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Adenovirus-mediated down-regulation of miR-21-5p alleviates experimental autoimmune uveoretinitis in mice

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ABSTRACT

MiR-21-5p has been found to be up-regulated in the retina of experimental autoimmune uveoretinitis (EAU) mice and correlated with the pathogenesis of EAU. The objective of the present study is to explore the role of miR-21-5p in EAU. C57 mice were immunized with residue1–20 ($IRBP_{1–20}$) in complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* H37Ra to induce EAU, and miR-21-5p was knocked down via subretinal injection of anti-miR-21-5p adenovirus. The pathological score, TUNEL positive cells and the expression of pro-inflammatory factors in the retina were reduced, and the expression of IL-10 was increased by down-regulation of miR-21-5p. Up-regulation of miR-21-5p significantly decreased the mRNA and protein levels of IL-10 in ARPE-19 cells. The binding activity of miR-21-5p on the 3′UTR of IL-10 mRNA was confirmed by luciferase reporter assay. Moreover, the miR-21-5p level in splenic lymphocytes of EAU mice was increased at the 7th day after immunization and reached its peak at the 14th day, that was in accordance with the changing trend with the Th17 cell frequency in the spleen. Besides, lentivirus-mediated down-regulation of miR-21-5p reduced the Th17 cell frequency and increased the Treg cell fraction of $IRBP_{1–20}$-stimulated lymphocytes in vitro. Taken together, in situ down-regulation of miR-21-5p attenuates EAU by inhibiting inflammatory responses and reducing retinal cell apoptosis. miR-21-5p may also participate in the progress of EAU by affecting Th17/Treg balance via the regulation of IL-10. Therefore, we demonstrate that miR-21-5p can serve as a therapeutic target in the management of uveitis and other autoimmune diseases.

1. Introduction

Uveitis is an intraocular inflammatory disease that affects people at any age, especially in the working-age populations. Uveitis is a major cause of visual loss, which accounts for about 10–15% of the total blindness patients in the developed world [1] and about 25% in developing countries [2]. The etiology of uveitis is unknown in most cases, but bacterial/viral infections and autoimmunity to retinal are believed to be closely associated with it [3]. Experimental autoimmune uveoretinitis (EAU) is a T cell-mediated autoimmune disease of the eye induced by retinal-specific antigens [4]. EAU has been wildly used as an animal model of human ocular diseases including uveitis. Consistent with uveitis [4,5], the typical characteristics of EAU are inflammatory infiltration and destruction of retinas. Thus, it is necessary for further investigation of the pathogenesis of uveitis and the development of effective strategies to treat this disease by using the EAU animal model.

MicroRNAs (miRNAs) are a class of endogenous, micromolecular and noncoding RNAs, and involved in a large number of cell biological processes and human diseases. Previous studies have suggested that miRNAs play pivotal roles in immunity and autoimmunity [6,7]. MiR-21 is considered as an oncomiR that participated in apoptosis and oncogenesis by the regulation of various tumor suppressors [8]. Recently, miR-21 has been considered as a diagnostic biomarker for several autoimmune diseases, such as systemic lupus erythematosus [9], rheumatoid arthritis [10], multiple sclerosis [11] and ulcerative colitis [12]. Moreover, specific knock-down of miR-21 is beneficial to experimental autoimmune encephalomyelitis [13], lupus-like autoimmunity in the chronic graft-versus-host disease models [14] and autoimmune lymphoid hyperplasia syndrome [15]. In particular, Ishida et al. [16] found that miR-21 was strongly up-regulated in the eyes of EAU mice compared with the naïve controls, and the changes in miR-21 expression were positively correlated with the expression of IL-17 in autoimmune

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diseases [17]. To further study the molecular mechanisms underlying the pathogenesis of EAU, the role of miR-21 in EAU was investigated in the present study.

2. Material and methods

2.1. Animals

Adult female C57 mice with the license number: SCXK (Jing) 2014-0004 were purchased from Beijing Huafukang bioscience co., Inc. (Beijing, China). The animals were maintained in a standard environment at 20 ± 2 °C with a 12 h/12 h-light/night cycle. All animal experiments were approved by the Laboratory Animal Care and Use Committee of the Qilu Hospital of Shandong University and conformed to the Association of Research in Vision and Ophthalmology animal policy (the Statement for the Use of Animal in Ophthalmic and Vision Research).

2.2. EAU induction and treatment

EAU was induced in mice as previously reported [18]. In brief, mice were immunized on one footpad and tail tip with residue1–20 ($IRBP_{1-20}$) containing 300 μg human interphotoreceptor retinoid-binding protein epitope emulsified in 0.2 ml complete Freund's adjuvant at 1:1, supplemented with 2 mg/ml *Mycobacterium tuberculosis* H37Ra. Concurrently, mice were injected intraperitoneally with 1 μg *Bordetella pertussis* toxin. Animals were randomly divided into three groups: Control group, EAU control group, and EAU + anti-miR-21-5p group. For EAU control group and EAU + anti-miR-21-5p group, mice were subretinally injected [19] with 10^8 pfu adenovirus vector (GenePharma, Shanghai, China) or 10^8 pfu anti-miR-21-5p adenovirus (GenePharma, Shanghai, China) before EAU induction. Mice were treated with an equal volume of saline via subretinal injection as a negative control in this study. The treatment schedule of each group was shown in Fig. 1a. The eyes were collected on 0, 7, 14 or 21 days after immunization, and fixed in 10% formaldehyde or preserved at −80 °C for subsequent experiments.

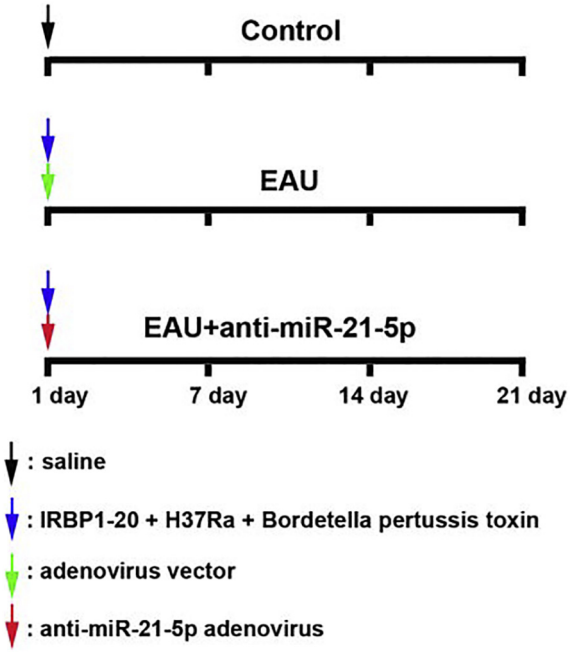

Fig. 1. Treatment schedule of each group. EAU was induced in C57 mice by immunization with residue1–20 ($IRBP_{1-20}$) in complete Freund's adjuvant supplemented with *Mycobacterium tuberculosis* H37Ra, and miR-21-5p was knocked down via subretinal injection of anti-miR-21-5p adenovirus.

2.3. Histopathological analysis

The fixed retina tissues were embedded in paraffin and cut into 5-μM thickness. Then the sections were stained with hematoxylin and eosin (H&E) according to the standard procedures. Histologic grading was performed as Caspi et al. [4] described previously: 0, no inflammatory cell infiltration and normal retinal architecture; 1, mild inflammatory cell infiltration but no retinal structural destruction; 2, partial or mild destruction of the outer retina; 3, moderate destruction of the outer retina; 4, extensive and severe destruction of the outer retina and partial destruction of the inner retina; 5, complete destruction of the entire retina. Cell apoptosis was detected by terminal deoxynucleotide transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay using a commercial kit (Roche, Indianapolis, IN, USA) strictly according to the manufacturer's protocol.

2.4. Human retinal pigment epithelial cell culture and transfection

Human retinal pigment epithelial cells ARPE-19 were obtained from Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). Cells were cultured in DMEM/F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, BI, Kibbutz, Israel), 100 IU/ml penicillin and 100 μg/ml streptomycin in the 37 °C incubator. For transfection, cells were seeded in 12-well plates at a density of 1.5×10^5 cells/well. After growing to subconfluence, the cells were transfected with 20 nM scrambled miRNA mimic (NC mimic) or 20 nM miR-21-5p mimic by using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) reagent following the user's instructions.

2.5. Western blot assay

Total proteins were extracted by RIPA lysis buffer (Beyotime) and quantified by bicinchoninic acid (BCA) protein assay (Beyotime). Then an equal amount of proteins was separated by 5%, 8% or 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes. Next, the membranes were blocked with 5% (m/v) non-fat milk for 1 h at room temperature, and then incubated with primary antibodies against IFN-γ (1: 250 diluted, Wanleibio, Shenyang, China), IL-10 (1: 200 diluted, Santa Cruz Biotechnology, Santa Cruz, CA, USA), IL-17A (1: 750 diluted, Abcam, Cambridge, MA, USA) or TNF-α (1, 250 diluted, Biorbyt, UK) at 4 °C overnight. The membranes were then immuno-blotted with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Beyotime) at 37 °C for 45 min. The specific protein bands were finally visualized using an enhanced chemiluminescence reagent kit (Beyotime).

2.6. Real-time quantitative PCR

Total RNA was extracted with TRIpure Total RNA Kit (Bioteke Corporation, Beijing, China). Next, the RNA was reverse transcribed with Super M-MLV reverse transcriptase as per the manufacturer's instructions. Real-time quantitative PCR reactions were performed with 2 × Power Taq PCR MasterMix (Bioteke Corporation) and SYBR Green (Bioteke Corporation). GAPDH was used as an internal control. The primer sequences used in the present study were as follows: IL-10, sense: 5′-accaagacccagacatca-3′, antisense: 5′-ttcacagggaagaaatcg-3′; GAPDH, sense: 5′-gaaggtcggagtcaacggat-3′, antisense: 5′-cctggaagatggtgatgggat-3′.

2.7. Luciferase reporter assay

The wild-type mouse IL-10 3′UTR sequence containing the miR-21-5p target site or 3′ sequence with mutated miR-21-5p target site was inserted into the *NheI*/*SalI* site downstream of the *luc2* gene of pmirGLO Vector (Promega, Madison, USA). The miR-21-5p mimic and NC mimic

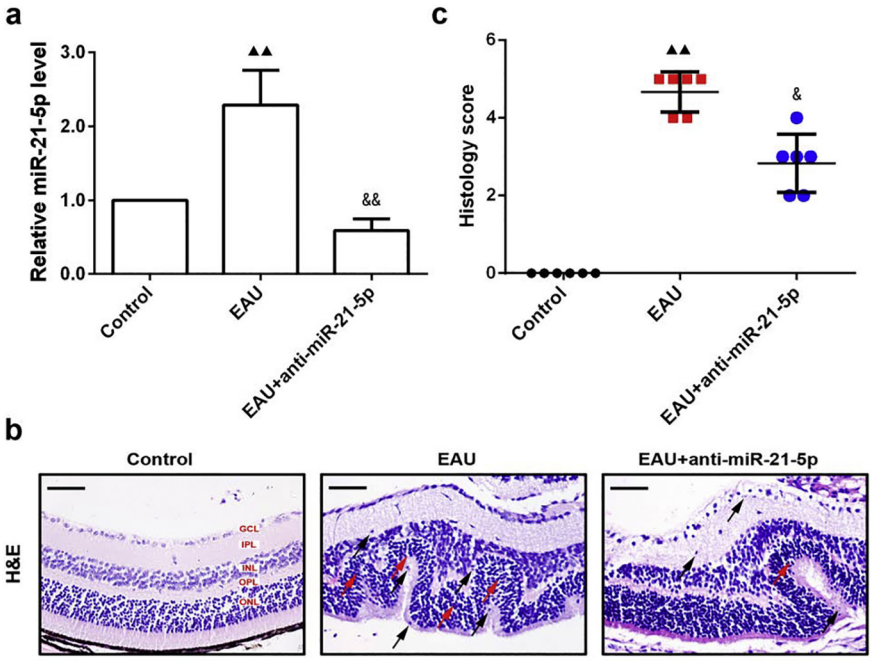

Fig. 2. In situ knock-down of miR-21-5p alleviated EAU-induced retinal injury. The expression of miR-21-5p in retinal homogenates was measured by real-time PCR (a) at 21 days after immunization. Histological changes in retinal tissues were analyzed by H&E staining assay at 14 days after immunization (b) and the histology score was showed (c). Data are expressed as mean ± SD. $^{▲▲}P < 0.01$ vs. Control group, $^{\&\&}P < 0.01$ vs. EAU group, $^{\&}P < 0.05$ vs. EAU group. Typical photographs from 6 independent repetitions are shown. Scale bar = 50 μm. GCL: the ganglion cell layer, IPL: the inner plexiform layer, INL: the inner nuclear layer, OPL: the outer plexiform layer, ONL: the outer nuclear layer. The red arrows indicate the destroyed retinal structure, the black arrows indicate the infiltrated inflammatory cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

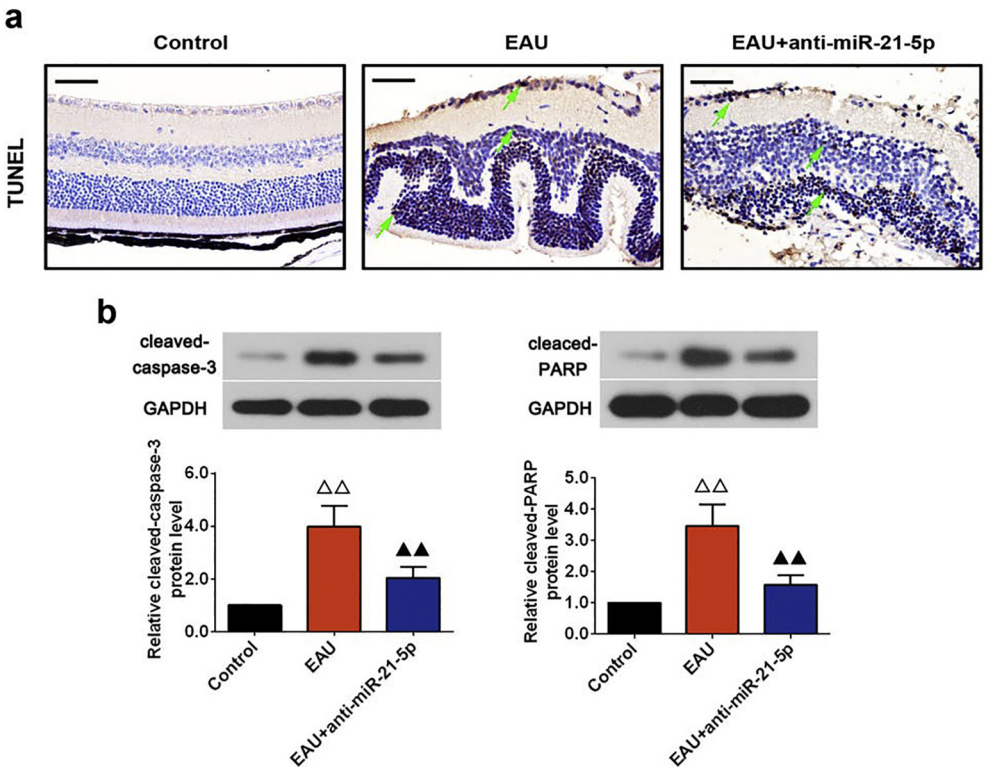

Fig. 3. In situ knock-down of miR-21-5p inhibited EAU-induced apoptosis of retinal cells. The apoptosis cells were detected by TUNEL staining assay (a) and the expression of cleaved-caspase-3 and cleaved-PARP of retinal tissue were detected by Western blot at 14 days after immunization (b). Representative images from 6 independent repetitions are shown. Scale bar = 50 μm. The green arrows indicate the typical apoptosis cells. $^{▲▲}P < 0.01$ vs. Control group, $^{△△}P < 0.01$ vs. EAU group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

were purchased from GenePharma (Shanghai, China). For the luciferase assay, HEK293T cells (Zhong Qiao Xin Zhou Biotechnology Co., Ltd) were seeded in 6-well plates at the density of 3×10^5 cells/well and allowed to grow to subconfluence. Then the cells were transiently co-transfected with 150 ng wild-type IL-10 3′UTR or mutated 3′UTR and 20 nM NC mimic or miR-21-5p mimic by using the Lipofectamine 2000 reagent (Invitrogen, CA, USA). The luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega) 24 h after transfection.

2.8. Lymphocytes culture and cytokine ELISA

Lymphocytes from the spleens at 0 d, 7 d, 14 d or 21 d after immunization and draining cervical lymph nodes at 14 d after immunization were isolated as previously reported [20,21]. The cells were cultured in RPMI 1640 media (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (FBS, BI, Kibbutz, Israel) and 2 mM glutamine. For cytokine detection, the splenic lymphocytes were stimulated with 10 μg/ml $IRBP_{1-20}$ for 72 h at 37 °C and 5% CO_2. The cells from cervical lymph nodes were infected with anti-miR-21 lentivirus (GenePharma) at 25 multiplicity of infection (MOI) in the presence of polybrene

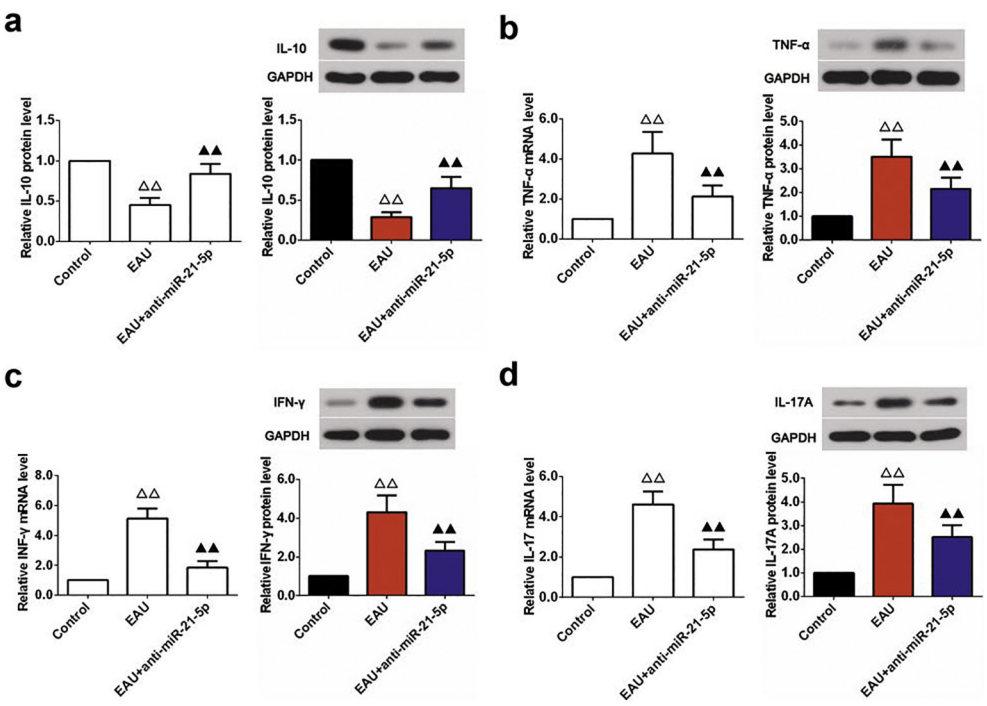

Fig. 4. Adenovirus-mediated down-regulation of miR-21-5p suppressed inflammatory responses in the retina of EAU mice. The mRNA and protein levels of IL-10 (a), TNF-α (b), IFN-γ (c) and IL-17A (d) of retinal homogenates at 21 days after immunization were determined by real-time PCR or Western blot. Typical protein bands from 6 independent repetitions are shown. Data are expressed as the mean ± SD. $^{\triangle\triangle}P < 0.01$ vs. control group, $^{\blacktriangle\blacktriangle}P < 0.01$ vs. EAU group.

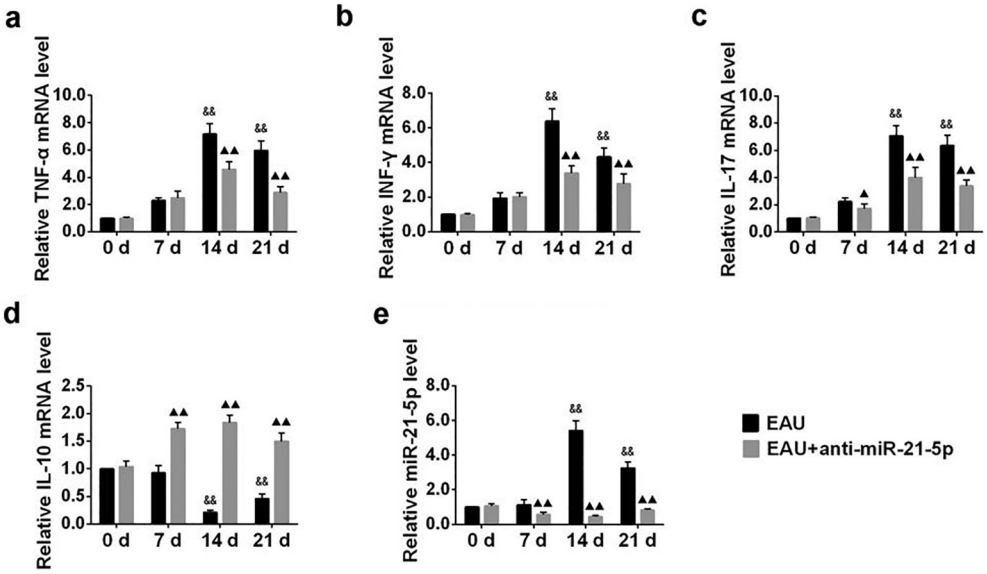

Fig. 5. Effects of miR-21-5p on cytokines expression at different time points. The expression of TNF-α (a), IFN-γ (b), IL-17 (c), IL-10 (d) and miR-21-5p (e) of retinal homogenates at 0, 7, 14 and 21 days after immunization were determined by real-time PCR. Data are expressed as the mean ± SD. $^{\&\&}P < 0.01$ vs. 0 days after immunization, $^{\blacktriangle}P < 0.05$ vs. EAU group, $^{\blacktriangle\blacktriangle}P < 0.01$ vs. EAU group.

(Sigma), 48 h before $IRBP_{1–20}$ stimulation. Then the TNF-α, IFN-γ, IL-17 or IL-10 levels in the supernatants were respectively detected by ELISA kits following the user's instructions (USCN Life Science Inc., Wuhan, China).

2.9. Flow cytometry

Lymphocytes from the spleens or draining cervical lymph nodes were stimulated with 50 ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich Int, Louis, MO, USA) and 750 ng/ml ionomycin (Sigma-Aldrich Int) in the presence of 10 μg/ml Brefeldin A (Sigma-Aldrich Int) for 4 h or 10 μg/ml $IRBP_{1–20}$ for 72 h. Subsequently, the cells were separated by CD4 MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), then these cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 monoclonal antibody (eBioscience, San Diego, CA, USA) and allophycocyanin (APC)-conjugated anti-IL-17A monoclonal antibody (eBioscience) or FITC-conjugated anti-CD4 monoclonal antibody and phycoerythrin (PE)-conjugated anti-forkhead box protein3(Foxp3) antibody (eBioscience) for 30 min in dark. Finally, the stained cells were analyzed with the Novocyte flow cytometry (ACEA Bioscience, San Diego, CA, USA).

2.10. Statistical analysis

Data were expressed as the mean ± standard deviation (SD), and differences between groups were analyzed by the Kruskal-Wallis test followed by Dunn's multiple comparisons test or One-way ANOVA followed by Bonferroni's multiple comparisons test using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA, USA). A P value < 0.05 was considered statistically significant.

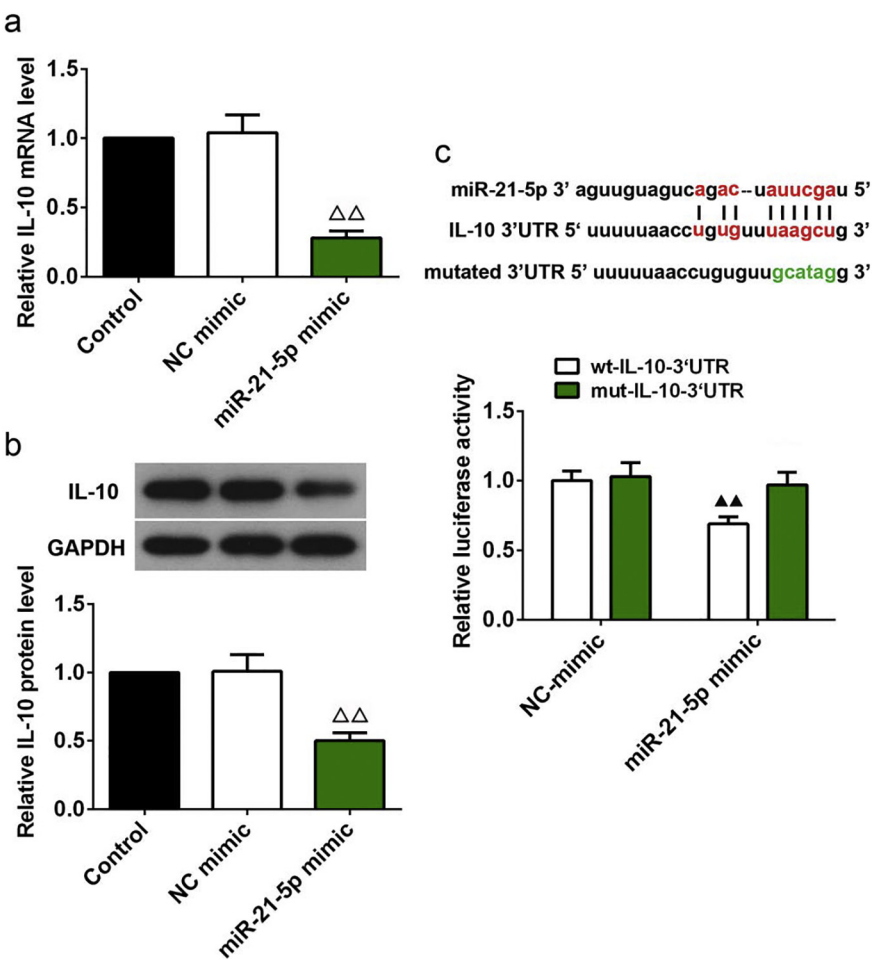

Fig. 6. Up-regulation of miR-21-5p inhibited the expression of IL-10 in vitro. Human retinal pigment epithelial cells ARPE-19 were transfected with NC mimic or miR-21-5p mimic. The mRNA level of IL-10 was measured by real-time quantitative PCR (a), the protein level of IL-10 was determined by Western blot (b). The specific regulation of miR-21-5p on the expression of IL-10 targeting the 3′UTR of IL-10 was detected by luciferase reporter assay (c). Data are expressed as the mean ± SD, and analyzed by One-way ANOVA followed by Bonferroni's multiple comparisons test. $^{\triangle\triangle}P < 0.01$ vs. NC mimic group, $^{\blacktriangle\blacktriangle}P < 0.01$ vs. mut-IL-10-3′UTR and NC mimic co-transfected group.

3. Results

3.1. Inhibition of miR-21-5p alleviates histopathological injuries and apoptosis of the EAU-retina

After EAU induction and treatment with anti-miR-21-5p adenovirus in mice following the schedule shown in Fig. 1, the expression of miR-21-5p in the retinal tissues was measured by real-time PCR. As shown in Fig. 2a, the expression of miR-21-5p was significantly increased in the retina of EAU mice compared to that of control mice and was markedly reduced by subretinal injection of anti-miR-21-5p adenovirus. The retina tissues were stained by H&E to analysis the histopathological changes. As shown in Fig. 2b, the microscopic appearance of retinal tissues in EAU mice showed aberrantly retinal destruction and inflammatory cell infiltration. In comparison, the retinas from the EAU + anti-miR-21-5p group showed ameliorated pathologic changes. Similarly, the histologic grades determined from H&E staining were markedly reduced by the inhibition of miR-21-5p in the EAU + anti-miR-21-5p group compared to that in the EAU group (Fig. 2c, 2.83 ± 0.75 vs. 4.67 ± 0.52, $P < 0.05$). Apoptotic cells were detected by TUNEL assay. As shown in Fig. 3a, the TUNEL-positive cells were obviously increased in the retina of EAU mice, and the population of positively stained cells was decreased by the treatment of anti-miR-21-5p adenovirus. The up-regulated expression of apoptotic proteins, cleaved-caspase-3 and cleaved-PARP, was also inhibited by knockdown of miR-21-5p (Fig. 3b). Collectively, inhibition of miR-21-5p could protect eyes from retina injury and apoptosis induced by EAU.

3.2. Inhibition of miR-21-5p reduces the inflammatory reactions of EAU-retina

To investigate the effect of miR-21-5p on inflammatory reactions in EAU mice, the mRNA and protein levels of inflammatory-related factors were measured using real-time PCR and Western blot at 21 d after immunization. As shown in Fig. 4, the mRNA and protein expression levels of pro-inflammatory mediators, including TNF-α, IFN-γ and IL-17, were significantly increased in the retina of EAU mice. Moreover, the mRNA and protein levels of the anti-inflammatory mediator, IL-10, were strongly decreased. Compared with that in the EAU group, mice treated with anti-miR-21-5p adenovirus had lower levels of TNF-α, IFN-γ and IL-17 and a higher level of IL-10. In order to further define the correlation between miR-21-5p and inflammatory reaction in the EAU retina, the kinetics of the miR-21-5p expression and these cytokines were also detected. As shown in Fig. 5a–c, the mRNA levels of TNF-α, IFN-γ and IL-17 increased significantly at the 14th day after immunization and decreased slightly at the 21st day. As expected, the changing trend of miR-21-5p is consistent with these pro-inflammatory factors (Fig. 5e), however, the IL-10 mRNA was distinctly reduced at the 14th and softly increased at the 21st day after immunization (Fig. 5d). Further, subretinally injection of anti-miR-21-5p adenovirus not only keeps miR-21-5p at a low level throughout the study but also suppressed the expression of TNF-α, IFN-γ, IL-17 and up-regulated the expression of IL-10. Collectively, these results suggest that miR-21-5p has participated in EAU-related inflammatory reactions, and inhibition of miR-21-5p could suppress inflammatory responses in the retina of EAU mice.

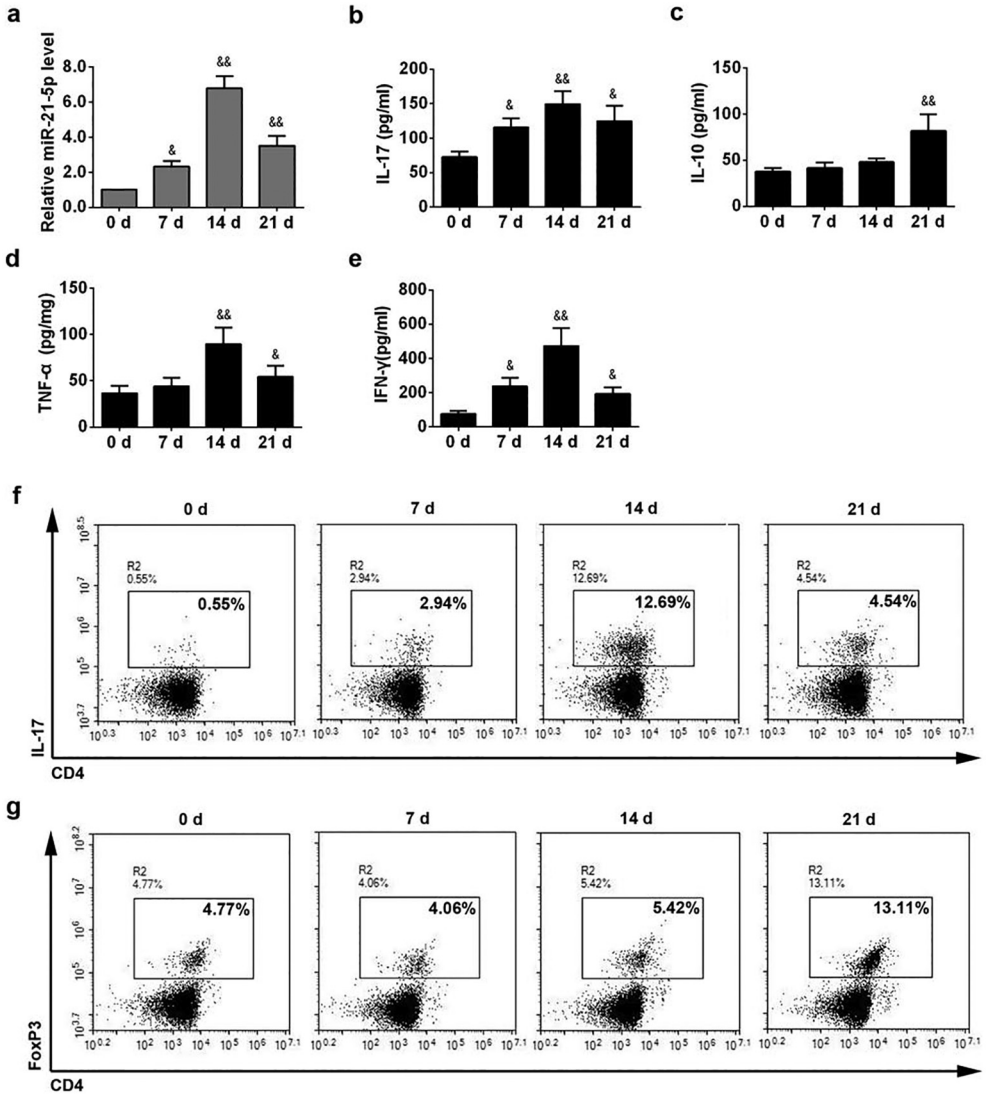

Fig. 7. miR-21-5p involved in the Th17 and Treg balance in the splenocyte of EAU mice. The lymphocytes from the spleens at 0 d, 7 d, 14 d or 21 d after immunization were isolated and the expression of miR-21-5p (a) was detected by real-time PCR, the levels of IL-17 (b), IL-10 (c), TNF-α (d) and IFN-γ (e) were measured by commercial kits. The frequency of IL-17 + CD4 + T cells (f) and Foxp3 + CD4 + T cells (g) was detected by flow cytometry. Data are expressed as the mean ± SD. $^{\&\&}P < 0.01$ vs. 0 days after immunization, $^{\&}P < 0.05$ vs. 0 days after immunization.

3.3. MiR-21-5p regulates the expression of IL-10

In order to investigate the mechanisms underlying the regulatory effect of miR-21-5p on inflammatory responses in the eyes of EAU mice, human retinal pigment epithelial cells were transfected with miR-21-5p mimic or NC mimic. The expression of the anti-inflammatory cytokine, IL-10, was examined after transfection. As shown in Fig. 6a & b, the mRNA and protein levels of IL-10 were significantly reduced in cells after transfection of miR-21-5p mimic. Luciferase reporter assay was used to further validate the binding activity between miR-21-5p and IL-10. As shown in Fig. 6c, a significant reduction in relative luciferase activity was observed in wt-IL-10 3′UTR and miR-21-5p mimic co-transfected cells. However, there was no difference in luciferase activity among the other three groups. Thus, miR-21-5p could regulate the expression of IL-10 by targeting its 3′-UTR.

3.4. The expression of miR-21-5p is related to the Th17 and Treg balance in the splenocyte of EAU mice

Considering the regulatory effect of miR-21-5p on T cell differentiation in other immunological diseases [22,23], we further examined the cytokine levels and the expression of miR-21-5p in splenic lymphocytes from EAU mice. As shown in Fig. 7a–e, the levels of IL-17, TNF-α, IFN-γ, as well as miR-21-5p expression were increased and reached its peak at the 14th day after immunization, the IL-10 level was significantly up-regulated at the 21st day after immunization. Besides, the frequency of Th17 and Treg cells were also detected (Fig. 7f & g), the results showed that the Th17 cell proportion began to increase on the 7th day after immunization and reached a peak on the 14th day, however, the frequency of Tregs was not changed on the 7th and 14th day but increased on the 21st day.

3.5. miR-21-5p regulates the Th17 and Treg balance of cervical lymph node cells

To verify the effect of miR-21-5p in the Th17 and Treg balance in vitro, the $IRBP_{1-20}$ primed draining cervical lymph node cells were isolated and transfected with anti-miR-21 lentivirus prior to in vitro stimulation with $IRBP_{1-20}$. The miR-21-5p level was strongly up-regulated by the stimulation of $IRBP_{1-20}$ and markedly reduced by anti-miR-21 lentivirus (Fig. 8a). Meanwhile, the IL-10 level was decreased and the IL-17 level was increased by $IRBP_{1-20}$ stimulation, and these were

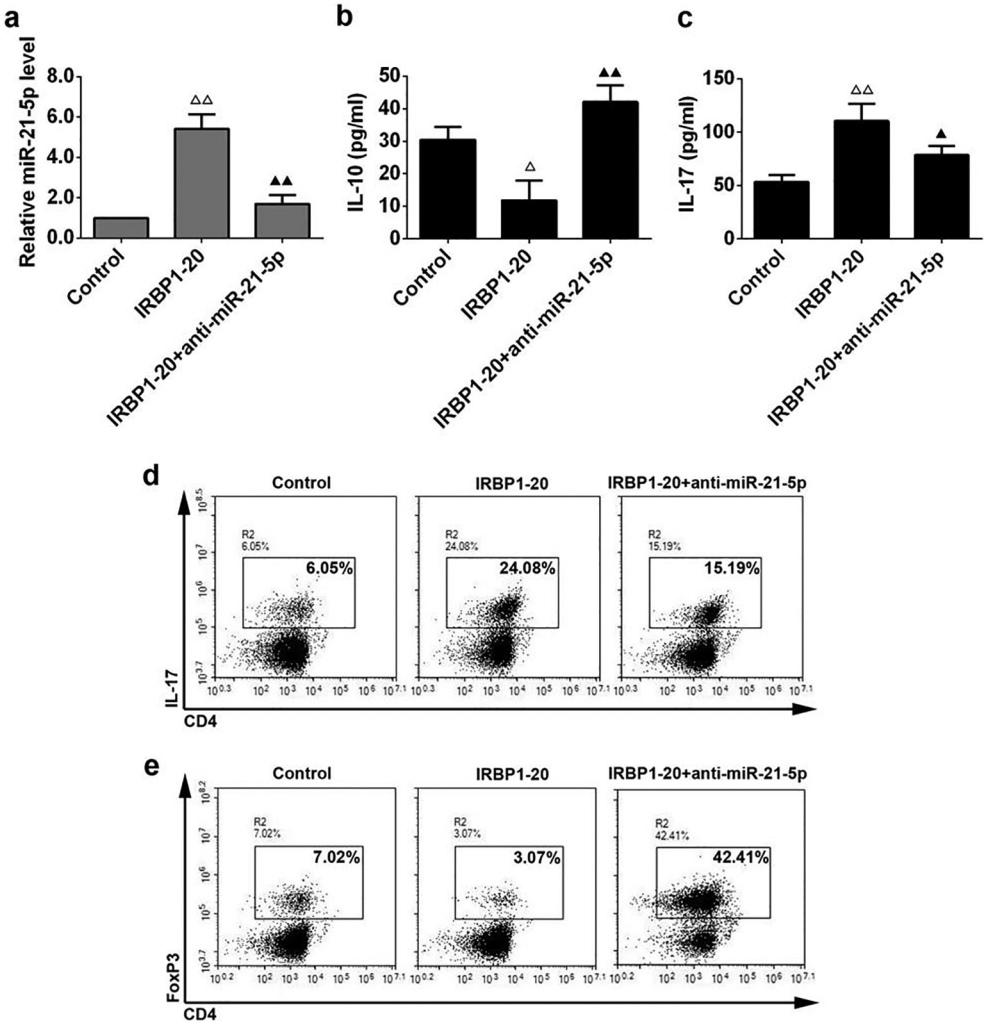

Fig. 8. Down-regulation of miR-21-5p regulates the Th17/Treg balance of IRBP1–20 stimulated cervical lymph node cells. The $IRBP_{1–20}$ primed draining cervical lymph node cells were isolated and transfected with anti-miR-21 lentivirus prior to in vitro stimulation with $IRBP_{1–20}$. Then the expression of miR-21-5p was detected by real-time PCR (a), the IL-10 (b) and IL-17 (c) content in the supernatant were also measured. The frequency of IL-17 + CD4 + T cells (d) and Foxp3 + CD4 + T cells (e) was detected by flow cytometry. Data are expressed as the mean ± SD. $^{\triangle\triangle}P < 0.01$ vs. control cells, $^{\blacktriangle}P < 0.05$ vs. $IRBP_{1–20}$ stimulated cells, $^{\blacktriangle\blacktriangle}P < 0.01$ vs. $IRBP_{1–20}$ stimulated cells.

both reversed by down-regulation of miR-21-5p (Fig. 8b & c). Further, knock-down of miR-21-5p suppressed $IRBP_{1–20}$ induced up-regulation of Th17 cell frequency and increased the Treg cell fraction in vitro (Fig. 8d & e). Thus, down-regulation of miR-21-5p could alter the balance of Th17/Treg cells.

4. Discussion

EAU is an organ-specific antigen-induced autoimmune disease and extensively used to study basic mechanisms of uveitis at present. In the current study, EAU-induced retinal architecture disruption, inflammation and cell apoptosis were all alleviated by inhibition of miR-21-5p in the retina. The regulatory effect of miR-21-5p on IL-10 was confirmed by luciferase reporter assay. The dynamic changes of miR-21-5p in spleen lymphocytes were in line with those of Th17 cells. Additionally, knock-down of miR-21-5p in $IRBP_{1–20}$ stimulated lymphocytes reduced the frequency of Th17 cells and increased Tregs proportion. Our data revealed that in situ inhibition of miR-21-5p is helpful to relieve eye injury caused by EAU, and miR-21-5p may also contribute to the progress of EAU by regulating Th17/Treg balance via inhibition of IL-10.

The EAU induced by active immunization can be generally divided into two stages, the afferent phase and the effector phase. The main pathological events of the afferent phase are antigen recognition and immunocompetent cells activation, and this phase lasts 5–7 days. In the effector phase, the immune cells migrate to retinal tissues, trigger the inflammatory cascade, and result in the onset of EAU symptoms within another 5–7 days. After that, the inflammatory level of the target tissue decreases and the disease starts to recede in the next 5–7 days, but the lesion may last for several weeks, depending on the animal species, peptides position of pathogenic antigens and immune dose [4,16,24]. Studies have shown that the peaks of EAU histology score and retinal apoptotic cells proportion both occurred on the 11th to 15th days after immunization with IRBP [16,25–27]. Poulaki et al. [5] suggest that apoptotic nuclei appeared in the inflammatory areas and most of these apoptotic nuclei belonged to the resident cells resulting in significant cell loss in EAU. Our data showed that in situ inhibition of miR-21-5p relieves retinal damage, reduced apoptosis cells and inhibited inflammatory responses. Further, we found that miR-21-5p could bind to the 3′-UTR of IL-10 and negatively regulate its expression. IL-10 is a pivotal anti-inflammatory cytokine [28], and numerous studies emphasized its anti-inflammatory and immunosuppressive activity in the eyes [29,30]. De et al. [31] also found that subconjunctival injection of IL-10 adenovirus significantly reduced pathological symptoms of EAU without affecting the systemic immune response. Thus, in situ down-regulation of miR-21-5p may alleviate the EAU-induced retinal injury by the suppression of inflammatory response partly via up-regulation of IL-10.

Cytokines and chemokines in the inflammatory sites recruit T-lymphocytes to exacerbate ocular injury during EAU [32]. Early studies have demonstrated that Th17 cells are the aetiological contributors to autoimmune diseases [33,34], including EAU. Tregs can produce abundant anti-inflammatory cytokines to modulate the immune system and play critical roles in preventing immune aggression. Decreased frequency and diminished function of Tregs are closely related to active

uveitis [35]. Considering the involvement of miR-21-5p in the differentiation of the Th17 and Treg cells [23,36,37], we further examined the kinetics correlation between miR-21-5p and immune response during EAU. Our data showed that the expression of the miR-21-5p and the pro-inflammatory cytokines in the retinal tissues were slightly elevated at the 7th day and strongly up-regulated at the 14th day after immunization, however, the pro-inflammatory cytokines, the Th17 frequency, as well as the miR-21-5p level in the splenic lymphocyte were significantly increased at the 7th day and reaches its peak at the 14th day after immunization, but the frequency of splenic Treg and the IL-10 level were not changed at the 7th and 14th day after immunization, and increased at the 21st day. It is reported that the expression of miR-21 was maintained at a low level in normal T cells and antigen-presenting cells, but significantly increased in activated T cells [38–40]. Besides, miR-21-5p could promote Th17 differentiation and negatively regulates Tregs frequency [22,41]. These well explains our results, that is, the highly expressed miR-21-5p promotes splenic Th17 differentiation and suppresses Tregs differentiation in the afferent phase of EAU, then at the effector phase, the Th17 cells infect to the retinal tissues, promotes inflammation responses, destroy the retinal structure and induce cell apoptosis. After that, the reduced miR-21-5p level leads to a decrease in the proportion of Th17 cells and an increase in the proportion of Tregs in the spleen. At the same time, due to the regulation of miR-21-5p, the IL-10 level is inhibited in the afferent phase and up-regulated after the effector phase of EAU.

Th17 cells led to autoimmunity and inflammation, whereas Tregs plays an opposite function in EAU [42,43], thus, unraveling the effect of miR-21-5p on Th17/Treg balance is critical to better reveal the role of miR-21-5p in the progress of EAU. Over-expression of miR-21-5p promotes Th17 differentiation via activation of the STAT3 pathway [44]. Knock-down of miR-21-5p protect lupus-like autoimmunity disease by decreased Th17 and increased Tregs in the mice model [14]. miR-21-5p contributed to Th17 differentiation and negatively regulate the frequency of Treg cells by targeting a negative regulator of TGF-β signaling, SMAD-7 [22,41]. Our in vitro data showed that knock-down of miR-21-5p suppressed IRBP1–20 induced up-regulation of Th17 cell frequency and increased the Treg cell fraction. These results indicating that miR-21-5p may involve in the regulation of Th17/Treg balance during EAU. In addition, the negative regulation of miR-21-5p on IL-10 expression may also be one of the reasons why miR-21-5p inhibits the differentiation of Tregs.

In conclusion, our study revealed that in situ inhibition of miR-21-5p alleviate EAU-induced retinal injury by its anti-inflammatory activities. In the afferent phase of EAU, miR-21-5p seems to promote the differentiation of Th17 and inhibit Treg differentiation. Down-regulation of miR-21-5p converted the differentiation of naive T cells from Th17 cells toward Tregs in vitro. These effects may be due to the regulatory effects of miR-21-5p on IL-10 expression. Therefore, we suggest that miR-21-5p may represent a potential therapeutic target in the treatment of uveitis as well as other autoimmune diseases.

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Author contributions statement

LS and YC conceived of the presented idea. LS, HG, ZL, YWW carried out the experiment. LS wrote the manuscript with support from YJW, HG, ZL. LS, ZL, YWW, and YJW analyzed the data. All authors discussed the results and contributed to the final manuscript.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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