After MA Injection We observed the changes of the ocular surface of the mice through the anterior segment photography. As a result, allergic conjunctivitis was not found in the eyes of mice in NC, NC+PBS, and NC+MA. While mice in Control and Control+PBS showed swelling of eyelid, difficulty of opening eyes, hyperemia and edema of bulbar conjunctiva, and a large amount of mucinous secretion of conjunctival sac. Compared with Control, the eyelid of mice in Control+MA is slightly swollen, the bulbar conjunctiva is slightly congested but not accompanied by edema, and mucous secretion is seen in the conjunctival sac. For mice in IT and IT+PBS, there is a mild swelling of the eyelid, a mild edema and hyperemia of the bulbar conjunctiva, and a little secretion in the conjunctival sac. Mice in IT+MA had no obvious swelling of eyelid, no hyperemia and edema in bulbar conjunctiva, only showed a small amount of mucinous secretion in conjunctival sac (Figure 3).

Reaction and Differentiation of TH-17 Cells were Restrained IL-17 is a inflammatory factor secreted by Th17 cells, it takes a significant place in allergic diseases. Therefore, we utilized flow cytometry to detect the percentage of IL-17 in CD4+ cells in the splenocyte supernatant. We found that after treating with MA, the percentage of IL-17 in the IT was substantially reduced (P<0.05), and the percentage of IL-17 in the Control+MA was also significantly reduced compared to the Control+PBS (P<0.01). After MA treatment, compared with the Control, the percentage of IL-17 in CD4+ cells in the IT was substantially decreased (P<0.05). Compared with the Control+PBS, the percentage of IL-17 in CD4+ cells in the spleen cell supernatant of the IT+PBS group was significantly reduced (Figure 4A-4D, P<0.01). Compared with the IT+MA, the percentage of IL-17 among the Control+MA was reduced, but it was not statistically significant (Figure 4E). The expression of IL-17 was also detected by ELISA. ELISA results showed that compared with the Control+PBS, the IL-17 concentration in the Control+MA reduced markedly (P<0.01), and there was no significant difference in the other two groups (Figure 4F). In the spleen cell supernatant, both in the Control and the IT, the concentration of IL-17 decreased after treatment with PBS and MA, but there was no statistical significance (Figure 4G). These data suggest that when the NF-κB/P65 signal pathway is blocked, the reactivity of Th17 cells decreases, resulting in a decrease in IL-17 production.

Enhances the Reaction and Differentiation of CD4+CD25+ Cells To analyse the expression of anti-inflammatory factors after the NF-κB signal pathway was blocked under immune tolerance, we tested the contents of CD4+CD25+ and IL-10, respectively. The data revealed that after treatment with PBS and MA, compared with the Control, the percentage of CD4+CD25+ in the IT group increased significantly (Figure 5A-5E, P<0.001). Also, we tested the expression of IL-10 in the serum and splenocyte supernatant of allergic conjunctivitis immune tolerant mice by ELISA. The results showed that in the immune tolerance state, compared with the use of PBS, the IL-10 concentration in the Control group and IT group increased after using MA, but there was no statistical significance (Figure 5F and 5G).

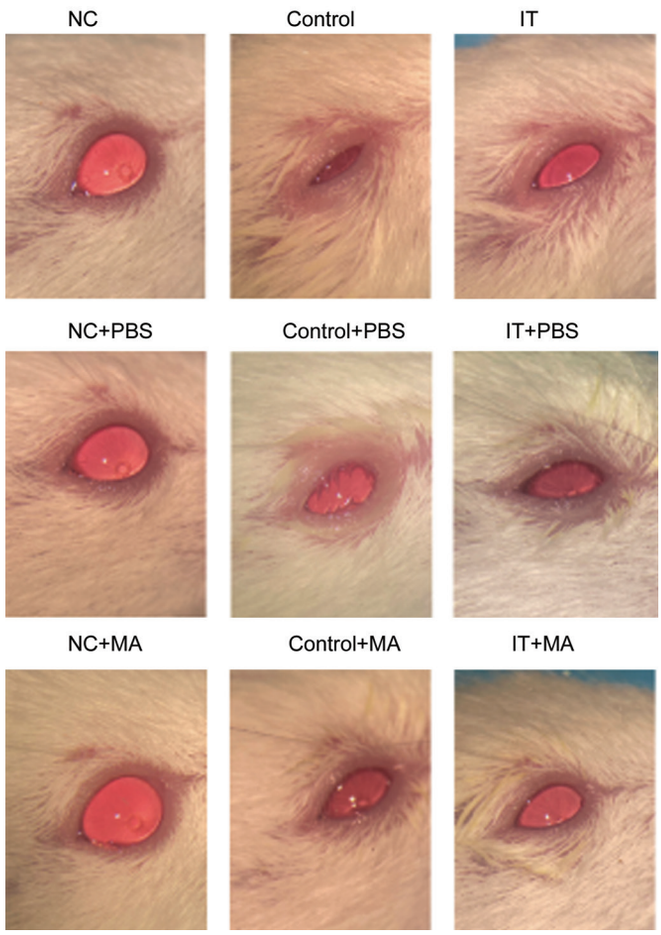

Figure 3 Effect of MA treatment and induction of immune tolerance on the development of allergic conjunctivitis in mice Ocular surface photos of the mice in NC, Control, IT, NC+PBS, Control+PBS, IT+PBS, NC+MA, Control+MA, and IT+MA groups. NC: Negative control; Control: Positive control; IT: Immune tolerance; MA: Maslinic acid.

DISCUSSION

At present, artificially induced immune tolerance has become an effective way to treat a series of allergic diseases[10-11]. Conjunctival tissue can also migrate to lymph nodes through antigen presenting cell's (APCs) and activate specific T cells effectively, thus regulating immune response. Studies have demonstrated that conjunctival tissue can actively regulate immunity, exert the function of immune tolerance and regulate immune response by migrating antigen-presenting cells to lymph nodes and activating specific T cells[12]. The immune tolerant mice induced by a high dose of RW showed no response or lack of response to T cells, and the release of

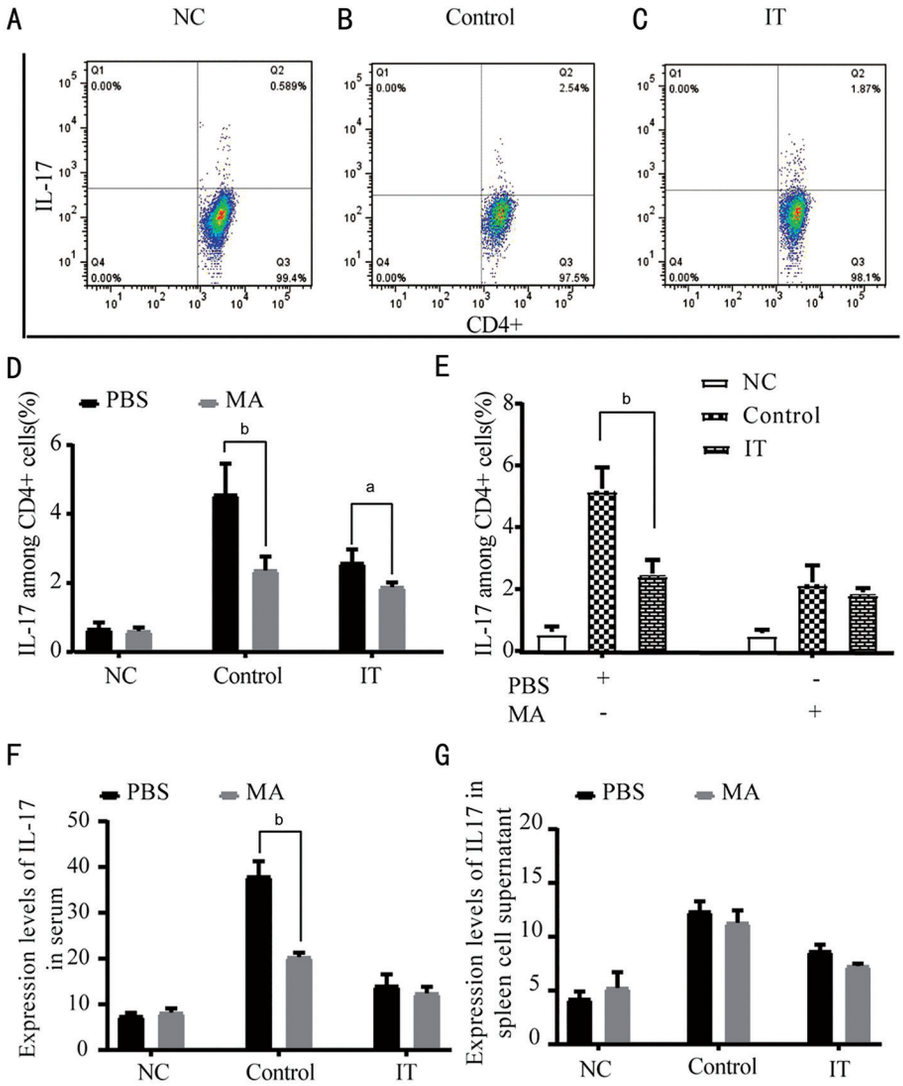

Figure 4 Expression changes of IL-17 in allergic conjunctivitis immune tolerance mice model after treatment with PBS and MA A-E: The percentage of the IL-17 among CD4+ cells; F-G: Expression level of IL-17 in serum and splenocyte supernatant. NC: Negative control; Control: Positive control; IT: Immune tolerance; MA: Maslinic acid. n=6/group, ^aP<0.05, ^bP<0.01.

pro-inflammatory factors decreased. However, T cells can still produce IL-10, transforming growth factor (TGF)-β, interferon (IFN)-γ, and other cytokines thus can play a role of Treg cells[13]. Our data revealed that the expression of the anti-inflammatory factors, such as IL-10 and CD4+CD25+, were on the rise, which is in line with the previous study.

The results of this experiment show that in the state of immune tolerance, the number of Treg cells and the expression of IL-10 increased. Studies have shown that the increase of IL-10 could significantly inhibit the production of specific IgE and promote the expression of IgG4[14], decrease the pro-inflammatory factor[15], and induce T lymphocytes tolerance through CD28 costimulatory pathway[16]. The study of Ishida *et al*[17] revealed that the number of eosinophils in conjunctival tissues of mice increased significantly after thymectomy of allergic conjunctivitis immune tolerant mice and injection of CD25+ antagonist. If only IL-10, TGF-β antagonists were used, there was no significant difference. It is implied that Treg cells exert a more considerable regulatory role in allergic conjunctivitis immune tolerant mice. Our result showed that in allergic conjunctivitis immune tolerant mice, the concentration of IL-10 in splenocyte supernatant and serum, the expression of CD4+CD25+ in splenocyte supernatant was close to or even higher than that of Control, which was consistent with the above results.

IL-17 has significant pro-inflammatory and chemotactic effects and shows a considerable role in allergic diseases. Qiu *et al*[18] reported that the degree of allergic symptoms and IL-17 level in allergic rhinitis patients who accept antigen-specific treatment for 2y lower than those in treated for 1y significantly. Li *et al*[19] also confirmed that in the state of immune tolerance, the content of IL-10 rised while the content of IL-17 in serum reduced. In this experiment, the ELISA was selected to detect the content of IL-17 in serum and splenocyte supernatant,

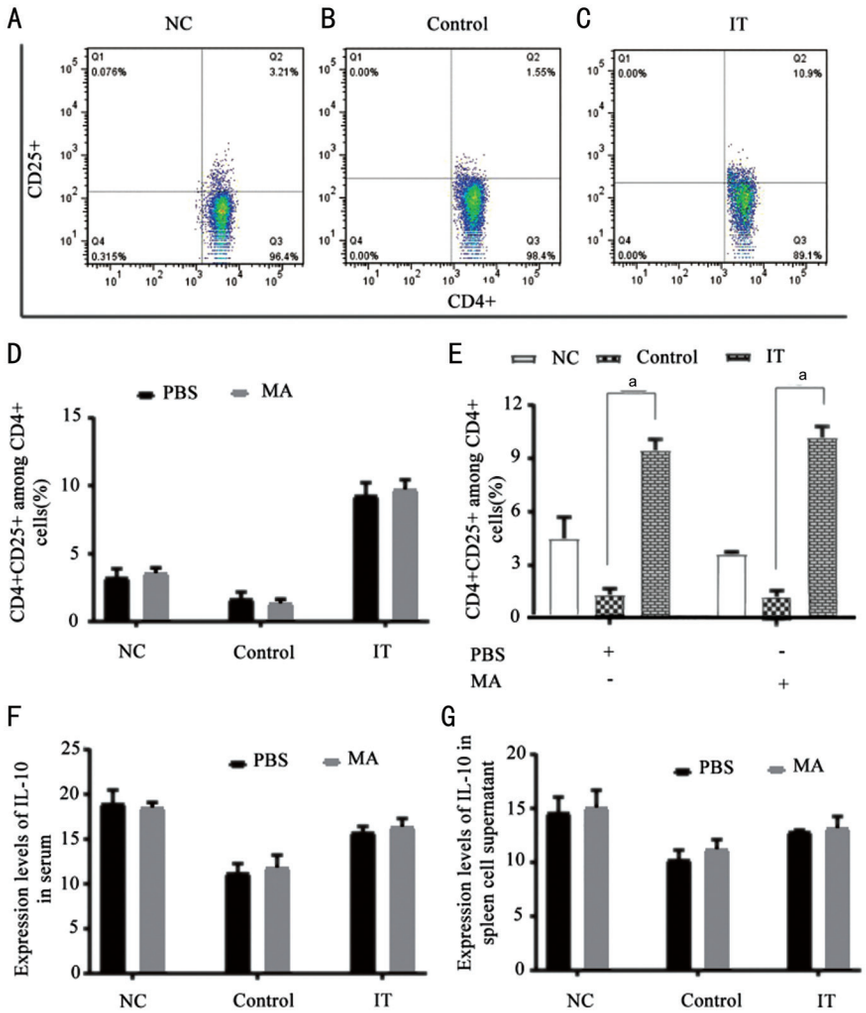

Figure 5 After treatment with PBS and MA, the expression changes of CD4+CD25+ and IL-10 in allergic conjunctivitis immune tolerance mice model A-E: The percentage of CD4+CD25+ among CD4+ cells; F-G: The concentration of IL-10 in serum and splenocyte supernatant. NC: Negative control; Control: Positive control; IT: Immune tolerance; MA: Maslinic acid. n=6/group, ^{a}P<0.001.

and flow cytometry was selected to analyse the percentage of IL-17 in cell suspension of spleen. It was found that the level of IL-17 in the IT was lower than that in the Control, suggesting that the level of IL-17 in the immune tolerance state has good reproducibility. On one hand, the decrease of IL-17 is due to T lymphocyte non-responsiveness, active inhibition and clonal deletion[20], and on the other hand, The higher the ratio of Th17/Treg, the more severe the inflammatory injury of tissues and organs. The immune tolerance state can induce the increase of the number and activity of Treg cells, decrease the ratio of Th17/Treg, and enhance the immune tolerance state, so as to avoid the damage of tissues and organs due to excessive immune response[21].

Immune tolerance can be divided into central tolerance and peripheral tolerance. Central tolerance, which is mainly composed of thymus and bone marrow tissues, mainly relies on two special antigen-presenting cells: dendritic cells derived from hematopoiesis and medullary thymic epithelial cell (mTEC)[15], which can express or present tissue-specific antigens to T cells under the mediation of antoimmune regulator (AIRE)[22]. The reactive T cells that have not been cleared by thymus tissue constitute a peripheral T cell group and are distributed in peripheral lymphoid tissue, which mainly prevents excessive immune damage through peripheral tolerance[23]. Studies have shown that several kinds of cells involved in immune response and immune regulation can be detected simultaneously in secondary lymphoid organ (SLO)[24]. In this study, we mainly tested peripheral immune tolerance and did not test the immune response and immune regulation in mice thymus tissue. We will use it as part of the next research plan.

Transcription factor NF-κB family exerts a significant role in immune response and immune regulation. It can be activated by the canonical pathway or the noncanonical pathway. The activators of the canonical pathway are mainly inflammatory cells and their related inflammatory cytokines, Toll-like receptor ligands and so on. Ikk kinase plays an important role in this pathway. Noncanonical pathway can be activated by

CD40, LTβR, *etc.*, which mainly maintains the function of peripheral lymphoid organs, acquired immune response and immune regulation.

Although there are differences in activation conditions and biological functions between canonical pathway and noncanonical pathway, there is interaction and regulation between them[25]. Ikkα activated by NF-κB induced kinase (NIK) can activate nonclassical pathway by phosphorylating p100, but Ikkα injected *in vitro* cannot effectively activate nonclassical pathway, it suggested that Ikkα must be activated by NIK. Ikkα is an important component of Ikk kinase complex, it shows momentous functions in the phosphorylation of IκBα, and thus participates in the regulation of classical pathway[26].

Besides, when T cell receptors bind to MHCII complex with high affinity, apoptosis of T cells can be induced[22]. In the stromal-derived medullary thymic epithelial cells low expression transgenic mice, the level of CD4+ was low and the expression of Treg cells was relatively increased[27]. When MHCII was completely deficient, Treg cells shwed low expression[28]. In this experiment, the expression of CCL5 and P65 was increased in allergic conjunctivitis mice, but the expression of CCL5, P65 decreased when NF-κB signal pathway was suppressed, suggesting that NF-κB signal pathway is very important in allergic conjunctivitis mice and played a positive regulatory role in CCL5.

It is reported that the lymph node function of patients with abnormal P65 and Ikk gene is significantly decreased[29]. Lymphoid tissue not only exerts a significant function in the initiation of acquired immunity, but also shows a regulatory function in peripheral auto-specific T cells[10]. Besides, SLO exerts an essential function in the maintenance and homeostasis of Treg cells[30]. In peripheral lymphoid DCs, CD40 can not only sensitize the noncanonical NF-κB, but also promote the produce of immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO), it can participate in pregnancy tolerance, immunosuppression, and mucosal immune tolerance. There are two isomers of IDO (IDO1 and IDO2), which can decompose tryptophan to inhibit the proliferation of T cells. Meanwhile, when the canonical pathway is blocked, the differentiation of Treg cells is also promoted by IDO. It can also exert immune tolerance by directly activating FOXP3, but when IDO is inhibited, Treg cell activity decreases significantly[31].

The CD40 gene-deficient mice, anti-CD40-treated wild-type mice, and Ikk kinase-deficient mice have low expression of Treg in peripheral lymphoid tissues[32], it may imply that the noncanonical NF-κB signal pathway can maintain Treg expression levels. However, the direct regulation mechanism between them needs to be further studied. Also, the canonical NF-κB signal pathway plays an indispensable role in regulatory mechanism for Treg cell proliferation and differentiation[33], and the Ikk kinase maintains the normal physiological state of Treg by regulating the level of IL-10[34].

MA can inhibit the DNA binding activity of the NF-κB signal pathway, and inhibit the phosphorylation of IκB-α required for P65 activation, thereby blocking the canonical NF-κB activation[35]. In this study, according to flow cytometry, when NF-κB signal pathway was inhibited, the level of IL-17 in the Control and the IT was significantly different. There is no significant difference between IL-17, IL-10, and IgE. However, the data from qRT-PCR and Western blot revealed that there was no significant difference in the level of CCL5 and P65 in conjunctival tissue of mice detected, suggesting that immune regulation in mice has played a role in immune tolerance. This may be because the noncanonical NF-κB signal pathway has an effective maintenance effect on peripheral tolerance volatility[36]. Besides, noncanonical pathways can exert immune regulatory effects by leading the loss of effector T cells and activating Treg cells[37]. Although the low level of IL-17 in the IT was not detected in this experiment, the CD4+CD25+ expression was significantly increase.

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