

# Interleukin-35 Suppresses Pyroptosis and Protects Neuronal Death in Retinal Ischemia/Reperfusion Injury

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## Research

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# Abstract

**Background:** Retina ischemia-reperfusion (I/R) is a pathological process in many eye disorders. Neuroinflammation and cell pyroptosis have been recognized as important in the pathogenesis of tissue damage in retina I/R. Interleukin (IL)-35 is a novel heterodimeric cytokine that exhibits anti-inflammatory activity in various autoimmune diseases, but its role in retina I/R and the underlying molecular mechanisms remain unexplored. This study investigated the effect of IL-35 on retina I/R and the inhibition of pyroptosis and neuronal death.

**Methods:** A murine retina I/R model was used to explore the neuroprotective effect of IL-35 recombinant protein *in vivo*. The primary murine microglial cells of pyroptosis and the retinal ganglion cells (RGCs) of oxygen and glucose deprivation/reoxygenation (OGD/R) models were employed to test the anti-pyroptotic and anti-apoptotic effects of IL-35 *in vitro*.

**Result:** We found that IL-35 decreases retinal damage, RGC death, and inner plexiform layer (IPL) thinning in mice with retinal I/R injury, with significant attenuation of pyroptosis in the retina. The data also demonstrated the anti-pyroptosis action of IL-35 in primary microglia stimulated with lipopolysaccharide (LPS) and adenosine triphosphate (ATP). Furthermore, primary RGC apoptosis induced by OGD/R was directly suppressed by IL-35, and the IL-35-mediated neuroprotection was abrogated when miR-21 was blocked.

**Conclusion:** Our findings identify potential underlying mechanisms of RGC apoptosis and suggest a new therapeutic target, IL-35, which exerts a robust neuroprotective effect against retina I/R.

## Introduction

Retina ischemia-reperfusion (I/R) injuries are related to many diseases of the eye, including glaucoma, diabetic retinopathy, retinal arterial occlusion and so on<sup>1-4</sup>. These kinds of diseases are usually accompanied by retinal ganglion cell (RGC) apoptosis, which inevitably leads to a loss of visual field.

Although the pathogenesis of retina I/R injuries is not fully understood, inflammation is considered critical in the pathogenesis of RGC death during retinal I/R injury<sup>5</sup>. Pyroptosis has more recently been identified as a particular proinflammatory form of programmed cell death<sup>4</sup>. Our previous study also suggested that pyroptosis occurs in ischemic injured retinas<sup>5,6</sup>.

IL-35 is a newly described heterodimeric cytokine of the IL-12 family, composed of an  $\alpha$  chain of 12p35 and a  $\beta$  chain of Epstein-Barr virus-induced gene3 (EBI3)<sup>6</sup>. It appears to have an anti-inflammatory effect by regulating the differentiation and function of immune cells, such as T cells, B cells, macrophages, and dendritic cells<sup>7-10</sup> but its role and mechanisms to microglia and retina I/R remain unclear. Thus, the present study was conducted to experimentally confirm the role and mechanisms of IL-35 in retina I/R injuries.

# Materials And Methods

## Animals and treatment

Male C57BL/6 mice aged 6 to 8 weeks were procured from the Guangzhou Animal Testing Center. Our study adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. This research was approved by the Committee of Animal Care of the Zhongshan Ophthalmic Center. The retinal I/R model of mice was prepared as described previously.<sup>11</sup> Before operating, a single intraperitoneal dose of ketamine (100 mg/kg) and xylazine 10 mg/kg) was used to induce anesthesia. The corneas were topically anesthetized with a drop of 0.5% tetracaine and the pupils dilated using 1% tropicamide. A 30-gauge needle containing infuse sterile saline was used to maintain high intraocular pressure (IOP) of 70 mmHg for 1 hour. For the control, the same procedure was applied to the contralateral eye with normal IOP. The needles were carefully withdrawn after 1 hour, and tobramycin ointment was used to prevent infection.

An intravitreal injection of IL-35 recombinant protein (2 $\mu$ l), IL-35 recombinant protein mixed with antagomir solution (2 $\mu$ l), or a control solution (2 $\mu$ l) was administered to the eyes of the mice immediately after the I/R model was completed. The IL-35 solution was prepared by dissolving IL-35 recombinant protein (Sigma-Aldrich, USA) in sterile PBS to make a 0.1mol/L stock solution, which was stored at 4°C and used within 3 months. The antagomir (20 $\mu$ M; Ribobio, China) of miR-21 and the control solution were mixed immediately before injection.

## Primary RGC cultures and the oxygen and glucose deprivation/reperfusion (OGD/R) model

Murine primary RGCs were isolated in accordance with a previous procedure<sup>12</sup>. In brief, single-cell suspensions were generated from the retinas of neonatal mice. To dislodge the adherent macrophages, flasks coated with anti-mouse macrophage antibodies (Cedarlane, USA) were used to incubate the suspensions. The adherent cells were then collected by transferring the non-adherent cells into flasks coated with Thy1.2 monoclonal antibodies (Millipore Chemicon, USA). Finally, the RGCs were collected with a growth medium supplemented with factors at 37°C for 24 h in 5% CO<sub>2</sub> until they were completely adherent.

The OGD/R model was constructed by first washing the cells two times with PBS (Gibco) and then substituting the medium used for the culture with glucose-free dulbecco's modified eagle medium (DMEM)(Gibco). Next, the cells were placed in a modular incubator chamber (Billups-Rothenberg, Inc. USA) set at 37°C and containing 5% (vol/vol) CO<sub>2</sub> and 95% (vol/vol) N<sub>2</sub> for 3 h. Incubation of the control cells was performed in normoxic conditions (5% [vol/vol] CO<sub>2</sub> and 95% [vol/vol] air) in a medium that was free of serum but contained 4.5 g/L D-glucose, also for 3 h. After the exposure period, the cells were placed in normoxic conditions and incubated for 12 h.

## Isolation of the primary retinal microglia and establishment of a cell pyroptosis model in vitro

Microglia were harvested from mixed primary glial cultures that were obtained from the retinas of newborn C57BL/6J mice, as previously reported<sup>13</sup>. Following mechanical- chemical dissociation (papain comprising 180 units/mL DNase; Sigma-Aldrich, USA) for 10 min at 38°C, the cells were suspended in DMEM that was supplemented with 10% (vol/vol) Foetal Bovine Serum (FBS), penicillin, and streptomycin and cultivated for two to three weeks. After confluency was achieved, isolated primary microglia cells were obtained by shaking at 200 rpm at 37°C for 6 h in an orbital shaker.

Culturing of the cells was conducted in high-glucose (4.5 g/ml) DMEM containing 10% FBS, 100 U/ml streptomycin, and 100 µg/ml penicillin, which was placed in a humidified incubator with an atmosphere of less than 5% CO<sub>2</sub> and at 37°C. To induce cell pyroptosis, cells were stimulated with 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich, USA) in DMEM for 4 h, with or without co-treatment with different concentrations of IL-35, and then challenged with adenosine 5'-triphosphate (ATP; Sigma-Aldrich, USA) (1mM) for 1 h.

### **RNA reagent delivery**

Microglia or RGC cells at 70% confluency were chosen for the process of transfection. In accordance with the manufacturer's instructions, miR-21 inhibitor was added to the reagent complexes and transfection buffer (Ribobio, China) until a concentration of 200 nM was reached. A minimum of 24h of transfection was required to achieve maximal transfection efficiency.

### **Quantitative real-time PCR and qPCR**

TRIzol Reagent (Invitrogen, China) was used to extract the total RNA from the cultured and retina cells in accordance with the manufacturer's instructions. cDNA synthesis was performed with a PrimeScript RT Master Mix (TaKaRa, China). The amplification reaction was established with 25 cycles for β-actin and 30–32 cycles for the other transcripts. The products of PCR were transferred onto 2% (wt/vol) agarose gels in order to assess the relative expression. A Light Cycler 480 Real-Time PCR System (Roche Molecular Systems, Inc., SUR) was used to perform quantitative analysis. The expression of the target mRNA was first determined and then normalized to GAPDH. The primers of pyroptosis and neuronal death used in the present study are shown in Table 1.

### **Western blot**

The total amount of protein was obtained from the retinas and transferred onto 10% (wt/vol) polyacrylamide gels according to the standard protocol. The target proteins' expression was normalized to GAPDH, then retrieved from the corresponding sample (taken as 1.0) and quantified with ImageJ Software. The primary antibodies used in the present study are listed in Table 2.

### **Histological examination**

On the seventh day after the I/R model, the mice were sacrifice and eyeballs were collected for formalin fixation and paraffin embedding. Three slices (4 µm) around the optic nerve were sectioned and

hematoxylin and eosin (H&E) staining was conducted. Three sections were measured and averaged for each eye.

### **Immunofluorescence (IF)**

The mice were killed seven days after the model was established and intracardially perfused through the left ventricle with 4% paraformaldehyde. Following enucleation, the retinas were first dissected and then stained as both whole-mounts and slices. A Zeiss LSM 510 confocal laser scanning microscope (Carl Zeiss, USA) was used to capture images, which were processed with Adobe Photoshop CS8. Antibodies for Brn3a and Tuji-1 were used to label the RGCs for the assessment of RGC death. Data for three separate sections per eye was used to calculate the mean outcome. The primary antibodies used in the present study are listed in Table 2.

### **Flow cytometry**

To measure the apoptosis of the RGCs induced by OGD/R, flow cytometry and a propidium iodide (PI) and AnnexinV-FITC (fluorescein isothiocyanate) detection kit were used in accordance with the manufacturers' instructions. FlowJo 7.6.2 (FlowJo, LLC, USA) was used for flow cytometric analysis according to standard protocols. Analysis of IL-1 $\beta$  was also conducted with the monoclonal antibody according to standard protocols, and FlowJo Software was used to perform the data analysis.

### **Statistical analysis**

The data is reported as mean  $\pm$  SD. SPSS software version 22 (SPSS Inc, USA) was used to perform one-way ANOVAs and Bonferroni's post hoc test. The statistical analyses were two-tailed, with  $p < 0.05$  considered to be statistically significant.

## **Results**

### **Neuroprotective effects of intravitreal IL-35 in retinal I/R injury**

In the present study, intravitreal injection of IL-35 was administered to evaluate its role in retina I/R. We first investigated the effects of various concentrations of IL-35 on IOP-induced RGC damage. Representative IF of specific stains (Tuj-1+ and Brn3a+) showed a dramatic decrease in RGCs seven days after the experimental I/R injury. Intravitreally administered IL-35 reversed the damage to the RGCs caused by I/R, compared with the non-treated mice (control  $100.0 \pm 5.89\%$ , vs. I/R  $19.27 \pm 0.37\%$ ,  $p < 0.001$ ). Specifically, minor RGC mortalities were observed with treatment with IL-35 at the optimum concentration of 20 ng/ml ( $49.77 \pm 8.65\%$ ) (Fig 1A-B). Based on this, 20 ng/ml of IL-35 was selected to conduct the subsequent in vivo experiments.

We then quantitatively estimated the change in retinal thickness in all groups using histological sections. In the IL-35 treatment group, the observed damage to the retina, specifically to the IPL—induced by I/R was robustly mitigated by IL-35 (control  $54.91 \pm 4.09\%$ , I/R  $35.24 \pm 2.83\%$ , IL-35 group  $48.18 \pm 6.13\%$ )

(Fig 1C-D). Taken together, the intravitreal IL-35 treatment substantially lowered the retina damage levels and RGC apoptosis rate.

### **IL-35 inhibited increases in IOP-induced pyroptosis**

We previously reported that pyroptosis is involved in the inflammatory process of retinal I/R and plays an important role in the apoptosis of RGC [6-8] but that the role of IL-35 in pyroptosis remain unclear. We therefore tested whether IL-35 inhibits the pyroptosis induced by retina I/R.

IL-35 was administered intravitreally immediately after the acute elevation of IOP. As measured by qRT-PCR, the mRNA levels of Nod-like receptor family, pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing C-terminal caspase-recruitment domain adaptor protein (ASC), caspase-1, gasdermin D (GSDMD), IL-1 $\beta$ (including interleukin-1 $\beta$ ), and IL-18(including interleukin-18), increased in the retinas of the I/R group, compared to the normal group, and declined in the IL-35-treated group (Fig 2A-F). Western blot (WB) results for NLRP3, cleaved GSDMD, and cleaved caspase-1 (Fig 2G-H) confirmed the qRT-PCR results. In IF analysis, we observed that IL-35 treatment decreased the regulation of NLRP3 and ASC inflammasome in retinas following I/R (Fig 2I-J).

### **IL-35 inhibits microglia pyroptosis in vitro**

The cell counting Kit-8 (CCK8) assay showed no cytotoxic effects of IL-35 (from 0 to 100 ng/ml) on the microglia (Fig 3A). Then, we then examined the effects of IL-35 on ATP-induced pyroptosis in LPS-primed microglia. Flow cytometry revealed a remarkable increase of IL-1 $\beta$  in the LPS + ATP group ( $6.23 \pm 0.89\%$ ), but treatment with IL-35 especially the concentration of 50 ng/ml attenuated this effect ( $3.60 \pm 0.34\%$ ) (Fig 3B-C). Relative expression of NLRP3, ASC, and IL-18 mRNA increased in the OGD/R group, and decreased in the IL-35-treated group (50 ng/ml) (Fig 3D-G).

### **IL-35 directly protects RGC apoptosis induced by OGD/R**

To explore the mechanisms underlying the effect of IL-35 on RGC apoptosis, primary murine RGCs were isolated and purified for the OGD/R experiments. First, the RGCs were identified by IF on the second day of cultivation (Fig 4A). Then, a CCK8 assay showed that IL-35 (from 0 to 100 ng/ml) did not exhibit significant toxicity (Fig 3B). Cells were then divided into five groups: (a) a control group, (b) an OGD/R group, and (c) three IL-35 groups (5, 10, and 20 ng/ml). We then exposed the RGCs to OGD for 2 h followed by recovery for 2 h to mimic the in vivo I/R injury induced by elevated IOP. The percentage of viable cells (PI-Annexin V-) decreased after OGD/R exposure (NC  $100.00 \pm 3.03\%$ , OGD/R  $51.68 \pm 3.68\%$ ), which was significantly increased by doses of IL-35 ranging from 1 to 20 ng/ml, with 10 ng/ml( $90.31 \pm 5.70\%$ ) and 20 ng/ml( $90.68 \pm 2.08\%$ ) exerting similar anti-apoptotic effects that were both greater than the others (Fig 4C-D).

### **miR-21/PDCD4 axis is essential for IL-35-mediated anti-pyroptosis effect**

We next assessed whether the miR-21/PDCD4 axis is also involved in the anti-pyroptosis effects of IL-35. As shown in Fig 6a, the miR-21 antagomir significantly, but incompletely, reversed the IL-35-mediated inhibition of the mRNA of NLRP3, caspase-1, and IL-18. Similarly, the in vitro flow cytometry results showed that administration of the miR-21 inhibitor significantly, but incompletely, reversed the IL-35-mediated mitigation of IL-1 $\beta$  production induced by LPS and ATP (LPS+ATP 9.52  $\pm$  0.43%, IL-35 group 3.39 $\pm$  0.42%, IL-35+miR-21 inhibitor 7.20 $\pm$  0.49%).

### **miR-21/PDCD4 axis conferred neuroprotection to RGCs**

We then examined the expression levels of miR-21 and its target gene, programmed cell death protein 4 (PDCD4), in the retina after I/R damage. The mRNA level of miR-21 was decreased in the I/R group (Fig 5A), while the protein level of PDCD4 increased compared to the control group (Fig 5B-C), and IL-35 significantly altered this tendency.

IF staining showed that the I/R-induced activation of PDCD4 expression in Iba-1+ microglia could be inhibited by IL-35. However, administration of miR-21 antagomir significantly, but incompletely, reversed the IL-35-mediated inhibition of PDCD4.

The results showed that miR-21/PDCD4 signaling plays a critical role in the IL-35-mediated neuroprotective effect in retina I/R (Fig 5D-E). Our results showed that the inhibition of miR-21 significantly, but incompletely, reversed the IL-35-mediated neuroprotection of RGCs, both in vivo (control 100.00  $\pm$  6.19%, IR 27.11  $\pm$  7.90%, IL-35 group 41.99  $\pm$  4.31%, IL-35+miR-21 antagomir 41.99 $\pm$  4.31%) (Fig 5F-G) and in vitro (control 95.81  $\pm$  9.05%, OGD/R 62.43  $\pm$  7.89%, IL-35 group 86.11  $\pm$  2.09%, IL-35+miR-21 inhibitor 62.29 $\pm$  3.54%) (Fig 5H-J). Collectively, these findings indicate that the miR-21/PDCD4 axis plays a critical role in the IL-35-mediated neuroprotection of RGCs.

## **Discussion**

Retina I/R is a pathological condition in various ocular diseases that results in severe eye injury and potentially vision impairment or blindness<sup>14-17</sup>, and neuroinflammation is widely recognized as a critical factor in the injury induced by retinal I/R<sup>18, 19</sup>. In recent years, IL-35 has been found to have anti-inflammatory effects, as it can regulate immune responses in immune cells, like macrophages and dendritic cells<sup>10, 20, 21</sup>. However, the effect of IL-35 on retina I/R remains unclear. The present study found that IL-35 dramatically alleviated retinal damage and RGC apoptosis following retina I/R, with a significant inhibition of inflammation. These results indicate that IL-35 could serve as a treatment for diseases involving retinal I/R.

Pyroptosis is a particular inflammatory programmed cell death that is initiated by inflammasomes<sup>22</sup>. During inflammatory responses, inflammasomes, such as ASC and NLRP3, are activated and subsequently activate inflammatory caspase-1, resulting in cleaved GSDMD. The N-terminal domain of cleaved GSDMD translocates to the cell membrane, forming the transmembrane pores that facilitate the

release of cleaved IL-1 $\beta$  and IL-18, to stimulate inflammatory responses<sup>23</sup>. Accumulating evidence has shown that pyroptosis occurs in virous inflammatory diseases<sup>24,25</sup>, and our previous studies also found that pyroptosis participates in the pathological process of retina injuries following high IOP<sup>18,26</sup>. This study, for the first time, found that IL-35 can lower the expression of inflammatory molecules and attenuate pyroptosis in vivo. Furthermore, our vitro investigations have confirmed that IL-35 can alleviate pyroptosis in the microglia directly.

The progressive loss of RGCs caused by retina I/R remains a significant cause of irreversible vision loss in blinding diseases like acute glaucoma and retinal vascular occlusion<sup>16,27</sup>. However, the pathogenesis and mechanism of retina I/R injury is complicated. RGC death can occur from pathological processes triggered by retina I/R, such as neuroinflammation, oxidative stress, and mitochondrial malfunction<sup>5</sup>, and it is therefore vital to develop pharmacological interventions that can enhance RGC survival and protect the optic nerve. Here, we presented evidence that IL-35 produces neuroprotective effects by protecting RGCs from apoptosis directly at the level of cell biology. The findings suggest that IL-35 produces neuroprotective effects by dual mechanisms and that it might be considered a candidate agent for the treatment of damage in the context of retina I/R.

miR-21(MicroRNA-21) is a multifunctional miRNA that is central to inflammation and apoptosis in many diseases<sup>14,28,29</sup> and can down-regulate the promoter activity of PDCD4 and participate in anti-inflammatory signaling.<sup>29</sup> The miR-21-PDCD4 axis was also shown in our previous study to be involved in retina injuries triggered by high IOP.<sup>11</sup> Here, we found that the anti-pyroptotic and anti-apoptotic properties of IL-35 are likely conferred by miR-21, both in vivo and in vitro. Specifically, these results suggest a key role for the miR-21-PDCD4 axis in IL-35-mediated protection from retina I/R.

## Conclusion

In summary, the present study demonstrates that IL-35 inhibits inflammasome-induced pyroptosis triggered by high IOP. Furthermore, IL-35 could directly protect RGCs from apoptosis. Most notably, we further established the neuroprotective mechanisms of IL-35 by showing that the miR-21-PDCD4 axis is essential for IL-35 inhibition of microglia pyroptosis and RGC apoptosis. These results demonstrate that IL-35 can protect against retina I/R and could serve as a potential therapeutic drug for retina I/R.

## Abbreviations

I/R: ischemia/reperfusion; IL-35:Interleukin-35; RGCs: retinal ganglion cells; OGD/R : Oxygen and glucose deprivation/reoxygenation ; LPS: lipopolysaccharide; ATP: adenosine triphosphate; EB13: Epstein-Barr virus–induced gene3; IOP: intraocular pressure; DMEM: glucose-free dulbecco's modified eagle medium; FBS Foetal Bovine Serum; PBS: Phosphate-buffered saline; H&E: hematoxylin and eosin

IF: Immunofluorescence; PI:propidium iodide; FITC :fluorescein isothiocyanate;IPL: inner plexiform layer; GCL: ganglion cell layer; NLRP3: Nod-like receptor family, pyrin domain containing 3; ASC: apoptosis-

associated speck-like protein containing C-terminal caspase-recruitment domain adaptor protein GSDMD: gasdermin D; IL-1 $\beta$ : including interleukin-1 $\beta$ ; IL-18: including interleukin-18; miR:-21:microRNA-21 PDCD4:programmed cell death protein 4;qPCR: quantitative polymerase chain reaction; CCK-8: Cell Counting Kit-8

## Declarations

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### Availability of data and materials

The data during this study are available from the corresponding author.

### Authors' contribution

B.L and Y.L were responsible for the conception and design, data collection and assembly, manuscript writing, and final manuscript approval. N.J and S.H were responsible for data collection and assembly. W.S and Y. Z were responsible for conception and design, manuscript writing, and final manuscript approval.

### Ethics approval and consent to participate

All animal experiments were performed according to the institutional guidelines of the Committee of Animal Care of the Zhongshan Ophthalmic Center.

### Consent for publication

Not applicable.

### Competing interest

All authors claim that they have no competing interests.

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## Tables

### Table1 Primer sequences for RT-qPCR.

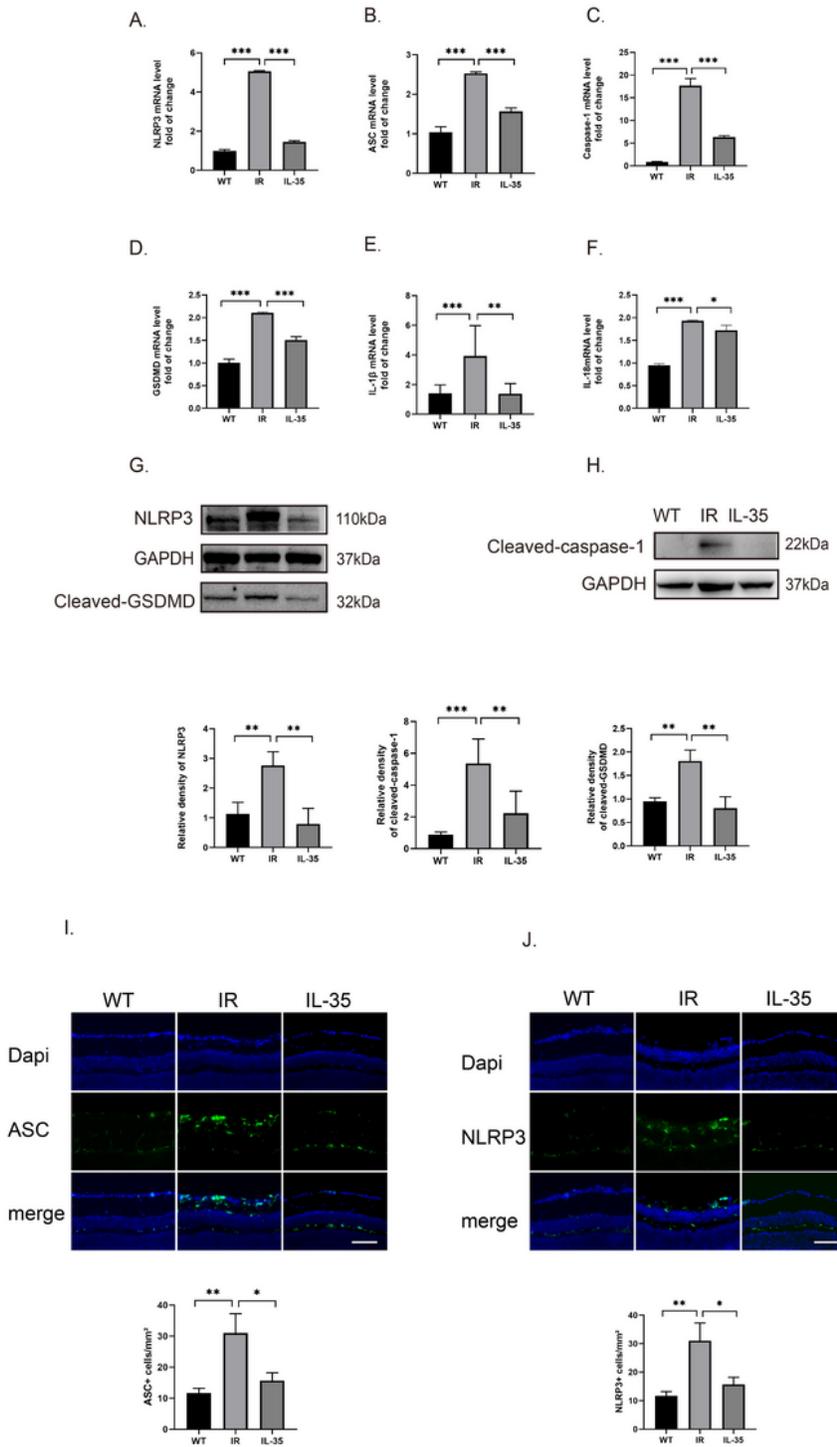
Primer	Sequence
GAPDH-F	TGCCACTCAGAAGACTGTGG
GAPDH-R	GTCCTCAGTGTAGCCCAGGA
ASC-F	AGACATGGGCTTACAGGA
ASC-R	CTCCCTCATCTTGTCTTGG
NLRP3-F	GGTCCTCTTTACCATGTGCTTC
NLRP3-R	AAGTCATGTGGCTGAAGCTGTA
Caspase-1-F	TGAAAGAGGTGAAAGAATT
Caspase-1-R	TCTCCAAGACACATTATCT
IL-1 $\beta$ -F	GGGCCTCAAAGGAAAGAATC
IL-1 $\beta$ -R	CTCTGCTTGTGAGGTGCTGA
IL-18-F	CTGTACAACCGCAGTAATACGG
IL-18-R	ACTCCATCTTGTTGTGTCCTGG

F, forward; R, reverse.

**Table 2 Primary antibodies and dilutions.**

Primary antibody	Manufacture	Dilution
GAPDH	CST	WB(1:1000)
NLRP3	CST	WB(1:1000) IF(1:200)
Cleaved-caspase-1	CST	WB(1:1000)
Cleaved-GSDMD	CST	WB(1:1000)
PDCD4	CST	WB(1:1000)
ASC	CST	IF(1:200)
Brn-3a	SANTA CRUZ	IF(1:500)
Tuj-1	Abcam	IF(1:500)
Dapi	Sigma-Aldrich	IF(1:200)
IL-1 $\beta$	eBioscience	FC(1:500)
FITC	eBioscience	FC(1:500)
PE	eBioscience	FC(1:500)

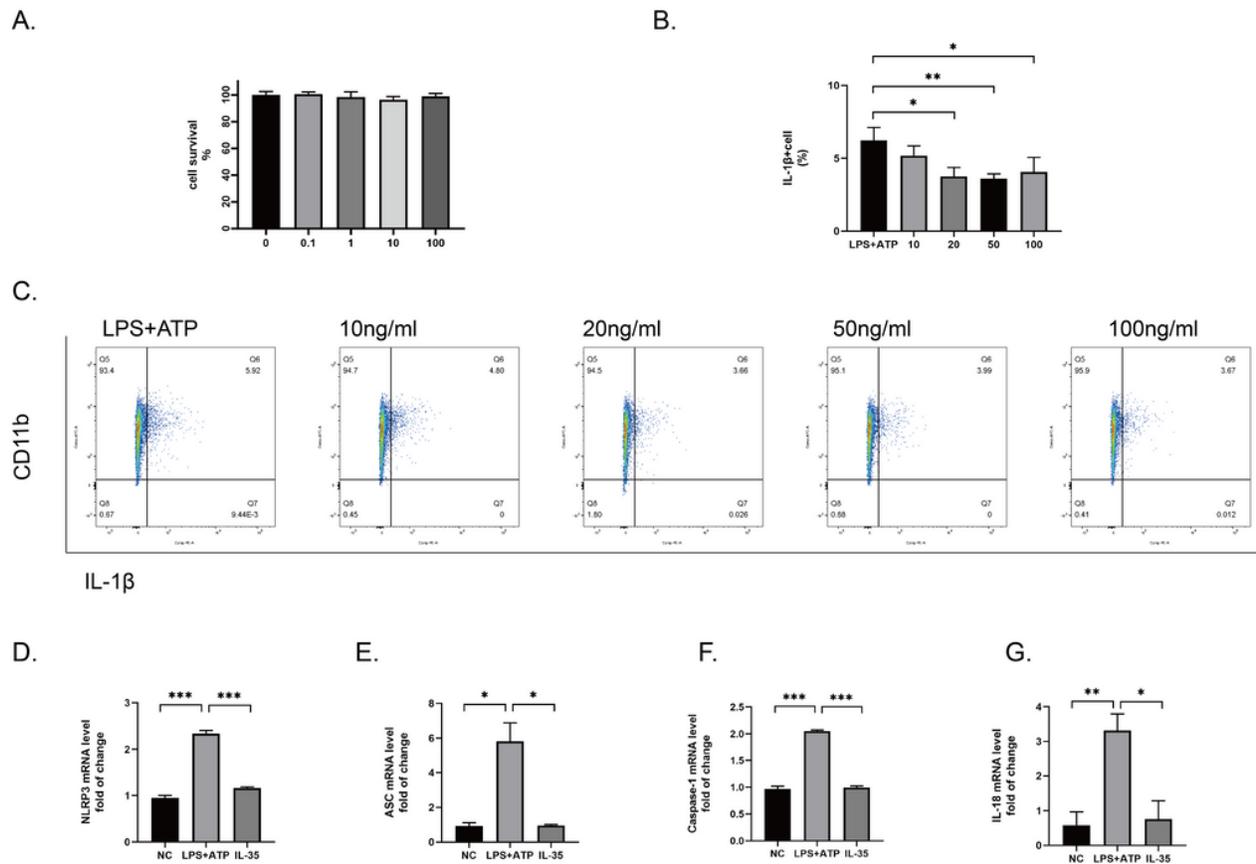
# Figures



**Figure 1**

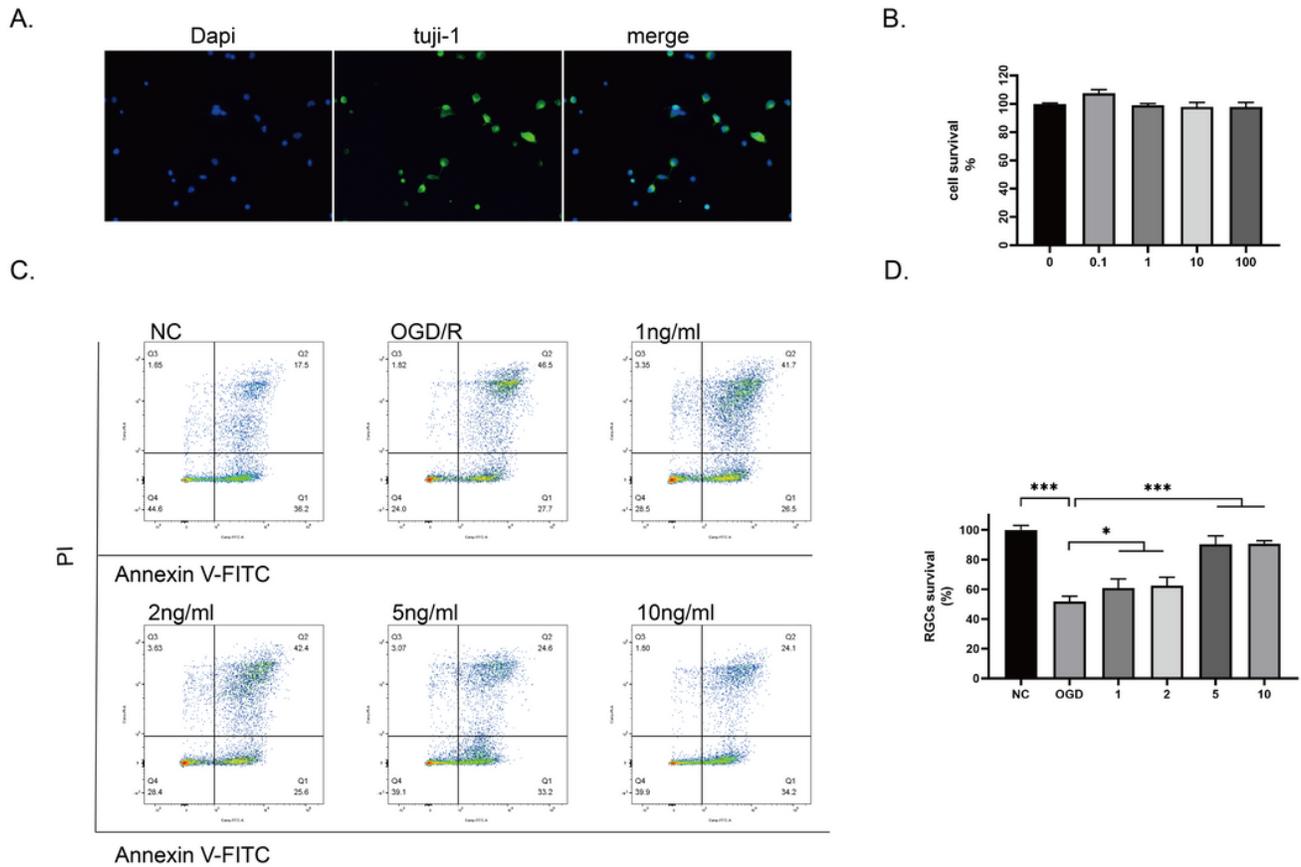
IL-35 inhibits RGC loss and the modification of IPL thickness resulting from I/R. On the seventh day following I/R injury, the mice were sacrificed. (A) IF image of the retina stained with Tuj-1 in green and Brn3a in red (scale bar = 50 μm). Intravitreal injection of IL-35 decreased the loss of RGCs, particularly in

the group that was treated with 20 ng/ml IL-35. (B) Statistical analysis of RGC count. (C) Representative images of H&E-stained sections of the retina are presented. (scale bar =50  $\mu$ m). IPL thinning was reduced by 20 ng/ml of IL-35. (d) Statistical analysis of IPL thickness. Data is reported as mean  $\pm$  SD (n =5). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



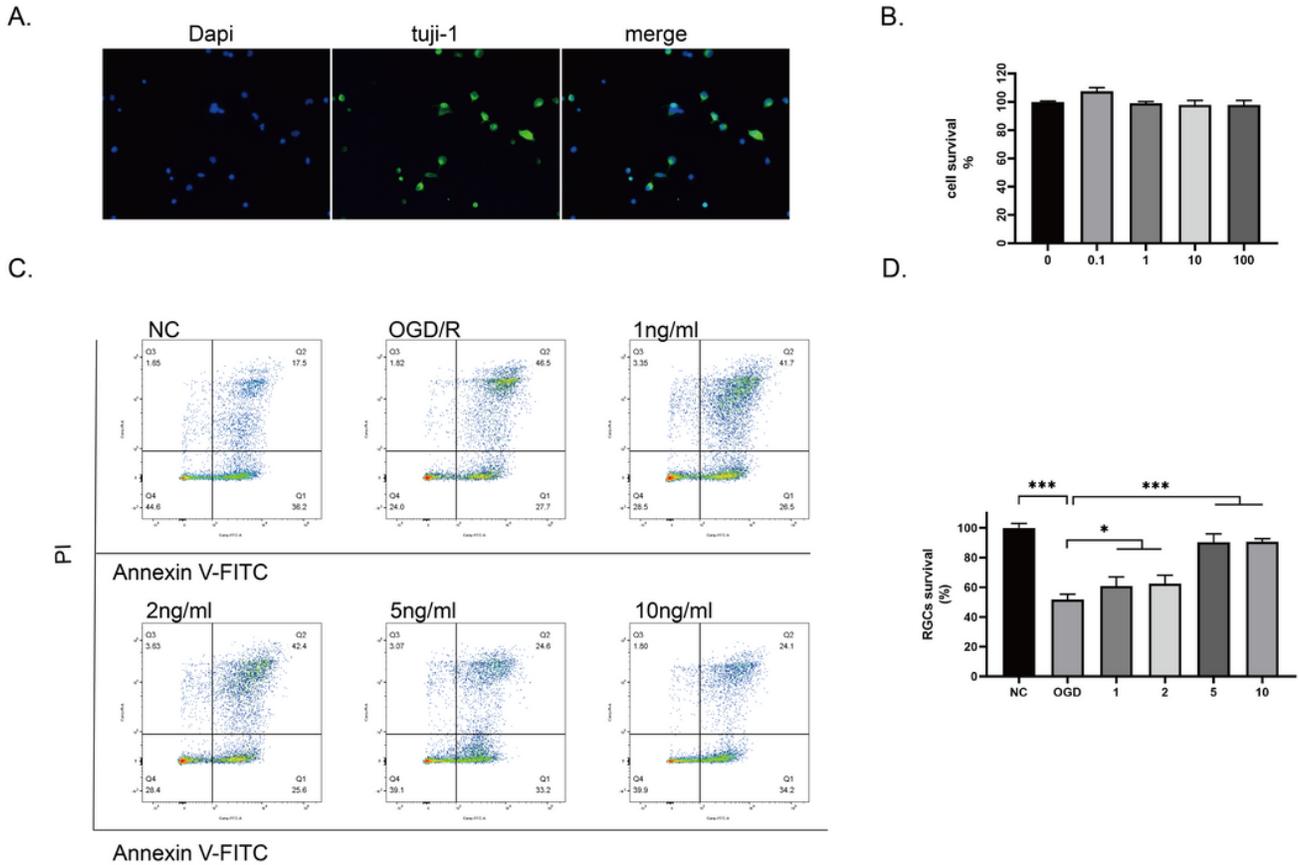
**Figure 2**

IL-35 mitigates the microglia pyroptosis resulting from retina I/R. (A) Intravitreal injection of IL-35 reduced the amount of retinal mRNA expression of NLRP3, ASC, caspase-1, GSDMD, IL-1 $\beta$ , and IL-18 one day after I/R. (B-C) Treatment of IL-35 effectively prevented the increase of NLRP3 induced by I/R and the cleavage of GSDMD and caspase-1 at the protein level. (D-E) IF analysis shows higher protein levels of ASC and NLRP3 in the retinas of mice that underwent I/R and lower levels in the retinas of IL-35 treated mice. Data is reported as mean  $\pm$  SD (n = 4). \*p < 0.05, \*\*p < 0.01.



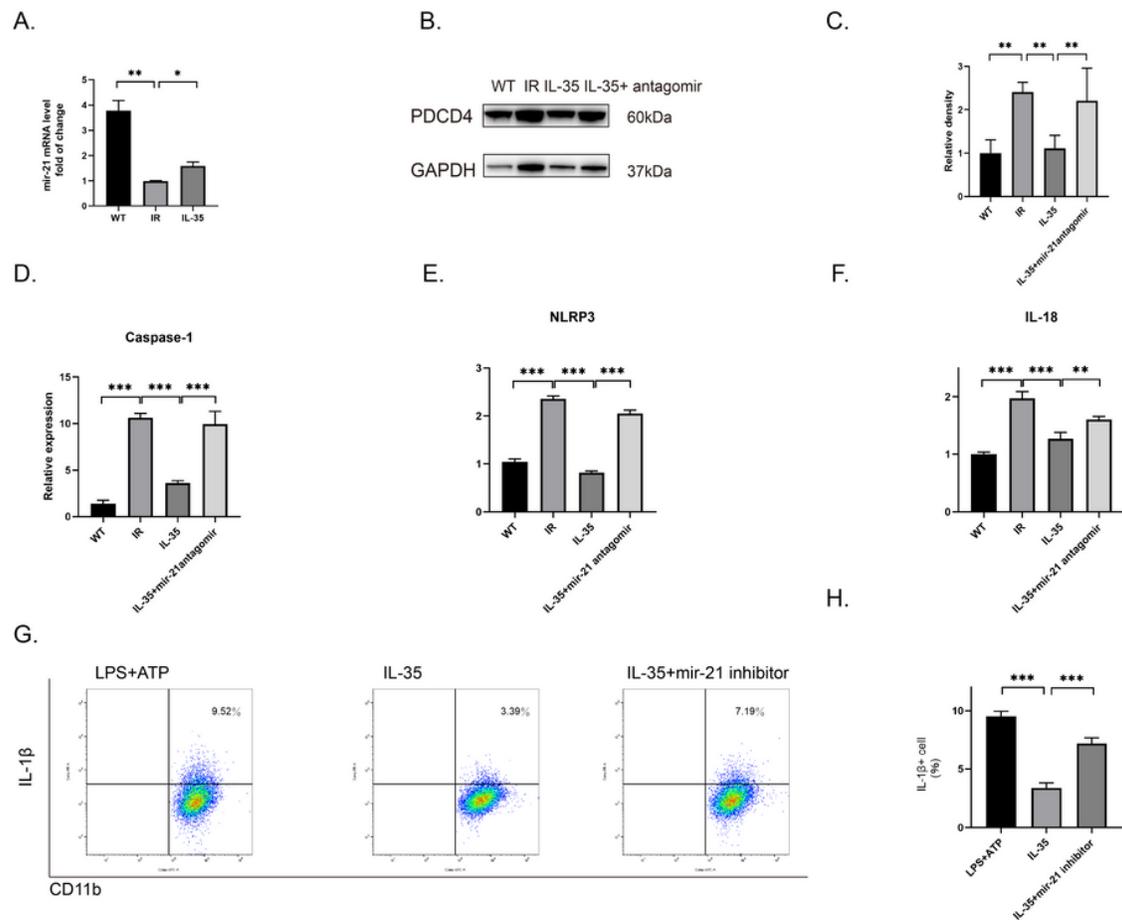
**Figure 3**

IL-35-induced RGC survival after exposure of the cells to OGD/R. (A) IF staining (scale bar = 50  $\mu$ m) showing the primary RGCs specifically stained with cytoplasmic Tuj-1 (green) and nuclear DAPI (blue). (B) CCK8 assay shows that IL-35 had no significant effect on RGC viability. (C-D) Primary RGC apoptosis after OGD/R treatment was established with flow cytometry. The survival of primary RGCs was increased after treatment with 5 ng/ml and 10 ng/ml of IL-35 in the course of OGD (2 h)/R (6 h). Data is reported as mean  $\pm$  SD (n = 5). \*p < 0.05, \*\*\*p < 0.001.



**Figure 4**

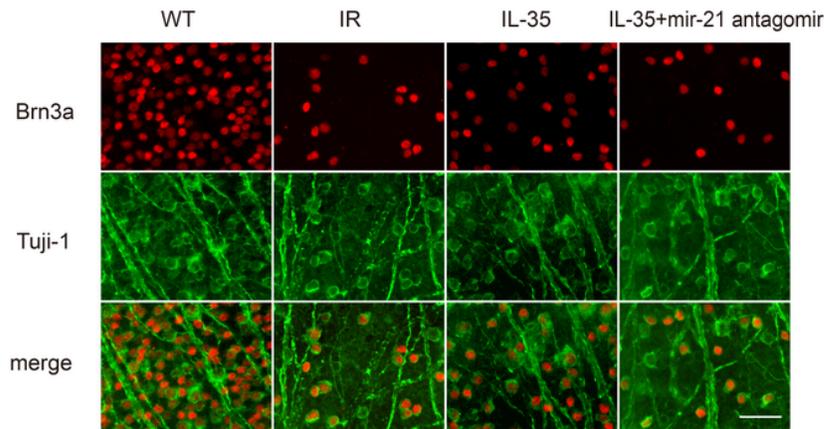
IL-35 suppresses the expression of inflammatory factors in the in vitro model of microglia pyroptosis. (A) IL-35 had no adverse effect on cell viability in microglia. (B-C) The microglia cellular production of IL-1 $\beta$  was established by flow cytometry. Less inflammatory factors were observed after treatment of the microglia with 50 ng/ml IL-35. (D) As evaluated by RT-qPCR, IL-35 significantly suppressed the mRNA levels of NLRP3, ASC, caspase-1, and IL-18 in retinas. Data is reported as mean  $\pm$  SD (n = 5). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, vs. LPS + ATP group.



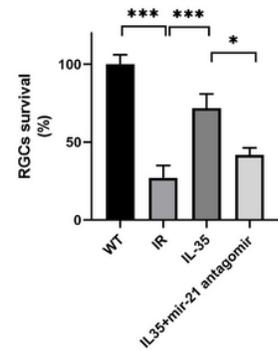
**Figure 5**

The miR-21/PDCD4 axis is vital for the effects of IL-35-mediated anti-pyroptosis. (A) The retinal mRNA expression of miR-21 was elevated. (B-C) The protein expression of PDCD4 declined. (D-F) The miR-21 antagomir significantly, but incompletely, reversed the IL-35-mediated suppression of NLRP3, caspase-1, and IL-18 production, as assessed by real-time PCR in the in vivo model of I/R. (G-H) Treatment with miR-21 inhibitor significantly undermined the suppressive effect of IL-35 on the release of IL-1β in vitro, as measured by flow cytometry.

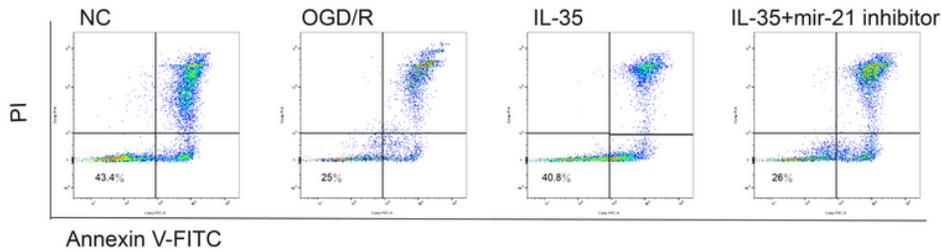
A.



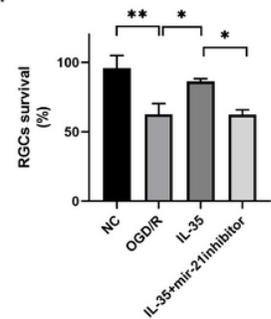
B.



C.



D.



## Figure 6

IL-35 protects RGCs from apoptosis via the miR-21/PDCD4 axis. The results of treatment with IL-35 on day three following I/R and intravitreal injection. (A-B) miR-21 antagomir dramatically suppressed the IL-35-mediated neuroprotective effects in IOP-induced RGC apoptosis. (C-D) IL-35 inhibited the apoptosis of primary RGCs induced by OGD/R in vitro. miR-21 inhibitor significantly reduced the IL-35-mediated cytoprotective effect on RGCs.