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Original article

MiR-146a overexpression effectively improves experimental allergic conjunctivitis through regulating CD4⁺CD25⁺T cells



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ABSTRACT

Objective: To study the mechanism of miR-146a in the regulation of allergic conjunctivitis (AC) through CD4⁺CD25⁺T cells.

Methods: BALB/c mice were sensitized with ragweed pollen (RW) and alum, and then challenged with RW. Eosinophil infiltration was determined using Giemsa assay. ELISA assay was performed to examine the level of antigen-specific IgE in the serum and cytokine levels in splenocytes. The expression of miR-146a was measured by qRT-PCR. Flow cytometric analysis was used to analyze the percentage of CD4⁺CD25⁺T cells and Tregs.

Results: In this study, we found that miR-146a overexpression could effectively improve the symptoms of AC in mouse models. Moreover, *in vitro* experiments, the proliferation of splenocytes was controlled and the expression of IL-5 and IL-13 was also decreased after transfected with miR-146a mimic. In addition, the inhibitory effect of Tregs on Tcons was affected by the change of miR-146a content in Tcons and had a positive correlation effect. When the content of miR-146a in Tregs changed, the inhibition was not affected. In addition, when inhibited miR-146a, NF-κB signaling pathway was activated, and the expression of IL-5 and IL-13 in Tcons increased. While after added Bay (NF-κB blocker), the NF-κB activity was decreased, and the expression of IL-5 and IL-13 in Tcons also reduced.

Conclusion: MiR-146a decreased in Tcons was contributed to the development of AC through regulating the inhibitory effect of Tregs on Tcons and NF-κB signaling pathway.

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1. Introduction

Allergic conjunctivitis (AC) is a common ocular surface allergic disease and is a hypersensitivity of the conjunctiva of an external allergen. Seasonal allergic conjunctivitis (SAC) and perennial allergic conjunctivitis (PAC) have mild symptoms and shorter duration, while vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKP) have severe symptoms and longer duration [1,2]. In North America, 20% to 30% of the total population have allergic diseases, 50% of which have AC [3,4]. Fungal allergens are one of the causes of allergic diseases. According to statistical data, aspergillus fumigatus (AF) is one of the four seasons common fungal allergens [5]. The main clinical symptoms of AC have eye itching, eyelid edema, conjunctival hyperemia, increased secretion and photophobia.

AC is mainly caused by type I and IV type of allergic reactions. In the development of conjunctivitis, a large number of eosinophils infiltrated in the pathological tissue of the conjunctiva, and antigen-specific T cells predominate in the progression of the disease [6–9]. CD4⁺CD25⁺T (Tregs) cells are one of antigen-specific T cells, which has been first discovered in 1995 by Sakaguchi et al. [10]. Tregs have immunomodulatory function. It can monitor the excessive activation and proliferation of lymphocytes in the body, and can effectively maintain the balance of the immune system. The main function of Tregs is to inhibit the activation, proliferation and differentiation of CD4⁺CD25⁺T (Tcons) cells. Many studies have been carried out on the mechanism of Tregs in immunosuppression. Studies have shown that Tregs can inhibit Tcons by secreting soluble cytokines with inhibitory effect. However, the molecular changes of Tcons after Tregs inhibition have not yet cleared.

Recently, miRNAs have been identified as an important regulator of many biological systems, including mammalian immune system. MiRNAs have a unique expression in the immune cells, and its expression pattern varies with different T cell subsets and developmental stages [11]. The development, proliferation,

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differentiation, migration and effect of immune cells are regulated by miRNAs. Studies have shown that miR-146a is associated with inflammatory responses, and is regulated by inflammatory cytokines and specific antigens [12].

In this paper, we investigated the mechanism of miR-146a in the regulation of experimental allergic conjunctivitis (EAC) through CD4⁺CD25⁺T cells, and discussed the role of miR-146a in inhibition of Tcons by Tregs.

2. Material and methods

2.1. Animals

Twenty BALB/c mice (4–6 weeks old female) were randomly divided into 4 groups (control group, AC group, AC + miRNC group, AC + miR-146a group) and each group had 5 mice. The Institutional Animal Care and Use Committee approved all procedures.

2.2. Experimental allergic conjunctivitis (EAC) models

The mouse model of allergic conjunctivitis was induced by ragweed pollen (RW). On the first day of the experiment, the mice in AC group, AC + miRNC group and AC + miR-146a group were immunized with 50 μ l of RW combined with Alum Adjuvant (0.5 mg RW in 20 mg Alum) by the left-hind footpad and the tail base injection, while control group was only injected equal Alum. From day 10 to 14, the mice in the control group were challenged with 0.2 g/L of PBS in the conjunctival sac, and the other three

groups were challenged with 0.2 g/L of RW (200 mg RW in 1 ml PBS). On day 10, mice in AC + miRNC group were intravenously injected with miRNC at 2 h before RW challenge in the conjunctival sac, and mice in AC + miR-146a group were intravenously injected with miR-146a overexpressing vector packaged with lentiviral vector. On day 13, the mice in the two groups were intravenously injected with miRNC and miR-146a again. Twenty-four hours after RW challenge, all mice were sacrificed. The conjunctivas were harvested; peripheral blood was obtained for measurement of IgE levels; and spleens were harvested to culture splenocytes for transfer, cytokine and proliferation assays. The grouping experimental method was shown in Fig. 1A.

Construction of miR-146a overexpressing lentiviral vector: The double-stranded oligonucleotide was connected with the GV309 vector (Genechem, Shanghai, China) which was digested by AgeI/NheI (Genechem), and then transfected into DH5 α Escherichia coli (Genechem). Next, the positive colony was selected and recombinant plasmids were extracted. Sequence analysis was carried out by Genechem.

2.3. Giemsa assay

The conjunctivas were fixed in 10% buffered formalin. Vertical 2- μ m-thick sections were cut and stained with Giemsa. Count the number of eosinophils infiltrated into the conjunctiva in each slice.

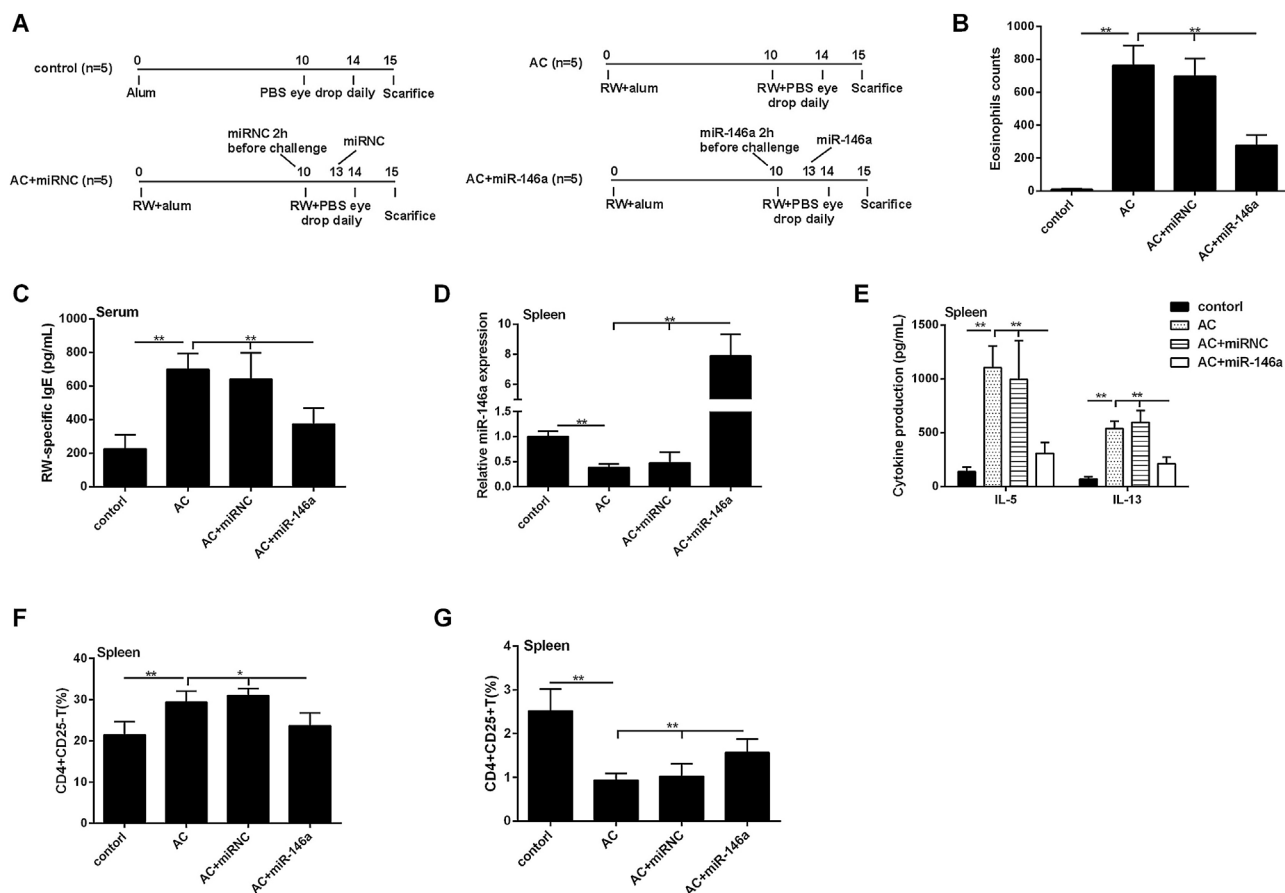


Fig. 1. The condition of the mouse after tail base intravenous injection of the miR-146a overexpressing vector packaged with lentiviral vector. (A) Experimental protocol of four groups. (B) The number of eosinophils (the mean $\pm$ SD). (C) RW-specific IgE levels in serum. (D) Relative miR-146a expression in spleen. (E) Cytokines (IL-5, IL-13) production in spleen. (F&G) The percentage of CD4⁺CD25⁺T cells and CD4⁺CD25⁺T cells in spleen. *P < 0.05, **P < 0.01.

2.4. Measurement of RW-specific IgE in serum

Peripheral blood was collected in the centrifuge tube. Then, the supernatant serum was collected after centrifugation (3000 r/min, 15 mins) at 4 °C. ELISA assay was used to detect RW-specific IgE level.

2.5. Cell transfection

According to the manufacturer's instructions, miR-146a mimic and inhibitor were transfected into RBC-depleted splenocyte (RBC: red blood cells), Tcons and Tregs using Lipofectamine 2000 (Invitrogen), respectively. After 48 h of transfection, the cells were collected for related indicators detection.

2.6. Splenocytes culture

RBC-depleted splenocytes were collected from WT mice and a single cell suspension was cultured in RPMI1640 medium (containing 100 mg/L fetal bovine serum, 100 mg/L penicillin and 100 mg/L streptomycin; FCS, ICN Biomedical Japan Co., Tokyo, Japan). The cell concentration was adjusted to 1×10^6 cells/ml. The number of viable cells was identified by trypan blue staining (above 98% available). 100 μ l of splenocytes suspension were added to 96-well flat-bottom plates (Nunc, Rochester, NY, USA) and stimulated with RW at a final concentration of 25 μ g/ml. Then the cells were incubated in a constant temperature (37 °C, 5% CO₂) for 72 h.

2.7. Tcons and Tregs co-culture

Co-culture was performed in 96-well round-bottom plates (Nunc, Rochester, NY, USA). Purified Tcons (1×10^5 cells/well), Tregs (1×10^5 cells/well) or both populations were cultured with irradiated APCs (1×10^5 cells/well) in triplicate for 3 days at 37 °C in complete RPMI 1640 medium (0.2 ml/well). Cultures were stimulated with 1 μ g/ml anti-CD3/CD8 (BD PharMingen, San Diego, CA).

2.8. Cellular proliferation assay

Cells were pulsed for 16 h with 0.5 μ Ci/well ³H-thymidine, harvested, and the incorporated radioactivity was measured by standard techniques. The number of pulses per minute (cpm) represented cellular proliferation reaction.

2.9. Cytokine analysis

IL-5 levels were measured by ELISA kit (Cayman, Ann Arbor, Michigan, USA). Samples and standards (100 μ l/well) were added to the ELISA plates coated with anti-IL-5 monoclonal antibody and incubated at room temperature (20–25 °C) for 120 mins. Then the plate was washed for 4 times, added horseradish peroxidase labeled anti-IL-5 monoclonal antibody (100 μ l/well) and incubated at room temperature for 60 mins. After that the plate was washed for 4 times again, added chromogenic agent and incubated for 10–30 mins in dark room. Finally, stop solution was added to the ELISA plates and mixed. The light density values were measured by microplate reader in 5 mins. According to the values, the standard curve was drawn and the specimen concentration was detected. The IL-13 level was measured as described above.

2.10. Quantitative real-time PCR (qRT-PCR)

According to the instructions of the kit (RNeasy Micro or Mini Kit, USA), the total RNA was extracted and reversely transcribed to

cDNA (QuantiTectReverse Transcription Kit, USA) for qRT-PCR (StepOne Plus, USA). PCR reaction conditions were as follows: 95 °C pre-denaturation for 15 mins, 94 °C denaturation for 15s, 60 °C annealing for 1 min, a total of 40 cycles. The relative expression of miR-146a was analyzed by 2^{−ΔΔCt} method and β -actin was used as the control for normalization.

2.11. Flow cytometric analysis

Freshly isolated or *in vitro* stimulated splenocytes were incubated with FITC-labeled anti-mouse CD4 antibody (GK1.5, eBioscience, San Diego, CA) on ice for 30 mins. After washing with cold 2% FCS-PBS, the cells were incubated with biotin-labeled anti-CD25 antibody (PC61, eBioscience) followed by incubation with streptavidin-PE (BD Biosciences, Franklin Lakes, NJ). The cells were then washed with cold 2% FCS-PBS and detected on a flow cytometer (Becton Dickinson, Mountain View, CA). CellQuest software was used to analyze the data.

2.12. Western blot assay

Western blot was performed with anti-cytoplasm-p65, anti- β -actin, anti-TRAF6, anti-Thublin, anti-nuclear-p65 and anti-Lamin B. Cells were harvested for 24 or 48 h post transfection, the total protein was extracted from the cells with lysis buffer and the protein concentration of each sample was measured using BCA kit (Bolingkewei, Beijing) according to the instructions. To analyze the protein content, 50 μ g of total protein was electrophoresed in 10% SDS-PAGE gel and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) and then incubated with the corresponding antibodies described above. Immunoblot was performed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.13. Statistical method

SPSS 17.0 software was used for statistical analysis. All data were presented as mean $\pm$ standard deviation (SD). $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Effects of miR-146a in AC mice

To investigate the effect of miR-146a in conjunctival inflammation triggered by allergens, we evaluated the pathological indexes of AC mice after miR-146a overexpression. BALB/c mice (4–6 weeks old female) were randomly divided into 4 groups (control group, AC group, AC + miRNC group, AC + miR-146a group). The grouping experimental method was shown in Fig. 1A.

The analysis showed that the eosinophil counts in the conjunctiva of AC group was significantly higher than that of control group, while the eosinophil counts in the conjunctiva of AC + miR-146a group was significantly lower than that in AC group and AC + miRNC group ($P < 0.01$, Fig. 1B).

The levels of IgE in serum of 4 groups were detected by ELISA assay. The results showed that compared with control group, the serum level of IgE in AC group was significantly increased ($P < 0.01$, Fig. 1C). The level of IgE in AC + miR-146a group was significantly lower than that in AC group and AC + miRNC group ($P < 0.01$, Fig. 1C), which indicated that overexpression of miR-146a could decrease the level of IgE in serum.

The expression of miR-146a in the spleen was significantly increased by injection of miR-146a mimic through vena caudalis ($P < 0.01$, Fig. 1D). ELISA assay was used to detect the expression level of IL-5, IL-13 in the spleen and the results showed that the

level of IL-5 and IL-13 in AC group increased significantly, while the level of IL-5 and IL-13 in AC+miR-146a group was significantly lower than that in AC group and AC+miRNC group ($P < 0.01$, Fig. 1E). Moreover, Tcons in spleen were higher in AC group than in control group, while the percentage of Tcons in AC+miR-146a group was lower than that in AC group and AC+miRNC group ($P < 0.01$, Fig. 1F). Tregs had the opposite trend with Tcons (Fig. 1G).

3.2. Effects of miR-146a on splenocytes

Compared with control group, miR-146a had a low expression in RW-treated RBC-depleted splenocyte, as well as in Tregs and Tcons (Fig. 2A). Thus, after RW treatment, splenocyte proliferation was accelerated. However, miR-146a overexpression could significantly inhibit cell proliferation ($P < 0.01$, Fig. 2B).

The expression level of cytokines (IL-5 and IL-13) in supernatant of splenocyte in RW group was significantly higher than that in control group ($P < 0.01$, Fig. 2C & D). And the expression level was significantly decreased after miR-146a overexpression ($P < 0.05$, Fig. 2C & D). The above results illustrated that miR-146a could inhibit the proliferation of RW-treated RBC-depleted splenocyte and the expression of cytokines (IL-5 and IL-13).

3.3. Effects of miR-146a expression in Treg-suppressed Tcons

Tcons and Tregs were purified from WT mice spleen and transfected with miR-146a inhibitor or negative control (NC), respectively. The results showed that miR-146a inhibitor indeed could decrease the expression of miR-146a in Tregs and Tcons (Fig. 3A & B). Then, wild type Tcons and Tregs were transfected with inhibitor of miR-146a or NC and then co-cultured. The suppressive activity of Tregs on Tcons was measured by using ^3H -

thymidine at $0.5 \mu\text{Ci}/\text{well}$ for the final 16 h of co-culture as described in 2.6 section. The data showed that wild type Tregs could effectively inhibit the proliferation of Tcons. However, after transfected miR-146a inhibitor, the inhibition effect was significantly decreased ($P < 0.05$, Fig. 3C). Tcons were transfected with mimic or inhibitor of miR-146a or NC and then co-cultured with wild type Tregs. Results in Fig. 3D showed that miR-146a mimic significantly increased the suppression of Tcons proliferation triggered by Tregs, whereas miR-146a inhibitor reduced the inhibitory effect of wild type Tregs on Tcons. Interestingly, when wild type Tregs were transfected with mimic or inhibitor of miR-146a or NC and then co-cultured with Tcons, the results showed that no matter miR-146a was low expression or overexpression, the changes of the inhibition effect of Tregs on Tcons were not significant (Fig. 3E). These indicated that the inhibitory effect of Tregs on Tcons was affected by the change of miR-146a content in Tcons.

3.4. Effects of miR-146a on NF-Kappa B (NF- κB) activity and cytokines expression in Tcons

Tcons were purified from WT mice spleen and transfected with miR-146a mimic or inhibitor or miRNC. Results showed that miR-146a mimic decreased the expression of IL-5 and IL-13 in Tcons significantly. However, miR-146a inhibitor dramatically increased their expression (Fig. 4A & B). Furthermore, miR-146a mimic could significantly reduce the activity of NF- κB , while miR-146a inhibitor enhanced NF- κB activity (Fig. 4C). After overexpression of miR-146a, the expression of p65 in the cytoplasm increased, while the expression of p65 in the nucleus decreased. Inhibited miR-146 showed the opposite trend (Fig. 4D & E). In addition, the expression of TRAF6 (miR-146a targeting molecule and NF- κB activating

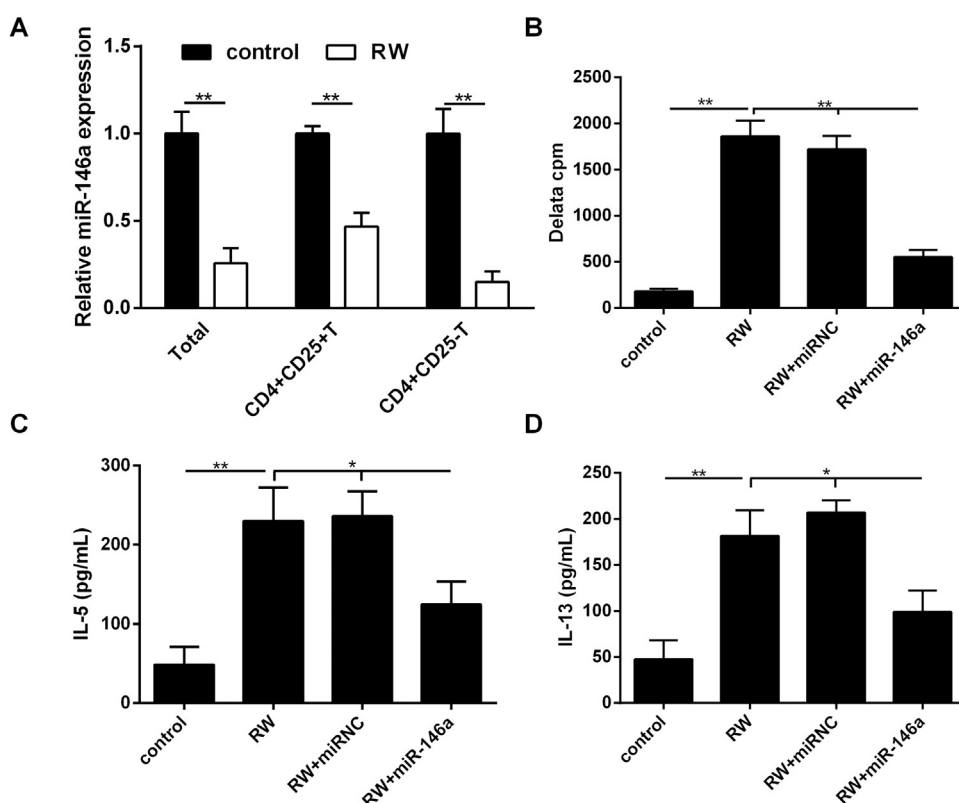


Fig. 2. Effect of miR-146a on the proliferation of RW-treated RBC-depleted splenocyte and the expression of cytokines (IL-5, IL-13). (A) Relative miR-146a expression in splenocyte. (B) The proliferation of splenocyte. (C&D) The expression of cytokines (IL-5, IL-13). * $P < 0.05$, ** $P < 0.01$.

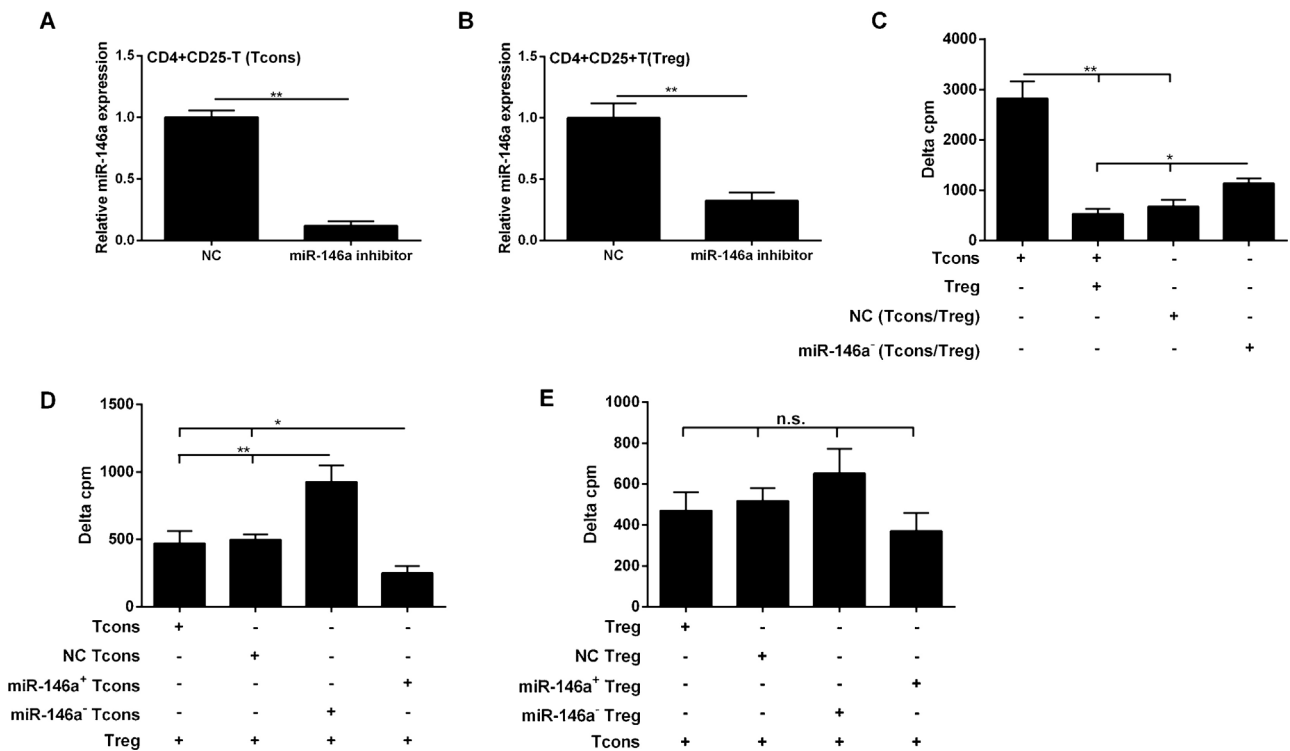


Fig. 3. MiR-146a participates in Treg-mediated regulation of Tcons proliferation. (A&B) Tcons and Tregs were purified from WT mice and transfected with miR-146a inhibitor or negative control (NC), respectively. Expression of miR-146a was detected in transfected Tcons and Tregs. (C-E) Tcons and Tregs were transfected with inhibitor of miR-146a or NC and then co-cultured (C). Tcons were transfected with mimic or inhibitor of miR-146a or NC and then co-cultured with Tregs (D). Tregs were transfected with mimic or inhibitor of miR-146a or NC and then co-cultured with Tcons (E). The inhibition effect of Treg on Tcons was measured. * $P < 0.05$, ** $P < 0.01$.

molecule) was contrary to that of miR-146a (Fig. 4D & E). IL-5/IL-13 expression was increased after inhibited miR-146a in Tcons, while the up-regulation was reversed by Bay (11-7082, NF- κ B blocker) (Fig. 4F).

Tcons were isolated and stimulated with anti-CD3/CD28 beads for a total of 2 days. In response to anti-CD3/CD28 stimulation, NF- κ B activity decreased dramatically in Tcons. However, the activity of NF- κ B was significantly enhanced by inhibition of miR-146a (Fig. 4G). The activity of NF- κ B was significantly attenuated by the addition of NF- κ B blocker Bay (Fig. 4G).

4. Discussion

The type of AC has many, and pollen allergic conjunctivitis in SAC has the highest incidence of clinical. AC is one of the refractory ophthalmic diseases and often associated with allergic rhinitis and allergic asthma. Due to the high incidence, long disease duration, easy to recurrent, and limited efficacy, AC affects the quality of life of patients seriously. The incidence of allergic conjunctivitis in the world is 15% to 20% (even 40% reported), and the incidence is increasing [13]. According to reports, the proportion of allergic conjunctivitis in central China was the highest (45.1%), the proportion in the north, south, southwest and north central region of China were 8.0%, 18.1%, 1.1% and 18.1% [14].

The cause of AC is very complex and is the result of multi-cell and multi-factor. Studies have shown that the pathogenesis of AC is the result of allergen-specific IgE-mediated type I hypersensitivity and T cell-mediated cellular immune responses [15,16]. Recent studies have shown that IL-5 could synergize IL-4 to stimulate B cells to synthesize IgE, induce eosinophil activation, proliferation, differentiation and migration; inhibit eosinophil apoptosis and prolong survival time of eosinophils [17]. IL-13 is involved in the

development of T cells and B cells. It can regulate the function of Th2 cells, promote the synthesis of IgE, induce eosinophil migration, and play an important role in the pathogenesis of allergic diseases [18,19].

Recently, miRNAs have become key negative feedback mechanisms in numerous immune responses. It has been reported that a variety of miRNAs have been used as activators or inhibitors in the immune response. In this study, we found that miR-146a overexpression could alleviate the symptoms of AC in mice. It could effectively reduce the infiltration of eosinophils in conjunctiva, RW-specific IgE level in the serum, the production of cytokines (IL-5 and IL-13), and the proportion of Tcons. Moreover, *in vitro* experiments of splenocytes, we also found that miR-146a overexpression could inhibit the inflammatory response in cells. Therefore, we further studied the mechanism of miR-146a in AC.

In addition, recent studies have shown that immune disorder in patients with AC leads to decreased expression of Tregs and increased expression of activated CD4⁺Tcons cells [20]. According to the surface marker and function of T lymphocyte subsets, CD4⁺Tcons cells can be further differentiated into Th1 and Th2 cells [21–23]. Th1 and Th2 cells are indispensable for host resistance to pathogens and tumors. However, excessive production of T cells can cause autoimmune diseases and inflammatory responses. Wild type Tregs can effectively inhibit the proliferation of Tcons, but in the inhibition of Tregs, the exact molecular changes in Tcons are difficult to ascertain. Wills-Karp [24] suggested that IL-13 was involved in maintaining the expression of Th2-type cytokines in allergic reactions. In this paper, we studied the mechanism of miR-146a in inhibition of Tcons by Tregs.

It was found that the inhibitory effect of Tregs on Tcons was affected by the change of miR-146a content in Tcons and had a positive correlation effect. When the content of miR-146a in Tregs

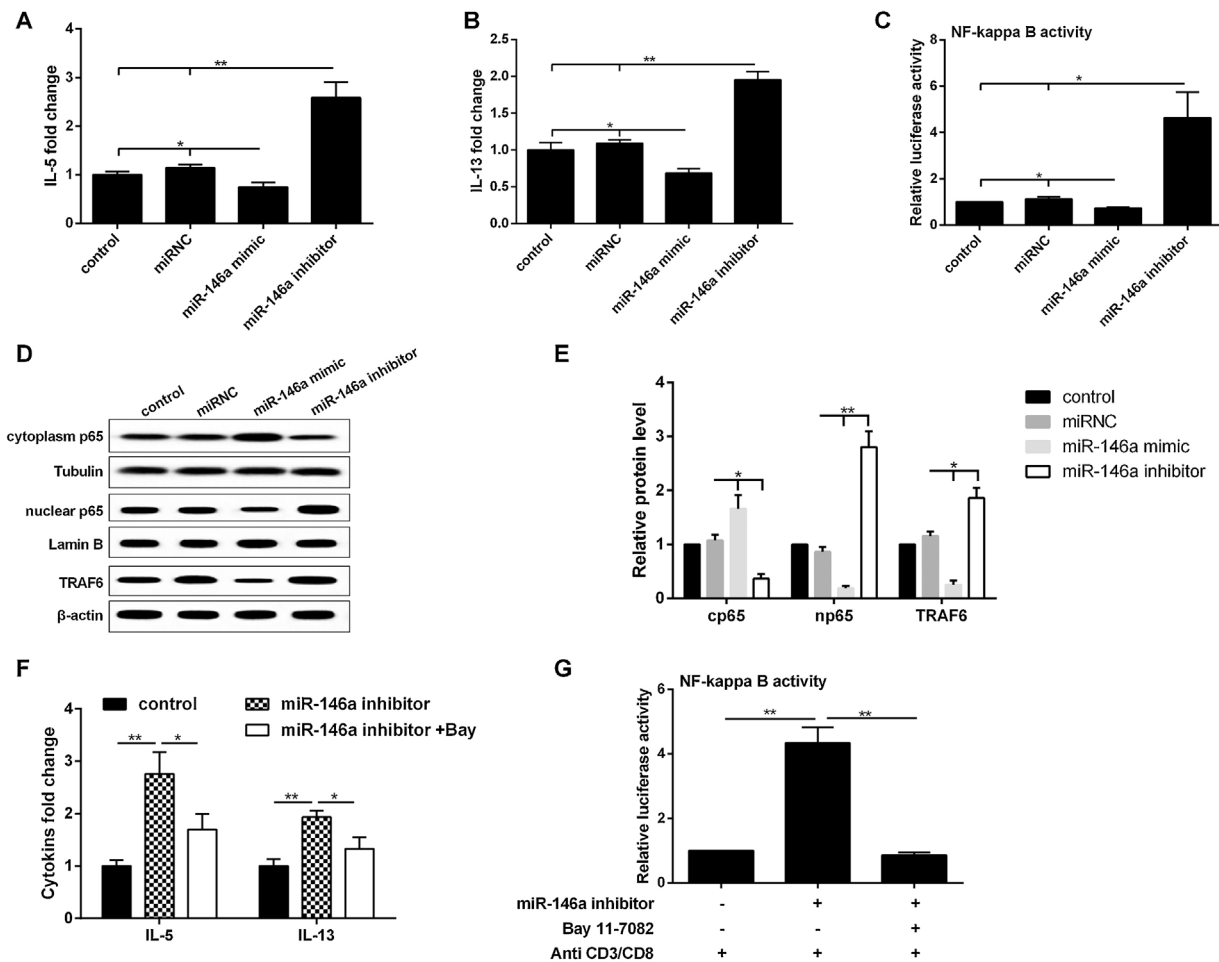


Fig. 4. MiR-146a regulated IL-5 and IL-13 expression in Tcons cells by NF-κB. (A&B) Tcons were purified from WT mice and transfected with miR-146a mimic or inhibitor or miRNC. The expression of IL-5 and IL-13 in Tcons was measured. (C&G) NF-κB activity. (D&E) Relative protein level. (F) The expression of cytokines (IL-5, IL-13) in Tcons. * $P < 0.05$, ** $P < 0.01$.

changed, the inhibition was not affected. It was speculated that the content of miR-146a in Tcons could regulate the inhibitory effect of Tregs on Tcons. Moreover, this study also revealed that miR-146a could regulate the expression of IL-5 and IL-13 in Tcons by regulating NF-κB signaling pathway, and then regulate the inflammatory reaction of AC.

In conclusion, our results confirmed that increased the expression of miR-146a in CD4⁺CD25⁻T cells could effectively improve the symptoms of AC, which may be a new way to treat AC.

Conflict of interest

The authors have no actual or potential conflicts of interest to declare.

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