

Cytotoxic effect of specific T cells from mice with experimental autoimmune uveitis on murine photoreceptor cells

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Abstract

• **AIM:** To investigate the cytotoxic effect of specific T cells from mice with experimental autoimmune uveitis (EAU) as well as their secreted interferon (IFN)- γ and interleukin (IL)-17A on murine photoreceptor (661W) cells.

• **METHODS:** An EAU model was established in female mice by injection of interphotoreceptor retinoid binding protein (IRBP) emulsion supplemented with complete Freund's adjuvant (CFA) and *Mycobacterium tuberculosis* (TB). On day 12 after induction of EAU, specific T cells from spleen and lymph node tissues were isolated and cultured for 4d and the levels of IFN- γ and IL-17A in the supernatants were determined by enzyme-linked immunosorbent assays (ELISAs). T cells and their supernatants were added to 661W cells to observe the alteration of cell morphology; IFN- γ and IL-17A were separately added to 661W cells to observe the effect of IFN- γ and IL-17A on cell proliferation.

• **RESULTS:** The levels of IFN- γ and IL-17A in the T cell supernatants were 1568.64 ± 38.79 pg/mL and 1456.57 ± 46.98 pg/mL, respectively. The supernatants apparently inhibited 661W cell proliferation ($P < 0.05$). T cells could also attach to the surface of 661W cells, and IFN- γ showed a more serious cytotoxic effect on 661W cells than IL-17A, inhibiting cell proliferation ($P < 0.01$).

• **CONCLUSION:** IFN- γ and IL-17A from T cells of EAU mice model can exert cytotoxic effects on murine photoreceptor cell proliferation, and IFN- γ shows more serious cytotoxic effects on murine photoreceptor cells than IL-17A.

• **KEYWORDS:** T cell; murine photoreceptor cell; IFN- γ ; IL-17A; experimental autoimmune uveitis; mouse

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INTRODUCTION

Uveitis is an autoimmune eye disease that is mainly mediated by T cells^[1-2]. Uveitis can lead to vision loss and mostly occurs in young adults and children. Consequently, it results in heavy burden on society and families. With an incidence of approximately 50/100 000 people and a prevalence of 0.1%^[3], uveitis remains one of the leading causes of blindness among all eye diseases. In developing countries, approximately 25% of irreversible blindness is caused by uveitis and its complications^[4].

Usually, uveitis is divided into anterior uveitis, intermediate uveitis, posterior uveitis, and panuveitis on the basis of structure. Intermediate uveitis or posterior uveitis, which is associated with other systemic immunological diseases, is characterized by inflammation of the ciliary body, vitreous, retina or choroid. Intermediate uveitis or posterior uveitis might be secondary to ocular or systemic infections such as tuberculosis (TB) and toxoplasmosis. Moreover, intermediate uveitis or posterior uveitis can also develop in specific ocular or systemic conditions, including Vogt-Koyanagi-Harada disease (VKH), Behçet's disease (BD), sympathetic ophthalmia,

sarcoidosis, and birdshot chorioretinopathy^[5]. Currently, the mechanism of uveitis is still unclear.

The vast majority of uveitis is caused by the autoimmune response. Due to the lack of knowledge of the human pathology, an animal model of experimental autoimmune uveitis (EAU) has been established *in vitro*^[6-7]. EAU is a model of human posterior uveitis, a potentially blinding ocular inflammatory condition that influences the choroid and the neural retina^[8-9]. EAU can be induced by immunization with several retinal autoantigens, of which retinal S-antigen (S-Ag) and interphotoreceptor retinoid binding protein (IRBP) are the best studied^[10].

Initially, EAU was considered a disease that is mediated by CD4⁺ T helper 1 (Th1) cells, and evidence has shown that autoreactive Th1 cells mediate the development of EAU^[11]. EAU is closely correlated with the level of IFN- γ generated by Th1 cells^[12]. Recently, Th17 cells, a CD4⁺ T-cell subset, were shown to play a crucial role in several autoimmune diseases, including uveitis. Th17 cells can produce interleukin (IL)-17 and are closely associated with the pathogenesis and development of EAU^[13]. The roles of interferon (IFN)- γ and IL-17 have been confirmed to be pathogenic in EAU^[14-15]. Recent studies have shown that innate immune cells in EAU secrete inflammatory cytokines^[16].

Cell lines are often used to help study the molecular basis of eye diseases to design preventive or therapeutic interventions^[17]. Photoreceptor cells are very important for vision, although they are very susceptible to the outer microenvironment. A mouse photoreceptor cell line, the 661W cell line, has been described that is derived from a retinal tumor formed in a transgenic mouse expressing SV40 large T-antigen under the control of the IRBP promoter^[18].

Our previous results suggest that the persistent recurrent EAU model established in C57BL/6 mice may be a good model that can represent different aspects of human uveitis^[12]. In clinical practice, we also noted that the visual acuity of the patients with uveitis had no apparent improvement after treatments, although they did not show inflammatory symptoms. Therefore, in the present study, to investigate the effect of T cells from mice with uveitis and IL-17 as well as IFN- γ on 661W cells, we first established an EAU model in mice with IRBP emulsion, and then, the spleen and lymph node tissues from the mice with EAU were isolated to collect T cells to explore the interaction between the T cells and 661W cells. Furthermore, the levels of IFN- γ and IL-17A in the supernatants from cultured T cells were determined, and the cytotoxic effects of IFN- γ and IL-17A on 661W cells were investigated. Our investigations will help elucidate the interaction between T cells and murine photoreceptor cells and the consequence of vision damage.

MATERIALS AND METHODS

Ethical Approval The experiments were approved by the Ethics Committee of Shandong University of Traditional Chinese Medicine. Principles for the care and use of laboratory animals in research based on the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research were strictly followed.

Animals and Treatments Pathogen-free female C57BL/6 (B6) mice (6-8wk, purchased from Peking Vital River Laboratory Animal, Ltd., Beijing, China) were maintained in specific pathogen-free conditions according to the guidelines of the China National Institutes of Health. The induction of EAU in C57BL/6 mice was performed as described previously^[17,19-20]. Briefly, C57BL/6 mice were subcutaneously immunized at six different locations (footpads, tail base, and flanks) with 350 μ g of human IRBP 1177-1191 (China Peptides Co., Ltd., Suzhou, Jiangsu Province, China) that was emulsified in complete Freund's adjuvant (Sigma-Aldrich Company, MA, USA) and *Mycobacterium tuberculosis* (TB, strain H37RA; Difco Laboratories, Detroit, MI, USA). A single dose of 500 ng of pertussis toxin (PTX; Enzo Life Sciences, Farmingdale, YN, USA) was injected intraperitoneally.

Cell Culture 661W cells used in the present study was kindly provided by Dr. Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, USA). The 661W cells were cultured in 25-cm² flasks (NEST Biotechnology, Wuxi, China) as described previously^[21]. In brief, 661W cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Oklahoma City, OK 73190, USA) supplemented with 1.0 g/L glucose, 10% fetal bovine serum (FBS; Gaithersburg, MD, HyClone, Logan, UT, USA), 100 μ g/mL streptomycin and 100 U/mL penicillin. All cells were cultured at 37°C in a water-saturated incubator containing 5% CO₂ plus 95% air. Cell counts were performed using an automated cell counter (TC10; Bio-Rad, Hercules, CA, USA).

Preparation of Specific T Cells from the Mice with EAU The T cells were obtained according to previous methods^[19-20,22]. On day 12 after immunization, the mice with EAU were sacrificed, and the lymph node and spleen tissues were isolated to collect T cells by a nylon wool column. Antigen-presenting cells (APCs) from the mice with EAU were irradiated by X-rays (3000 mGy) and mixed with T cells (1:1). Further, 1 \times 10⁷ cells in 1 mL medium mixed with β -CM (containing DMEM, 0.000002% β -mercaptoethanol, 10% FBS and 1% streptomycin) were stimulated with 10 mg/mL IRBP 1177-1191 and recombinant mouse IL-2 (10 ng/mL) in a 6-well plate (NEST Biotechnology, Wuxi, China) for 2d. Subsequently, the activated T cells were purified by Ficoll reagent (Beijing Solarbio S&T Co., Ltd., China) and cultured for another 2d.

Flow Cytometric Analysis For cell surface molecule staining, T cells were first purified using Ficoll reagent and then cultured for 2d. Further, T cells were stained with anti-CD₃-FITC buffer and were determined by a flow cytometer (Accuri C6, USA). T cells stained with anti-CD4-FITC and anti-CD8-PE buffer were stored at 4°C for 40min and then washed with phosphate buffered saline (PBS) three times. Finally, the treated T cells were analyzed using a flow cytometer (Accuri C6, USA).

Enzyme-linked Immunosorbent Assay For determination of the levels of IFN- γ and IL-17 secreted by T cells, 100 μ L of the supernatants was collected after T cells were cultured for 2d. The levels of IFN- γ and IL-17A were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (both were purchased from Dakewe Biotech Company, China) and were determined according to the manufacturer's instructions.

Morphological Alterations in 661W Cells Cultured either with T Cells or with Supernatant *in vitro* T cells were cultured for 2d after purification with Ficoll reagent. Further, T cells (1×10^7 cells per mL) and 200 μ L of the supernatants from T cells (1×10^7 cells per mL) were added to 24-well plates (NEST Biotechnology, Wuxi, China) with 661W cells (final volume: 500 μ L). Subsequently, 661W cells were cultured for an additional 2d. At the indicated times, morphological alterations in 661W cells were observed using an inverted fluorescence microscope (Olympus IX71, Japan).

Real-time Cell Electronic Sensing Assay The real-time cell electronic sensing (RT-CES) assay (the xCELLigence system, Roche, Germany) can provide dynamic information that can be used to identify the interplay between adherent cells and chemicals^[23-24]. In the present study, the RT-CES assay was used to monitor the proliferative effect of T cells after treatment with the supernatants, IFN- γ and IL-17A on 66W cells. In brief, 8×10^3 661W cells were seeded in each well in an E-plate, cultured overnight, treated under different conditions and further cultured for an additional 3d.

Interactions Between 661W and T cells T cells and 661W cells were cocultured for 2d, and then, the supernatants were discarded and washed 3 times with PBS. Further, anti-CD₃-PE was added to the wells of 6-well plates (NEST Biotechnology, Wuxi, China) to label 661W cells. The plates were placed at 4°C for 40min, and then, T cells and 661W cells were fixed by glutaraldehyde prior to staining. Finally, the morphology of T cells was observed by an inverted fluorescence microscope (Olympus IX71, Japan).

Alterations in 661W Cells after Adding IFN- γ and IL-17A The 661W cells (8000 cells per well) in log phase were either seeded in a 96-well plate (NEST Biotechnology, Wuxi, China) or seeded in an E-plate and then cultured overnight; further, either recombinant mouse IFN- γ (100 ng) or 100 ng of

IL-17A was added to each well and cultured for an additional 3d. Control samples were seeded in the same plate and treated with medium only. The morphology of the 661W cells was observed by an inverted fluorescence microscope (Olympus IX71, Japan), and the dynamic response was recorded using the RT-CES system and cultured for an additional 3d after 661W cells were treated either with IFN- γ or with IL-17A.

MTT Assay Cell viability was evaluated using the 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay^[25]. The principle of the MTT assay is that succinate dehydrogenase in the mitochondria of living cells can reduce exogenous MTT to water-insoluble purple formazan and deposit in cells, whereas dead cells have no such function. For quantification of the viable cells after exposure to recombinant mouse IFN- γ (100 ng) or 100 ng of IL-17A, cells were first seeded in 96-well plates (NEST Biotechnology, Wuxi, China) at a density of 8.0×10^3 cells per well, grown overnight, and then treated with different concentrations of recombinant mouse IFN- γ (100 ng) or 100 ng of IL-17A for 24, 48 and 72h. At the indicated times, the medium was gently discarded, and another 180 μ L of culture medium supplemented with 20 μ L of MTT (5 mg/mL, Sigma, USA) was added to each well for additional incubation for 4h at 37°C in a 5% CO₂ incubator. At the indicated times, the treated cells were observed using a bright field microscope with 200-fold amplification, and six nonoverlapping fields were randomly observed by two independent researchers, and the viable cells were calculated and averaged.

RESULTS

Characterization of T Cells and CD4⁺ T Cells As shown in Figure 1, after surface molecule staining, all of the treated cells were analyzed by flow cytometry, and the level of T cells was $96.8\% \pm 1.9\%$, in which the level of CD4⁺ T cells was $88.3\% \pm 2.6\%$.

Measurement of IFN- γ and IL-17A The levels of IFN- γ and IL-17A in the supernatants secreted from T cells after culture for 2d were 1568.64 ± 38.79 pg/mL and 1456.57 ± 46.98 pg/mL, respectively (Figure 2). This result demonstrated that the abundant inflammatory cytokines secreted by T cells may affect adjacent cell physiological functions.

Morphology and Proliferation of 661W cells After treatment with either T cells or T cell supernatants, 661W cells became spindly, yet after treatment with T cell supernatants, the morphological alteration was more apparent than that of the cells treated with T cells (Figure 3). Moreover, the RT-CES results revealed that treatment with T cell supernatants exerted a greater inhibitory effect on 661W cell proliferation than treatment with T cells (Figure 4).

Interaction Between T Cells and 661W Cells Typical images were captured after 661W cells were cocultured with T

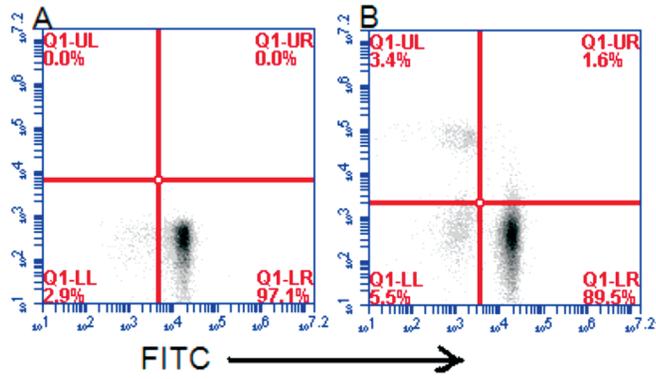


Figure 1 Determination of the proportion of T cells After staining either with anti-CD3-FITC or with anti-CD4-FITC/anti-CD8-PE for surface molecules, the proportion of T cells was analyzed with a flow cytometer.

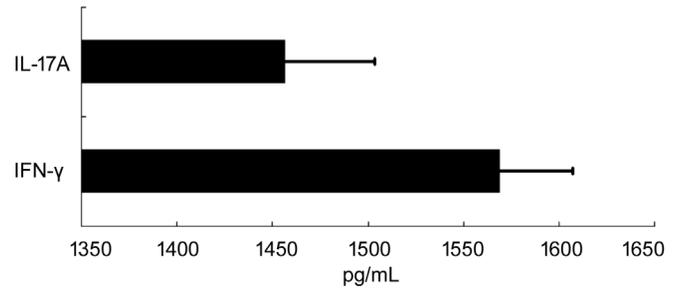


Figure 2 Measurements of IFN-γ and IL-17A T cells were purified by Ficoll reagent and further cultured for 2d, and the levels of IFN-γ and IL-17A in supernatants secreted by T cells were determined by ELISAs.

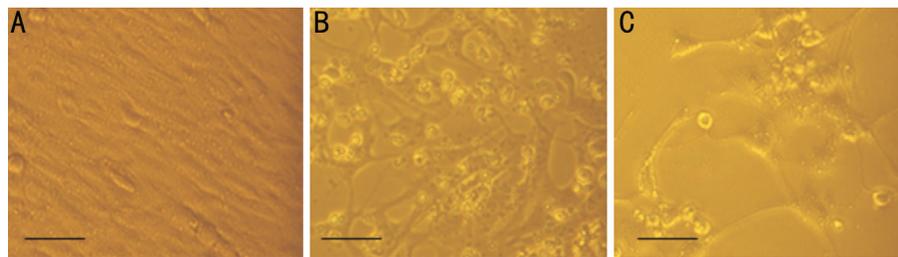


Figure 3 Morphological alterations in 661W cells after treatment either with T cells or with their supernatants A: Control cells; B: Treatment with T cells; C: Treatment with supernatants. Bar=20 μm.

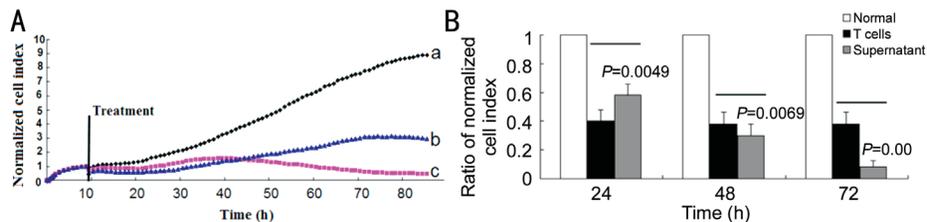


Figure 4 Dynamic determination of 661W cell proliferation after treatment with either T cells or with the supernatants A: The response of 661W cells after treatment either with T cells (curve b) or with their supernatants (curve c) determined by an RT-CES system. Curve a is the proliferative curve of 661W cells treated with neither T cells nor the supernatants (control). B: Comparison with the relevant control samples at 24, 48, and 72h.

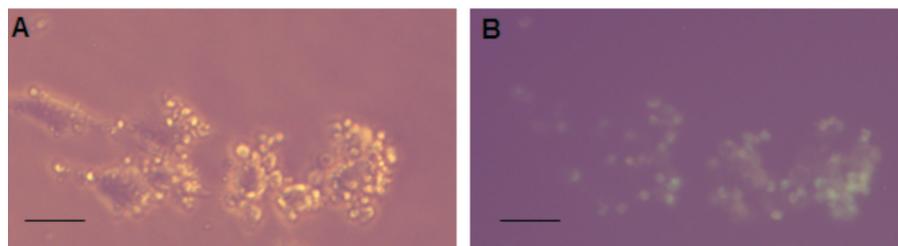


Figure 5 Morphological images of 661W cells incubated with T cells Typical images were captured after 661W cells were treated with T cells and stained with anti-CD4-FITC- and anti-CD8-PE-labeled T cells in the absence (A) and presence (B) of fluorescence. It was noted that T cells could be attached to 661W cells, indicating that T cells show chemotaxis to 661W cells. Bar=20 μm.

cells by a microscope with or without fluorescence. As shown in Figure 5, T cells were apparently attached to the surface of 661W cells, indicating that T cells could exert chemotactic effects on 661W cells.

Effect of Recombinant Mouse IFN-γ and IL-17A on 661W Cells The cytotoxic effects of IFN-γ and IL-17A on 661W

cells were further explored using both microscopy and RT-CES techniques. The results indicated that both IL-17A and IFN-γ can exert cytotoxic effects on 661W cells (Figures 6-8), *i.e.*, both IL-17A and IFN-γ can inhibit 661W cell proliferation *in vitro*. Importantly, IFN-γ exerted a more serious inhibitory effect on 661W cells than IL-17A. The RT-CES and

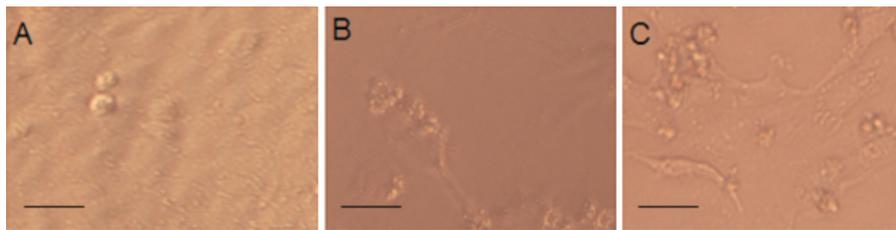


Figure 6 Typical images of 661W cells after treatment with either recombinant rat IFN- γ (B) or IL-17A (C). Control cells (untreated cells) were also cultured in the same plate (A). IFN- γ exerted a greater inhibitory effect on 661W cells than IL-17A. Bar=20 μ m.

MTT results (Figures 7-8) were also consistent with those of microscopy (Figure 6). In the present study, we noted that the same concentrations of IFN- γ and IL-17A showed different cytotoxic effects on photoreceptor cells. IFN- γ exhibited a more severe cytotoxic effect on photoreceptor cells than IL-17A.

DISCUSSION

The blood-retinal barrier is made of retinal endothelial cells and retinal pigment epithelial cells. When inflammation occurs in eyes, the blood-retinal barrier can be destroyed. In this situation, peripheral activated T lymphocytes can pass through the blood-retinal barrier because of the T cell receptors and similar polypeptides in the retina, resulting in endophthalmitis^[7,16].

In a previous study, the utility of CD4⁺ T lymphocytes in autoactivation was associated with the pathogenesis of autoimmune disorders. CD4⁺ cells were divided into Th1 and Th2 subsets. IFN- γ is secreted by the Th1 cell subset, which is a major subset of pathogenic T cells in various autoimmune diseases that has been confirmed to be pathogenic in autoimmune uveitis in both patients and animal models^[26]. In an earlier study, Th1 cells were shown to be autoimmune inflammatory effector cells, whereas Th2 cells could inhibit this effect^[27]. Tarrant *et al*^[28] considered that the regulation of the Th1 cell response plays a major role in the pathogenesis of uveitis. Early lymphocyte adoptive transfer experiments also confirmed that EAU can be successfully induced by antigen-specific T lymphocytes producing abundant IFN- γ with a Th1 cell phenotype^[28]. It was reported that mice in which the IFN- γ gene was deleted (elimination of Th1 cells) showed more severe inflammation in the eye after EAU induction. Nevertheless, IFN- γ is not the only response to cytokines^[29-30]. IL-23 and Th17 cells could also explain this contradiction. The Th17 cell subtype is essential for the pathogenesis of autoimmune uveitis. In the EAU model, Th17 cells and IL-17 seem to play an important role in inducing inflammation^[31]. Th17 cells resist pathogens by secreting Th17-related cytokines. The Th17 reaction obviously leads to inflammation-related autoinflammatory diseases^[29]. Recent evidence suggests that newly recognized IL-17, which is produced by Th17 cells, plays a crucial role in the progression of EAU^[26], and Th1 and Th17 responses are differentially required for EAU

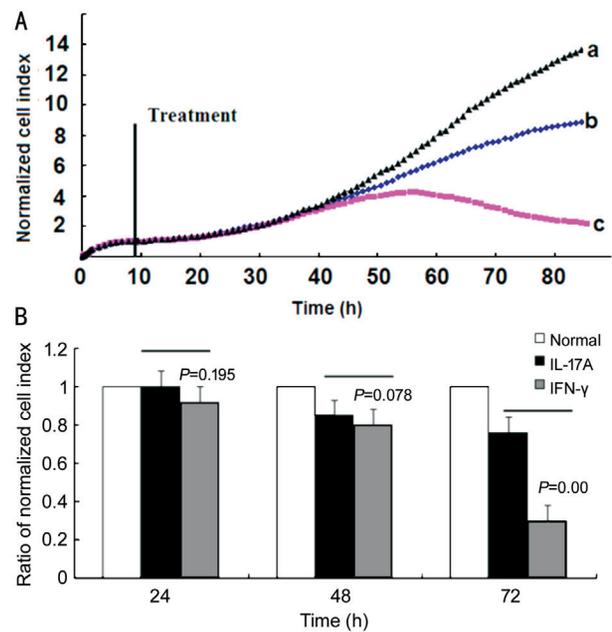


Figure 7 Dynamic determination of 661W cell proliferation after treatment with medium, IL-17A or IFN- γ A: Dynamic response of 661W cells after treatment with medium only (a; untreated cells), IL-17A (b), and IFN- γ (c). All cells were cultured overnight in the E-plate and then treated with either IL-17A or IFN- γ and further cultured at 37°C for an additional 72h. B: Comparison with the relevant control samples at 24, 48, and 72h.

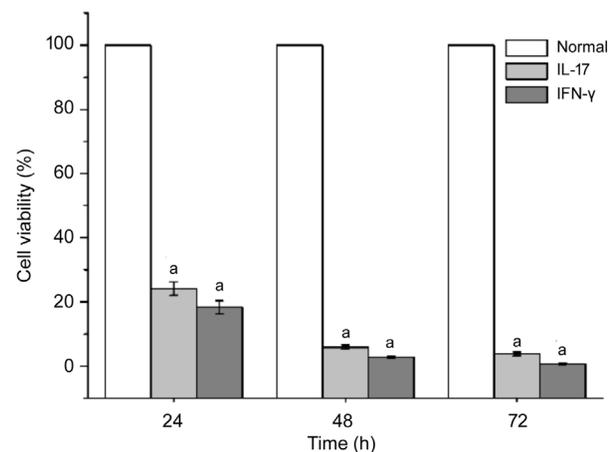


Figure 8 Results of the MTT assay The 661W cells were treated with 100 ng of recombinant mouse IFN- γ or 100 ng of IL-17A for 24, 48, and 72h and then cultured with 5 mg/mL of MTT solution for another 4h. The cell viability is presented as the mean \pm SD. ^a*P*<0.01 vs normal samples.

development^[32]. Studies have revealed that Th17 and Th1 cells could be induced in the pathogenesis of EAU^[33]. Moreover, studies on EAU and human diseases such as VKH disease imply that activated CD4⁺ T cells, including Th1 and Th17 cells, play an effector role in intraocular inflammation^[34]. Therefore, both Th1 and Th17 cells can play a role in the development and pathogenesis of uveitis.

In the present study, after the successful induction of EAU in mice, the anterior chamber showed empyema, hyperemia and occlusion of the pupil, and our findings were consistent with the literature^[35]. We also found that murine photoreceptor cell proliferation can be inhibited by T cells and their supernatants, and inverted fluorescence microscopy and RT-CES assays also showed that the supernatants of T cell medium inhibit 661W cell proliferation more seriously than T cells. Based on these findings, we hypothesized that both IFN- γ and IL-17 can inhibit 661W cell proliferation. To validate this hypothesis, we added the same amount of IFN- γ and IL-17 to 661W cells. Using microscopy and RT-CES techniques, we found that IFN- γ exerted a more serious inhibitory effect on 661W cell proliferation than IL-17. In the mice with EAU, IFN- γ and IL-17 secreted by IRBP-induced specific T cells exerted cytotoxic effects on 661W cells. Consequently, the vision of EAU mice was impaired.

Uveitogenic T cells cause chronic or recurrent uveitis, in which severe retinal damage is a common pathological feature^[36]. Based on our findings and clinical practice, we can infer that even after patients have been cured, their vision still cannot be restored to normal. This phenomenon can be attributed to the fact that photoreceptor cells can be damaged by IFN- γ and IL-17 secreted by inflammatory T cells in the mice with EAU. In other words, IFN- γ and IL-17A can exert cytotoxic effects on murine photoreceptor cells. Nevertheless, the mechanism by which IFN- γ exhibits more serious cytotoxic effects on photoreceptor cells than IL-17A should be further studied.

In summary, we have successfully established a mouse model of EAU, and we found that both IFN- γ and IL-17A secreted by specific T cells from the mice with EAU can exert cytotoxic effects on murine photoreceptor cells and inhibit their proliferation. IFN- γ can exhibit more severe cytotoxic effects on murine photoreceptor cells than IL-17A.

Nevertheless, the limitations of the present study should be mentioned. First, we only demonstrated that the cell culture medium (*i.e.*, T cell supernatants containing IFN- γ and IL-17) could exert an inhibitory effect on 661W cell proliferation. The mechanism of the cytotoxic influence of IFN- γ and IL-17A on murine photoreceptor cells is still unclear and needs to be further studied. Second, although anti-IL-17 antibody is sufficient to block the development of disease^[37], whether anti-IFN or anti-IL-17 antibody protects photoreceptor

cells needs to be further investigated. Third, IL-17 is not only a proinflammatory cytokine but also causes lipid peroxidation that may be toxic to photoreceptor cells; thus, the effect of IL-17 on lipid peroxidation needs to be further studied.

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